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Virus Carrier Cells and Virus-Free Cells in Fetal Rubella* (33348)

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Certain viral infections of animals occurring *in utero* are characterized by a persistence of the virus. In these animal models, the majority or totality of the cells may exhibit a state indicative of prior experience with the virus. Avian and murine leukemia viruses appear to be present in the majority of the cells of the host (1–3). Lymphocytic choriomeningitis virus (LCM) antigens are demonstrable in the majority of the cells of the early embryo of the mouse while relatively few cells of older animals contain viral antigen (4). *In vitro* studies have shown that previously infected cells which have become LCM antigen-negative are resistant to superinfection; this resistance to superinfection is not due to selection of insensitive clones of cells (5). In congenital rubella, however, it has been postulated that only a limited number of cells give rise to clones of persistently infected cells (6). The purpose of the present study was to directly determine the proportion of cells of the rubella-infected fetus which are producing virus and to see whether cells which do not produce virus exhibit evidence of prior experience with the virus.

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Materials and Methods. Tissue culture. Fibroblasts from lung tissues of fetuses from women not experiencing rubella, from normal newborn foreskins, and from skin and muscle tissue of fetuses exposed to rubella virus *in utero* were obtained by trypsinization with 0.25% trypsin (Difco 1:250) at 37° for 45–60 min. Fresh trypsin was added twice during this period. The cells derived from these sources were maintained in cultivation by several passages at 3–4-day intervals. The BSC-1 cells (7) and primary African green monkey kidney cells (GMK) (8) were cultivated and used for rubella virus assay. With the exception of GMK cells (8), all cells were grown in Eagle's basal medium supplemented with 10% fetal bovine serum; penicillin, 100 units/ml; kanamycin, 100 μg/ml; and 0.75 g/liter of sodium bicarbonate. Maintenance medium was the same except that 2% fetal bovine serum and 1.50 g/liter of sodium bicarbonate was used. Medium for cells grown in plastic petri dishes in a 5% CO_2 in air atmosphere contained 2.25 g/liter of sodium bicarbonate and nystatin (Mycostatin) 50 units/ml as an additional antibiotic. Agar overlay consisted of Eagle's basal medium, 10% fetal bovine serum, 2.25 g/liter of sodium bicarbonate, antibiotics, 1.2% Bacto-agar and 25 μg of neutral red/liter.

Viruses and virus assays. Rubella virus R-1, isolated from the thyroid of an infant with congenital rubella (6) was prepared in

BHK $_{21}$ cells (9). The virus was assayed in BSC-1 cells utilizing a previously published (10) modification of the hemadsorption-negative plaque test (11), and the titers expressed as pfu/0.2 ml. The virus was also assayed by interference of echovirus 11 CPE in GMK, in which case the titers are expressed as inhibition dose$_{50}$ (ID_{50}) (12). Virus stocks of Newcastle disease virus (NDV), California strain, (American Type Culture Collection), were prepared in the allantoic sac of embryonated hen eggs, and the virus was assayed by plaque formation in chick embryo fibroblasts. Vesicular stomatitis virus (VSV), Indiana strain, was grown in green monkey kidney cells, and assayed by the plaque counting method.

Interferon production and assay. Interferon was produced by inoculating monolayers of cells grown in 16-oz (80 cm^2) flat bottles with 2 ml of NDV containing 1 pfu/cell. For cells known to contain rubella virus, an inoculum of 50 pfu/cell was also used. The virus was allowed to adsorb at 37° for 4 hr onto the monolayers from which the medium had been drained. Thirty ml of medium without serum were added to the cultures after the adsorption period, and the cultures were incubated for 36–48 hr at 37° in 5% CO_2 atmosphere. The fluid was harvested and rendered virus-free by acidification and ultracentrifugation. Interferon activity was assayed in the cultures of human fibroblasts by determining the dilution at which there was a 50% reduction in plaque forming units (pfu) of VSV (13).

Results. Determination of number of infected cells. Fetal tissues were obtained from women who had contracted clinical rubella between 3 and 10 weeks of gestation and had undergone therapeutic abortion 20–60 days later. The illness of the women was proven serologically to be rubella (14). Fetal parts, easily identified from placental tissue and consisting of skin and muscle, were washed and monodispersed in trypsin as described. The trypsinized cells were washed and resuspended in growth medium. One portion of the cells was counted and serially diluted. Aliquots of the diluted cells were inoculated into tubes containing monolayers of GMK cells.

