The Measurement of Specific Antibodies in Bolivian Hemorrhagic Fever by Neutralization of Virus Plaques (33711)

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Complement-fixing (CF) antibodies to Machupo virus, etiologic agent of Bolivian hemorrhagic fever (BHF), have been detected in human and animal sera obtained from certain confined areas of Bolivia, particularly the Beni Province (1). It was found, however, that such antibodies were not sufficiently specific to distinguish Machupo virus infections from those induced by other viruses of the Tacaribe group (1), particularly Junín virus, the causative agent of Argentine hemorrhagic fever. Measurement of antibodies in animal neutralization (N) tests using illness or death of the animal as an end point has been unreliable (2). The CF technique proved to be of limited value for testing many rodent sera because of poor fixation of complement and the high frequency of anticomplementary activity in sera, especially from the rodent *Calomys callosus*. This is of particular significance since this rodent has been incriminated in the transmission of the virus of BHF (3, 4). To be able to detect naturally acquired antibody in this species seemed of obvious importance in understanding the natural history of infection in the *Calomys*, and a reliable method to measure specific antibodies was sought. The finding by other workers that plaques were formed under agar in certain types of cultured cells (5–7) led to the recognition of four distinct members of the Tacaribe virus group (8), and provided the basis for a simple method to measure type-specific antibodies to the Tacaribe-group viruses by neutralization of virus plaques in Vero cell cultures. Using this test, a comparison was made between temporal patterns of N and CF antibodies in humans infected with Machupo virus. The technique also was useful for the detection of type-specific antibodies in various animals, and for identification of virus strains.

Materials and Methods. Vero, a continuous line of cells derived from *Cercopithecus aethiops* kidney (9) was received from Dr. N. H. Wiebenga, NIH, Bethesda, at passage (P) 104. A pool of cells was frozen in culture medium containing 10% glycerine and stored in liquid nitrogen at P 106. Sublines were started at regular intervals in order that all work was done with cells between the limits of P 116 and P 146. Frequent attempts to isolate mycoplasma from cell cultures utilizing methods outlined by Hayflick (10) were uniformly negative. Cells were cultivated and maintained as described previously (11). Monolayers were prepared either in 60 × 15 mm plastic petri dishes or in 8 × 12 in. disposable plastic trays (Linbro 96CF clear) hereafter termed panels. Roller tube cultures of diploid human embryonic fibroblasts (strain WI-26) were grown and maintained as previously described (12), except that chicken serum was used in place of calf serum in the maintenance medium.

Virus strains. Two substrains of Machupo virus, strain Carvallo, were used. One had been isolated and passaged 7 times in infant hamsters and the other was employed after isolation and 2 further intracerebral passages in infant mice. Mouse brain pools of Junín virus, XJ strain, Tacaribe virus, strain 11573, and Amaparí virus, strain BeAn 70563 were used at passage levels of 18, 22, and 12, respectively. These agents were obtained from Dr. Jordi Casals, Yale Arbovirus Research Unit; Dr. Leslie Spence, Trinidad Regional Virus Laboratory; and Dr. Robert Shope, Belém Virus Laboratory. All viruses were stored at −60° as 10% centrifuged brain suspensions in phosphate buffered saline (PBS), pH 7.4, containing 25% normal rabbit serum.

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Immune reagents. Adult mice received a total of four weekly intraperitoneal (i.p.) inoculations of 0.1 ml of viral antigen prepared as a 20% suckling mouse brain suspension in borate saline buffer, pH 8.0, mixed with 0.1 ml of Freund's complete adjuvant. One week later, 0.1 ml of a 10% suspension of Sarcoma 180/TG cells (13) was given, followed in 3 days by inoculation of 0.1 ml of virus without adjuvant. Ascitic fluids were harvested 8–20 days thereafter, and sera were obtained by exsanguination. All sera and ascitic fluids were heated (56°, 30 min) prior to use in neutralization tests.

