# RUBELLA VIRUS CARRIER CULTURES DERIVED FROM CONGENITALLY INFECTED INFANTS

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Clinical rubella in the first trimester of pregnancy may result in congenital anomalies of the infant, as was first noted in Australia by Gregg (1). In 1964 and 1965 widespread epidemics of congenital rubella occurred in the United States in which thousands of infants were born with more severe malformations than had previously been described (2–4). Before methods were available for isolation of rubella virus, the anomalies were attributed to the effect of the virus upon the developing fetus during a critical period of organ formation. More recently, however, studies have demonstrated excretion of virus by the infants and persistence of the virus in infant's tissues for at least a year after birth (2–8). This virus persistence occurs despite the development of neutralizing antibodies to rubella in the infant's serum (9–13).

During a study of postmortem tissues from infants with congenital rubella, we found that tissue cultures could be derived from various organs and that these cultured tissues, even though they appeared normal, were infected with rubella virus (6). In some instances the infected tissue cultures developed into cell strains which resulted in the establishment of spontaneous rubella carrier cultures. This in vitro model of the symbiotic relationship between the infant cells and rubella virus was investigated in an attempt to obtain a possible explanation for the in vivo observation of persistent infection.

## *Materials and Methods*

*Tissue Cultures.*—The preparation of rubella infected cell strains from infants with congenital rubella has been previously described (6); the method followed was that used by Benyesh-Melnick et al. (14) for isolating cytomegalovirus, adenovirus, measles, coxsackie, and varicella viruses from a variety of infant organs obtained at postmortem. Bone marrows were grown as described by Benyesh-Melnick et al. (15). Similar techniques were applied for the preparation of normal, noninfected cell strains from postmortem tissues of infants without evidence of rubella. Primary African green monkey kidney (GMK) cells were prepared as described (16), and in addition a line of monkey cells BSC-1 (17) was used. Constricted culture tubes were used, as they produced more uniform monolayer cultures which were conveniently restricted to one part of the tube (18). 1 oz flat bottles were used for some experiments and for all plaque assays.

*Media.*—Growth medium for cell strains derived from infants consisted of Eagle's basal

medium supplemented with 10% fetal bovine serum, antibiotics (penicillin, 100 units/ml and streptomycin: 100 μg/ml), and 0.75 g/liter of bicarbonate. Maintenance medium for cell strains derived from infants and for GMK cells consisted of Eagle's basal medium with 2% fetal bovine serum, antibiotics, and 1.5 g/liter of bicarbonate. This medium was also used for growth of BSC-1 cells.

*Viruses and Virus Assays.*—Virus stocks were prepared in monolayers of cells grown in 16-oz. prescription bottles. The growth medium was removed from the monolayers, 0.5 to 1 ml of seed virus was added and after an adsorption period of 1 hr at 37°C, the monolayers were washed once with maintenance medium. The cells were incubated in the presence of maintenance medium after which the viruses were harvested by freezing the cultures in a dry ice–alcohol bath and thawing. Virus-containing materials were clarified by low speed centrifugation, ampuled, and stored at −35°C.

Rubella virus assays were performed utilizing the inhibition of the cytopathic effect (CPE) of echovirus 11 in GMK cells (19). Rubella virus titers are expressed as inhibitory dose$_{50}$ ($InD_{50}$) which represents the dilution of virus necessary to prevent echovirus 11 CPE in 50% of the tubes upon challenge. Echovirus 11 (Gregory) was obtained from the World Health Organization Enterovirus Reference Centre, Houston, and passed in GMK cells. The virus was assayed by the plaque-counting method in GMK cell monolayers as used in this laboratory (20). In some instances the virus was assayed in culture tubes containing monolayers of GMK cells or human lung fibroblasts. Four tubes were employed per 10-fold dilution and the tissue culture infectivity dose ($TCD_{50}$) was calculated by the method of Reed and Muench (21). Herpes simplex virus (Black) was a laboratory strain. Virus stock was prepared in human lung fibroblasts, ampuled, and stored at −90°C. The virus was assayed by titrations in culture tubes containing monolayers of human lung fibroblasts. Vesicular stomatits virus (VSV) used in this laboratory has been described by McCombs et al. (22). The virus had been passed several times in human lung fibroblasts before preparation of virus stock in GMK cells. The virus was assayed by the plaque-counting method in BSC-1 cells. Following virus inoculation and a 1 hr adsorption period, the cells were overlayed with Eagle's basal medium, 1.5% Bacto-Agar (Difco Laboratories, Inc., Detroit), antibiotics, neutral red, 2.25 g/liter bicarbonate, and 3% fetal bovine serum. Plaques were counted in 2 or 3 days.

*Antiserum.*—Rubella antiserum was prepared in baboons. Animals were inoculated intramuscularly twice, 3 wk apart, with 3 ml virus stock (Ellis strain, $10^{3.5}$ $InD_{50}$/ml) mixed with an equal volume of Freund's complete adjuvant and at the same time intravenously with 3 ml of virus fluid. Serum drawn 8 wk after the second inoculation was found to have a titer of 1/256. The neutralization test was essentially that described by Parkman et al. (23).

## EXPERIMENTAL

Sixty-eight attempts were made to establish cell strains from infants with congenital rubella in Houston (2). Primary cell monolayers developed in 11 instances and 10 of these were found to be spontaneously infected with rubella virus. Five of the infected cell monolayers were successfully subcultivated into cell strains. These included cell strains derived from thyroid, umbilicus, lung, bone marrow, and kidney which were designated as rubella infected cell strains R-1 through R-5, respectively. Noninfected cell strains were also developed from tissues of infants without evidence of congenital rubella and from one infant with congenital rubella. The noninfected cell strains were designated as control cell strains C-1 through C-4, and included lung from the infant with congenital rubella and lung, umbilicus, and thyroid from infants without rubella, respectively.

