Multiplication of Machupo Virus, the Etiological Agent of Bolivian Hemorrhagic Fever, in Cell Cultures* (33560)

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(Introduced by Thomas G. Ward)

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Cytopathic effects (CPE) of Machupo virus (1), the etiological agent of Bolivian hemorrhagic fever, in human embryonic lung (WI-26) has been reported (2). However, others failed to confirm (3). Machupo virus has been shown to produce plaques in continuous embryonic rabbit kidney (MA-111) (4) cell culture under agar overlay.

This report presents the results of screening various cell culture systems for susceptibility to Machupo virus. It was found that Machupo virus not only multiplies to high titer in a line of African green monkey kidney (MK) cells (Vero), but also forms well defined plaques. We also studied some characteristics of Machupo virus infection in Vero cells.

Materials and Methods. Virus. The Machupo virus used was the Carvallo strain, which was obtained from the Middle America Research Unit, Canal Zone. It was isolated in suckling hamster (SH), and had been passed five times in SH. The strain was cloned once by plaque selection in Vero cells, and used in most experiments.

Immune sera. Antiserum for Machupo, Tacaribe, and Junin viruses was obtained from rabbits inoculated intravenously 3–5 times at 7–10-day intervals with a suspension of infected SH or suckling mouse (SM) brain diluted 10^{-3} in medium 199 (M-199) (5). Rabbits were bled prior to inoculation.

Tissue cultures. Cell cultures of primary hamster kidney, continuous embryonic rhesus monkey kidney (MA-111), MA-104, and human embryonic lung (WI-26 and WI-38) were obtained from Microbiological Associates, Inc. A line of African green monkey kidney (BSC-1) (6) (passage 34) was received from Dr. H. E. Hopps, Bethesda, Maryland. These cell cultures were maintained in Eagle's minimum essential medium in Earle's balanced salt solution containing 3% fetal calf serum and antibiotics (penicillin, 100 units; and streptomycin, 100 μg /ml). Vero cells (7) were obtained from Dr. Y. Yasumura, Chiba, Japan, in 1964. In this laboratory, the cells have been subcultured and maintained in M-199 containing 5% fetal calf serum and antibiotics, as previously described (8). Passage levels 107–165 of Vero cell cultures were used in this study. Serological confirmation of simian origin was carried out by Dr. C. S. Stulberg, the Child Research Center of Michigan, Detroit, Michigan (9). Medium 199 without serum was used as virus and serum diluent. The agar overlay medium consisted of 0.5% lactalbumin hydrolysate in Earle's salt solution, 2% fetal bovine serum, 0.23% $NaHCO_3$, neutral red (1:45,000) antibiotics, and 1.5% agar.

Virus assays. Cell cultures in tubes were drained and inoculated with 0.2 ml of 10-fold serial dilution of virus. Four tubes were usually used for each dilution. After 1 hr of incubation at 37°, 1 ml of medium was added. The tubes were incubated in stationary racks at 37°. Observations for CPE were made for 14–21 days following inoculation, and fluids were changed every 5–7 days. The 50% end point was calculated by the Reed–Muench formula (10).

Plaque assay in 2-oz bottles was carried out as described elsewhere (8). Plaques were counted on days 10–12 after inoculation and results were expressed as plaque-forming units (pfu).

Neutralization tests. Immune and preim-

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mune rabbit sera were heated at 56° for 30 min. Neutralization tests were performed by mixing 0.5 ml of a virus dilution, containing an estimated 100 pfu/0.2 ml, with 0.5 ml of a 2-fold serial dilution of serum. After incubation at room temperature for 1 hr, 0.2 ml of the mixture was inoculated per bottle, and the overlay was added as described above. Titers were expressed as the highest dilution producing plaque reduction of at least 80% of the simultaneous control assay in a 1:8 dilution of preimmune or normal rabbit serum.

Relationship of virus dilution to plaque number. Starting at 10^{-5} dilution of virus stock, 0.2 ml of serial 2-fold virus dilutions were inoculated into each bottle. After 1 hr, bottles were removed from the incubator, washed twice with diluent, and overlaid with agar medium as described above.

Rate of virus multiplication in Vero cells. The methods employed in experiments on the rate of virus multiplication have been previously described in detail (8). Multiplicity of infection was 0.003.