Infection and overlay of cultures. Petri dish monolayers were aspirated and washed three times with 5 ml of PBS, pH 7.2, or with appropriately buffered PBS in the case of pH absorption studies. Two tenths ml was added to each dish with a hand-operated automatic pipette employing disposable plastic tips (Becton-Dickinson Co., Biopette no. 1100, Biotip no. 1101). Monolayers grown in plastic panels were drained by rapid inversion and residual medium was wiped from the top surface with sterile gauze. Individual wells were inoculated with 0.05 ml of virus or virus–serum mixture. After absorption for 1 hr at 36° in a humidified atmosphere containing 4% CO_2, wells and dishes were overlaid with 0.5 or 3.0 ml of a modified Husing-Melnick medium as described previously (11). A second overlay containing 1:20,000 neutral red was added after 5 days of incubation at 36° and plaques were counted 12–24 hr later.

Neutralization test in WI cell culture tubes. Serial fourfold dilutions of test sera were made in 12 × 75 mm sterile tubes. Unheated guinea pig complement (Markham) which had been previously diluted 1:5 in PBS, pH 7.4, was used as serum diluent. Equal volumes of virus, diluted so as to contain from 30–100 $TCID_{50}$, were added to the serial serum dilutions and incubated for 2 hr at room temperature. A virus control tube containing equal volumes of virus and diluent was incubated simultaneously. At the end of the 2-hr period, 0.1 ml volumes of virus–serum mixtures were inoculated into 2–4 WI-26 cell culture tubes and incubated on a rotary drum at 33–34°. Titration of the virus was carried out at the end of the incubation period by making three serial tenfold dilutions from the virus control in PBS containing 10% complement. Three tubes were inoculated per dilution. Cell cultures were examined daily for appearance of cytopathic effect (CPE). End points were determined 10 days after inoculation of cultures and were expressed as the highest dilution (50% basis) which completely prevented appearance of viral CPE.

Plaque reduction N test in Vero cell panels. Serial fourfold dilutions of serum were made in microtiter disposable plates made of heavy clear plastic (Linbro Chemical Co.; Model 1S-MRC-96, Clear, 3.5 × 5 in.) with a cup capacity of 0.3 ml, using wire loops and droppers calibrated to deliver 0.025 ml. Initially, test sera were diluted in PBS supplemented with 10% complement, but subsequently PBS supplemented with 10% "normal"[2] unheated human serum was found to be equally suitable. When double volumes were required, dilutions were made in 5.75 × 8 in. plates (Model 96 SC Clear) with a cup capacity of 1 ml, using loops, and droppers calibrated to deliver 0.05 ml/drop. At the time diluent was dropped into the plate, 20 drops were placed into a sterile rubber-stopped 12 × 75 mm glass tube for use as the virus control. An equal volume of virus, either 0.075 or 0.15 ml per three drops was added to the plate and to the virus control tube. The virus was diluted in PBS–0.7% bovine albumin to contain 80–100 plaque-forming units (pfu)/0.05 ml. Plates were protected by styrene covers and incubation was carried out for 1 hr at room temperature with frequent gentle shaking. Duplicate wells were infected with 0.05 ml of virus–serum mixture as described above. Three fourfold dilutions were made from the virus control tube and each was inoculated into 6 wells.

Virus identification test. Field specimens were inoculated intracerebrally into suckling hamsters (SH) less than 5 days old. The

[2] Free of Tacaribe-group antibodies.

brain was removed from the sick or dead animal and a 20% suspension was made in borate saline, pH 8.0, and divided into 2 aliquots. One was kept at 4° for CF testing; normal rabbit serum was added to the second (25%) and the material was frozen at −60°. The unknown crude antigens which fixed complement when tested against 4–8 antibody units of Machupo, or one of the other Tacaribe-group immune ascitic fluids, were then considered candidate isolates. The frozen aliquot was used for virus typing. Each unknown was tested against hyperimmune mouse ascitic fluid (MAF) to Machupo, Junín, Tacaribe, and Amaparí viruses containing 12–24 homologous antibody units and against a normal MAF. Master decimal dilutions of each unknown were made in tubes and three drops of each dilution was transferred to the microdiluting trays in five replicate lines to which equal volumes of antibody were added. Known virus pools of specific titer were included and the test was carried out as described for plaque neutralization. Results were expressed as $\log_{10}$ pfu reduction.