Attachment and growth of cells following trypsinization of tissues from congenital rubella infants were usually not as good as with cells derived from infants without evidence of congenital rubella. Once monolayers formed, it was possible to perform serial passages readily twice weekly for 8 to 10 subcultures. Serial passage of cells, which was performed when a complete monolayer developed (5 to 6 $\times$ $10^6$ cells), was then possible at weekly intervals for an additional 8 to 10 passages. Thereafter the monolayers required many weeks to develop and subcultivations were performed infrequently. It was possible to

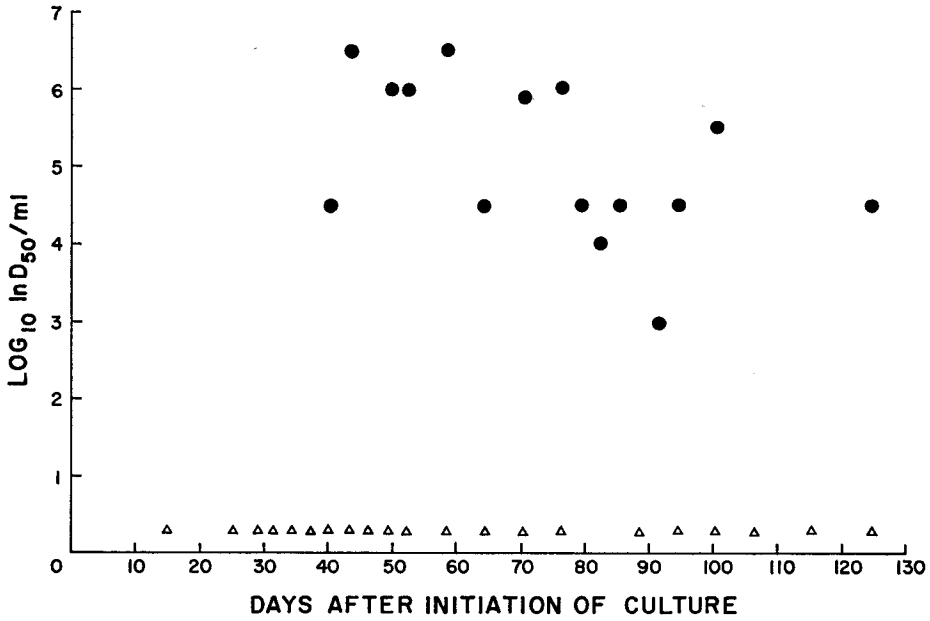


FIG. 1. The passage history of R-1 thyroid chronically infected cell strain in relation to spontaneous production of rubella virus. (△, indicates day of subcultivation; ●, indicates rubella titers in tissue culture fluid).

maintain R-1 thyroid cells for 11 months through 23 subcultivations. The passage history of the first 21 subcultivations of these cells is shown in Fig. 1 and is representative of the passage histories found with the other chronically infected cell strains. R-2 umbilicus was also maintained for 11 months and 23 subcultivations. R-3 lung survived for 3 months and 14 subcultivations while R-4 bone marrow survived 7 months and 14 subcultivations. The infected cell strain derived from kidney tissue has been developed more recently and has undergone 12 subcultivations over a 3 month period. It appears that the chronically infected cell strains may enter a slow growth phase (24) more rapidly than the noninfected cell strains. Morphologically the cells were fibroblasts and could not be distinguished from noninfected cells. Staining with acridine orange

and with hemotoxylin and eosin stains did not reveal any specific characteristic features of the infected cells.

Rubella virus could be demonstrated at concentrations of $10^{4.0}$–$10^{6.5}$ $InD_{50}$/ml in the tissue culture fluid throughout the life of the cultures. This represented about 1 to 10 $InD_{50}$ of rubella virus in the culture fluid per cell.

The multiplication of chronically infected cell strains was examined and compared with that of the noninfected cell strains.

*Experiment.*—Cells were dispersed by trypsinization and suspended in growth medium at 3.5 to 5 × $10^4$/ml. Constricted culture tubes were seeded with 1 ml cell suspension and incubated in stationary racks with medium changes performed at 3- to 5-day intervals. At intervals of 1 to 3 days, 3 tubes of each cell strain examined were drained of their medium and washed once with salt solution. The monolayers were drained of the wash fluid then covered with 1 ml of 0.2% trypsin. The tubes were incubated at 37°C for 30 min, when the cells were dispersed by pipetting, and were counted in a hemocytometer. The average of the cell numbers obtained for the 3 tubes was taken as the final count.

Representative growth curves of early and late passages of chronically infected cells, together with the growth curves of noninfected cultures, are shown in Fig. 2. Multiplication of chronically infected cell strains was found to be impaired. The 4 noninfected fibroblast cell strains doubled their cell number in 24 hr during the rapid growth phase. This generation time was obtained repeatedly and is in agreement with reports of others (24). Early in the life of the chronically infected cultures a generation time of about 48 hr was obtained as shown in Fig. 2 but beyond 10 subcultivations multiplication was more retarded with a doubling time of about 72 hr.

Carrier states of animal viruses in cell cultures have been shown in many instances to depend upon the presence in the culture fluid of a viral inhibitor (see reviews by Ginsberg (25) and Walker (26)). These have included both specific viral antibodies and nonspecific serum factors which inhibit virus multiplication (27). Therefore, a possible inhibitory effect of the fetal bovine serum used in the medium of chronically infected cell strains was sought.

*Experiment.*—R-2 umbilicus cells passage 18 were planted in culture tubes and allowed to develop into monolayers. The tubes were divided into 3 groups. The monolayers were washed with serum-free medium and each group of tubes received 1 ml of (*a*) medium with 10% fetal bovine serum, (*b*) medium with 2% fetal bovine serum, and (*c*) medium with no serum supplement. Tubes were observed daily for CPE and at intervals the fluid from 3 tubes was pooled and titered for virus. Cell counts were performed at the onset, 3rd, 7th and 10th day of the experiment. Noninfected C-1 lung cells were placed on similar media and observed concurrently with the infected cells.

Rounding and mild degenerative changes were noted at 7 to 10 days in some of the chronically infected cells maintained on serum-free medium; however, similar changes were observed in control cells on the same medium. No cellular degeneration was noted in the monolayers maintained on media containing

serum. Rubella virus concentrations in the culture fluid increased over the first 3 days then remained fairly constant thereafter at a ratio of about 10 $InD_{50}$ virus/cell. As shown in Fig. 3, virus yield was no greater in the absence of serum. The cell numbers appeared to decrease slightly in the absence of serum, increase slightly in the presence of 10% serum, and remain about the same in

