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Recovery of Rubella Virus from Army Recruits. (27750)

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(Introduced by J. E. Smadel)
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During February and March 1961, a new agent was isolated repeatedly from military recruits hospitalized with rubella at Fort Dix, N. J.(1,2). This agent, recovered from throat washings, has been found to propagate only in a limited number of cell lines. In these it fails to hemadsorb, or to produce cytopathologic effect (CPE) or sufficient antigen to react in *in vitro* serological tests. It is recognizable only by its ability to interfere with ECHO virus, Type 11 (E-11). This preliminary report describes methods used for recovery of this agent, its association with rubella, and certain characteristics which identify it presumptively as a virus.

Materials and methods. Between 7 February and 27 March 1961, 79 male recruits between 17 and 25 years of age were hospitalized with febrile exanthems at Walson Army Hospital, Fort Dix. In 20 of these a definite clinical diagnosis of rubella was made; in 21 others rubella seemed the most probable diagnosis. Twenty-five had scarlet fever, and 13 had atypical exanthematous disease. The criteria used for these diagnoses are shown in Table I. Throat washings were obtained from each patient within 24-48 hours of appearance of rash; patients gargled with 15 ml of Hanks' balanced salt solution (BSS), containing 0.4% bovine plasma albumin (BPA), and no antibiotics. Throat washings were stored at −65°C until tested. Throat cultures obtained simultaneously were plated on sheep blood agar for recovery of β hemolytic streptococci (3). Acute phase sera were collected upon admission; convalescent specimens were obtained 1-26 weeks later in all but 5 patients. All sera were stored at −20°C and were heated at 56°C for 30 minutes before use in neutralization tests. *Cell cultures,* experimental animals, and challenge virus.* Primary cultures of African Green monkey kidney (*Cercopithicus aethiops*, GMK) and Hep2 cells in continuous passage were maintained with Eagle's basal medium containing 2% chicken serum and penicillin and streptomycin in final concentrations of 100 u or μg/ml. Monolayers of human embryonic kidney (HEK) were maintained with serum free medium 199 containing the same antibiotics. Media were changed in inoculated tubes at 3-4-day intervals. Embryonated eggs, suckling mice, and adult rabbits were obtained through the Dept. of Animal Husbandry of this Institute. E-11 virus used for challenge of infected cultures was in 9th or 10th rhesus kidney passage and was received as strain Gregory in 7th passage in 1956 from Dr. A. B. Sabin. *Virus isolations.* Throat washings as collected were incubated at room temperature for 20-30 minutes with penicillin (1000 u/ml), streptomycin (1000 μg/ml) and Mycostatin® (500 u/ml). One-half ml of this mixture, without further treatment, was added to each of 2-4 tubes of HEK, GMK, and Hep2 cells. Acute serum specimens in 0.2 ml volume were similarly inoculated. Initially, duplicate cultures were incu-

* Cell cultures and media were obtained from Microbiological Associates, Bethesda, Md. and Flow Laboratories, Arlington, Va.

TABLE I. Diagnosis of Exanthems, Fort Dix 1961.

Diagnosis	Clinical and laboratory criteria
Rubella	
20 patients	1) Morbilliform rash 2) Posterior cervical, auricular or occipital lymphadenopathy 3) Maximum temperature $\leqq$ 102.2°F 4) No other agent implicated by laboratory test
Probable rubella	
6 patients	As above, but without lymphadenopathy
15 "	As above, but harboring other agents*
Scarlet fever	
25 patients	1) Scarlatiniform rash 2) Pharyngitis, with or without exudate 3) Temperature $\geqq$ 101°F (19 patients) 4) Streptococci, Group A, and/or ASO titer rise
Atpical exanthem	
13 patients	Febrile rash disease not classified by above criteria

* These include by recovery of pathogen:
2 adenoviruses type 4, 1 COE virus, 1 parainfluenza virus, 1 herpes sixplex virus and 3 β hemolytic streptococci, Group A.
and/or by serology:
7 adenovirus, 2 parainfluenza virus, and 3 antistreptolysin O titer increases.

bated in stationary racks both at 35° and 32°C; later, after lower incubation temperatures failed to yield additional agents, only at 35°. Cultures containing the original inoculum, (1st passage) and one passage made at 7-10 days in the same cell line, (2nd passage) were observed for CPE daily for 7-14 days, and were tested for guinea pig red cell hemadsorption(4) after 7 days.

