Virus Isolations from Human Cases of Hemorrhagic Fever in Bolivia. (29772)

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An acute febrile disease with frequent hemorrhagic diathesis was recognized in the eastern prairie Department of Beni, Bolivia, in 1959. Clinical and serological evidence obtained in 1962(1,2) suggested that this disease was remarkably similar to that first reported in Argentina in 1955(3). The Argentine syndrome was subsequently shown to be caused by an agent designated Junin virus (4). In May 1963 a laboratory was established in San Joaquin, Bolivia, to study the etiology, epidemiology and ecology of the disease which was epidemic in that town. It is our purpose here to report the isolation of several strains of a virus from hemorrhagic fever patients, and to describe certain properties of the isolate selected as the prototype virus strain.

Materials and methods. Laboratory facilities. Since virus isolation was to be attempted within the confines of the small town where the disease was epidemic, and since it was suspected that the infectious agent might have a natural cycle involving infection of rodents and arthropods which were abundant in the town, all feasible measures were taken to exclude the inadvertent introduction of such an agent into the laboratory, and to protect workers from potential infection by aerosols. Laboratory buildings with brick floors and tight roofs were fitted with screens on windows and doors. Floors, walls and ceilings were sprayed with 10% DDT. A separate building housed the animal breeding colony. Zinc phosphide baits were maintained in all quarters to prevent wild rodents from entering, or laboratory rodents from escaping, the laboratory. Food and bedding were fumigated with methyl bromide, sealed in plastic bags and shipped by air from the Canal Zone. All water was boiled. Separate boxes and water bottles were maintained for breeding and inoculated animals. Processing and inoculation of specimens were carried out in specially designed plastic isolettes (Reyniers & Son, Chicago, Ill.), which were maintained at a mild negative pressure and exhausted to the air at roof level. This chamber was decontaminated before being opened at the end of each procedure by spraying it with 40 ml of a 12.5% solution of peracetic acid in a 0.1% aqueous solution of sodiumalkylaryl sulfonate. Inoculated animals were kept in a separate room; cages were fitted with special lids lined with FG-50 filter material (supplied by American Air Filter Co., Bethesda, Md.). Inoculated animals were examined daily using forceps inside standard Reyniers type B plastic isolettes also maintained at mild negative pressure. Primary identification and sorting of wild animals and arthropod materials were carried out in separate buildings 75 yards from the virus laboratory.

Collection and inoculation of specimens. Blood specimens were collected in 7 ml Ten-Broeck grinders, refrigerated and macerated, or placed in sterile tubes, containing 0.03 mg of heparin in sterile saline per ml of blood. Tissue specimens were prepared as 10% suspensions in phosphate buffered saline pH 7.5 (PBS) containing 25% heat inactivated normal rabbit serum, 500 units penicillin, and 500 μg streptomycin per ml. After centrifugation at 2500 rpm for 15 minutes, the supernatant fluids were saved as specimens. All specimens were inoculated into animals without prior freezing, after which the remaining portions were rapidly frozen and stored in liquid nitrogen (—196°C). Infant mice not more than 2 days of age and/or infant hamsters less than 5 days old were inoculated intracerebrally (IC) and intraperi-

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toneally (IP) with 0.02 ml and 0.03 ml, respectively, of specimen fluids. Animals were examined daily for signs of illness for a period of 17-21 days. Sick animals were harvested and 10% organ suspensions prepared in PBS with rabbit serum for passage. All other studies, described below, were done in the Bethesda and Canal Zone laboratories.