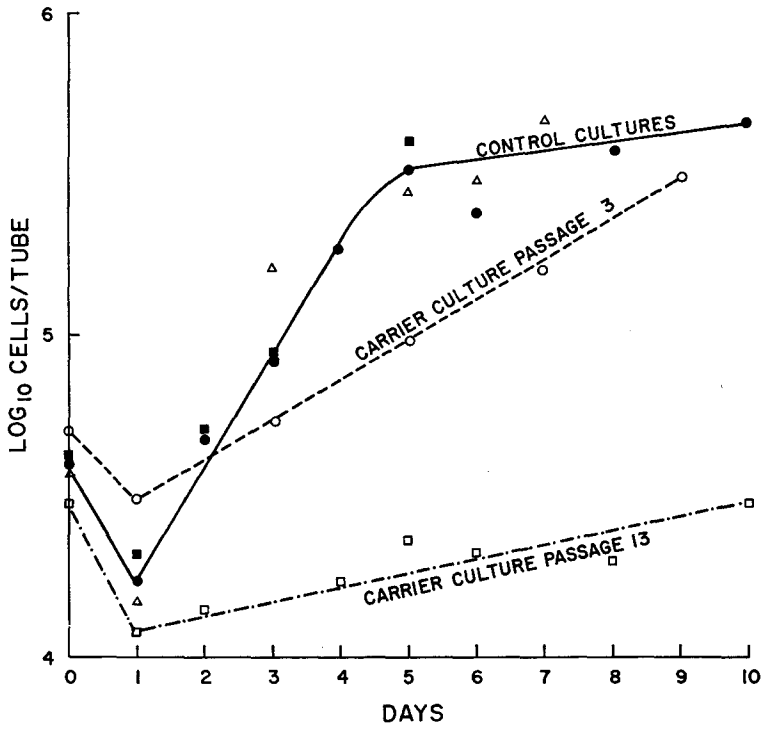


FIG. 2. Growth curves of rubella carrier culture cells and control cells. (●, C-1 lung passage 13; △, C-1 lung passage 17; ■, C-3 umbilicus passage 6).

the presence of 2% serum. These findings reveal no inhibitory effect of fetal bovine serum on rubella virus.

Interferon produced by affected cells of a carrier culture may protect other cells from infection by the persistent virus thus allowing a balance of virus production and cell survival (28). Our attempts to demonstrate interferon in tissue culture fluid from rubella carrier cultures were unsuccessful. These attempts included assay by plaque reduction of the indicator vesicular stomatitis virus (VSV), as well as suppression of CPE in tubes challenged with 100 $TCD_{50}$ VSV, after overnight exposure of human lung fibroblasts or human embryonic kidney cells to carrier culture fluid which had been centrifuged at 25,000 RPM

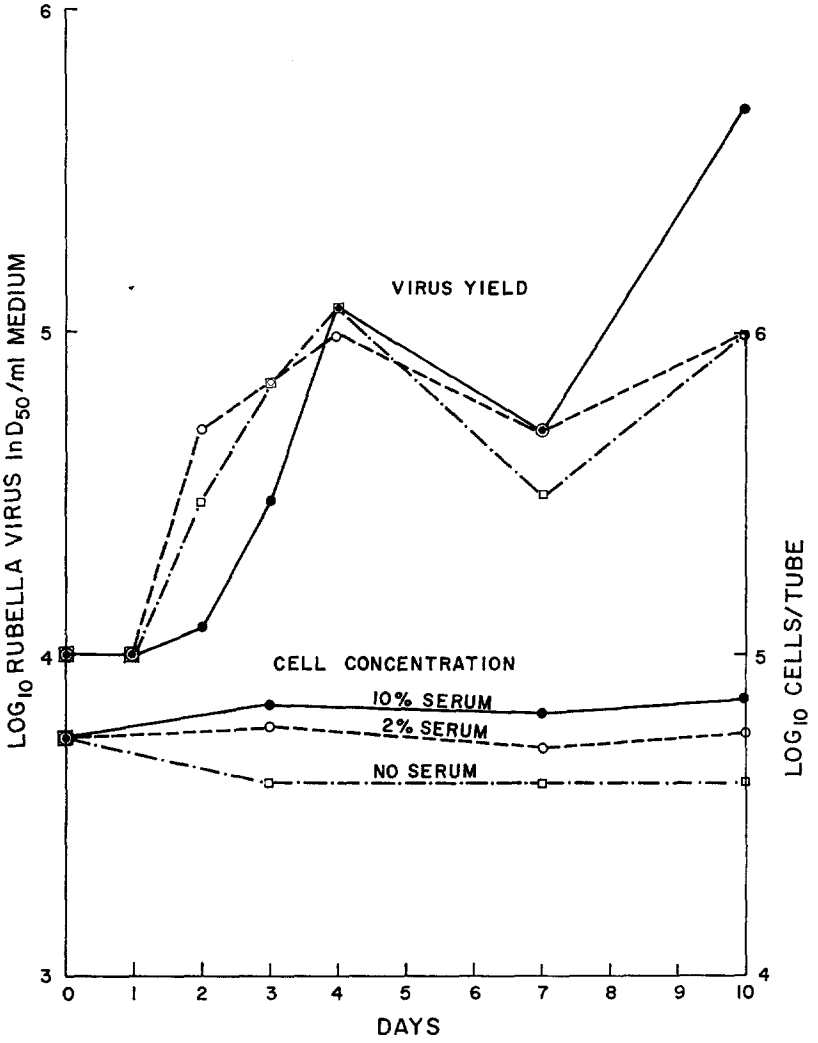


FIG. 3. The effect of different concentrations of fetal bovine serum on rubella virus production in chronically infected cells.

for 1 hr (to remove virus but not interferon). Interferon prepared by infecting human embryonic kidney cells with attenuated measles virus (Edmonston strain) protected human lung fibroblasts from VSV challenge, with the tissue culture fluid yielding a titer of 1:8. This material was used as a control. Incubation of human lung fibroblasts for 2 hr with undiluted, uncentrifuged tissue culture fluid from chronically infected cells did not alter the single cycle growth curves of VSV (29) or echovirus 11.

Carrier cultures have been described by Henle et al. (30) in which interferon was not demonstrated in undiluted tissue culture fluid but could be found after concentration. Therefore, the possible role of interferon in the maintenance of the rubella infected cell strains was further investigated by applying methods known to destroy or interfere with the action of interferon (31). These methods included the incorporation into the medium of trypsin which destroys interferon and cortisone acetate which inhibits interferon activity (32).

*Experiment.*—Culture tubes containing monolayers of infected and noninfected tubes were drained of their medium and washed with Eagle's medium without serum. Portions of each group received the following medium: (*a*) growth medium without serum containing 0.005% trypsin (Difco, 1/250); (*b*) growth medium without serum; (*c*) growth medium containing 2 μg/ml of cortisone acetate; and (*d*) standard growth medium with serum. Tubes were observed daily for CPE and 2 tubes of each group were frozen, thawed, and assayed for rubella virus.

