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Spontaneous Virus Carrier Cultures and Postmortem Isolation of Virus from Infants with Congenital Rubella.* (30608)

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Infants born to mothers contracting rubella in the first trimester of pregnancy often have congenital anomalies. These anomalies may be due to the effect of the virus upon the fetus during a critical period of organogenesis or they may result from the effect of the persistence of rubella virus in the developing tissues. The critical period of infection which results in a persistent viral infection also seems to be the first trimester of pregnancy. Studies of the products of conception obtained by therapeutic or spontaneous abortions from women who experienced clinical rubella early in pregnancy have demonstrated the presence of virus in the placenta as well as in multiple organs(1-5). Postnatally, infants born with the rubella syndrome may continue to excrete virus for many months(3,6,7). A study of postmortem material was therefore undertaken to determine the extent of tissue involvement of infants who had been born with the rubella syndrome and who had subsequently expired. In addition, the investigation was designed to compare two techniques for isolating viruses from postmortem tissues. These included the *in vitro* propagation of tissues obtained at autopsy and the direct inoculation of suspensions of postmortem tissues into appropriate tissue culture systems.

An epidemic of rubella occurred in the Houston, Texas area in the spring of 1964(8). Seventy infants born with anomalies consisting of low birth weight, cataracts, congenital heart defects, thrombocytopenic purpura, hepatosplenomegaly and evident dysfunction of the central nervous system, came to the attention of Baylor investigators in the winter of 1964(6). Ten of these infants expired and comprise the basis of the present study. Postmortem material from 6 infants without the above mentioned complex of anomalies was also studied.

Materials and methods. Procurement of postmortem material. Portions of bone marrow, brain, heart, kidney, liver, lung, lymph node, spleen, thymus and thyroid were obtained at autopsy with care taken to avoid cross-contamination. Tissues were placed in separate sterile jars containing Melnick's lactalbumin hydrolysate medium and antibiotics (penicillin 500 U/ml, streptomycin 500 μg/ml). The tissues were processed within several hours in most cases; however, occasional specimens were held at 4°C for 24-48 hours before being processed.

Virus isolation from tissue suspensions. 10-20% suspensions of tissues in Eagle's basal medium were prepared by grinding with alundum followed by centrifugation at 3000 rpm for 30 minutes. The supernate was inoculated into 4 tubes of African green monkey kidney cell cultures (GMK) prepared as previously described(9). Each tube received 0.2 ml of supernate. Following adsorption for 1 hour, 1 ml of Eagle's basal medium with 2% fetal bovine serum and 1.5 g per liter of bicarbonate was added. After one week of incubation at 36°C, 100 TCD_{50} echovirus 11 was added to one-half of the tubes. When control tubes were found to have developed 2-3+ CPE (*i.e.*, 50 to 75% of the cells in

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the culture showed enteroviral cytopathic changes), the inoculated tubes were examined for presence or absence of interference. Fluid from the inoculated tubes which had not been challenged with echovirus 11 was passed to additional GMK cultures, incubated and challenged. All specimens were passed twice before being considered negative for interfering agents. Companion tubes of those demonstrating interference were passed at least once before neutralization was attempted.

Virus isolation from tissue cultures of infant organs. Portions of organs were trypsinized into cell suspensions and dispensed into 1 oz prescription bottles or culture tubes. The method for preparation and subculture of kidney cells has been previously described in detail(10). Bone marrow was removed from ribs or femurs. The bone marrow fragments were washed 3 times in trypsin diluent and minced into fine pieces with scissors. Five ml of Eagle's basal medium with 20% fetal bovine serum and 0.75 g bicarbonate per liter was added and the material pipetted vigorously. The resulting cell suspension was passed through double-layered sterile gauze and placed into a 1 oz bottle. The medium was changed in 24 hours and thereafter when the pH fell to 7.

Other tissues were divided into 2-4 mm fragments, washed 3-4 times in trypsin diluent and trypsinized with 0.2% trypsin for 20 minutes at 37°C. The initial trypsin solution was decanted, fresh solution added and trypsinization continued for 45 minutes. Resulting cell suspensions were centrifuged at 800 rpm for 10 minutes and the cells were resuspended in Eagle's basal medium with 10% fetal bovine serum and 0.75 g bicarbonate per liter. Resuspended cells were then handled in the same manner as bone marrow cells. Subculture of these cells was performed by a trypsinization process recently described (11).

Periodically, culture fluid from tissue cultures was inoculated into culture tubes containing GMK monolayers and handled in the same manner as tissue suspensions. Monolayers of the tissue culture cells were also grown in tubes and challenged directly with echovirus 11; however, interference was found to be less complete than with GMK cells, similar to the findings of Parkman *et al* (12).

Identification of rubella virus. Hyperimmune rubella antiserum was prepared in rabbits by biweekly intravenous injection for 7 weeks of clarified tissue culture fluid containing $10^{3.5}$ interfering doses (InD_{50}/ml) of rubella virus, Ellis strain, previously isolated and identified in this laboratory(8). Equal volumes of appropriately diluted antiserum and tissue culture fluid containing 50-100 InD_{50} of interfering agent were mixed and incubated for 2 hours at room temperature. Four tubes of GMK cells were then inoculated with 0.2 ml of the mixture and challenged with 100 TCD_{50} of echovirus 11 after 6 days of incubation. Interference of echovirus CPE in control tubes, but not in tubes containing the antiserum, was considered positive evidence that the interfering agent was rubella virus.