Results. Cytopathic effects. In WI-26 and WI-38 cultures inoculated with Machupo virus in a low dilution, early CPE was evident within 5–7 days. Morphological changes in cells can be described as nonspecific degeneration as reported by Webb (2), and resulted in complete destruction of the cell monolayer 4–7 days after initial appearance of early focal lesions. However, titers obtained by CPE end points in both cell cultures were very low, ranging from 10^{-3} to $10^{-3.5}$/ml. No CPE was observed in monolayer tube cultures of primary hamster kidney, MA-111, MA-104, BS-C-1, or Vero cells.

Plaque assays. Figure 1 shows the plaques produced by Machupo virus at 10^{-5} dilutions in Vero bottle cultures, 12 days after seeding. Small, irregularly shaped plaques were visible as early as days 6 and 7 after inoculation. They increased in diameter to 0.5–2.0 mm by day 12 at that time, the plaques were easily counted when the bottle culture was held against a white background. There was little or no change in counts after day 12. The plaques were a little larger than those of Tacaribe or Junin viruses (8, 11), and often included a number of cells which retained the neutral red stain in the center of the plaques (Fig. 1). Machupo virus plaques were also obtained in MA-111 bottle cultures by using the overlay described above. However, the plaques were not as clear, and the boundaries were not as distinct, as those in the Vero monolayers. Repeated plaque titration of the same stock virus indicated that the titers obtained in Vero cells give results at least 0.5 log higher than those obtained in MA-111 cells.

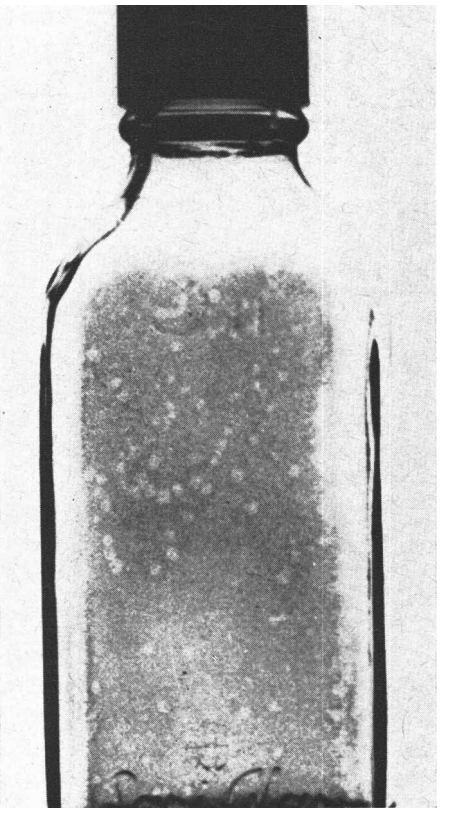

FIG. 1. Plaques produced by Machupo virus at 10^{-5} dilutions in Vero cell cultures, 12 days after inoculation.

Serial passages. Over a period of a month, 5 serial subcultures of Machupo virus were made in Vero cultures, resulting in a cumulative final dilution of the original inoculum of 10^{-16} (Table I). Subcultures from infected to new cultures were carried out at 7-day

TABLE I. Serial Passages of Machupo Virus (Carvallo strain) in Vero Cells.

Passage level	Cumulative $\log_{10}$ dilution of original inoculum[a]	Cumulative no. of days in tissue culture	Log_{10} virus titer (pfu/ml)
SH/6[c]			7.6[b]
SH/6, Vero/1	4	7	7.6
Vero/2	7	14	7.3
Vero/3	10	21	6.8
Vero/4	13	28	7.2
Vero/5	16	35	6.0

[a] Original inoculum was a suspension of infected hamster brain.

[b] ($10^{-7.1}$ pfu/ml) in MA-111 cells.

[c] SH = suckling hamster.

intervals, and no definite CPE was observed during these passages.

The relationship between plaque count and virus concentration is shown in Table II. A linear relationship was observed between the number of plaques and the virus concentration, a result indicating that each plaque was produced by a single virus particle (12).

Rate of virus adsorption. Vero cell monolayers in drained bottle cultures were inoculated with approximately 100 pfu of virus in 0.2 ml aliquots. These were incubated at 37° with intermittent turning to prevent drying of cells and to improve plaque distribution of virus seed. At various intervals bottles were removed from the incubator, washed, and overlaid with agar medium.

