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Growth of High Titered Rubella Virus in Roller Bottle Cultures of Vero Cells* (33477)

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(Introduced by F. L. Black)

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Attempts to isolate and fully characterize the structural components of rubella virus (RV) and associated virus specified nonstructural proteins have been seriously restricted by the relatively low yields of virus obtained from infected tissue cultures. Suspended cultures of BHK-21 cells which have yielded infectivity titers as high as $10^{7.5}$ ID_{50}/ml have been the most efficient producers of rubella virus available to date (1).

Replication of RV with concomitant production of cytopathic effects (CPE) in Vero cells (continuous African green monkey kidney) was reported in a previous publication (2). Though the data presented in that study dealt primarily with the use of these cells for primary isolation and direct assay of RV infectivity, it was shown that they supported the growth of RV to relatively high titers. The present communication describes a method of growing RV in roller bottle cultures of Vero cells which yields 1–2 liter quantities of virus suspension titering in excess of 10^9 $TCID_{50}$/ml. The procedure provides in addition a correspondingly large yield of hemagglutinin (HA) and complement fixing (CF) antigen.

Materials and Methods. Cylindrical bottles 10 cm in diameter × 40 cm in length (840 cm^2 surface area)[2] were seeded with 3 × 10^7 Vero cells in 150 ml of prewarmed HMEM–5% CS[3]. The bottles were placed in a roller apparatus[2] housed in a 37° warm room and rolled at 0.5 rpm. After 2 or 3 days the medium was replaced with EMEM–5% CS medium (3).[3] The monolayers were confluent and ready to use 2–3 days later. At this stage the average number of cells per bottle was 2 × 10^8. Before virus inoculation, growth medium was removed and the cell sheet was washed with 100 ml of balanced salt solution. The RV inoculum (10–15 ml) was allowed to adsorb to the monolayer on the roller apparatus for 60–90 min. The unadsorbed inoculum was then removed and replaced with 50 ml of prewarmed EMEM containing 5% kaolin treated newborn agamma calf serum. At 24-hr intervals the culture fluid was harvested and replaced with 50 ml of fresh prewarmed medium. Daily harvests were continued for up to 10 days. Culture fluids were assayed for HA (4) and infectivity by CPE in Vero cells and by interference in primary African green monkey kidney cultures (2). For extraction of HA and CF antigen from the infected monolayer, 10 ml of 0.1 *M* glycine buffer, pH 9.0, containing 0.4% bovine serum albumin (4) was added to the culture bottle after the final daily harvest of infectious fluid was removed. The bottle was then rolled at 37° for periods of 8 or 30 hr. The extract was freed of gross

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[1] Liebhaber, H., Pajot, T., to be published.

[2] Purchased from Bellco Glass Co., Vineland, New Jersey.

[3] (MEM) minimal essential medium; (H) indicates Hanks balanced salt solution; (E) indicates Earles balanced salt solution; 5% CS indicates 5% calf serum.

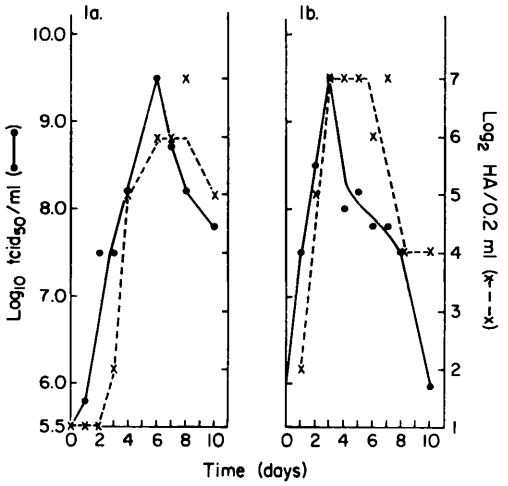

FIG. 1. Time course of appearance of rubella virus infectivity and hemagglutinin (HA) in roller bottle cultures of Vero cells inoculated with virus at an input multiplicity of (a) ~ 4 $TCID_{50}$/cell, and (b) ~ 100 $TCID_{50}$/cell.

cellular debris by low speed centrifugation and was dialyzed in the cold, for 8 hr against distilled water and for 24 hr against 20 vol of Veronal buffered saline (5). The CF antigen titration was carried out according to the procedure of Schmidt and Lennette (5).

Results. Representative growth curves at two levels of input multiplicity of rubella virus are presented in Fig. 1. The time course of appearance of extracellular virus and hemagglutinin paralleled one another quite closely during the period of exponential increase, after which infectivity titers dropped quite rapidly whereas HA titers remained essentially unchanged for at least 3 days. As previously observed (2) increasing the multiplicity of input does not appear to influence the magnitude of the peak infectivity titer but rather the rate at which it is reached. Infectivity titers of $10^{9.5}$ $TCID_{50}$/ml have consistently been obtained.