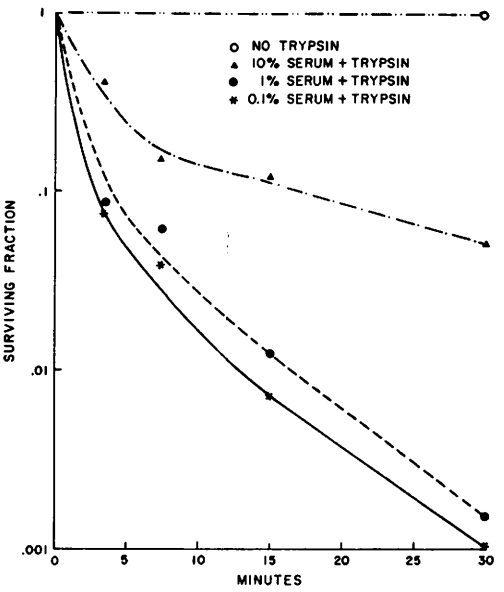

FIG. 1. Inactivation of rubella virus by trypsin in the presence of different concentrations of serum protein.

These cultures were incubated for 2 weeks and assayed for the presence of rubella virus by interference with the echovirus 11 cytopathic effect (12). Another aliquot of the diluted cells was planted in culture tubes and observed for growth of the cells. When monolayers developed, the culture fluids were assayed for rubella virus. The remaining monodispersed cells of the fetuses were planted in 16-oz flat bottles at a concentration of approximately 3×10^7 cells/bottle.

The number of infectious units determined by the methods used represents infected cells of the fetus, with negligible contribution of extracellular virus. This conclusion is based on several pieces of evidence. Extracellular virus is promptly inactivated by trypsin treatment similar to that used for dispersion of fetal cells. When crude rubella virus stock was exposed to trypsin of the same concentration, in the same diluent and at the same temperature as used for cell dispersion, the virus was rapidly inactivated (Fig. 1). This mixture contained 0.1% residual serum, which was present in the virus stock. The addition of 1% fetal bovine serum did not result in a significant difference in inactivation. With 10% serum added, there was a

90% loss of infectivity in the presence of trypsin in 30 min. Thus, under the conditions of cell dispersion with an exposure to trypsin of 45 min, few if any extracellular virions may survive.

The possibility that cell-to-cell contact of infected with uninfected cells during the trypsinization procedure may artificially increase the number of infected cells was considered. 3×10^6 rubella carrier cells, obtained by subculture of infected tissues of a fetus with congenital rubella (6), were resuspended in 4 ml of trypsin and brought into contact with a cell sheet containing 1×10^7 cells of normal human fibroblasts. This dispersed the monolayer and the mixture was resuspended and agitated repeatedly during 1 hr at 37°. After this, the number of infectious centers was determined on GMK cells, and it was found that 2×10^6 infectious centers were present in the test mixture. A similarly treated suspension of carrier cells without contact with virus-free fibroblasts also yielded 2×10^6 infectious centers. These data confirmed the earlier-finding (6) that the number of rubella virus producing cells in natural carrier cultures approximates the total number of cells, and show that cell-to-cell or extracellular transmission of rubella virus does not occur in the presence of trypsin.

Determination of infectious centers by assaying serial dilutions of fetal cells for rubella virus on GMK cells indicated that few fetal cells contained infectious virus (Table I). The proportion of cells producing virus varied considerably from fetus to fetus with the range being from one per $10^{3.0}$ to $10^{5.4}$ cells.

A minimum of about 10,000 fetal cells/tube was required for the development of monolayers. Assuming that one virus infected cell per tube would result in an infected culture, the minimum incidence of infected to uninfected fetal cells was found to vary between 1/10,000 to less than 1/100,000 (Table II). The results obtained by planting fetal cells in culture tubes and by assaying for infectious centers were in agreement for the 4 fetuses analyzed by both methods.

Rubella virus-free cells from infected tissues. Cultures of fetal cells in tubes which were found not to contain rubella virus were subdivided and by serial subculturing, it was possible to develop cell strains free of virus. Other fetal cells which had been planted in 16-oz flat bottles provided virus carrier cultures. The virus-free cells were examined for an altered ability to support the replication of rubella virus, NDV, and VSV, and for their ability to produce interferon and for their sensitivity to this substance.