Results. Rubella virus was isolated from tissues of 8 of 10 infants with rubella syndrome but not from any of the tissues of 6 infants without rubella syndrome who died during the period of this study and who were examined concurrently. The infants with rubella lived from 11 to 110 days with an average age at death of 45 days. Virus isolation from postmortem material was not related to age at death; both the youngest and oldest infants yielded recoverable interfering agents (Table I). In 6 of 10 cases (60%) the virus was recovered from tissue suspensions and in 5 cases (50%), it was recovered from tissue cultures. The isolation rates of the 2 techniques employed were essentially the same. However, in 2 cases virus was isolated only from the cultures (kidney, thyroid, bone marrow) and not from any of the tissue suspensions.

Ten of 57 tissue culture attempts were successful. These tissue cultures could be subcultured for varying periods and several chronically infected cell strains were developed. They seemed to have properties similar to those described by Plotkin *et al*(13) for experimentally induced carrier cultures in human diploid fibroblasts. The chronically infected cells were found to have a doubling

TABLE I. Results of Virus Studies of Postmortem Material.

Infant No.	Virus isolated antemortem	Age at death, days	Virus isolated from tissue suspensions	Organ tissue cultures		
				Attempts	Organs grown	Virus isolated
1	+	11	+	2/5*	kidney	+
					umbilicus	+
2	+	22	—	0/6		
3	+	23	+	0/4		
4	+	27	—	1/5	lung	—
5	+	28	+	2/5	"	+
					bone marrow	+
6	not done	28	—	2/9	thyroid	+
					bone marrow	+
7	+	47	+	0/4		
8	+	55	—	1/7	kidney	+
9	+	96	+	0/4		
10	+	110	+	2/8	spleen	+
					bone marrow	+

* No. of successful attempts/No. organ cultures attempted.

TABLE II. Virus Isolation from Tissue Suspensions.*

Infant No.	Tissues tested as 10% suspensions									
	Bone marrow	Brain	Heart	Kidney	Liver	Lung	Lymph node	Spleen	Thymus	Thyroid
1			—	—	—			+		
2				—	—			—		
3					—	+		—		
4		—		—		—	—	—	—	—
5				+		+		+	+	
6				—	—	—				
7	+	+	—	—	—			—		
8	—			—	—	—	—	—	—	
9				+		+			+	
10			+	+		+	+	+		

* + indicates rubella virus isolated; — indicates organ tested but rubella virus not isolated; blank space indicates organ was not tested.

time during the rapid growth phase of 48-72 hours which is 2-3 times that of non-infected human diploid cell strains. One chronically infected cell strain was successfully maintained through 21 subcultivations over a period of 5 months. Rubella virus at titers of $10^{3.5}$ to $10^{5.5}$ InD_{50}/ml was demonstrable in the tissue culture fluid of this cell strain throughout the entire period of observation. All tissues which grew *in vitro* produced virus except lung tissue from Case No. 4. In this case virus could not be recovered from tissue suspensions although an interfering agent isolated from spinal fluid obtained antemortem was neutralized with rubella antiserum.

The results of studies of tissue suspensions are shown in Table II. In 3 of the 6 infants from which rubella virus was isolated by this method, all organs examined were found to contain virus. Rubella virus was recovered from 2 organs in a 4th case and from one organ in 2 other cases. The highest rate of virus isolation was from lung tissue, 57%, while only the liver failed to yield virus. This latter observation is in agreement with studies of fetal tissues recently reported by Monif *et al*(5). Suspension of thyroid was negative in the one case examined; however, tissue culture of thyroid from another case yielded rubella virus (Table I).

Discussion. The results of this investigation demonstrate that 80% of infants born with the congenital rubella syndrome who ex-

pired continued to harbor virus until death. Since the infection was acquired in the first months following conception, the infecting agent persisted for at least 6-9 months after acquisition. The duration of persistence of the virus in surviving infants with rubella has not been established. However, our attempts to isolate virus from a cell strain derived from bone marrow obtained from a 22-year-old man who had clinical features compatible with congenital rubella were unsuccessful.

The data presented indicate that the virus persists in multiple organs. Agonal viremia might be considered an alternate explanation for these findings. In this regard, rubella virus was isolated from 1 of 7 antemortem blood specimens drawn from the infants studied (Phillips *et al,* unpublished data). A postmortem blood specimen from Case No. 2 was found to contain virus although virus could not be isolated from tissues. No attempts were made to titrate the amount of virus in the tissues studied.

Neutralizing antibodies to rubella virus have been demonstrated in the cord blood of infants with the rubella syndrome(14). Presumptive evidence has been presented that these infants are capable of producing antibodies to the virus(15). This response appears analogous to that of newborn mice receiving small doses of lymphocytic choriomeningitis virus. Even though these mice may produce antibodies, they may simultaneously harbor virus for several months(16). The immunologic response of infants to intrauterine acquired rubella requires further investigation.

From the information available it seems reasonable to conclude that the defects of congenital rubella could be related to a persistent rubella infection. The factors allowing the virus to persist in those exposed soon after conception are not understood, and indeed the role of cellular resistance to virus disease has not yet been clarified(17). Whether or not congenital rubella is an example of a lack of viral induced cellular resistance can only be speculated.

Summary. Rubella virus was isolated from 8 of 10 infants who died of congenital rubella. Several of the infant organs were grown in tissue culture and 10 cell lines were established. They were characterized by their spontaneous chronic infection with rubella virus in the absence of cytopathic changes, and thus they behaved as true virus carrier cultures. The organs that yielded the carrier cultures included kidney, bone marrow, lung, spleen, thyroid and umbilicus. Suspensions of tissues yielded virus in 6 cases; however, in 2 cases, virus was isolated only from the carrier cultures but not from the tissue suspensions.

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