

Association of Virus with Cases of Rubella Studied in Toronto: Propagation of the Agent and Transmission to Monkeys

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IT WOULD appear from recently published reports that the long-awaited and confidently anticipated isolation of a virus from cases of rubella has been achieved. Buescher *et al.*¹ and Parkman *et al.*^{2, 3} have isolated, from military recruits suffering from rubella, a virus that grows in African green (grivet) monkey kidney cultures and interferes with the subsequent multiplication of enterovirus ECHO 11 in these cultures. No definite cytopathic changes in the kidney cultures could be found in these experiments. Sever and Schiff⁴ have confirmed the interfering action of rubella virus in tests in which enterovirus Coxsackie A9 was used as challenge. Weller and Neva⁵ have also recovered viruses from cases of rubella. These viruses were serially propagated in primary human amnion cultures; they produced unique cytopathic changes consisting of an aggregation of nuclear material and the presence of cytoplasmic inclusion bodies. These changes only occurred two to four weeks after inoculation and progressed slowly for some weeks more. On passage the cytopathic changes developed in a much shorter period of time.

Our interest in attempts to isolate rubella virus was stimulated several years ago during the studies of Laforest *et al.*⁶ and Robinson, Doane and Rhodes⁷ in Toronto on rubelliform infections caused by certain enteroviruses, especially ECHO 9 and Coxsackie A16. From these as well as several unpublished studies, it became apparent that although the virus of rubella caused clinical features resembling in some respects those associated with enteroviruses, it could not be recovered by conventional methods in tissue cultures or suckling mice.⁸

The isolation of the Salisbury common cold or "rhino" viruses by Tyrrell and associates,^{9, 10} in which they made use of the ability of these agents to interfere with the subsequent growth of ECHO 11 virus in tissue cultures, suggested to one of us (A.J.R.) that this technique could be used to attack the unsolved problem of the etiology of rubella. This approach, however, led to no definite results until we adopted the suggestion of Buescher and his associates^{1, 2} that African green (grivet) monkey kidney cultures be employed.

This report describes the isolation in Toronto of rubella virus from nine of 18 cases of clinical

ABSTRACT

Using an interference test with indicator virus Echo 11, a virus has been isolated in nine of 18 specimens from cases of typical rubella. The virus will interfere with the development of cytopathology in green monkey kidney cells with viruses Echo 11, Coxsackie B1 and B4, Poliovirus I and III (Sabin strains) and simian virus SV4. In four of five paired sera this virus was neutralized by convalescent but not by the acute phase serum, tested by interference inhibition. No cytopathology was observed in unstained cultures or in sequential cultures stained with acridine orange or fluorescent antibody. The virus was destroyed by exposure to 56° C. for 30 minutes and 15% ether at 4° C. for 24 hours, but survived with some reduction in titre at 4° C. for 24 hours. Green monkeys infected by this virus developed a macular rash, lymphadenopathy and modest rise in white blood cell count.

rubella by the use of an interference test in grivet monkey kidney tissue cultures. Three of these strains have been propagated through five to nine passages in tissue cultures. The neutralization of the interference-inducing activity of the virus by convalescent serum is described, and some additional biological characteristics of the virus are presented, including infectivity for African green monkeys. It is considered that our results provide confirmation of the work of the United States investigators cited above and point to the likelihood that the agents isolated by the various North American workers are indeed the cause of rubella.

METHODS

Collection of Specimens from Cases of Rubella

Cases of rubella were reported to our laboratory by interested physicians in the Toronto area, whose help is gratefully acknowledged. These patients were visited as early as possible by one of us (B.S. or K.F.G.) and, after a review of the clinical diagnosis, specimens were taken. Specimens were taken only on the first or second day of illness. Throat swabs were obtained from young children and

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immediately placed in a balanced salt solution containing penicillin and streptomycin. Washings of a balanced salt solution were obtained from older patients. Acute phase blood specimens were also drawn at this time; convalescent phase blood was obtained some three to four weeks later. All specimens were immediately brought to the laboratory and were kept frozen at -20° C. until tested.

Tissue Cultures

African green monkey kidney cells in suspension were obtained from the Connaught Medical Research Laboratories (C.M.R.L.) of the University of Toronto. These cells were grown at 37° C. for five days as monolayer cultures, in Hanks' saline solution supplemented with 0.5% lactalbumin hydrolysate (Difco) and 2.0% calf serum. The medium was then changed to C.M.R.L. medium No. 597 with 4% sheep serum, and the cultures were kept for two more days before use in the interference experiments.