TABLE I

*The Effects of Cortisone and Trypsin on Chronically Infected Cells**

| Medium | Titer‡ after incubation period of | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 day | 2 days | 3 days | 4 days | 5 days | 6 days | 7 days |
| Trypsin 0.005% in serum free medium | $10^{3.4}$ | $10^{3.6}$ | $10^{3.4}$ | $10^{3.5}$ | $10^{3.8}$ | $\geq 10^{3.5}$ | $10^{4.5}$ |
| Serum-free medium | $10^{4.2}$ | $10^{3.8}$ | $10^{3.2}$ | $10^{3.4}$ | $10^{3.8}$ | $10^{3.8}$ | $10^{4.3}$ |
| Cortisone 2 μg/ml growth medium | $10^{4.5}$ | $10^{4.5}$ | $10^{4.6}$ | $10^{4.5}$ | | $10^{4.4}$ | |
| Growth medium | $10^{4.6}$ | $10^{4.8}$ | $10^{4.6}$ | $10^{4.8}$ | | $10^{4.8}$ | |

* No cytopathic effect was seen during the period of observation.

‡ Rubella titer, inhibitory $dose_{50}$/ml.

The results of the experiments are shown in Table I. Neither cytopathic effect nor higher rubella virus titers developed in the cultures treated with either cortisone or trypsin. These data suggest that the maintenance of persistent infection in rubella infected cell strains is not dependent on interferon.

Essential in the characterization of a virus carrier state is the determination of the proportion of the cell population infected by the virus (26). Hypothetically a single virus-producing cell might be able to produce enough rubella virus to confer interference in GMK cells. Consequently studies were undertaken to establish the number of chronically infected cells needed to produce such interference.

*Experiment.*—Monolayers of chronically infected cells were washed with salt solution, trypsinized, and resuspended in growth medium. The cells were sedimented by centrifugation at 800 RPM for 10 min, washed with salt solution, resuspended in the Earle's solution, and divided into two equal parts. To one portion was added 10% normal baboon serum and to the other was added 10% rubella antiserum prepared in baboons. The mixtures were held at room temperature for 1 hr after which the cells were sedimented and resuspended in Earle's

solution. Cell counts were performed and further dilution of the cells was made to provide the desired number of cells per 0.1 ml. Cells were inoculated into culture tubes containing GMK monolayers from which the medium had been drained. 1 hr later 1 ml of maintenance medium was added and the tubes incubated in a stationary rack at 37°C. The medium was changed in 7 days and the tubes inoculated with 100 $TCD_{50}$ echovirus 11 after 14 days.

The results of an experiment performed with R-2 umbilicus cells are shown in Table II. All GMK monolayers receiving more than a calculated 15 cells and some of the tubes receiving less cells were resistant to echovirus 11. The number of cells producing interference in 50% of the inoculated tubes was 3.8 and 1.4 for cells treated with normal baboon serum and rubella antiserum, respectively.

TABLE II

*Determination of the Number of Cells Infected in R-2 Umbilicus Carrier Culture*

| Cells plus normal baboon serum | | Cells plus baboon antiserum | |
|---|---|---|---|
| Average No. of cells per tube | Results | Average No. of cells per tube | Results |
| 15 | 5/5* | 30 | 5/5 |
| 1.5 | 2/5 | 3 | 3/4 |
| 0.15 | 0/5 | 0.3 | 1/5 |
| 0.015 | 0/5 | 0.03 | 0/5 |
| $InD_{50}$‡ (cells) | 3.8 | $InD_{50}$ (cells) | 1.4 |

* Number tubes showing interference/number tubes tested.

‡ Inhibitory $dose_{50}$ calculated by method of Reed and Muench.

Similar experiments were performed with R-3 lung cells at 2 passage levels (Table III). The number of cells capable of conferring interference was essentially the same at both passage levels. Studies of the same R-3 cell strain at its 2nd passage level from the patient also showed interference in all tubes inoculated with 5 cells/tube, the least number of cells tested. Other experiments, such as that shown in Table VI with the R-1 cell strain, indicated that a single cell from the carrier culture could be found to confer interference. No interference was produced in GMK monolayers inoculated with 3000 control lung cells (C-1). These data suggest that virtually all cells of the population are producing rubella virus and that this high proportion of infected cells is established early in the life of the cultures.

The number of cells capable of conferring interference to GMK cells was noted to be the same in the presence or absence of rubella antiserum. This implied a nonneutralizable or intracellular position of the virus, which was further supported by the following experiment.

*Experiment.*—Infected cells from the R-3 lung cultures were prepared as above and one half of the cells were disrupted by sonication. To one half of the sonicated cells was added 10% normal baboon serum while the other half received 10% rubella antiserum. After 1 hr at room temperature the mixtures of sonicated material and whole cells were diluted and inoculated onto GMK monolayers. Following an incubation period of 2 wk the GMK monolayers were challenged with echovirus 11.

It was found that the disrupted cells treated with rubella antiserum did not produce interference at concentrations derived from an equivalent of 120 cells (Table IV). Thus the virus appears to be in an intracellular position.

TABLE III

*The Number of R-3 Lung Cells Infected at Different Passage Levels*

| Passage 7 | | Passage 10 | | | |
|---|---|---|---|---|---|
| Cells without serum | | Cells without serum | | Cells plus antiserum | |
| Average No. cells per tube | Results | Average No. cells per tube | Results | Average No. cells per tube | Results |
| 230 | 7/7* | 270 | 5/5 | 180 | 5/5 |
| 23 | 7/7 | 27 | 5/5 | 18 | 5/5 |
| 2.3 | 5/7 | 2.7 | 2/5 | 1.8 | 1/5 |
| 0.23 | 0/7 | 0.27 | 0/5 | 0.18 | 1/5 |
| $InD_{50}$‡ (cells) | 1.5 | $InD_{50}$ (cells) | 7 | $InD_{50}$ (cells) | 6 |

* Number tubes showing interference/number tubes tested.

‡ Inhibitory $dose_{50}$ calculated by method of Reed and Muench.

The perpetuation of virus in the carrier state necessitates its spread from cell to cell. Viruses which are cytolytic usually are transferred from infected cell to new host cell by means of the fluid phase of the culture. However, noncytolytic viruses may be passed to daughter cells during cell division. The ability to rid chronically infected cells of persistent virus implies a fluid phase in the cell-to-cell transfer of virus and not a passage of virus during cell division. Several attempts to cure the spontaneously rubella infected cell strains were unsuccessful. Amantadine-HCl has been reported to inhibit rubella replication (33), the apparent mechanism of action being prevention of virus penetration (34). However, amantadine-HCl at concentrations adequate to suppress virus production was toxic to the chronically infected cells. Similar toxic effects have been reported for human diploid cells experimentally infected with rubella (35).