TABLE I. Number of Skin and Muscle Cells of Fetuses Infected by Rubella Virus as Determined in GMK Cells.

No. of fetal cells/tube	Fetus 1	Fetus 2	Fetus 3	Fetus 4
$10^{5.5}$			4/4	2/3
10^5	5/5[a]	5/5		1/3
$10^{4.5}$			0/4	1/3
10^4	5/5	0/5		0/3
$10^{3.5}$			0/4	0/3
10^3	2/5	0/5		0/3
$10^{2.5}$			0/4	0/3
10^2	1/5	0/5		0/3
$10^{1.5}$			0/4	
10^1	0/5	0/5		
Cell ID_{50}[b]	$10^{3.0}$	$10^{4.5}$	10^5	$10^{5.2}$

[a] Number of tubes showing interference to echovirus 11 CPE/no. of tubes tested.
[b] Method of Reed and Muench.

The ability of rubella virus to replicate in virus-free cell strains and virus carrier cultures was examined by infecting monolayer cultures in tubes with the virus at a multiplicity of infection of 0.01 ID_{50}/cell. After an adsorption period of 2 hr, excess virus was removed and the cultures were incubated for 6 days at 37°. Triplicate tubes of each cell strain examined were then frozen, thawed, and assayed for virus. The results presented in Table III indicate that the replication of rubella virus in the virus-free cells was similar to that observed in cells derived from the lung of a fetus which had not been exposed to rubella virus, as well as in newborn foreskin cells. The cultures of fetus 1 were tested soon after they were established *in vitro* and are thought to represent a mixture of virus-free and virus-infected cells. Other strains

TABLE II. Number of Skin and Muscle Cells of Fetuses Infected by Rubella Virus as Determined by Growth of Fetal Cells in Tissue Culture.

	Fetus									
	1		2		3		4		5	
No. of fetal cells	Growth of cells	Presence of virus	Growth of cells	Presence of virus	Growth of cells	Presence of virus	Growth of cells	Presence of virus	Growth of cells	Presence of virus
300,000	3/5[a]	3/3[b]			5/5	5/5			5/5	5/5
100,000					5/5	5/5	3/3	0/3		
60,000	3/5	3/3					3/3	0/3	5/5	5/5
20,000					2/5	0/2				
10,000	4/5	4/4	5/5	4/5			3/3	0/3	5/5	5/5
5000	0/5	—	0/5	—	0/5		1/3	0/1	0/5	—
1000	0/5	—	0/5	—	0/5		0/3	—	0/5	—
Minimal no. of infected cells	1 per 10,000		1 per 10,000		1 per 100,000		<1 per 100,000		1 per 10,000	

[a] Number of tubes developing monolayers/no. of tubes seeded with cells.
[b] Number of tubes yielding rubella virus/no. of tubes containing monolayers of cells.

TABLE III. Replication of Rubella Virus in Tubes Containing Monolayers of Cells Derived from Fetuses Infected by Rubella Viruses *in Utero*.

Cell source	Virus carrier state	Rubella virus titer[a]	
		Not superinfected with rubella virus	Superinfected with rubella virus
Fetus 1	Present	200	800
Fetus 5	Present	20,000	20,000
Fetus 3	Present	10,000	10,000
Fetus 3	Absent	0	1300
Fetus 4	Absent	0	200
Fetal lung	Absent	0	2500
Newborn foreskin	Absent	0	1500

[a] Rubella virus titer (pfu/0.2 ml of culture fluid) present 6 days after superinfection with rubella virus.

were used in passages 5–8. The virus-free cells were also found to support rubella virus replication as demonstrated by the development of hemadsorption-negative plaques (Table IV). The number of plaques and size of plaques were essentially the same in the virus-free cells as in cells derived from foreskins of normal infants. The ability of sheep red blood cells to adsorb to the virus-free cells in areas of the cultures not infected with rubella virus demonstrated that the virus-free cells supported the production of the hemagglutinin of NDV. In contrast, the virus carrier cultures did not support the replica-

TABLE IV. Replication of Rubella Virus in Monolayers of Cells Derived from Fetuses Infected with Rubella Virus *in Utero* as Determined by Development of Hemadsorption-Negative Plaques.

