# Isolation in Tissue Culture of an Interfering Agent from Patients with Rubella.* (27826)

J. A. VERONELLI, H. F. MAASSAB, AND A. V. HENNESSY
(Introduced by Thomas Francis, Jr.)
*Department of Epidemiology and Virus Laboratory, School of Public Health and Department of Pediatrics, University of Michigan, Ann Arbor*

Isolation of the causative agent of rubella has been attempted several times(1,2,3,4). Until the report of Buescher and Parkman (5,6), such attempts were unsuccessful or failed to receive confirmation. These investigators reported the isolation of an interfering agent from 19 of 28 recruits affected with rubella-like disease. The rubella-associated (R.A.) agent could be recognized only by the resistance it conferred on primary tissue cultures of grivet (green monkey) kidney cells to superinfection with ECHO 11 and other enteroviruses. Nevertheless, the etiological association of the R.A. agent with rubella-like disease was suggested.

This report deals with the isolation of similar interfering agent(s) from a group of 7 university students affected with clinical rubella. The isolations were obtained by the use of a continuous line of cells derived from rhesus monkey kidney (LLC-$MK_2$) instead of primary green monkey kidney cell cultures. The recovery of interfering agent(s) from a majority of the tested specimens of acute stage sera was of particular interest in the studies presented here.

*Materials and methods. Clinical findings.* The patients were 7 university students, aged 18 to 22 years, who became affected with rubella during the months of February to May 1962. Diagnosis was made by the physicians of the University Health Service and all of the patients were also examined by one of the authors.

Two patients had a history of contact with other members of the group 15 and 21 days before onset of rash, and an incubation period of 21 days could be traced in an additional patient.

The disease was characterized in all cases by an erythematous macular or maculopapular eruption of approximately 3 days' duration. Prodromal symptoms were present in 6 of the 7 patients with a duration of one to 5 days and consisted of malaise, headaches, and nausea. Diarrhea was present in 3 patients and fever in 2 instances during the prodromal period and in 3 during the early course of the disease. Occipital, retroauricular or marked posterior cervical lymphadenopathy, usually tender to palpation, was present in all patients.

*Collection and handling of specimens.* Routine technics were employed in obtaining material for viral studies. Only a few points deserve special mention: nasopharyngeal swabbings were obtained with a small dry cotton swab placed at the end of a flexible piece of wire introduced parallel to the floor of the nasal cavity until it touched the posterior pharyngeal wall; the patient was then encouraged to cough. The swab was immediately immersed in 3 to 5 cc of beef broth infusion containing penicillin and streptomycin.

Blood obtained during the acute illness was promptly centrifuged at 2000 rpm for 10 minutes and the serum was frozen within 2 hours. All other samples were frozen and stored at −70°C within one hour after collection. The interval between collection of acute and convalescent serum varied from 2 to 9 weeks.

*Tissue cultures.* The continuous line of rhesus monkey kidney cells (LLC-$MK_2$) was obtained from Dr. Robert Hull of Eli Lilly Research Laboratories(7). Cultures were grown in medium 199 with 1% horse serum and maintained in Eagle's medium(8) with double concentration of amino acids, gluta-

* This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board and was supported in part by Office of the Surgeon General, U. S. Army, Washington, D. C., and in part by USPHS General Research Support Program.

mine, and vitamins (double strength Eagle's) and 1% horse serum.

Primary grivet kidney cell cultures (PGK) were purchased from Microbiological Associates and maintained either in Eagle's medium with 5% chicken serum or in a medium composed of Hanks' BSS, lactalbumin hydrolysate 0.5%, glutamine 1%, and chicken serum 2 to 4%.

Continuous grivet kidney cells (BS-C-1) were obtained from H. E. Hopps, Division of Biological Standards, Nat. Inst. of Health (9). Growth medium consisted of medium 199 with 20% fetal bovine serum and 0.01% yeast extract; maintenance medium was double strength Eagle's with 2 to 4% chicken serum.

*Inoculation procedure.* Cell monolayers were incubated at room temperature in the presence of 0.3 cc of serum-free medium and 0.2 cc of the inoculum. After 2 hours' adsorption, 0.5 cc of medium containing double concentration of serum was added and the cultures were incubated at 37°C.

Passage material consisted usually of fluid and cells. After the first passage, material from the control tubes was used as inoculum for the control group of the next passage.

*Maintenance of cultures.* Cultures were maintained from 10 to 14 days before challenge. Usually 0.2 cc of fresh medium was added 5 to 6 days after inoculation and half the fluid phase was replaced for fresh medium on the 10th day after inoculation.