Results. Formation of plaques. All four Tacaribe-group viruses produced grossly visible discrete plaques in Vero cell monolayers under agar 5–8 days after inoculation when the staining overlay was added on day 5. Junín and Machupo plaques averaged 1–1.5 mm in diameter and had sharp borders on day 6. No significant increase in number was observed after incubation for an additional 4 days. The plaques formed by Tacaribe and Amaparí viruses were 1 mm or less in size, with less distinct edges. Frequently Amaparí plaques were not countable until days 7 or 8 of incubation, at which time they had reached their maximum size. Incorporation of 200 mg/ml of DEAE-dextran into the overlay medium caused no obvious beneficial effects, whereas sodium dextran sulfate depressed both plaque size and sharpness.

Several comparative titrations of each agent in infant hamsters or mice and cell cultures revealed that the plaque assay was as sensitive as animals for measurement of virus infectivity using the virus pools described in this paper. Furthermore, it was much more reproducible. Ten consecutive weekly plaque titrations of a Machupo virus pool, for example, averaged 6.1 (± 0.9) × 10^7 pfu/ml.

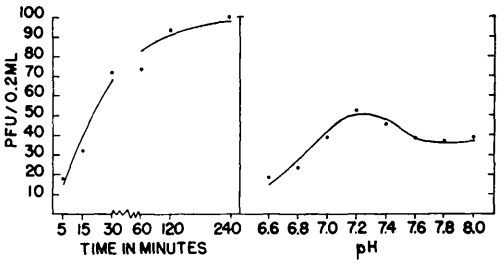

FIG. 1. Effect of time and pH on adsorption of Machupo virus in Vero cell monolayers. Temporal study was done at pH 7.2, adsorption was carried out for 2 hr in pH experiments.

Factors affecting Machupo virus plaque formation. Time and pH of adsorption. Adsorption of Machupo virus into Vero cell monolayers grown in petri dishes was carried out in a CO_2 incubator and assays were made at 5, 15, 30, 60, 120, and 240 min. The optimal time of adsorption, as shown in Fig. 1, was 4 hr. Adsorption times in excess of this were impractical due to drying out and resultant destruction of the cell sheet. Counts obtained after 2 hr varied only slightly from the optimum. The curve of adsorption at varying pH values is also shown in Fig. 1. Maximum adsorption was found at pH 7.2, but variations in plaque counts over a pH range of 7.0–8.0 was minimal. Below pH 7.0, a sharp decline in efficiency was observed.

Comparisons of the reproducibility of panel and petri dish plaque counts are summarized in Table I. Slightly higher and more

TABLE I. Comparative Plaque Assay of Machupo Virus in Petri Dishes and Plastic Panels.

	pfu[a] in indicated system			
	Panel (64 wells/dil.)		Petri dish (11 dishes/dil.)	
Dilution	Range	Av	Range	Av
10^{-4}	36–40[b]	>40	80–113	99
10^{-6}	3–13	7.3	7–15	10

[a] In 0.05 ml.

[b] Maximum countable number = 40.

TABLE II. Comparison of Plaque and Cell Culture Tube Assay of Machupo Virus Neutralizing Antibody.

Antibody source	Reciprocal titer in indicated system: Plaque reduction (Vero)[a]	Culture tube (WI-26)[b]
Human HF, acute	<4	<2
Human HF, convalescent	16	8
Human HF, convalescent	64	64
Immune hamster	512	256
Immune mouse ascitic fluid	64	64

[a] 60–80 pfu.
[b] 32–100 $TCID_{50}$.

reproducible counts were demonstrable in dishes than panels. The small surface area of the wells may account for this effect and, in any case, limited the number of plaques which can be accurately assayed to about 25.