Rubella antisera incorporated into the medium were also not effective in curing the chronically infected cell strains. One such attempt is exemplified in the following experiment.

*Experiment.*—Chronically infected cells were planted in 1-oz. bottles. To one half of the bottles was added regular medium while the other half regularly received growth medium plus 10% human convalescent rubella antiserum having an antibody titer of 1/64. Medium was changed twice weekly and the fluid removed was periodically titered for virus. 3 wk after the onset of treatment the number of cells capable of conferring interference with echovirus 11 in GMK cells was determined by the terminal dilution method described above.

TABLE IV

*Rubella Antibody Neutralization of Interfering Agent Obtained from Ruptured Cells of Carrier Culture*

| Average No. cells per tube | Results | | |
|---|---|---|---|
| | R-3 cells plus rubella antiserum | R-3 sonicated cells | |
| | | Normal serum | Rubella antiserum |
| 120 | 7/7* | 7/7 | 0/7 |
| 12 | 7/7 | 4/7 | 0/7 |
| 1.2 | 2/7 | 2/7 | 0/7 |
| 0.12 | 0/7 | 0/7 | 0/7 |

* Number of tubes showing interference/number tubes tested.

TABLE V

*The Effect of Antiserum on Tissue Culture Fluid Virus*

| Cultures | Days of treatment | | |
|---|---|---|---|
| | 3 | 7 | 14 |
| With antiserum | <1.0* | 1.2 | 1.4 |
| Without antiserum | 2.5 | 3.4 | 4.2 |

* Reciprocal of log $InD_{50}$.

The results of rubella virus titers are summarized in Table V and it can be seen that complete suppression of virus in the tissue culture fluid was not achieved. No evidence of virus was found at a 10-fold dilution of the tissue culture fluid after 3 days of treatment with antiserum; however, at 7 and 14 days interference was produced in GMK cells inoculated with fluid at this dilution. The decrease of 2 and 3 log $InD_{50}$ of virus in the tissue culture fluid at 7 and 14 days does not appear to be due to a change in the proportion of cells capable of producing virus (Table VI). Similar results were obtained using the hyperimmune antiserum prepared in baboons. In this experiment rubella virus was not detected in the undiluted culture fluid for 6 days, but thereafter the virus titer gradually increased.

The ability of infected cells to survive and produce virus without undergoing

death would be anticipated in a viral carrier state in which the virus was transferred to daughter cells during cell division. An effort was made to isolate individual cells and maintain them for a period of time which would allow any viral cytopathic effects and inactivation of the virus produced to occur. This was accomplished in capillary tubes.

TABLE VI

*Effect of 3-wk Treatment of Cultures with Antiserum on the Number of Cells Capable of Producing Interference*

| Cells from control cultures | | Cells from treated cultures | |
|---|---|---|---|
| Average No. cells per tube | Results | Average No. cells per tube | Results |
| 120 | 7/7* | 130 | 7/7 |
| 12 | 7/7 | 13 | 7/7 |
| 1.2 | 1/7 | 1.3 | 3/7 |
| 0.12 | 1/7 | 0.13 | 3/7 |
| $InD_{50}$ (cells) | 1 | $InD_{50}$ (cells) | 0.8 |

* Number tubes showing interference/number tubes tested.

TABLE VII

*Interference Produced by Single R-3 Cells after Incubation for 1 Month in Capillary Tubes*

| Infected R-3 cells | Control cells | Control rubella virus from fluid phase of R-3 cultures |
|---|---|---|
| 13/32* | 0/17 | 0/21 |

* Number tubes producing interference/number tubes tested.

*Experiment.*—R-3 lung cells in passage 7 were prepared in cell suspensions as described above for determining the proportion of cells infected. They were diluted in growth medium to a concentration of 20 cells per ml. Approximately 0.1 ml portions of the cell suspensions were drawn into 1.6 to 1.8 × 100 mm capillary tubes which were then flame sealed. The tubes were incubated at 37°C for 1 month after which they were broken and the contents of each capillary tube emptied into a culture tube containing GMK cells. The medium was changed in 7 days and after 2 wk the GMK monolayers were inoculated with 100 $TCD_{50}$ echovirus 11. Capillary tubes containing control C-2 cells and tubes containing cell-free R-3 tissue culture fluid were handled similarly.

Small opaque areas were noted in some capillary tubes; however, a proper optical system to delineate individual cells within the tubes was not available. The results of the production of interference in GMK cells by the capillary tube contents are shown in Table VII. Cells from the early subcultures of R-3 apparently survived for 1 month in the capillary tubes, for 40% of these tubes

contained sufficient virus to produce interference against echovirus 11 in GMK cells. These results also imply a high proportion of the cells introduced into the capillary tube were infected with the virus. Of importance is the fact that chronically infected cells could be isolated in capillary tubes where they apparently survived and produced virus. Control noninfected cells and cell-free virus containing fluid did not confer interference in GMK cells after an incubation period of 1 month under similar circumstances. The interfering agent produced in the capillary tubes by the chronically infected cells could be passed and was neutralized with rubella antiserum.

Definite evidence of passage of virus to daughter cells during cell division can be obtained in developing clones of infected cells under circumstances preventing transfer of virus through tissue culture fluid. Although 22 cell doublings "or splits" are theoretically necessary to produce $10^6$ cells from a single cell (36), and the number of splits of chronically infected cell strains before the pattern of slow growth occurred were far fewer, attempts to clone infected cells were made. One such attempt is described below.

*Experiment.*—R-5 kidney cells, passage 3, were trypsinized, washed with growth medium, and individual cells (or double or triple cell aggregates) were placed in constricted tubes using a microcapillary technique. ½ ml of growth medium was added to the tubes and the pH of the medium adjusted with $CO_2$. The tubes were incubated in a stationary rack and they were periodically examined for cells. After 3 wk of incubation the medium was transferred to GMK monolayers which were incubated for 2 wk and then challenged with 100 $TCD_{50}$ echovirus 11.

Examination of the tubes revealed 13 of 41 tubes in which the cells apparently did not survive. Fluid from these tubes failed to produce interference in GMK cells. There were 15 tubes in which individual cells developed into clones of cells ranging from 4 to 16 in number. The number of cells in each clone reached a maximum after which the cells gradually underwent degenerative changes. Fluid from 7 of the 15 tubes produced interference in GMK cells. Two or more cells were inoculated into 13 tubes and the fluid from 12 of these produced interference in GMK cells.