*ECHO 11 challenge.* Challenge with ECHO 11 was accomplished by inoculation of the same cultures with 100 to 1000 tissue culture infectious dose 50($TCID_{50}$) of ECHO 11 virus in 0.1 cc volume. Only one-half of the tubes corresponding to any given sample were challenged, while the rest were used for further passages. Final readings were taken at the time the ECHO 11 cytopathic effect (CPE) was complete in the control tubes. Confirmation of the presence of ECHO 11 virus was obtained by hemagglutination with group O human erythrocytes. For this test 0.5 cc of tissue culture fluid from each challenged tube was mixed with an equal volume of a 0.5% (v/v) suspension of human O cells. Results were read after one hour incubation at room temperature.

*Neutralization tests.* All tests were carried out using the LLC-$MK_2$ cell line. Acute and convalescent sera from each patient were tested simultaneously. Cells derived from the same passage were used for retitration of the agent at the same time. The method employed consisted of mixing decreasing amounts of R.A. agent with a constant amount of each serum under test(10). The detailed procedure follows: a 1:2 dilution of each serum was inactivated for 30 minutes at 56°C and then 0.4 cc was added to an equal volume of each dilution of R.A. agent. After incubation at room temperature for 2 hours, 0.2 cc of mixture was inoculated into each of 3 tubes of cultures containing 0.8 cc of serum-free medium (Eagle's double strength). Tubes were cared for as described above. Ten days after inoculation of the R.A. agent the cell monolayers were washed with BSS, the medium replaced, and a dose of 1000 $TCID_{50}$ of ECHO 11 virus inoculated into each tube. Readings for ECHO 11 CPE were taken daily during the 5 days following challenge. A hemagglutination test using 0.5 cc of fluid from each tube and 0.5 cc of a 0.5% suspension of human group O erythrocytes gave confirmatory evidence of the presence or absence of ECHO 11 CPE.

Two types of controls were employed: 1. Serum controls were included to rule out the existence of nonspecific inhibitors or specific antibodies against ECHO 11 or of toxic effects on the culture cells. To each of 3 tissue culture tubes containing 0.8 cc of serum-free medium was added 0.2 cc of a 1:4 dilution of the serum under test.

2. A control titration of the R.A. agent was carried out simultaneously to each test. The 2 higher dilutions of R.A. agent employed in the test were mixed with an equal volume of BSS instead of serum. After incubation at room temperature for 2 hours, 0.2 cc of each mixture was inoculated into each of 3 tissue culture tubes containing 0.8 cc of double strength Eagle's medium with 1% horse serum. Maintenance, challenge with ECHO 11 virus, and readings were accomplished in the manner described above for the neutralization tests.

TABLE I. Summary of Studies on Isolation of Interfering Agents from 7 Patients with Rubella.

| Patient | Time after onset spec. obtained (hr) | Type of specimen | Result on 1st passage and tissue culture employed | | Result on further passage |
|---|---|---|---|---|---|
| W.M. | 18–24 | Throat swab | Inc. | PGK | + |
| | | NP swab | N.T. | LLC-MK$_2$ | + |
| | | Acute serum | + | " | + |
| | | Throat washing | Inc. | " | – – – |
| G.P. | 24 | NP swab | + | LLC-MK$_2$ | + |
| | | Acute serum | Inc. | " | — |
| | | Throat washing | — | " | – – – |
| C.F. | 18–24 | Throat swab | + | PGK | + |
| | | NP swab | + | LLC-MK$_2$ | + |
| | | Acute serum | — | PGK and LLC-MK$_2$ | – – – |
| B.W. | 18–24 | Throat swab | — | LLC-MK$_2$ | – – – |
| | | NP swab | Inc. | " | + |
| | | Acute serum | — | " | – – – |
| | | Stool specimen | — | " | – – – |
| Ch. T. | 52 | Throat swab | + | LLC-MK$_2$ | + |
| | | NP swab | + | " | + |
| | | Acute serum | Inc. | " | + |
| D.K. | 12 | Throat swab | + | LLC-MK$_2$ | + |
| | | NP swab | + | " | + |
| | | Acute serum | Inc. | " | + |
| | | Throat washing | — | PGK | – – – |
| R.N. | 24–32 | Throat swab | + | LLC-MK$_2$ | – – – |
| | | Acute serum | + | " | – – – |
| | | Throat washing | — | " | – – – |

*Code:* +, complete interference; —, no interference; Inc., incomplete interference; N.T., passed without challenge; – – –, no further passages; PGK, primary grivet cultures.