When normal sera from various animal species were incubated with virus at 1:5 and 1:10 dilution, variable but significant plaque reduction was observed. The effect was particularly evident with sera from guinea pigs, rabbits, and 5–10% of humans. These inhibitors were heat-stable (56°, 30 min) and usually were not detectable at dilutions of 1:20 or greater. In view of this problem, a pool of normal human serum was used at 1:10 dilution in the diluent for test sera and virus titers were adjusted accordingly. When this effect was considered, it was found that complement (fresh guinea pig serum) gave no enhancement of virus neutralization, with one exception. After prolonged storage at —20°, certain hyperimmune Machupo MAF's showed a 4- to 16-fold reduction in titer, which was restored by adding 10% complement to the ascitic fluid diluent.

Virus neutralization. Using 80% or greater reduction in plaque numbers as evidence for virus neutralization, acute phase sera from cases of BHF failed to neutralize Machupo virus while convalescent sera had titers of at least 1:16. Further evidence for the specificity of the method was obtained by titration of paired sera obtained from animals experimentally infected with different Tacaribe-group viruses. Following i.p. inoculation with Machupo virus, a *Cebus* monkey, a cat, and various rodents (guinea pigs, *Proechimys guayenensis, Rattus rattus, Sigmodon* sp., *Calomys callosus*) developed homologous neutralizing antibody titers ranging from 1:32 to 1:512 or greater, 30–90 days after inoculation. Neutralizing antibodies following inoculation with Junín and Tacaribe viruses were detected in marmosets, rabbits, and the rodent *Calomys laucha.* Each animal was bled prior to inoculation, and all specimens were tested for N antibodies. None was positive. No evidence of heterologous N antibody was found in any of the postinoculation sera.

Comparison of the plaque method with the WI-26 tube method revealed close quantitative agreement (Table II). As previously reported, complement was essential in the tube test to prevent virus breakthrough and subsequent scoring of tubes as not neutralized (14).

Measurement of antibodies after Bolivian hemorrhagic fever. Complement-fixing antibodies usually appeared 3–4 weeks after the onset of BHF in humans, reached peak titers in 40–60 days, and declined over an interval

TABLE III. Comparison of CF and N Antibody Titers in Convalescent Sera from Confirmed BHF Cases.

Time after onset illness (months)	No. of sera tested	CF: GM[a]	CF: Range[b]	N: GM	N: Range	No. sera N+/CF—[c]
8–12	30	14	<2–128	64	32–512	4
13–24	36	5	<2–64	42	16–256	20

[a] Geometric mean titer.
[b] Reciprocal serum titer.
[c] CF negative = titer <1:8.