Cells of viral carrier states have often been found to have altered susceptibility to superinfection with a second virus; this has been especially true where interferon has been responsible for maintaining the carrier state. Studies of the viral susceptibility of the rubella virus carrier cultures were undertaken. Since noninfected clones of the spontaneously infected cell strains could not be established, the virus susceptibility of the chronically infected cell strains was compared with the normal cell strains derived from similar tissues of other infants.

*Experiment.*—Cells were planted at 5 to 10 × $10^4$/ml in constricted culture tubes. When confluent monolayers had developed the medium was removed and the tubes were inoculated with 0.1 ml of the appropriately diluted virus. 10-fold dilutions of each test virus were made in Earle's solution and 4 tubes were inoculated per dilution. After 1 hr at room temperature, 1 ml of maintenance medium was added to the tubes which were observed 3 times weekly for

2 wk. Cytopathic effect involving more than 25% of cells of the monolayer was considered as positive evidence of viral effect.

Chronically infected cells were found to be resistant to the cytopathic effects of VSV and herpes simplex virus, the differences in virus titers between noninfected and chronically infected cell strains being 3 $\log_{10}$ or greater (Table VIII). Echovirus 11 developed slightly more slowly in chronically infected cells than in noninfected cells but eventually similar titers were obtained in both cells, based on the appearance of enteroviral cytopathic changes.

The failure of VSV and herpes simplex but not echovirus 11 to produce cytopathic changes in the carrier cultures raised the question of replication of these different viruses in the cells. Therefore the growth characteristics of

TABLE VIII

*Virus Susceptibility of Rubella-Infected and Control-Noninfected Cell Strains*

| Virus | Rubella-infected cell strains | | | | Control noninfected cell strains | | | |
|---|---|---|---|---|---|---|---|---|
| | R-1 Thyroid | R-2 Umbilicus | R-3 Lung | R-4 Bone marrow | C-1 Lung | C-2 Lung | C-3 Umbilicus | C-4 Thyroid |
| Echovirus 11 | 6* | 5.3; 6.2 | 6.0; 6.3 | 5.3 | 5.7; 6.5 | 5.5; 5.7 | 7.0 | 7.0 |
| Herpes simplex | <1 | <1; <2 | 2.5; 2.5 | <1 | 5.7; 6.7 | 5.5; 5.5 | 7.3 | 6.3 |
| Vesicular stomatitis | <1 | <1; <1 | <1; 1.5 | <1 | 4.5; 5.5 | 5.3; 5.5 | 6.3 | 5.7 |

* Reciprocal of highest dilution ($\text{Log}_{10}$) producing CPE/0.1 ml.

echovirus 11 and VSV were examined in chronically infected and noninfected cell strains.

*Experiment.*—Cell monolayers in constricted culture tubes were washed with Earle's solution and inoculated with an input multiplicity of about 2 to 4 PFU virus/cell. Virus was allowed to adsorb 1 hr at 37°C. The monolayers were washed once with Earle's solution and aliquots of wash material were titered for unadsorbed virus. Maintenance medium was added to the culture tubes which were then incubated at 37°C. Three tubes for each cell strain were removed at appropriate intervals and frozen. The total virus was determined by 3 cycles of freezing and thawing of the tubes, pooling their contents, and assaying the pooled material.

Replication of both viruses was examined in 3 chronically infected cell strains, and in 3 control noninfected cell strains. Fig. 4 contains a representative growth curve of echovirus 11 in spontaneously infected R-3 lung cells, control C-2 lung cells, and experimentally infected GMK cells. The only difference noted between chronically infected and control noninfected human cells was 0.5 to 1 log less virus yield in the chronically infected cells. No evidence of echovirus 11 replication was found in GMK cells which had been infected with rubella virus

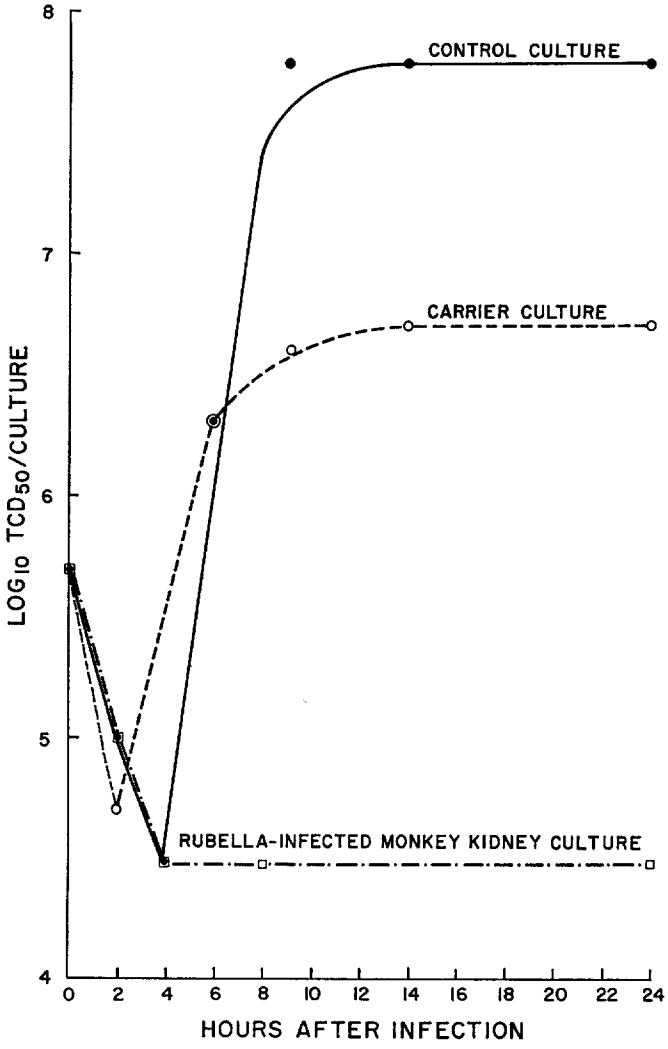


FIG. 4. The replication of echovirus 11 in R-3 lung rubella carrier cultures ($1.4 \times 10^5$ cells/culture), C-1 lung control cultures ($4 \times 10^5$ cells/culture), and GMK cultures ($4 \times 10^5$ cells/culture) infected with rubella virus from R-3 lung cultures.

from R-3 tissue culture fluid 5 days before the introduction of echovirus 11. Unlike echovirus 11, vesicular stomatitis virus did not replicate significantly in the chronically infected cell strains (Fig. 5).