Efforts to demonstrate interference with growth of E-11 virus were made in GMK and HEK cultures. Initial, or 2nd passage cultures were challenged 7-14 days after inoculation with an estimated 10,000 tissue culture infectious $doses_{50}$ ($TCID_{50}$) of E-11 virus contained in 0.1 ml; E-11 virus was added directly to inoculated and control cultures without changing medium, and daily observations for CPE were continued. When control cultures inoculated with E-11 virus were in an advanced state of degeneration, uncentrifuged culture fluids from both control and test cultures were assayed for E-11 virus hemagglutinins. Serial 2-fold dilutions of culture fluids (1:5 to 1:80) were made in unbuffered physiological saline in 0.5 ml. Equal volumes of 0.5% human red cells, Type O, were added immediately and patterns read after 90 minutes sedimentation at 4°C. *Immune sera.* After the agent had been isolated, adult rabbits were immunized with tissue culture fluids harvested at 7 days containing rubella virus (strains M-32, M-33) from which cellular debris had been removed by low speed centrifugation. The 1st dose of 0.5 ml was given intravenously; subsequent inoculations of 0.5 ml were given subcutaneously at weekly or bi-weekly intervals for 10 doses.

Results. Recovery of rubella virus. In the initial experiment, 8 of 10 throat washings from patients with classical rubella yielded an agent which interfered with propagation of E-11 virus in GMK, but not HEK cultures. Interference was complete in 7 and partial in 1, and was transmissible. In none of 8 interfering cultures was CPE observed before E-11 challenge, nor did guinea pig red cells hemadsorb to cells after 7 days incubation in 1st or 2nd passage. No interference was observed in GMK or HEK cultures inoculated with sera obtained from the patients on the 1st or 2nd day of exanthem.

Cultures with complete interference showed no CPE after challenge with E-11 virus. Partial interference was characterized by focal CPE at margins of cell sheets which failed to progress. To minimize subjective differences in interpretation of CPE, supernatant media were assayed for E-11 hemagglutinins. In this and later experience, no hemagglutinins were shown in tubes with complete interference. On the other hand, hemagglutinin titers of 1:20 or less were associated with partial interference, and of 1:80 or greater with no interference.

After the interfering agent was demonstrated, re-isolation attempts were made from 1 of the negative and 5 of the positive throat washings; 4 of the 5 positive throat washings again yielded the interfering agent, and the single negative specimen failed to do so. During 20 serial passages of 3 strains at 7-10-day intervals the amount of interference increased, and the interval between inoculation and detectable interference became shorter. With undiluted 1st passage inocula, partial inter-

TABLE II. Influence of Passage Level and Time of Challenge Upon Interference Titers of Rubella Virus.

Strain	Passage	Titer rubella virus /0.1 ml* after indicated day of incubation 5	10	15
M-30	2	1.4	3.5	—
	10	4.0	4.0	4.0
M-33	2	—	3.5	4.0
	17	3.5	3.5	—

* Neg $\log_{10}$ as measured by interference with 10^4 TCD_{50} of E-11 virus.