Approximately 40% of the virus was adsorbed within 30 min, and the maximum adsorption occurred in 120 min at 37° (Fig. 2). The same rate of adsorption of Tacaribe and Junin virus was obtained in Vero cells (8, 11).

TABLE II. Relationship between Plaque Count and Virus Concentration.

Virus conc ($\times 10^{-5}$)	Plaques/bottle		Log_{10} virus titer (pfu/0.2 ml)
	No.	Av	
1	71, 66	68.5	6.8
½	33, 31	32	6.8
¼	18, 15	16.5	6.8
⅛	9, 6	7.5	6.8

Virus replication. The results of the growth experiment are shown in Fig. 3. These data were obtained by titrating the entire group of specimens together on two different days and averaging the titers. Although different lots of Vero cells were used, the titers obtained on the two occasions were nearly the same. The peak titer of 4.0×10^7 pfu/ml was obtained on day 5 when Vero cultures were infected at the input multiplicity of 0.003.

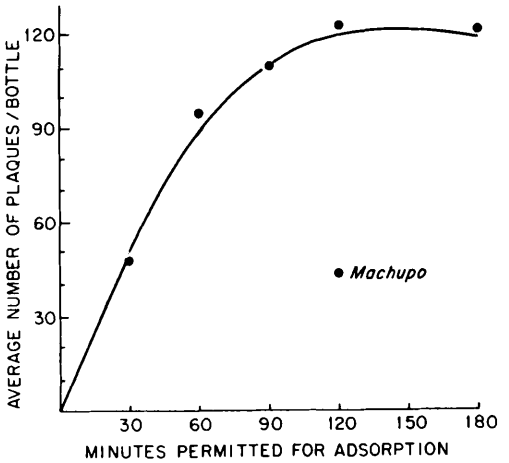

FIG. 2. The adsorption of Machupo virus to Vero cells as a function of time.

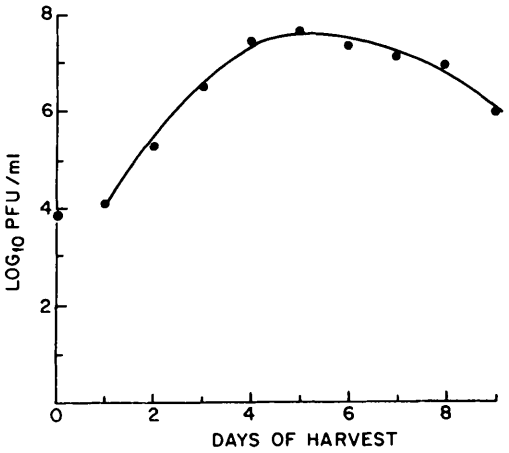

FIG. 3. Growth curve of Machupo virus in Vero cells. The multiplicity of input = 5×10^5 PFU/1.6×10^6 cells, or 0.003.

Neutralization tests. The development of plaques by Machupo virus was inhibited by type-specific immune rabbit serum. When an