Cell cultures. A HeLa cell line was obtained from Dr. R. P. Hanson, Dept. of Veterinary Science, University of Wisconsin, and serially propagated in Eagle's Basal Medium in Hanks' salt solution (HBME) with 10% heated calf serum. Diploid human embryonic lung fibroblasts (strain WI-26) were grown in Eagle's Minimum Essential Medium in Hanks' salt solution (HMEM), with 10% calf serum. A continuous cell line of newborn rabbit kidney (MA-111), supplied by Mr. Monroe Vincent (Microbiological Associates, Inc., Bethesda, Md.), was grown in M-199 with 5% fetal calf serum. For maintenance of cultures, serum concentration was reduced to 2%. In certain instances, chicken serum was substituted for calf serum. All media contained 100 units penicillin and 100 μg streptomycin per ml. Cultures in fluid medium were incubated at 33-34°C, and tubes were rotated in a drum at 12 revolutions per hour. MA-111 plaque bottles were incubated at 36.5°C after agar overlay.

Thioglycollate broth cultures were inoculated with samples of both virus and cell culture preparations for bacterial sterility control. Agar plates and broth tubes of medium for Mycoplasma (PPLO)(5) were also inoculated and the broth cultures subinoculated onto agar plates after incubation at 37°C for one week.

Chloroform sensitivity and size estimation. Infected brain suspensions were mixed with 1/10 volume of chloroform and incubated 30 minutes at room temperature with frequent agitation on a vortex mixer. Suspensions were then centrifuged at 2500 rpm for 20 minutes and supernatant fluids inoculated into newborn hamsters employing 10-fold dilutions in PBS containing 10% rabbit serum. Size estimation was carried out using gradocol membranes according to a previously described procedure(6). Starting material was clarified 1% infected brain in borate saline pH 8.

Serological tests. The complement fixation (CF) test used was a modified Bengston procedure employing overnight reaction of antigen, complement and antibody at 4°C. Two full units of complement were used. Antigens were prepared as crude tissue extracts in borate saline pH 9, or were extracted with sucrose and acetone. The XJ strain of Junin virus and TR 11573 strain of Tacaribe virus were obtained from the Rockefeller Foundation Virus Laboratory, New York. Virus seed pools and sucrose acetone extracted antigens were prepared from infected mouse brains by methods already described(7). Control antigens were made from similarly treated uninfected brains. Antisera were taken from hamsters after 3-4 injections of infected hamster brain suspension that did not contain rabbit serum. Immune ascitic fluids were made in mice by repeated IP injections (usually 4-5) of infected mouse brain suspension emulsified with complete Freund's adjuvant. Antisera were obtained from rabbits by bleeding mature males after 4 weekly injections of infected hamster brain suspension. Hemagglutination and hemadsorption trials were carried out according to methods described elsewhere(7,8).

Plaque neutralization tests were done in 2 oz bottle cultures of MA-111 cells. Serial dilutions of virus suspension were mixed with equal volumes of heat-inactivated (56°C for 30 minutes) and pre-diluted antiserum (1/5), and incubated for one-half hour at 37°C. Bottle cultures were washed, drained, inoculated with 0.2 ml of serum-virus mixture and incubated for one hour at 37°C. The monolayers were then washed 5 times, drained, and 5 ml of an Earle's-lactalbumin-agar overlay medium was added. This medium contained 2% fetal calf serum and neutral red at final concentration of 1:45,000.‡ Cultures

‡ This medium was modified from the basic formula of Hsiung and Melnick(9) by Drs. W. Downs and J. Henderson, Yale University School of Med., New Haven., Conn., and used for studies of both plaque production and neutralization of Tacaribe and Junin viruses in primary rhesus monkey kidney cell cultures.

TABLE I. Isolation of Serologically Similar Virus Strains from Human Cases of Hemorrhagic Fever, San Joaquin, Bolivia, May-July 1963.

Subject	Age	Sex	Specimen	Day disease obtained	Clinical course	Isolation animal	Reisolation: Hamster	Reisolation: Mouse	Reisolation: WI-26 cell culture
O.C.	2	♂	Spleen	8	Died day 8	Hamster	+	+	+
T.V.	8	♀	"	9	" " 9	Mouse	+	+	+
C.G.	53	♂	"	12	" " 12	"	+	+	+
S.M.	60	♂	Blood	4	" " 10	"	+	—	+
D.E.	20	♀	"	2	Survived*	Hamster	+	—	—

* Complement fixing antibody titers of <1:2 and 1:64 in acute and convalescent sera, respectively.

were incubated in the dark at 36.5°C, and plaques counted after 6-14 days.