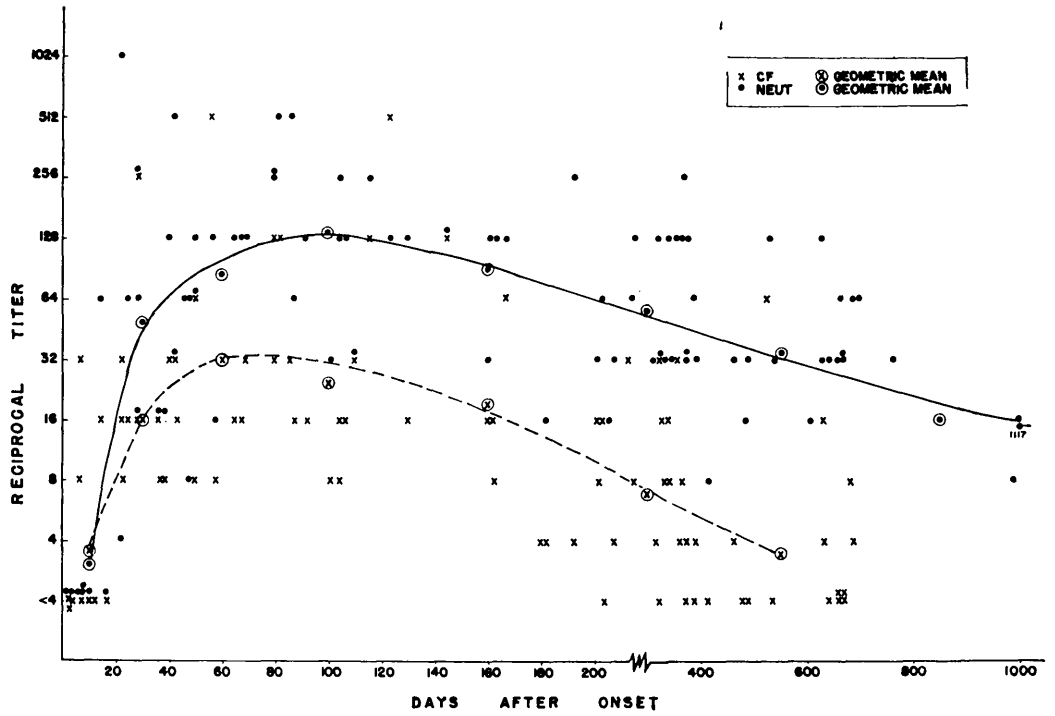

FIG. 2. Comparison of complement fixing and neutralizing antibody responses to Machupo virus in serial sera from 11 human cases of Bolivian hemorrhagic fever. Initial serum dilution tested by each method was 1:4.

of 1–3 years (1). As shown in Fig. 2, a similar temporal pattern of appearance and elaboration was found for N antibodies, with the exception that the slope of antibody decay was flatter than for CF. Indeed, as indicated by range and geometric mean titer, N antibodies persisted at detectable levels longer than CF antibodies (Table III). Titration of sera obtained from 6 persons showed that N antibodies were detectable 4 years after infection in 5 instances. Four sera from these individuals were still positive a year later. Moreover, as shown in Table IV, incubation of virus and serum for 16–18 hr at 4° clearly increased titers and restored the 3 "negative" sera to positive levels.

The N and CF tests were compared for serological diagnosis of BHF. Serum pairs (acute phase less than 7 days after onset, convalescent phase 30–100 days after onset) from 200 clinically diagnosed cases of BHF occurring in and near San Joaquin, Bolivia, were titrated by both methods employing an initial serum dilution of 1:4. Thirty-two serum pairs contained no detectable anitbody. Results from 168 cases with antibody are given in Table V. Correlation between the two methods was 92% among sera demon-

TABLE IV. Machupo Virus Neutralizing Antibody in Long-Term Convalescent Sera from Bolivian Hemorrhagic Fever Cases.

Individual	Convalescent interval (years)	Reciprocal titer when virus–serum incubated: 1 hr 24°	Overnight 4°
AOM	4	16	64
	5	16	64
PAW	4	8	64
	5	8	32
GJ	4	<4	32
	5	<4	32
RV	4	16	64
	5	16	128
EP	4	8	32
	5	<4	32
KMJ	4	32	64
	5	8	32

TABLE V. Serologic Study of 168 Clinically Diagnosed Cases of BHF.

	No. of pairs of sera with >4-fold rise	No. of pairs of sera showing stationary titer
Neut.	137	23
CF	134	20
Neut. or CF	141	27
Neut. and CF	130	16
Correlation methods	130/141 = 92%	16/27 = 59%

strating a rise in titer, but only 59% in sera with stationary antibody titer. Many serum pairs in the latter group had N but not CF antibodies in both specimens, which we interpret as evidence of Machupo virus infection of more than 1-year duration. Other individuals had low-titered CF antibodies without N antibodies which are considered nonspecific reactions.

Virus identification. A number of hamster pathogenic agents were obtained from kidneys of *Calomys callosus* trapped in San Joaquin and other towns in Bolivia. Crude antigen preparations of first passage infant hamster brain material which fixed complement when tested against a pooled Tacaribe-group MAF were selected for typing by neutralization test. A virus was considered identified if at least 100 pfu were inhibited by a given immune ascitic fluid. Such a test is illustrated in Table VI, in which 3 isolates were found to be strains of Machupo virus and a fourth was not identified as any of the recognized serotypes.