ference was first observed after 3 days; in other cultures inoculated simultaneously, interference was complete when challenged at 5 days. With 4th and 9th passage virus, complete interference was seen by the 2nd day, and with 11th passage virus, by 24 hours. *Frequency of isolation.* In tests of remaining specimens from this outbreak, 27 additional interfering agents were obtained. These were recovered from throat washings of 16 of 20 (80%) patients hospitalized with classical rubella. Fourteen of 21 patients classified as probable rubella also yielded the agent; 3 strains were recovered from 6 patients without obvious lymphadenopathy, and 11 strains from 15 patients harboring other respiratory pathogens endemic to the recruit population (Table I). Rubella virus was recovered from but 1 of 25 patients with scarlet fever, and from 4 of 13 patients with atypical exanthems. Six of 35 strains were tested for interference upon initial inoculation; 4 were positive. The other 2 became positive in 2nd passage. The remaining 29 strains were tested and detected in 2nd passage. *Growth characteristics.* Two strains (M-30 and M-33) were studied for rate of development of interfering substance (growth of rubella virus), and maximum dilution of infected culture producing demonstrable interference (virus titer). Results are summarized in Table II. With both strains in 2nd passage, maximal virus titers as determined by interference (InD_{50}) were demonstrable only after 10-15 days of incubation. When duplicate titrations of M-30 virus were challenged at 5 days, InD_{50} titers were 100-fold less. However, by 10th to 17th passage, rate of development of interfering substance increased, so that maximal InD_{50} could be shown by the 5th day. During adaptation several observations suggested that 1 InD_{50} of early passage virus actually contained 100-1000 infectious $doses_{50}$ (ID_{50}). This difference between InD_{50} and ID_{50} may explain in part the early difficulties in measuring antibody responses of rubella patients (see below). However, with continued passage the difference between InD_{50} and ID_{50} doses became less. Thus, in another experiment with virus in 17th passage, a single InD_{50} at 5 days contained but 10 ID_{50} as measured by subculture. *Complement-fixing (CF) and hemagglutinating (HA) antigens.* It has not been possible to prepare CF and HA antigens using various technics for disrupting infected cells, or by concentration of culture materials by dialysis against polyethylene glycol (Carbowax®)(5). This may be due to low concentrations of infective virus or its relative instability; the subject deserves further study. *Host range.* Thus far, rubella virus propagates in primary or continuous† GMK, and in HEK, and rhesus kidney (RhMK), but not in Hep2 cells. However, interference with growth of E-11 is consistently shown only in GMK cultures. Moreover, titers of rubella virus in primary cultures are approximately 100 times greater than in continuous GMK cells. Propagation of the agent occurs in HEK and RhMK, as demonstrated by subculture in GMK cells. However, the interference phenomenon is unpredictable in HEK and RhMK. Hence these cultures are of limited value for primary isolation of rubella virus. No reproducible evidence for growth of the virus has been obtained in embryonated eggs, suckling mice or adult rabbits. Finally, no evidence for propagation of the agent on PPLO media was obtained. *Heat stability.* Heating for 1 hour at 56°C in Hanks' BSS without albumin reduced the InD_{50} titer from $10^{-3.5}$ to 10^0 or less. Rates of destruction of the agent at 37°C as measured by tests for interfering activity were less, and reduced still further if the test suspension contained 1% BPA. Thus, preparations without added albumin declined from initial InD_{50} titers of

† Continuous grivet cell line was obtained in sixteenth passage from Dr. Joseph E. Smadel, Nat. Inst. Health.

TABLE III. Neutralization of Rubella Virus by Paired Patients' Sera.

Patient	Day of disease	Tube	Interference with E-11 virus* in tubes containing indicated dilutions of sera and 10 InD_{50} of M-33 virus						Serum control	Reciprocal rubella antibody titer
			2	4	8	16	32	64		
M-30	1	1	+	+	+	+				
		2	+	+	+	+			0	<2
	14+	1	0	0	0	0	+	+		
		2	0	0	0	0	0	+	0	32
M-33	1	1	0	+	+	+				
		2	0	+	+	+			0	2
	15	1	0	0	0	+	+	+		
		2	0	0	0	0	0	+	0	16

* Test challenged after 5 days with 10^4 $TCID_{50}$ E-11 virus.