Virus titers in wash material from growth curve experiments were essentially the same for rubella-infected and control-noninfected cells implying similar adsorption dynamics. The nature of adsorption was examined more closely for

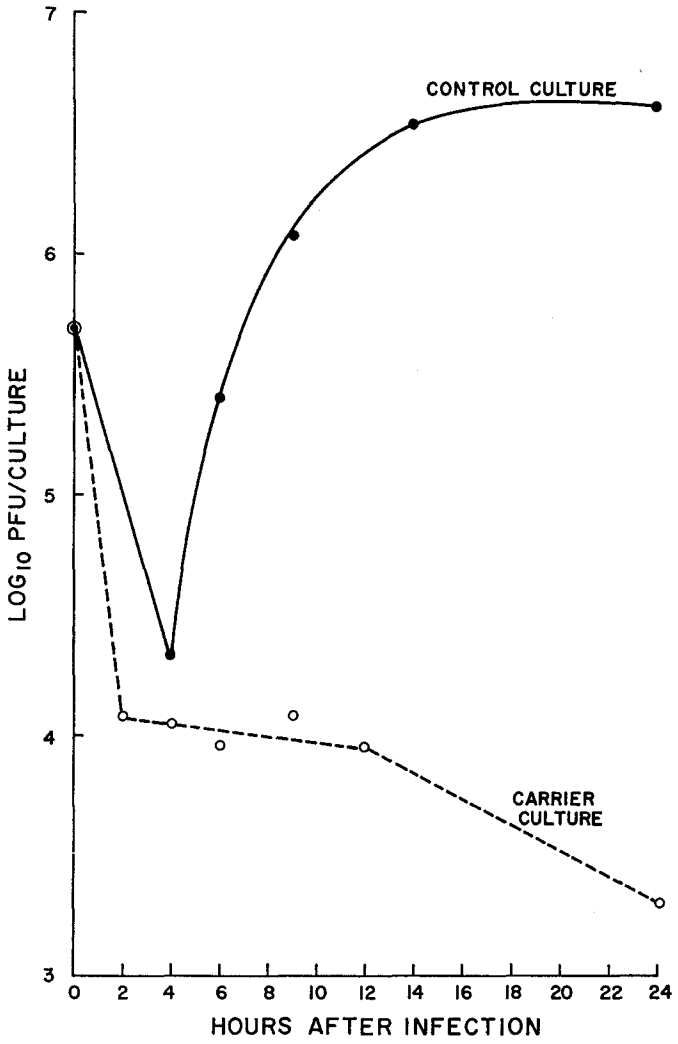


FIG. 5. The replication of vesicular stomatitis virus in R-2 umbilicus carrier cultures ($6 \times 10^4$ cells/culture), and C-3 umbilicus control cultures ($25 \times 10^4$ cells/culture).

vesicular stomatitis virus in two chronically infected cell strains, (R-3 lung and R-2 umbilicus) and a control noninfected cell strain (C-1 lung).

*Experiment.*—Monolayers of cells were dispersed by trypsinization, washed, and resuspended in maintenance medium at pH 8. To 2.5 ml of cells ($5 \times 10^5$/ml) were added $5 \times 10^5$ PFU of vesicular stomatitis virus in 0.1 ml. The suspensions were incubated at room temperature with periodic manual agitation. Portions (0.2 ml) of cell suspensions were removed at 15-min intervals and diluted 25 times in maintenance medium. The cells were immediately

TABLE IX

*Adsorption of VSV* on Rubella-Infected and Control-Noninfected Cells*

| Cell strains | 15 min | | 30 min | | 45 min | | 60 min | |
|---|---|---|---|---|---|---|---|---|
| | PFU/0.1 ml supernate | Adsorbed | PFU/0.1 ml supernate | Adsorbed | PFU/0.1 ml supernate | Adsorbed | PFU/0.1 ml supernate | Adsorbed |
| | | % | | % | | % | | % |
| R-3 lung | $2.4 \times 10^3$ | 25 | $1.7 \times 10^3$ | 47 | $1.1 \times 10^3$ | 66 | $1 \times 10^3$ | 69 |
| R-2 umbilicus | $2.3 \times 10^3$ | 28 | $1.6 \times 10^3$ | 50 | $1.2 \times 10^3$ | 62 | $9 \times 10^2$ | 72 |
| C-1 lung | $2.0 \times 10^3$ | 37 | $1.4 \times 10^3$ | 56 | | | $1.1 \times 10^3$ | 66 |

* Input virus titer, $3.2 \times 10^3$ PFU/0.1 ml.

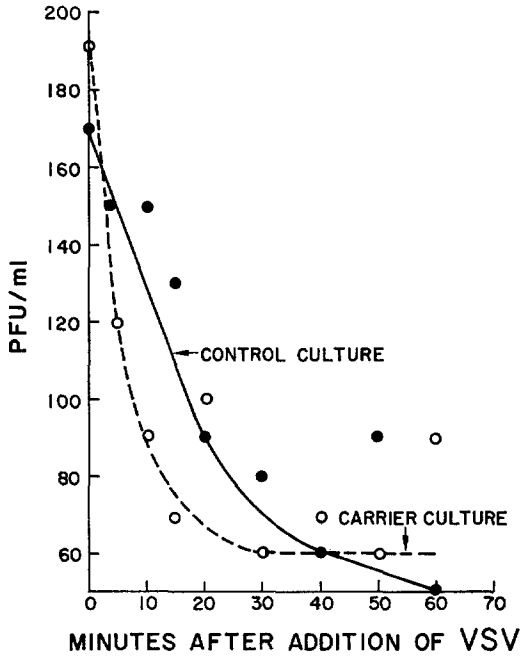


FIG. 6. Eclipsing of vesicular stomatitis virus in R-1 thyroid carrier culture cells and C-4 thyroid control cells.

sedimented by centrifugation at 1500 RPM for 10 min and residual virus in the supernatant fluid was assayed. Virus content of medium treated similarly but without the presence of cells was also determined.

No difference in adsorption was noted (Table IX). Consequently the lack of growth of VSV in chronically infected cells must be due to some other factor.

Further efforts to characterize the lack of replication of VSV were made by investigating the eclipse of the virus in infected and noninfected cells.

*Experiment.*—Infected and control cell monolayers were trypsinized and the cells sedimented by low speed centrifugation. The cells ($10^6$) were resuspended in 1 ml of cold growth medium containing $10^7$ $TCD_{50}$ VSV. The suspensions were agitated for a few minutes, then the cells were sedimented at 3000 RPM for 30 sec. The cells were washed 3 times in 10 ml of cold Eagle's medium without serum, resuspended in 10 ml of the same medium, and placed at 37°C. 1 ml portions were removed at appropriate intervals and immediately frozen in a dry ice–alcohol bath. When all samples had been collected, they were thawed and frozen 2 additional times and assayed for virus.