LLC-MK2, a continuous rhesus monkey kidney cell line, was obtained through the courtesy of Dr. R. N. Hull of the Lilly Research Laboratories, Indianapolis, and was grown in C.M.R.L. medium No. 597 with 10% of calf serum.

HEF, a human embryo foreskin cell line of fibroblastic morphology, was also employed. These cells were grown in Eagle's medium with 10% calf serum.

All the cell lines, both primary and continuous, were grown as monolayers in 16 x 150 mm. rubber-corked glass tubes.

Inoculation of Tissue Cultures with Pathological Specimens

Suitably cultivated monolayer cultures were inoculated with 0.2-ml. quantities of pathological specimens. One hour at 4° C. was allowed for adsorption of virus to the culture cells, then the appropriate maintenance medium was pipetted over the cell sheet. The cultures were then reincubated at 35° C. for nine days to allow virus growth, although no microscopic changes could in fact be seen.

Demonstration of Interference

In preliminary experiments the cultures were challenged six to 14 days after inoculation. Because interference could be demonstrated from the eighth day onward, the ninth day was adopted arbitrarily as the optimum day on which to conduct the challenge. On the ninth day after inoculation of the specimens each culture was accordingly infected with 100 TCD₅₀ of ECHO 11 or other virus employed as challenge. The cultures were examined two and three days later for evidence of cytopathic change induced by the challenge virus. Controls, in the form of uninoculated cultures as well as cultures inoculated either with ECHO 11 virus or rubella

specimen, were included in every experiment and were examined at the appropriate times.

The presence of interference was indicated by failure of the ECHO 11 or other challenge virus to cause cytopathic changes, provided that the challenge virus did produce such changes in control cultures uninoculated with specimens from cases of rubella.

If the interference test was negative and the challenge virus produced cytopathic effects, then further blind passages were tried. The replicate cultures of cells which had been inoculated with specimen material, but which were unchallenged, were frozen and thawed three times; 0.2 ml. of the cell lysate in medium was then transferred to fresh grivet kidney monolayer cultures as a second passage. One hour at 4° C. was allowed for virus adsorption, and the cultures were incubated and tested as outlined for isolation attempts at the first passage.

Adaptation of Strains of Virus to Tissue Culture

Supernatant fluids from cultures infected with specimens from patients R12, R13 and R23 were selected for attempts to propagate the agent responsible for interference, presumably the rubella virus. Supernatants were passed serially five to nine times in green monkey kidney cultures and interference was still regularly induced.

Cultures of green monkey kidney in "Blake" bottles were inoculated with each strain in order to provide quantities of material to constitute virus pools. Nine days after infection these cultures were frozen and thawed three times, and the resulting material was distributed to 1.0-ml. vials which were stored at -25° C. The virus content of several vials was titrated by inoculating serial dilutions into cultures which were subsequently challenged with ECHO 11 virus. The pools R12, R13, and R23 were found to have infectious titres ranging from $10^{3.0}$ to $10^{4.0}$ interference-inducing doses per 0.1 ml. (InD₅₀). The 50% interference-inducing endpoints were calculated by the Kärber method.¹¹ The experiments on other properties of the virus were performed on pool R23.

Tests for Interference Inhibiting (Neutralizing) Antibody

Acute and convalescent phase sera were inactivated at 56° C. for 30 minutes and were then diluted in maintenance medium by fourfold steps to yield dilutions 1:4 to 1:256; 0.5-ml. quantities of these dilutions were mixed with an equal volume of R23 virus pool. The virus inoculum had been adjusted to contain an estimated $10^{3.0}$ InD₅₀ per 0.1 ml. These mixtures were held at room temperature for one hour, and were then inoculated into four green monkey kidney cultures, 0.2 ml. per culture.

Nine days after inoculation of the serum-virus mixtures, each culture was challenged with 100

TABLE I.—INTERFERENCE BY SPECIMENS FROM 18 CASES OF RUBELLA: TESTS PERFORMED IN AFRICAN GREEN MONKEY TISSUE CULTURES AGAINST CHALLENGE OF ECHO 11 VIRUS

Patient's code No.	Age (years)	Clinical features		Specimens collected—days after onset	Interference produced in tissue cultures inoculated with throat washings or passage material		Titre of antibody for rubella isolate R23		
		Typical rash	Enlarged nodes		First positive passage	Total passages and final titre*	Acute serum	Convalescent serum	
R10	19	+	—	1	2nd	2			
R11	27	+	+	1	2nd	2	MV†		
R12	26	+	+	1	2nd	6	10 ^{3.2}	<1:4	1:4
R13	14	+	+	1	1st	6	10 ^{3.2}		
R14	8	+	—	1	1st	4	MV†		
R15	6	+	+	1	1st	1	MV†		
R17	12	+	+	1	1st	4		<1:4	1:16
R18	14	+	+	2	—	2			
R19	35	+	+	1	—	5			
R20	5	+	+	2	—	2			
R21	18	+	+	1	—	3			
R22	18	+	+	2	2nd	4		<1:4	1:64
R23	9	+	+	1	2nd	9	10 ^{3.8}	<1:4	1:16
R26	10	+	—	1	—	2		<1:4	1:64
R27	4	+	+	1	—	2			
R29	3	+	+	1	—	2			
R30	4	+	+	2	—	2			
R31	19	—	+	2	—	2			

*InD₅₀.