$10^{-3.3}$ to $10^{-1.6}$/0.1 ml after 3 hours at 37°, while samples with albumin declined from $10^{-3.8}$ to $10^{-2.9}$. The virus was stable with or without albumin for 18 hours at 4°C, and could be re-isolated after 1 year at −65°C from the original throat washings. *Sensitivity to organic solvents.* Each of 5 strains of rubella virus in 3rd or 4th passage with InD_{50} titers of $10^{-3.0}$ to $10^{-4.5}$/0.1 ml were completely destroyed by treatment with chloroform or ether(6,7). *Size.* In preliminary experiments 3rd or 5th GMK passage virus passed Millipore® filters of 450 and 300, but not of 100 and 50 mμ pore size. Ninety per cent of the interfering factor was recovered in the pellet produced by centrifugation at 15,000 rpm for 60 minutes in the Spinco #40 rotor. *Neutralization tests.* Initial attempts to demonstrate neutralizing antibody in sera of convalescent patients and in immunized animals using low passage virus were not interpretable. There were probably a number of factors which contributed to these early failures. However, after high titered preparations were attained by continued passage of strain M-33, a reproducible neutralization test was possible. In this test, equal volumes of serial 2-fold dilutions of sera (1:2 through 1:64 prepared in Hanks' BSS) were mixed with suspensions containing 1-10 InD_{50} of rubella virus. Hanks' BSS with 2% BPA was used to prepare control virus titrations and the 1-10 InD_{50} dose of virus. Virus dilutions and virus-serum mixtures were incubated at 37°C for 60 minutes before inoculation in 0.1 ml amounts into each of 2 GMK cultures. Five days later inoculated cultures were challenged with E-11 virus. Results of a typical test are shown in Table III. Pluses indicate cultures in which interference by rubella virus was demonstrated. With the 2 sets of paired sera illustrated, the patients displayed 8- and 32-fold increases respectively in antibodies during the 2 weeks between times when acute and convalescent specimens were obtained. None of the sera neutralized E-11 virus (serum controls, Table III); however, since such antibodies may be present in certain patients, this control was included in each test. *Antibody responses of recruits with rubella.* With adapted M-33 virus, significant increases in specific neutralizing antibody were shown during convalescence in 32 of 34 patients with rubella (Table IV). Ordinarily these increases were 8-fold or greater. Detectable antibody was frequently found in sera obtained at onset of rash (17/27 tested) and 1 or 2 days later, practically all sera were positive (6/7). Titers of acute phase antibody ranged from <1:2-1:32; of convalescent antibody, 1:4-1:64 or greater. The presence of specific antibody in most of the acute phase sera may account for our failure to recover virus from bloods of rubella patients examined to date. *Identity of recovered strains.* Twenty-six strains of rubella virus tested thus far have been shown to be similar, if not identical, by neutralization test. Ten-100 InD_{50} of each of the viruses was neutralized by a 1:4 dilution of rabbit antiserum prepared against the M-33 strain of virus; this antiserum diluted to 1:32 neutralized 10 InD_{50} of homologous virus. It failed to neutralize herpes simplex, Influenza A and B, parainfluenza I, II and III, respiratory syncytial viruses, and a simian hemadsorbing virus

TABLE IV. Antibody Responses in Rubella.

Clinical classification	Patients tested	No. of patients showing indicated fold increase in rubella antibody*					
		0	2	4	8	16	32
Rubella	19		1	1	12	3	2
Probable rubella							
Without nodes	4			2	1	1	
Other "infecting" agents	11	1		5	3		2

* Tested against approximately 10 InD_{50} rubella virus strain M-33.

similar to SV-5. Further, M-33 virus was not neutralized by antisera against parainfluenza I, II, III and SV-5. Thus, strains of rubella virus appear to be immunologically homogeneous and distinct from certain other respiratory viruses and cell culture contaminants of simian origin.

Discussion. These observations establish that this new agent is etiologically associated with rubella in young adults. It has many properties commonly ascribed to viruses. First, it multiplies in mammalian cell cultures. Second, it interferes with growth of another virus. Third, it is destroyed by heat and organic solvents. Fourth, filtration and centrifugation experiments indicate it is a particle between 100 and 300 mμ in diameter. Fifth, it has not grown on media known to support the growth of PPLO. Finally, all strains recovered thus far appear to be immunologically similar. Rubella virus differs from other solvent-sensitive animal viruses in its tested host range, and its failure to produce CPE, to hemadsorb, or to hemagglutinate, even after repeated passage. Moreover, no immunological relationship to other respiratory tract viruses has been found. "Common cold" viruses, also recognizable by interference with E-11 virus(8), are resistant to organic solvents and smaller in size(9). Thus, rubella virus is immunologically and biologically different from other known viruses.