Cell source	Virus carrier state	Titer of rubella virus pool ($\times 10^6$ pfu/ml)	
		Expt. 1	Expt. 2
Fetus 5	Present	Negative[a]	Negative[a]
Fetus 3	Absent	2.5	NT[b]
Fetus 4	Absent	NT	1.5
Newborn foreskin	Absent	1.2	1.2
BSC-1	Absent	NT	6.0

[a] No adsorption of sheep erythrocyte to monolayers infected with Newcastle disease virus.
[b] NT = not tested.

TABLE V. The Replication of Vesicular Stomatitis Virus in Cells Derived from Fetuses Infected *in Utero* with Rubella Virus as Determined by Plaque Formation.

Cell source	Virus carrier state	Titer of vesicular stomatitis virus pool[a] Expt. 1	Expt. 2
Fetus 3	Absent	96	NT
Fetus 4	Absent	100	123
Fetus 5	Present	<1	<1
Newborn foreskin			
Strain A	Absent	110	141
Strain B	Absent	127	150
Strain C	Absent	112	120

[a] Plaque forming units in 0.2 ml of a 10^{-4} dilution.

tion of NDV since the sheep red blood cells did not adsorb to these cells (11).

The replication of VSV was tested by determining the ability of the virus to form plaques in monolayers of the cells. As shown in Table V, VSV produced plaques at the same titers in virus-free cells from the infected fetuses as in cells derived from foreskins of normal infants and the plaques were the same size. No plaques formed in the virus carrier cultures, indicating the lack of VSV replication (6). Thus, while virus carrier cultures appeared to be resistant to superinfection with rubella virus and failed to support the replication of NDV and VSV, rubella virus-free cultures derived from infected infants supported the replication of rubella virus, NDV, and VSV in a normal manner.

Additional evidence of experience with rubella virus of the virus-free cells was sought by examining the sensitivity and ability to produce interferon in the virus-free cells and virus carrier cultures. A standard interferon preparation was assayed simultaneously on virus-free cells from fetuses 3 and 4 and on newborn foreskin cells. The 50% reduction of VSV plaques occurred at a dilution of 1:256 of the interferon preparation in all 3 cell strains. The production of interferon in virus-free cells was the same as in newborn foreskin cells as shown in Table VI. No interferon activity was detected in the medium of the virus carrier cultures. The addition of NDV did not result in the production of interferon nor did exogenous interferon reduce the yield of rubella virus in the carrier cultures (13).

Discussion. The persistence of virus following *in utero* infection is a feature of both rubella virus of humans and lymphocytic choriomeningitis virus (LCM) of mice. These two viruses also share many biophysical and biological similarities (15). Mims, in a study of LCM of mice, found LCM antigen in nearly every cell of 8–9-day-old and 12-day-old embryos. Near term embryos and older animals were found to possess far fewer LCM antigen containing cells (4). Cells from these adult animals when grown in tissue cultures, could not be superinfected with LCM virus although only a few of the *in vitro* grown cells contained viral antigen. In contrast, cells from animals not chronically infected with LCM were fully susceptible to infection. When macrophages from normal mice were infected with LCM *in vitro*, 95% developed viral antigen which was gradually lost over the following 3 weeks of cultivation. Superinfection by LCM of the infected macrophages after 3 weeks of cultivation was not possible while control cells not previously exposed to LCM, but held in culture for 3 weeks, could be infected. On the basis of these observations, the authors (5) suggested that the LCM genome was present in all of

TABLE VI. Production of Interferon by Cells Derived from Fetuses Infected *in Utero* by Rubella Virus.

Cell source	Virus carrier state	Interferon titers[a] Before induction with NDV	After induction with NDV Trial 1	Trial 2
Fetus 5	Present	<8	<8	<8
Fetus 6	Present	<8	<8	<8
Fetus 4	Absent	<8	128	256
Fetus 3	Absent	<8	64	128
Newborn foreskin	Absent	<8	256	128

[a] Titers represent dilution producing a 50% reduction in VSV plaques in newborn foreskin,

the cells of the chronically infected mice, but that virus could be detected in only a few cells.