The relationship of hemagglutinin titer to infectivity seen in Fig. 1 provides a simple means of determining which harvest contains the peak concentration of infectious virus without having to wait 14–21 days for infectivity assays to be completed. In repeated experiments, termination of the phase of exponential increase in HA titer was found to signal the presence of maximal concentrations of infectious RV.

The cell sheet, which remained adherent to the surface of the roller bottle for as long as 10 days after infection, contained significant quantities of HA and CF antigen which could be extracted by alkaline glycine buffer (5). The yield of both antigens, when extraction by this method was carried out over an 8-hr period, is listed in Table I. Increasing the extraction time to 30 hr increased the yield of HA, but resulted in an extract which had been rendered strongly anticomplementary.

Discussion. In addition to the reproducibly high yields of infectivity and HA and CF antigens this procedure has the advantage of requiring a minimum of time, medium, and manipulation. Purification of virus grown according to this method is rapid and efficient[1] since the high concentration of infectivity obtained directly in the culture fluids obviates the need for concentrating virus from large volumes of infected fluids. For example, the total infectivity in the fluids harvested from 10 Vero roller bottle cultures (500-ml total volume) at peak virus titer is equivalent to the total infectivity contained in 50 liters of virus suspension titering $10^{7.5}$ ID_{50}/ml from BHK-21 suspension cultures (1).

The identification of the virus grown in these cultures as rubella was established in

TABLE I. Hemagglutinin and Complement Fixing Antigen Titers in Alkaline[a] Extracts of Rubella Infected Vero Cells.

Duration of extraction (hr)	HA titer (HAU/0.2 ml)[b]	CF antigen titer (CFU/0.025 ml)[c]
8	512	16
30	≥1024	AC[d]
30	≥1024	AC

[a] 0.1 *M* glycine, pH 9.0, containing 0.4% bovine serum albumin.

[b] Reciprocal of end point dilution expressed as hemagglutinin units per 0.2 ml.

[c] Reciprocal of end point dilution of antigen which fixes 2 units of complement in a test with 8 units of antiserum. Result is expressed as units of CF antigen (CFU) per 0.025 ml.

[d] Anticomplementary.

neutralization tests with paired human sera from clinical cases of rubella and with rabbit sera pre- and post-immunization with rubella virus which had been grown in RK-13 cells and had never been through a passage in Vero cultures.

Summary. Roller bottle cultures of Vero cells yield rubella virus fluids with infectivity titers in excess of 10^9 $TCID_{50}$/ml. The cells, after having produced maximal virus yields contain large quantities of HA and CF antigen which can be extracted by pH 9.0 glycine buffer.

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Attempts to Transmit Infectious Mononucleosis to Rhesus Monkeys and Marmosets and to Isolate Herpes-Like Virus (33478)

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The viral etiology of infectious mononucleosis (IM) has been postulated for many years but attempts to isolate the etiologic agent or to transmit the disease to experimental animals have been unsuccessful (1, 2). Recently published serologic evidence (3–5) suggests a possible etiologic relationship of a herpes-like virus (EBV) to IM. Our previous serologic studies demonstrated that several species of nonhuman primates had a significant incidence of complement-fixing antibodies to EBV or to a closely related virus (6). These findings suggested that immunity to infection could account for the failures to transmit IM to nonhuman primates. This report gives an account of renewed efforts to transmit the illness to rhesus monkeys and marmosets free of detectable EBV antibodies and to isolate in cell culture EBV from clinical materials obtained from IM patients during the acute stage of illness.

Materials and Methods. Design of experiments. A schematic presentation of the design of transmission experiments is presented in Fig. 1.

Clinical specimens. Patients with infectious mononucleosis were hospitalized at Kimbrough Hospital, Fort Meade, Maryland and at Walter Reed Army Hospital, Washington, D. C. The clinical diagnosis in each case was confirmed by the following laboratory findings: positive heterophile antibody test after guinea pig kidney absorption of the serum, lymphocytosis, presence of atypical lymphocytes, and elevation of serum transaminase levels.

A swab of the tonsillar region and a throat gargle in 10 ml of medium RPMI 1640 (7) containing 10% heat inactivated fetal calf serum were obtained. In most cases 50 ml of blood was collected in ACD anticoagulant solution (0.8% citric acid, 2.2% sodium citrate, 2.45% dextrose) and another 10-ml portion of blood was allowed to clot for the collection of serum which was separated and refrigerated. The citrated blood sample was centrifuged at 1500 rpm for 10 min, the layer of buffy coat cells and some of the plasma were transferred to 16 × 100-mm test tubes and incubated in upright position at 37° for 3 hr during which time most of the erythrocytes settled. The buffy coat cells were aspirated and counted in a hemocytometer. Depending on the total yield of cells available a