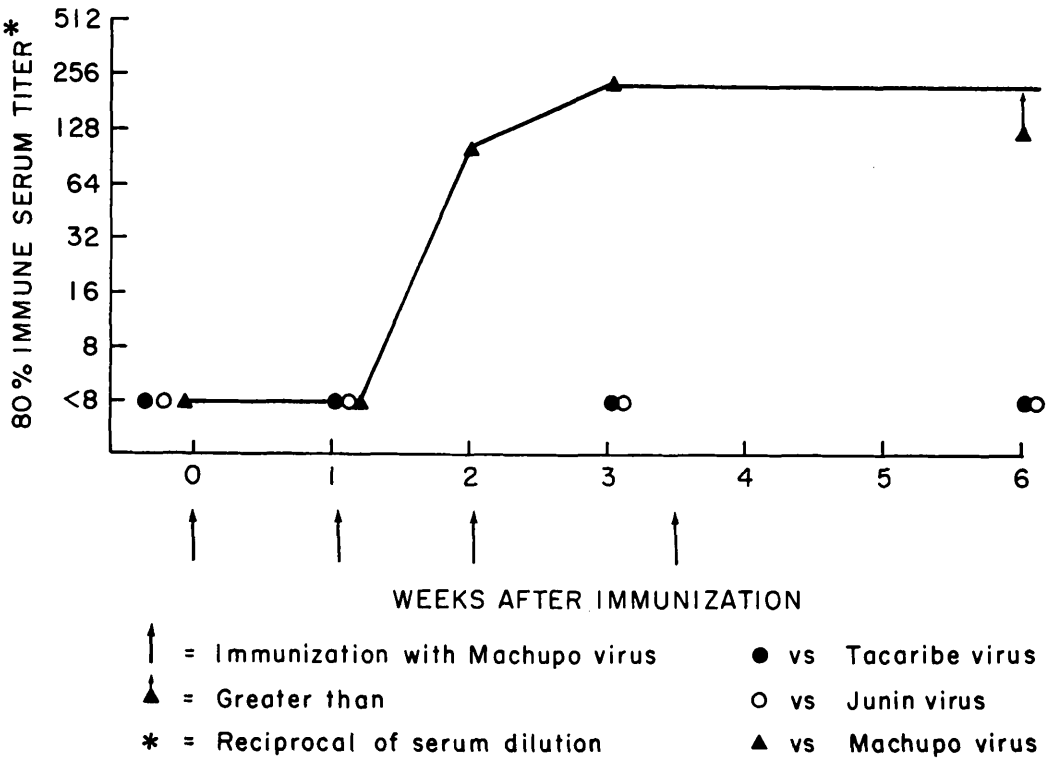

FIG. 4. Plaque neutralization with sera from Machupo immunized rabbit.

average dose of 62 pfu of Machupo virus was used, 80% plaque reduction was obtained with 1:220 dilution of Machupo virus antiserum. Plaque reduction was not obtained with a 1:8 dilution of normal (preimmunization) Tacaribe virus or Junin virus antiserum.

The rabbit serum prepared against Machupo virus and taken weekly during immunization was measured against Machupo, Tacaribe, and Junin virus antibodies. As shown in Fig. 4, no heterologous neutralization was observed in the rabbit sera taken at different periods during the immunization.

Five individual adult human sera have been tested against Machupo, Tacaribe, and Junin virus neutralizing antibodies. A serum from the convalescent, but not acute, phase of Bolivian hemorrhagic fever had neutralizing capacity against Machupo virus, and the others were negative. A convalescing human serum from a Korean hemorrhagic fever patient failed to show any neutralizing antibodies against Machupo, Junin, or Tacaribe virus.

Discussion. A line of African green monkey kidney (*Cercopithecus aethiops*) cells (Vero) appears to be the most useful *in vitro* system yet reported for Machupo virus. Machupo virus multiplied to high titer without CPE, and proved to be reproducible in 5 serial subcultures. Moreover, clear, well-defined plaques were produced. A linear relationship was observed between plaque counts and virus concentration, permitting precise quantitative virus assay.

Several investigators reported failure of Machupo virus to multiply in HeLa, WI-26 and continuous hamster kidney (BHK-21) cells (2, 3). Machupo virus CPE was reported in WI-26 cell cultures (2) and confirmed. However, titers obtained by CPE in WI-26 and WI-38 cells were very low. Other cell cultures, including primary hamster kidney, MA-111, MA-104, and BS-C-1, were examined without demonstration of CPE by Ma-

chupo virus infection. Machupo virus was also shown to produce plaques under agar overlay in MA-111 cell cultures (3, 4). It was reported that plaque counts in MA-111 cells were not constantly in direct proportion to virus concentration, but pfu assays were reproducible and comparable to SH assay (3). Machupo virus not only multiplies to high titer in Vero cells, but also forms well-defined plaques. The plaques produced in Vero cells were better defined and clearer than those produced in MA-111 cells; and the pfu titers obtained in Vero cells were higher than those obtained in MA-111 cells. Thus, under our laboratory conditions, the Vero cell line proved to be the most useful assay system for Machupo virus. Further, Vero cells are a continuous cell line easily maintained in the laboratory. In addition, titration in Vero cultures also provides a rapid differential diagnosis between Machupo and Junin virus in cases of hemorrhagic fever in South America. Junin virus, the etiological agent of Argentinian hemorrhagic fever, has been shown to produce plaques and CPE in Vero cells (11).