Results. Isolation of virus strains. Agents pathogenic for newborn hamsters or mice were recovered in San Joaquin from 5 of 20 human specimens taken from 16 patients. Source, isolation system and results of reisolation attempts on these strains in the Canal Zone laboratory are summarized in Table I. Each isolation was confirmed by reisolation. Virus was isolated from each of 3 cases in which autopsy tissue was tested. Only 2 isolations were obtained from bloods of 13 patients tested. Two of these patients ultimately succumbed. Thus, virus was isolated from the blood of one of 2 fatal cases, and from one of 11 who survived. It should be noted that the blood specimens had been obtained as early as possible after onset of illness, usually within the first 5 days.

Each of the isolates produced similar clinical disease in laboratory animals. Relationships by complement fixation test among these strains are shown in Table II. Since no evidence of significant strain variation was observed, the original isolate, designated Carvallo, was selected as the tentative prototype.

TABLE II. Relationships by Complement Fixation Test Among 5 Viruses Recovered from Human Cases of Hemorrhagic Fever.

Antigen	Antiserum O.C.	T.V.	C.G.	S.M.	D.E.
O.C.	*256**	128	256	256	128
T.V.	1024	*512*	512	1024	512
C.G.	512	512	*512*	1024	1024
S.M.	512	512	512	*512*	512
D.E.	512	256	256	512	*256*
Normal hamster brain	<2	<2	<2	<2	<2

* Reciprocal of antibody titer in immune hamster serum.

All further work reported here refers to this strain.

Animal pathogenicity. Infant hamsters and mice inoculated with infected brain or spleen suspensions typically exhibited signs of illness between 7 and 18 days later. These consisted of growth retardation, dull rough fur, and incoordination. After being twirled by the tail many exhibited tonic and clonic convulsions, ataxic circling gait and, at times, intermittent apnea and rigidity. Mortality, however, was highly variable even after 10 serial passages, and there were often numerous survivors in litters infected with large doses of virus. This pattern was more common in mice than hamsters, but was partially compensated by more conspicuous neurological symptoms in the mice. Relative "zoning" also was observed frequently in which initial dilutions of high titered inocula produced only sporadic illness and few deaths. After 3 to 4 weeks, surviving infected hamsters (CF antibody positive) were clearly smaller than uninfected control animals or littermates (CF antibody negative). Mice that recovered from infection could not be distinguished from controls. Although a quantitative difference in sensitivity between hamsters and mice was not established with serially passaged virus, variation in titer was clearly greater when assays were done in mice. For this reason, hamsters were considered the animal of choice for virus assay.

Virus titers averaging 10^7 to 10^8 LD_{50}/g were found in infected brains and spleens of sick animals. No evidence of gross hemorrhagic disease was noted at autopsy of sick or dead animals. The virus was pathogenic when administered by IC or IP routes, although preliminary studies suggested that pe-

ripheral inoculation was less efficient for virus detection. Both mice and hamsters of more than 4 weeks of age generally survived inoculation by either route without evidence of clinical illness, but CF antibodies were readily demonstrable. More detailed studies of pathogenicity of the virus in these and other hosts are in progress.

Observations in cell cultures. No definite CPE was seen in HeLa or MA-111 cell cultures in fluid medium despite inoculation of large or small amounts of virus at several different animal passage levels. Plaques were observed in bottle cultures of MA-111 cells overlaid with agar containing neutral red. The characteristics of Carvallo virus infection, plaque production and neutralization in this cell system will be described in detail elsewhere. Plaque production between 6-10 days was reproducible and assays of plaque forming units (PFU) were comparable to infectivity assays in suckling hamsters.