Discussion and Summary. The plaque-reduction, serum-dilution neutralization method satisfied most of the important criteria for efficient measurement of antibodies against Tacaribe-group viruses. This technique is type specific, economical to perform both in time and expenditure of reagents (0.025 ml of serum provides quantitative data), does not require special additives such as complement, is highly reproducible and easy to read. In practice, a team of two technicians easily tested 600 sera/week. By reversing the basic procedure and diluting virus rather than serum, it is simple to identify Tacaribe-group isolates.

It was found that N antibodies to Machupo virus appeared in the blood of humans convalescent from Bolivian hemorrhagic fever at about the same time as CF antibodies (3–4 weeks). Highest titers were achieved in parallel by both tests, but decay of CF activity was more rapid than for N antibody. Since individual Tacaribe-group viruses exhibit strong geographic localization and no two types have been found in the same area, either the CF or N test should provide accurate diagnosis of clinical disease associated with virus infection, and this prediction was substantiated in the 92% correlation observed between the two methods in a series of BHF cases. In addition, the N test appears to offer new possibilities for detection of past infection by a specific virus in given populations. This hope was supported by the ability to measure antibodies in each of a small number of persons 5 years after infection.

TABLE VI. Identification of Candidate Tacaribe-Group Virus Isolates.

	Log_{10} pfu/0.05 ml							
	Unknown viruses				Control viruses			
MAF	1	2	3	4	Mach.	Jun.	Tac.	Amap.
Machupo	<0.9	<0.9	<0.9	7.8	*3.2*	6.2	4.8	5.2
Junín	3.3	4.1	5.7	7.7	6.2	*<0.9*	4.5	5.0
Tacaribe	3.0	4.7	6.0	7.8	6.2	5.4	*<0.9*	5.1
Amaparí	3.0	4.5	6.1	7.8	6.2	5.6	5.0	*2.8*
Normal	3.5	4.6	6.7	7.2	6.6	5.9	5.0	5.0
Virus type:	Mach.	Mach.	Mach.	Not identified	Mach.	Junín	Tac.	Amap.

Preliminary observations suggest that the N test also can be used to assess the role of various vertebrates in the natural maintenance of Machupo and other Tacaribe-group viruses.

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Received Nov. 11, 1968. P.S.E.B.M., 1969, Vol. 130.

Variation of Myocardial Nucleolar Abundance with Heart Weight* (33712)

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The increased size of myocardial nuclei in hypertrophied human heart muscle is known to correlate with an increase in the DNA content of such nuclei (1, 2). The increment of DNA content appears to be in multiples of the diploid amount and in severely hypertrophied hearts corresponds to as much as 32N. Nuclei from hypertrophied myocardium can therefore be described as polyploid, and this suggests that such nuclei have a larger number of potentially active gene sites. It is entirely possible, however, that the additional DNA is either nonfunctional or represents only a small proportion of the genome which has been repeatedly duplicated, giving the semblance of polyploidy. In the present investigation we attempted to use the number of nucleoli per nucleus as an index of gene dosage, and found that nuclei from hypertrophied hearts do contain an increased number of nucleoli.

Materials and Methods. To avoid the problems associated with counting nucleoli in histologic sections, where only a portion of the nucleus is examined, nuclei were isolated and examined intact. Human hearts, obtained at autopsy, were used. Four g of myocardium, from the lateral wall of the left ventricle, midway between the apex and base, were used for each preparation. If the myocardium was involved in a pathological process other than hypertrophy, it was excluded from the study. The nuclei were isolated after homogenization of the muscle in 0.01 *M* citric acid, as described by Thomson *et al.* (3). Thin smears were made on glass slides, fixed, and stained as described by Shea and Leblond (4). In this method the preparation is incubated for 2 hr at 37° in DNase (Worthington, 1 × crystalized, 50 μg/ml), so that any intranuclear inclusions stained by toluid-

* Supported by United States Public Health Service Research Grant No. HE 10962 from the National Heart Institute.