†MV, contaminating monkey virus present.

TCD₅₀ of ECHO 11 virus. Controls were challenged at the same time. The presence or absence of interference was estimated two or three days later by the extent of the cytopathic changes caused by ECHO 11 virus. Absence of cytopathic change indicated that interference was induced by the R23 virus and that there was no antibody to rubella in the serum tested. *Vice versa*, the presence of cytopathic changes indicated that no interference had been caused by R23 and hence that the virus had been neutralized by antibody in the test serum.

Infectivity of Strain R23 of Rubella Virus for African Green Monkeys

Two healthy African green monkeys, a male and female aged between two and five years, were bled and each was inoculated with R23 virus pool, 1.0 ml. intraperitoneally and 0.5 ml. intranasally. The fur was clipped closely and the skin was examined twice daily for the presence of a rash. Lymph glands were palpated, and rectal temperatures were taken at the time of each examination. Every day, in addition, blood samples were removed for total leukocyte counts.

RESULTS

Of 18 throat washings from clinically typical cases of rubella inoculated in green monkey kidney cultures, nine prevented the development of the cytopathic action of ECHO 11 virus, presumably by the phenomenon of interference (Table I).

Convalescent phase serum of four out of five patients neutralized the R23 rubella strain and prevented the establishment of the interference phenomenon; antibody titres ranged from 1:16 to

1:64. In all cases the acute phase serum showed titres of less than 1:4 (Table I).

The results of tests for the heat and ether sensitivity of the interference-inducing property of R23 rubella virus are shown in Table II. It can be seen that R23 virus is sensitive to 56° C. for 30 minutes, for only residual activity remained after this exposure. Treatment with ether for 24 hours at 4° C. resulted in complete loss of virus activity. Storage at 4° C. for 24 hours, however, had relatively little effect on the activity of the virus.

Attempts to isolate pleuropneumonia-like organisms (PPLO) from virus pools in a medium known to grow these organisms were negative.

Adsorption of R23 virus to green monkey kidney cells at different temperatures was studied, and a slightly greater amount of virus was found to be adsorbed at 4° C.

The ability of R23 to grow in various cell lines and to interfere with challenge viruses other than ECHO 11 was studied. It was found that R23 virus growing in green monkey kidney cells not only interferes with ECHO 11, but also prevents the development of cytopathic changes of Sabin attenuated Poliovirus types I and III, Coxsackie B1 and B4, and Hull's simian virus SV4.¹² The SV4 virus

TABLE II.—TEMPERATURE AND ETHER SENSITIVITY OF ISOLATE R23 FROM CLINICAL RUBELLA

Experiment	Titre of R23 virus (expressed as InD ₅₀ per 0.1 ml.) after the following treatments			
	Control	56°C./30 min.	4°C./24 hours	15% ether/4°C./24 hours
1	10 ^{3.1}	10 ^{0.5}	10 ^{3.0}	0
2	10 ^{3.5}	N.T.*	N.T.	0
3	10 ^{3.5}	10 ^{0.5}	N.T.	0

*N.T.—not tested.

has been studied in detail in our laboratory; it appears to be a nanivirus and a member of the EMC subgroup of viruses.¹³ R23 virus did not prevent the development of A2 influenza virus hemagglutinins in green monkey kidney tissue cultures.

Multiplication of R23 virus in continuous rhesus monkey kidney line LLC-MK2 cells was observed as shown by the capacity to interfere with ECHO 11 virus, but no evidence of growth in HEF could be demonstrated.

We were unable to observe hemagglutination or hemadsorption with any rubella isolate at 4° C., 22° C., or 35° C. in tests with human O, fowl or guinea-pig red blood cells. Virus-infected culture fluid, of a titre between 10^{4.0} and 10^{5.0} InD₅₀ per ml., was concentrated 20-fold by ultracentrifugation, but still no hemagglutination could be demonstrated.