The results of an experiment utilizing R-1 thyroid and C-4 thyroid are shown in Fig. 6. There was no indication of a significantly impaired eclipsing of VSV in the 3 rubella-infected cell strains examined. These data indicate an intracellular site of interference with VSV replication in the chronically infected cell strains.

## DISCUSSION

Rubella virus carrier cultures have been induced in stable cell lines (37–39). Cell strains derived from certain tissues (skin and pharyngeal mucosa) of human embryos were also found to survive in a carrier state when experimentally infected with rubella virus (40). In addition, Kay et al. (41) and Goffe (42) have reported the isolation of rubella virus from tissue cultures derived from fetuses. Sufficient information is not available, however, to compare the artificially induced carrier cultures with those developing spontaneously from tissues of congenitally infected patients. The spontaneous carrier cultures reported in our paper are characterized by the following: (*a*) no rubella antiserum or serum inhibitors are necessary to maintain the cultures; (*b*) virtually the entire cell population is infected with rubella virus; (*c*) the virus does not appear to have a cytolytic potential in the cells which are able to survive and produce virus for long periods; (*d*) the infected cells are resistant to superinfection by some viruses but not by others; and (*e*) no evidence could be obtained to show that interferon plays a role in maintaining the carrier state.

Experimentally induced carrier cultures with similar virus-cell relationships have been described for mumps (43) and parainfluenza (44) in stable cell lines. In both of these systems direct evidence of the passage of virus from parent to daughter cells was demonstrated. Although direct evidence for this type of transfer of virus in cells chronically infected with rubella was not obtained, the development of clones of cells which were infected makes this type of cell transfer likely.

The absence of interference to superinfection of rubella-infected cell strains with echovirus 11 confirms the finding of Goffe (42). Variability in resistance to superinfection of carrier cultures with related and unrelated viruses has been noted by others (43, 45, 46). Crowell (46) found specific resistance to related

enteroviruses and this resistance was found to be an adsorption defect, which was not demonstrated to be the case in our rubella-infected cells. The mechanism of maintenance of the enterovirus carrier state reported by Crowell, however, was quite different from that operative in the rubella-infected cell strains. In the case of HeLa cells persistently infected with tick-borne encephalitis virus, Mayer (45) found them to be relatively resistant to infection with poliovirus 1 and coxsackievirus B1 but sensitive to infection with herpes simplex virus (45). The mumps carrier cultures described by Walker (43) were resistant only to superinfection with another strain of the same virus. The nature of the interference noted in the latter two instances is not clear. Whether they resemble the intracellular block of viral synthesis as noted for VSV in the rubella carrier cultures remains to be determined.

Interference produced by rubella virus in human amnion and bovine embryo cells has been attributed to interferon (47); however, no interferon was detected in GMK cultures infected with rubella virus (48). Interference of Newcastle disease virus by rubella virus in GMK cells has been described which does not appear to be mediated by an interferon (49). In this instance, as with VSV in chronically infected cells, there appears to be normal attachment and eclipse of the challenge virus. Evidence has been presented to suggest that the virus infection of a cell may result in either the production of virus or the production of interferon (50, 51). If a similar chain of events occurred with rubella-infected cells it would be difficult to postulate an interferon-mediated action as a mechanism for the viral carrier state in which virtually the entire population of cells were already infected with the virus (28).

Postulation of in vivo events from in vitro observations is hazardous; however, some comment on the possible nature of the in vivo rubella virus–cell relationship in congenital rubella is in order. In the cells derived from these infants the virus has been shown to survive and replicate without producing cell death. Virus appears to be transferred from parent to daughter cells during cell division, thus it can be envisioned that parent cells infected early in embryonic life may give rise to clones of infected cells. Cell transfer of virus under these circumstances would not be affected by circulating antibodies. Hayflick has suggested a possible relationship between the in vitro life span of cell strains and in vivo ageing (36). The slower growth and possible shortened survival of the chronically infected cells observed in vitro would suggest limited doubling potential of the in vivo infected cell clones, which upon dying out should result in spontaneous cure of the infants. Cessation of excretion of virus by infants with rubella apparently does occur (13, 52). Naeye and Blanc have recently demonstrated that organs of infants with congenital rubella contain subnormal numbers of cells (53). These findings are in keeping with the hypothesis that clones of infected cells have a limited doubling potential. Whether or not virus-infected cells in the congenital rubella infants themselves respond to produce

interferon is unknown, but our studies failed to reveal interferon production by the chronically infected cells in culture. Interferon production is a manifestation of the cellular response to foreign substances such as nucleic acids (54). Rubella virus infection of cells during the early developmental stages of the embryo could result in the incorporation of the virus into the cell at a time when it would not be recognized as foreign; and thus interferon production would not be stimulated. Experimental evidence for such a period of decreased production of interferon during early development has been demonstrated in chick embryos (55). In the human embryo, the number of cells which might not be capable of recognizing the rubella virus as foreign would have to be limited. Involvement of all the body cells including the antibody forming cells should result in complete immunologic tolerance similar to that observed in mice infected in utero with lymphocytic choriomeningitis virus (56). However, unlike mice which are reported not to receive significant amounts of maternal antibodies transplacentally (57), the extent of involvement in the human embryos might be confined to a limited number of clones by maternal antibodies (11).

## SUMMARY

Spontaneous rubella carrier cultures derived from tissues of infants with congenital rubella were studied in an attempt to elucidate a possible mechanism for viral persistence observed in these infants. Chronically infected cells were found to have a reduced growth rate and the cultures appeared to have a shortened life span. The rubella carrier state was not dependent on serum inhibitors or rubella antibodies. Virtually every cell in the carrier population was found to be producing virus. The carrier cultures could not be cured by rubella antibodies. The rubella-infected cells were resistant to superinfection with vesicular stomatitis virus and herpes simplex virus but were susceptible to infection with echovirus 11. The replication of vesicular stomatitis virus was apparently blocked at an intracellular site, for the virus readily adsorbed to the chronically infected cells and entered into an eclipse phase; however no infectious virus developed. No evidence of interferon production by these cells could be obtained. It is postulated that clones of rubella-infected cells in vivo, with properties similar to those in carrier cultures developed in vitro from tissues of in utero infected infants, might explain the observed viral persistence noted in congenital rubella.

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