Infected WI-26 cultures developed a definite, reproducible CPE. Initial changes consisted of fine dark granules in the cells accompanied by a general reduction of the refractility of the cell sheet. Within 2-3 days, nearly complete destruction of the sheet was observed. This degeneration was rather nondescript, marked by widespread cellular distortion and fragmentation, and the absence of rounding, enlargement or coalescence of affected cells. Progression of effect was more rapid in rotated, as compared to stationary, cultures. First changes were usually noted 4-6 days after inoculation; endpoints were reached by the 9th-14th day. Virus titers were comparable to those achieved in animals, and were more reproducible. Serial passages were easily made in WI-26 cultures, and a similar CPE was noted after cumulative dilution of starting material that represented a factor of greater than 10^{-20}. Quantities of virus produced in such cultures, however, were generally 100- to 1,000-fold lower than in animals, and CF antigens were not detected in tissue culture fluids. Tissue culture grown virus was reidentified by inoculation of newborn hamsters and testing of CF antigens prepared from their brains 7-10 days later.

Other properties. Infectivity was completely abolished after treatment with chloroform, whereas a simultaneous control titration in mice revealed presence of $10^{7.1}$ ID_{50}/g of brain. The virus passed through a gradocol membrane of 300 mμ APD but not 200 mμ or 110 mμ membranes. Applying Black's formula(10) for size estimation, this suggests the agent to be between 120 and 180 mμ. The data are to be construed as provisional, however, since the titer of the preparation filtered was only about 10^3 tissue culture doses (TCD_{50}). Passage through the smaller pore membranes took at least 24 hours under 15 psi N_2 pressure, suggesting that the brain suspension may have partially clogged the membranes. Further work may be expected to result in a smaller size value.

Since the provisional size range of the Carvallo agent was compatible with that of PPLO, the infected hamster brain pool (3rd passage) was tested on both solid and broth PPLO medium. There was no recognizable growth, even after subculture of broth medium onto agar plates. A preparation of second hamster passage virus was successfully lyophilized without apparent loss of infectivity. Hamster brain suspensions in PBS + 25% rabbit serum have not lost measurable infectivity during a 4-month period of storage at —70°C. More detailed studies of the biophysical properties of the agent are continuing.

Serological properties. Complement fixing antigens were readily prepared from brains of sick animals. Crude alkaline extracts were found to be stable for up to 3 months at 4°C. Such antigens were used for routine reidentification of the virus, using an immune hamster serum or ascitic fluid as source of antibody. It was also noted that chloroform treatment of such antigens destroyed infectivity without significantly altering antigen titers.

For comparison with other known viruses, sucrose-acetone extracted antigens were employed. The CF relationships of the Carvallo strain to Tacaribe and Junin viruses are presented in Fig. 1. The 3 agents were also tested in reciprocal plaque neutralization tests. As shown in Table III, these viruses

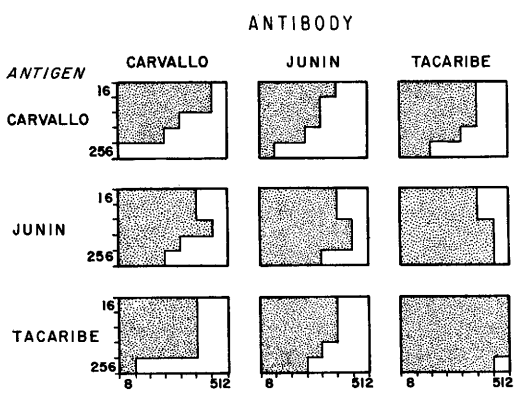

FIG. 1. Relationships by complement fixation test between Carvallo strain, Junin and Tacaribe viruses and hamster immune serum (Carvallo), and immune mouse ascitic fluids (Junin and Tacaribe). Shaded areas represent 2-plus or greater fixation of 2 full units of C′. The antisera did not react with normal brain antigens.

are distinctly different. The only observed cross reaction was slight reduction of Junin plaques by Tacaribe immune ascitic fluid.

Preliminary attempts to demonstrate a viral hemagglutinin were unsuccessful. Infected WI-26 cultures failed to hemadsorb guinea pig, chick, human or goose cells at 4° and 37°C.