In the growth experiment, the maximum titer was obtained on day 5 after inoculation when Vero cultures were infected at the input multiplicity of 0.003. This slow appearance of virus may reflect the low multiplicity of infection used in this experiment. It has been reported that the rate of virus multiplication in fluid cultures did not correspond with the rate of plaque development in arbovirus tissue culture systems (13). However, in this study, the rate of virus multiplication in fluid cultures did correspond to the rate of plaque development in Vero cells under agar overlay, as also in the cases of Tacaribe of Junin–Vero cell systems (8, 11).

It was reported that Machupo virus is closely related to Tacaribe and Junin viruses by the CF test (1, 3, 14). However, they can be differentiated by the plaque neutralization test (1, 3, 4). Plaque reduction neutralization tests of immune rabbit sera and human sera in Vero cells were readily performed and confirmed earlier findings (1, 3, 4). The development of Machupo plaques was not inhibited by a serum from a human patient convalescing from Korean hemorrhagic fever. It has been reported that human convalescing sera from Korean hemorrhagic fever failed to react with a Junin CF antigen (14).

Summary. Multiplication of Machupo virus, the etiological agent of Bolivian hemorrhagic fever, was studied in a variety of cell cultures. One line of African green monkey kidney cells (Vero) supported the multiplication of the virus to high titer, but developed no cytopathic effect. Machupo virus produced well-defined plaques in monolayer cultures of Vero cells under agar overlay. The plaques produced in Vero cells were better defined and clearer than those produced in continuous newborn rabbit kidney (MA-111) cells, and the pfu titers obtained in Vero cells were higher than those obtained in MA-111 cells. A linear relationship was observed between plaque counts and virus concentration. Maximum adsorption was obtained by 120-min incubation at 37°. When the cultures were infected with 0.003 pfu per cell, the maximum virus titer was obtained on day 5 after inoculation. Neutralization tests employing 80% plaque reduction were readily performed and indicated the absence of antibody for Machupo virus in the Junin and Tacaribe antisera that were tested. Neutralizing antibody response with human sera indicated the same.

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Uptake and Storage of Lung Histamine in Burn Shock* (33561)

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Apart from direct injury of the respiratory tract, the untreated patient with extensive burns is likely to develop pulmonary complications characterized by tachypnea, atelectasis, pulmonary edema, and pneumonia (1–3). Alterations in pulmonary compliance due to degeneration of lung surfactant has been among the causes proposed for pulmonary complications appearing in burn shock (4, 5). On the other hand, thermal injury of tissue is known to be accompanied by the endogenous release of histamine, serotonin, and bradykinin (6, 7), substances proposed as being responsible for vascular permeability responses to injury (8).

The rise in the blood level of histamine in burn shock (9, 10) may play a role in altering pulmonary function, since histamine in the experimental animal has often been cited as the humoral agent responsible for the production of pulmonary edema by epinephrine (11). The apparent lack of information on this aspect of burn shock led us to study the rate of rise in blood and lung histamine content in anesthetized dogs subjected to thermal injury.

Materials and Methods. The hair was shaved from the xiphoid to the base of the tail in 18 mongrel dogs anesthetized with 30 mg/kg of sodium pentobarbital. Burn shock was induced by exposing the lower half of the dog's ventral surface to heat from infrared lamps. A full thickness burn of the skin, verified by histological studies, was produced by exposure to the infrared heat for 10 sec/kg of dog weight. Systolic and diastolic blood pressures were measured with a pressure transducer connected to the left branchial artery. Changes in hematocrit during the course of burn shock were measured from arterial blood samples every 30 min. Blood samples for measuring arteriovenous blood differences of histamine (H) across the lungs were drawn simultaneously from catheters inserted into the right and left ventricles, via the right external jugular vein and left common carotid artery respectively. H levels in blood entering and leaving the lungs were determined during a control period and 15, 60, 180, and 240 min in the postburn period. After 4 hr of burn shock, approximately 2 g of lung tissue from the peripheral border of the left cardiac lobe was excised, frozen in liquid N_2 and analyzed in duplicate for H. Analysis of H in both blood and tissue samples was performed in accordance with the method of Shore *et al.* (12). The H concentration in blood-free lung tissue was calculated by subtracting the H content of blood trapped in the tissue sample from the total tissue concentration of H. Blood was determined by its conversion of hemoglobin to

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