The Reed-Muench formula(10) was used to calculate the 50% endpoints. The difference (without regard to sign) between the logarithm of the interference dose 50 (Int. D. 50) obtained in the presence of acute and convalescent serum was regarded as a neutralization index. This index, if converted to the antilogarithm, expresses the ratio between the number of Int. D. 50 neutralized by the convalescent and those neutralized by the acute serum.

*Results. Recovery of interfering agents.* The phenomenon of interference, which could be transmitted by serial passage, was demonstrated in cultures inoculated with specimens from all 7 patients. Table I lists the interval between onset of rash and securing of samples for virus studies, type of specimen tested and result. In 5 patients isolations were obtained from more than one type of sample.

Isolations were obtained from all of 6 available nasopharyngeal specimens; throat swabbings were positive in 5 of the 6 samples tested, while throat washings gave only one isolation out of 4 samples tested.

Isolations were obtained from 4 of the 7 samples of acute sera, obtained between 6 and 52 hours after onset. Routine bacteriological studies were done on all isolates with negative results. Culture of PPLO was not attempted.

*Interference and incomplete interference.* Interference with the growth of ECHO 11 or other enteroviruses is at present the only index of growth of a rubella-associated agent. The development of a complement fixation technic has been investigated by Buescher, Parkman and colleagues(6) and immunofluorescent technics using isothiocyanate-labeled convalescent sera were tried in our laboratory with no apparent success.

In the present study ECHO 11 virus was used exclusively as the challenging agent. Experimentally, doses of this virus in excess of $5 \times 10^5$ $TCID_{50}$/0.1 cc resulted in rapid CPE when added 10 days after inoculation

TABLE II. Neutralization Tests with Sera of Patients from Whom Interfering Agents Were Isolated.

| Patient* | RA titer control† | RA titer with Acute serum | RA titer with Conv. serum | Neutralization index (numerical) |
|---|---|---|---|---|
| W.M. | 2.07 | 2.30 | .77 | 33.5 |
| G.P. | 2.07 | 1.68 | .77 | 8.0 |
| C.F. | 1.55 | 2.07 | .82 | 17.8 |
| B.W. | 1.55 | 2.15 | 1.12 | 10.7 |
| Ch.T. | 1.55 | 1.80 | 1.00 | 6.3 |
| D.K. | .93 | 1.40 | .62 | 6.0 |

* Tests are arranged in the order in which they were performed.
† Titer = neg log of dilution.

of R.A. agent, while all doses at or below this level proved useful for the detection of interference. Doses of ECHO 11 between 100 and 1000 $TCID_{50}$/0.1 cc were usually employed; in most instances the lower dose was used when testing for primary isolation.

Even when employing low doses of the challenging virus, incomplete interference was a common finding in primary isolations. This phenomenon is characterized by delay in the appearance and slowness in progression of the ECHO 11 CPE, and it is probably an expression of the need of a certain titer of R.A. agent before complete interference becomes detectable. The frequency of incomplete interference in primary isolations, and occasionally in the first 2 passages, makes daily readings necessary after addition of the second virus; a single reading may not detect the difference between these tubes and the controls. Hemagglutination of group O human erythrocytes performed with the fluid from tubes showing incomplete interference gives + or ± results. Complete interference usually results upon further passage.

*Serologic evidence.* Convalescent sera were available from 6 patients and the results of neutralization tests are presented in Table II.

The 50% interference endpoint was, in all cases, displaced to lower dilutions by addition of convalescent sera. Absolute values of the neutralizing capacity of the acute and convalescent sera were not calculated. The results given as neutralization indices express the numerical ratio between the number of Int. D. 50 neutralized by the convalescent and those neutralized by the acute serum; in the paired sera studied neutralization indices varied between 6 and 33.5.

Certain aspects related to the technic employed are noteworthy: a. It is of interest that the R.A. agent titer in acute phase serum was, with one exception, higher than that of the titration control. It seems likely that the effect resulted from the fact that a smaller concentration of serum was present in the control tubes with a reduced multiplication of the agent. Consequently, the neutralization index was chosen as the applicable basis of measurement since the concentrations of acute and convalescent sera were the same.

b. A progressive loss of titer was evident in successive titrations of the pool of R.A. agent employed (Table II, 1st column). This is possibly the effect of repeated cycles of freezing and thawing. Such a decrease in titer does not affect a comparative value like the neutralization index.

c. The possibility of residual active virus being present in some of the acute serum specimens (even though all were heated at 56°C for 30 minutes) and of anti-ECHO 11 properties of the sera under test were considered. The consistent lack of interference in all the controls inoculated with those sera (without addition of R.A. agent) seems to rule out, under the experimental conditions employed, any effect attributable to such factors.

