Measurement of Antibodies to Machupo Virus by the Indirect Fluorescent Technique (37060)

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Bolivian hemorrhagic fever (BHF) was first recognized in the Province of Beni, Bolivia, in 1959 (1), and cases have continued to occur since then. Recent outbreaks have confirmed the fact that clinical manifestations can be quite nonspecific. Laboratory diagnosis is handicapped by a scarcity of facilities in the endemic region, as well as by the hazard to nonimmune personnel of working with the causative agent, Machupo virus. These problems also apply in varying degree to two other diseases caused by members of the arenavirus group (2, 3): Argentine hemorrhagic fever (AHF) caused by Junin virus (4) and Lassa fever caused by Lassa virus (5). The early recognition of outbreaks of these diseases is important since rodent control, immune serum therapy, and isolation of patients are thought to be of some use in treatment and prevention.

A plaque-neutralization test for detection of Machupo virus antibodies has been developed and proved to be specific and sensitive (6). However, these advantages are posited on two special considerations: the regular production of the necessary cell cultures, and the presence of trained immune personnel working in facilities designed to allow efficient and safe handling of Machupo virus. If inactivated antigens are used, the complement-fixation (CF) test (7) can be done by nonimmune workers, but this technique requires careful standardization and is less specific and less sensitive than the neutralization test. False-positive reactions occur and anticomplementary activity is often a major problem.

Direct or indirect fluorescent antibody staining of Tacaribe complex viruses in tissue culture or infected animal tissues, has been successful in a number of laboratories (8–12) as well as our own (13–15). Furthermore, the indirect fluorescent antibody test (IFAT) with human and animal sera proved to be a broadly reactive and extremely sensitive tool capable of demonstrating an immunological relation between the Tacaribe viruses and lymphocytic choriomeningitis (LCM) virus (15). This suggested that it might be possible to devise an IFAT to measure Machupo antibodies using a nonpathogenic Tacaribe complex virus as the antigen. Failing this, one would expect the danger of laboratory infection from pathogenic virus strains to be negligible if acetone-fixed viral antigen preparations were used (16, 17). Antigen slides could be prepared in special laboratories and then used elsewhere with ordinary laboratory facilities and careful precautions. With these considerations in mind, we have evaluated the IFAT for the diagnosis of BHF.

Materials and Methods. Viral antigens. Vero cell (continuous African green monkey kidney) culture methods were as described by Webb *et al.* (6), and virus strains used are detailed in Table I. A control antigen was prepared by inoculation of cell suspension with noninfected suckling hamster brain.

LCM-infected Vero monolayers on coverslips were provided by Dr. Wallace P. Rowe. All Tacaribe complex antigens were infected cell suspensions which were placed on glass slides, dried, and fixed. These cell suspensions were made by infecting Vero cell monolayers grown in 8- or 16-oz prescription bottles with stock virus suspensions diluted 1:10 in Dulbecco's phosphate-buffered saline solution (PBS) (pH 7.4) containing 0.7% bovine serum albumin, 200 units/ml penicillin and 200 μg/ml streptomycin. After decanting growth

TABLE I. Source and Passage History of Inocula Used for Preparation of Viral Antigens.

Virus	Strain	Passage	Origin
Machupo	Carvallo	$SHBr_2$	MARU
Junin	XJ	$?SM_1GP_2$ $SMBr_{16}Vero_6$	Dr. Jordi Casals, Yale Arbovirus Research Unit New Haven, Ct
Tacaribe	TRVL 11573	$SMBr_{24}$ $Vero_5SHBr_1$	Dr. Leslie Spence Trinidad Regional Virus Laboratory, Trinidad, W.I.
Tamiami	W-11075	$SMBr_{11}$	Dr. Charles Calisher Center for Disease Control Atlanta, Ga
Parana	12056	$SHBr_4$	MARU
Pichinde	AN 3739	$SMBr_{12}$	Dr. Carlos Sanmartin Universidad del Valle Cali, Colombia
Latino	10924	$SHBr_5$	MARU