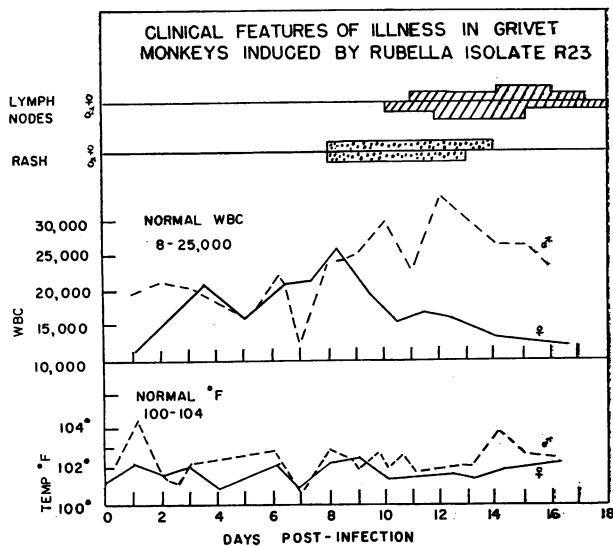


Fig. 1.—The clinical course of two African green monkeys, male and female, inoculated intranasally and intravenously with rubella isolate R23.

Attempts were made to detect cellular changes in culture cells by acridine orange staining¹⁴ and also by the indirect method of fluorescent antibody staining.¹⁵ No consistent differences between infected and control cells could be determined up to 15 days after infection.

R23 virus inoculated into two grivet monkeys produced a mild disease characterized by the appearance on the ninth day of a macular rash in the axillary and groin regions (Fig. 1). In the male monkey, lymphadenopathy of the axillary and groin nodes was first detected nine days after inoculation; in the female, the nodes were detected a day later. The male monkey, in addition, developed shotty submandibular nodes. The blood picture in the male animal revealed a modest rise in the white blood cell count lasting between day nine and day 13 after inoculation; in the female, the leukocytosis began in the incubation period. In neither monkey were the differential counts of significance. The

temperature of the animals showed little or no change from normal.

DISCUSSION

In confirmation of the results recently reported by Parkman and associates¹⁻³ we have isolated an interfering agent, which appears to be the rubella virus, from nine patients suffering from classical rubella. These patients ranged in age from six to 27 years. The fact that four of the patients yielding virus were aged 18 or over affords additional evidence that the clinical condition was, in fact, rubella. Three strains of the virus were propagated by serial passage in tissue culture. One of these viruses (R23) was used as antigen and was found to be neutralized by convalescent but not by acute phase serum of patient R23 and three other patients (R17, R22, R26).

Rubella virus isolate R23 in its seventh passage induced a mild illness in two grivet monkeys. This illness was characterized by the development of a macular rash and lymphadenopathy. The grivet monkeys did not appear to develop any obvious fever or desquamation of the rash, as reported earlier by Habel in the rhesus monkey.¹⁶ No evidence of enteric or respiratory disturbance was found. This apparent transmission of infection to the grivet monkey is regarded as additional evidence supporting the etiological association of isolate R23 with rubella.

We have not observed the development of cytopathic change in cultures as reported by Weller and Neva.⁵ This, however, is not in contradiction, since our observations of the cultures did not usually extend as long as was found necessary by these authors. The fact that our observations of the cultures inoculated with rubella specimens over periods up to 16 days showed no cytopathic changes makes it unlikely that a presently known virus causing changes in a shorter period of time was responsible for the interference induced by pathological specimens.

The heat and ether sensitivity of the virus R23 is similar to that reported by the other workers.^{1, 3} Work is presently in progress to characterize additional isolates, but they appear to be identical.

SUMMARY

The isolation, from nine of 18 patients with typical clinical rubella, of a virus presumed to be the etiologic agent of this disease is described. The virus is recognized by its property of inducing interference in African green monkey kidney cultures to subsequent infection with ECHO 11, Poliovirus types I and III (Sabin), Coxsackie B1 and B4, or SV4 virus.

The virus did not interfere with the production of hemagglutinin by A2 influenza virus. An isolate from patient R23 was neutralized by the convalescent but not by the acute phase serum of four out of five patients, one of whom was the patient R23. The interference-inducing activity of this isolate was destroyed by heating at 56° C. for 30 minutes, and by 15% ether at 4° C. for 24 hours, but it was relatively un-

affected by storage in culture fluid at 4° C. for 24 hours.

Inoculation of two grivet monkeys with seventh passage material of isolate R23 produced a mild illness characterized by the development of a rash and lymphadenopathy. The Toronto rubella virus strains appear to be similar in properties to those isolated independently in Washington, D.C., and Boston, Mass., by three other groups of workers.

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