*Discussion.* Rubella-like disease is a common ailment among recruits but presents certain epidemiological characteristics which has resulted in its designation as "recruit rubella." The studies presented here are confirmatory of the findings of Buescher and Parkman but were carried out in a group of students, among whom the disease presents a clear seasonal incidence, and histories of association with other cases and duration of incubation periods are frequently elicited. This stresses the likelihood that most of the cases clinically diagnosed as rubella are due to the same or similar agents.†

† Samples from a 4-year-old child affected with rubella on July 1961 yielded a positive isolation from the nasopharyngeal swab specimen. These samples had been stored in our laboratory at −70°C for a year before testing.

The multiple isolates recovered at the Walter Reed Institute for Medical Research have proven to be serologically identical(6). Similar studies are in progress with the isolates recovered from our patients.

While the etiological responsibility of the R.A. agent has not been established beyond doubt, there is increasing evidence to strengthen the likelihood of a causative association. In this respect the following findings in the present study deserve special mention: a. Isolation of the R.A. agent from members of a student population affected with typical rubella. b. Recovery of the agent from a majority of the blood specimens obtained during the acute phase of the disease. It has been reported that intramuscular inoculation of human volunteers with serum of rubella patients taken shortly after appearance of rash results in experimental transmission of the disease with more consistency than the spraying of infectious throat washings(11). c. A 6-fold or greater increase in the neutralizing properties of sera obtained in the convalescent stage.

In our hands the LLC-$MK_2$ continuous rhesus monkey kidney cell line has proven very satisfactory for isolations from clinical material. This cell line is more easily available and less expensive than primary grivet kidney cultures. Preliminary information as to the comparative characteristics of growth of this agent in the 3 cell lines employed in these studies is now being obtained. In parallel titrations the LLC-$MK_2$ line permitted the detection of interference in dilutions approximately one logarithm higher than the primary or continuous grivet monkey kidney cultures. Isolation from acute sera has been possible in all 3 lines but on the first passage, complete rather than incomplete interference was more frequent with the use of the LLC-$MK_2$. On further passages, the 3 cell lines can be used interchangeably.

*Summary.* The technic first described by Buescher and Parkman for isolation of an agent from patients with rubella was used in a study of 7 university students affected with this disease. 1. Agents which interfered with ECHO 11 in primary or continuous grivet monkey kidney cells or in a continuous rhesus monkey kidney cell line (LLC-$MK_2$) were recovered from all patients. 2. The agent was recovered from upper respiratory secretions of all cases and from blood drawn during the acute phase in 4 of 7 patients. 3. Rise in the neutralizing properties of the sera obtained in the convalescent period as compared with the acute sera was a constant feature in all samples tested. 4. The usefulness of the LLC-$MK_2$ cell line for primary isolation of this agent(s) was proven during this study.

ADDENDUM: Since this paper was submitted for publication, one of the isolates recovered in the present study has proven to be serologically identical with the virus recovered by Drs. Parkman and Buescher. The findings of these authors as well as the independent investigations by Drs. T. H. Weller and F. A. Neva have appeared in Proc. Soc. Exp. Biol. and Med., vol. 111, October, 1962.

The technical assistance of Miss Patricia Gosnell and Miss Ann C. Wilson and the cooperation of Mr. M. Murphy, Assistant to the Director, University Health Service, and of the students constituting the study group are gratefully acknowledged.

1. Habel, K., *Pub. Health Rep.,* 1942, v57, 1127.
2. Anderson, S. G., *Lancet,* 1954, v2, 1107.
3. ———, *ibid.,* 1957, v1, 1043.
4. Gutenkust, R. R., Heggie, A. D., *New Eng. J. Med.,* 1961, v264, 374.
5. Buescher, E. L., Parkman, P. D., Artenstein, M. S., Halstead, S. B., *Fed. Proc.,* 1962, v21, 466.
6. Parkman, P. D., Artenstein, M. S, McCown, J., Buescher, E. L., *ibid.,* 1962, v21, 466.
7. Hull, R. H., Cherry, W. R., Tritch, O. J., *J. Exp. Med.,* 1962, v115, 903.
8. Eagle, H., *ibid.,* 1955, v102, 37.
9. Hopps, H. E., Bernheim, B. C., Nisalak, A., Smadel, J. E., *Fed. Proc.,* 1962, v21, 454.
10. Lennette, E. H., in *Diagnostic Procedures for Viral and Rickettsial Diseases,* edited by T. Francis, Jr. *Am. Public Health Assn.,* 1956, 2nd Edit., pp. 1-51.
11. Schneider, B., Tottori, H., unpublished Thesis, Tulane University School of Med., 1959.

Received September 10, 1962. P.S.E.B.M., 1962, v111.