Cell population analysis of fetuses infected with rubella virus during the first trimester of pregnancy indicates a clear difference from the cell involvement described for mice infected *in utero* with LCM. While in LCM infections, the majority of cells of the early embryo appear to be infected, not more than 0.1% of the cells of the fetuses were infected with rubella virus. This difference in proportions of cells infected in the two diseases could possibly be accounted for by the time of infection, the infection of LCM being transovarial (4) while rubella virus is usually acquired during the multiple cell stage of development. Para-/or preconceptual rubella is often associated with fetal death and extensive involvement of the fetus by rubella virus may not be compatible with survival. The factor or factors responsible for the containment of rubella virus to a limited number of cells is speculative, both passively acquired maternal antibody and interferon having been suggested. Analysis of cell involvement by LCM virus of neonatally infected mice would be helpful in determining whether the degree of involvement was related to the stage of maturation at infection or to a basic difference in the pathogenesis of congenital rubella and the chronic infection of mice with LCM.

It could be argued that the number of cells producing rubella virus does not represent the true population of fetal cells affected by the virus. However, analysis of the virus-free cells from the infected fetuses demonstrated no evidence of prior experience with rubella virus as measured by their ability to support the replication of rubella virus, NDV, or VSV. In addition, virus-free cells derived from infected fetuses which were aborted at gestational ages of about 50–76 days were found to be capable of producing interferon and of responding to interferon. Thus, by the criteria of resistance to superinfection by the same or different viruses, it would appear that the majority of the cells of the fetuses infected *in utero* with rubella virus do not contain virus genome in a nonexpressed form as suggested in the LCM studies.

In cultures established from large numbers of cells of infected infants or fetuses, all of the cells of the cultures become infected and actively produce infectious virus. The virus does not produce a direct cytopathic effect, but does slow the growth rate of the cells and appears to reduce the ultimate doubling potential of the cells (6). Similar events occur in artificially infected cultures of fetal tissues (16). Studies of the chronically infected cultures derived from infants with congenital rubella and of chronically infected cultures produced in a stable line of monkey kidney cells have provided evidence that the virus can be transferred from parent to daughter cells during cell division (6, 17). It has been postulated that *in utero* infection during the first 3 months of gestation results in the infection of a limited number of cells and that these cells give rise to clones of infected cells. The clones of infected cells provide the source of virus in the persistent infection. The data obtained from the analysis of fetal cells reported in this study are compatible with this hypothesis.

Summary. The cells from fetuses infected *in utero* with rubella virus were analyzed for the presence or absence of virus. Cells that were virus-free were examined for evidence of prior experience with virus. By infectious center assay of fetal cells in GMK tubes, and by growing fetal cells in culture tubes, it was established that not more than 0.1% of the fetal cells contained infectious virus. No evidence of experience with rubella virus was found in virus-free cells, for such cells were able to support the replication of rubella virus, Newcastle disease virus, and vesicular stomatitis virus. The production of interferon and sensitivity to interferon was normal in virus-free cells, but not in virus-containing cells. The data obtained demonstrate that congenital rubella differs from congenital lymphocytic choriomeningitis virus infection of mice where all of the cells contain viral antigen or are resistant to superinfection by the virus.

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Cytotoxic Antibody Response to Skin Allografts in Calves: Effects of Extracorporeal Irradiation of Lymph* (33349)

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The immunological nature of skin allograft rejection has been firmly established. The mechanism responsible for this process, however, has not been fully resolved. Many investigators favor the view that rejection is based principally upon the action of sensitized mononuclear cells (1, 2). On the other hand, there are those who feel that humoral antibodies play a major role by exercising a primary cytotoxic action on the graft (3). Accumulating evidence for the participation of complement in the rejection of skin allografts (4, 5) indirectly supports the role of complement-dependent antibodies in the effector mechanism. Lymphocytotoxic antibodies (6) have been demonstrated in the sera of animals (7–9) and man (10) following skin allografting, and there is increasing evidence that these antibodies are directed against histocompatibility antigens (11).

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Previous studies from this laboratory have shown that extracorporeal irradiation of thoracic duct lymph (ECIL) will prolong the survival time of skin allografts in calves (12). Furthermore, when the skin grafts were placed within the drainage bed of the thoracic duct (posterior grafts), the grafts remained intact until ECIL was discontinued (13). The reason proposed for the survival of posterior skin grafts was that immunologically activated lymphocytes emerging from the regional lymph nodes were destroyed by ECIL prior to entry into the blood. In contrast, if the grafts were not within the drainage bed (anterior grafts), ECIL only delayed the graft rejection time.

Theoretically, ECIL should not interfere with the production or release of antibody from the lymph nodes draining posterior allografts, and certainly the amount of irradiation received by the antibody molecules would not alter their activity (14). The object of the present experiments was to exam-