Discussion. The isolation of several antigenically similar virus strains from persons suffering from hemorrhagic fever suggests that this agent is the etiologic agent of the disease. This conclusion is further strengthened by the following considerations: (1) each strain was successfully reisolated in another laboratory (MARU, Canal Zone); (2) no known related virus was under study in the isolation laboratory; (3) three of the strains, including the first one recovered, were obtained from tissues taken at autopsy; and (4) the single surviving virus donor developed CF antibodies to the prototype strain during convalescence.

TABLE III. Relationships Between Carvallo Isolate and Junin and Tacaribe Viruses by Plaque Neutralization* in Rabbit Kidney (MA-111) Cell Cultures.

Virus	Carvallo rabbit serum	Junin mouse ascitic fluid	Tacaribe mouse ascitic fluid
Carvallo	*3.0*	0	0
Junin	0	*4.0*	1.0
Tacaribe	0	0	*3.5*

* Constant serum plus varying virus dilutions incubated 37°C for 30 min and plaque reduction expressed as $\log_{10}$ neutralization index compared to normal rabbit serum or mouse ascitic fluid. Figures are mean values of repeated tests, rounded off to nearest 0.5 $\log_{10}$.

Further epidemiological documentation of the relationship between virus infection and clinical hemorrhagic fever will be presented elsewhere.

The characteristics of the Carvallo isolate were found to be generally similar to those described for Junin and Tacaribe viruses (11,12). The pattern and morphology of CPE produced in WI-26 cultures were indistinguishable from those of Junin virus. These findings, and the observed close relationship by CF among Carvallo, Junin and Tacaribe viruses, were not unexpected since human sera obtained from Bolivians convalescent from hemorrhagic fever in 1962 had been shown to react with Junin and Tacaribe antigens(2). The relationship between Junin and Tacaribe viruses had already been documented(13). The lack of relationship among the three viruses by neutralization test, however, suggests that they are clearly distinguishable agents. This conclusion also has been previously recorded in the case of Junin and Tacaribe viruses(13). Although the properties described for these 3 viruses are compatible with their designation as members of the arbovirus family, it is worth noting that direct evidence so far accumulated for such a hypothesis is very meager. It consists of a single isolation of Tacaribe virus from a mixed pool of 344 mosquitoes(12) and reports of recovery of Junin virus from one pool of mites subsequently identified as *Haemolaelaps glasgowi* (Ewing)(11,14). While solid evidence for the role of some arthropod or arthropods in transmission of these agents may ultimately be obtained, it should be remembered that the presently known properties of each of these viruses would also qualify them for candidate membership in a number of other virus families which infect man and/or animals. In any case, it is proposed to name the virus of Bolivian hemorrhagic fever Machupo Virus after the tributary of the Itenez River that

flows through the present epidemic area of the Department of Beni.

Summary. Five serologically similar virus strains were recovered in San Joaquin, Bolivia, in 1963 from humans suffering from a newly recognized febrile disease with hemorrhagic manifestations. Three isolates were from splenic tissue obtained at autopsy and 2 from blood specimens taken from acutely ill patients. The prototype strain (Carvallo) was pathogenic for newborn mice and hamsters, produced cytopathic effects in WI-26 cell cultures and plaques in MA-111 cell cultures, was inactivated by chloroform, and was estimated to be 180 mμ or less in size. It was nearly indistinguishable from Junin and Tacaribe viruses by complement fixation test, but completely distinct from these agents by neutralization test. It is proposed that these strains collectively be named Machupo Virus.

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Antibodies in Human Coccidioidomycosis: Immunoelectrophoretic Properties.* (29773)

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In clinically apparent coccidioidomycosis in humans there is a consistent appearance of precipitins and complement-fixing (CF) antibodies. While precipitins have only been useful diagnostically, the titer of CF antibodies, proportional to the severity of the infection, is also of value prognostically(1,2).

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