The mechanism by which rubella virus interferes with growth of E-11 virus has not been adequately studied. However, it seems unlikely that interferon-like substances(10) play a major role in its demonstration since the interfering factor is sedimentable, heat labile, and cannot be shown in inoculated cultures until after 24 hours of incubation.

That this virus is etiologically associated with rubella is clearly established by repeated recovery from patients with rubella, but rarely from those with scarlet fever in the same hospital at the same time. Further, a high correlation was shown between the recovery of virus and significant antibody responses to it in patients with rubella or probable rubella (Table V). The recovery of other pathogens from some of the rubella patients (*viz.*, adenoviruses, parainfluenza and Coe viruses, and β hemolytic streptococci, Table I) is not surprising, since these agents were responsible for other hospitalized respiratory disease without exanthems occurring at the same time in the same recruit companies. Finally, we have shown, but not reported here, that rubella in other military camps in 1960, 1961 and 1962, and in civilian populations, is also associated with viruses possessing similar properties. Indeed, each of 12 strains from these other outbreaks, including 2 of those of Weller and Neva(11), are immunologically similar to the original strains by neutralization test. This is consistent with the epidemiologic observation that classical rubella usually results in permanent immunity.

Summary. A virus recognized by its capacity to multiply in grivet monkey kidney cultures and to interfere in such cultures with subsequent multiplication of ECHO-11 virus, was isolated regularly from military recruits with rubella, but rarely from those hospitalized at the same time and place with scarlet fever. The newly recovered virus multiplied consistently in primary and continuous grivet

TABLE V. Correlation Between Antibody Increase and Recovery of Rubella Virus.

Virus recovered	Significant antibody increase	
	Yes	No
Yes	24	1*
No	8	1*

* Antibody present, no increase.

kidney cultures. The virus was filterable, sensitive to heat and organic solvents, and immunologically distinct from a number of other chloroform sensitive agents. Neutralizing antibody often was present in acute phase sera of patients with rubella and regularly increased significantly during convalescence in patients yielding the virus.

Ultracentrifugations were made by Dr. Richard Hartman, Dept. of Molecular Biology, of this Institute, and cultures for PPLO, by Dr. Ruth Whittler, Dept. of Bacteriology.

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Coenzyme Q and Succinate-Tetrazolium Reductase Activity of Neonatal Rat Kidney.* (27751)

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Fisher and Gruhn reported a very low succinate-tetrazolium reductase activity in the outer cortex of the neonatal rat kidney(1). The succinate-tetrazolium reductase system contains multiple components including a specific dehydrogenase, an electron transport mechanism and a tetrazolium salt which is reduced to a highly colored, water insoluble formazan. Initial evidence for this complexity was derived from studies with blue tetrazolium and neotetrazolium which could be shown to require a component which is removed or destroyed by incubating sections with lipase(2). More recently coenzyme Q has been demonstrated to be an intermediate in the succinate-tetrazolium reductase reaction(3). It is evident that a low succinate-tetrazolium reductase activity could result from a low activity of primary dehydrogenase, a deficiency of available coenzyme Q or a combination of these factors. That different factors actually do cause a low succinate-tetrazolium reductase activity has been shown for various types of proliferative lesions of the liver(4,5). The present work is designed to find the cause of the low succinate-tetrazolium reductase activity in the neonatal rat kidney. Evidence will be presented to show both primary dehydrogenase and coenzyme Q are involved in this low activity.

Methods and materials. The animal tissues were obtained from male and female Sprague-Dawley rats. The rats were sacrificed at age intervals of one day beginning with 2 days of age and continuing for 25 days. Animals of 60 days of age were also used. Both kidneys were removed, one was fixed in Zenkers-formalin solution and embedded in paraffin. The other kidney was mounted on a block holder and quick frozen in dry ice. The frozen tissue was then sectioned in a cryostat at 16 μ, mounted on a coverslip and dried. The sections were then stained for succinate-tetrazolium reductase activity with 2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′dimethoxy-4,4′-biphenylene)-ditetrazolium chloride (Nitro BT) being employed as the tetrazolium salt. Alternate sec-

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