medium, the virus was adsorbed 1 hr at 37° and then maintenance medium 199 containing 1% fetal bovine serum and antibiotics was added. Infected cell cultures were kept at 35–37° with medium changes every 3 to 5 days. The cells were harvested when 50 to 75% contained antigen, as early as 3 days (Machupo) and as late as 11 days (Latino)[1] after infection. At the time of harvest, the medium was decanted and replaced with 10 ml of warm 0.2% tetrasodium ethylenediaminetetraacetate (EDTA) in PBS. The bottle was kept at 37° until the cells became rounded and began to detach, usually within 5 to 20 min. Then the EDTA was decanted, leaving only a few tenths of a milliliter on the cell sheet. When the cells detached, 4 to 6 ml of medium 199 with 1% fetal bovine serum was added. The cells were further separated by rapid expulsion of the mixture from a pipette. The suspension was monitored microscopically to assure proper cell density and cell separation. Microscope slides were cleaned in detergent, running water, and 95% ethyl alcohol. After drying, they were marked with eight circles 5 mm in diameter, using a Mark-Tex pen (Mark-Tex Corp., Englewood, NJ). A small drop of cell suspension was placed in each circle with a Pasteur pipette, after which the slides were loosely covered with aluminum foil and kept at 37° in a forced draft CO_2 incubator until dry (usually 2 to 3 hr). The dry slides were placed in metal racks, immersed in precooled acetone, and stored in metal ammunition cases at —70°.

All manipulations of Machupo virus were carried out by immune personnel in a special isolated working area. After fixation the slides were removed to the general laboratory area and tested by nonimmune workers.

Slides stored in acetone at —70° were usable for more than 1 yr. Slides fixed in acetone at —70° for 1 wk or more and stored in ordinary slide boxes at —70° have been used after 6 mo with no deterioration in quality.

Conjugate. Throughout this study a single conjugate was used, a goat antiserum against human immunoglobulin G (IgG) conjugated with fluorescein isothiocyanate prepared by Dr. Roger Wilsnack, Huntington Research Laboratory, Baltimore, MD, (Lot No. 9061905) and kindly furnished to us by Dr. Wallace P. Rowe. Normal human serum produced only the typical IgG arc following immunoelectrophoresis and development with the conjugate. This conjugate was stored at —70° in aliquots. Working reagent was prepared by diluting the stock to 1:10 with Veronal-buffered saline solution (VBS) containing rhodamine bovine serum albumin

[1] Webb, P. A., Johnson, K. M., Peters, C. J., and Hibbs, J. B., unpublished data.

(18) at a final dilution of 1:40. Working reagent was kept at 4° for 1 to 4 wk.

Staining procedure. The slides were removed from acetone and allowed to dry at room temperature, washed in a stream of cold VBS (pH 7.9) and blotted dry. Sera were diluted with 0.025-ml microliter loops in plastic trays (Linbro Chemical Co.) using VBS as a diluent, and were dropped onto the circles containing the acetone-fixed antigen and incubated at room temperature for 30 min in a covered chamber. The slides were rinsed with cold VBS, washed in four changes of cold VBS for 1, 3, 3, and 3 min, respectively, using a rotary shaker, then were blotted and allowed to dry. Diluted conjugate was added to each circle, and after 30 min incubation at room temperature the washing procedure was repeated. Coverslips were mounted using glycerol–VBS, 9:1. Slides were read using a Leitz "Ortholux" microscope with neutral density and UG-1 exciter filters and 170/223 plus K-490 barrier filters. A 15× wide-field ocular and a dry dark-field condensor (NA = 0.8) were used. Slides were scanned with a 10× objective and, if necessary, the end point was verified with the 25× objective.

Sera. Acute and one or more convalescent serum specimens from neutralization (N) test confirmed cases of BHF were tested by IFAT. Eight were MARU laboratory personnel, and these persons contributed all sera obtained more than 2 yr from onset of disease. Twenty-five N- and CF-negative sera from individuals in San Joaquin, Bolivia, with no history of BHF were also tested. All acute phase sera from BHF cases were obtained in 1963; these and many convalescent sera had been stored in the MARU serum bank at −20° with several thaw–freeze cycles. Both N and CF tests had previously been performed on these sera (6). They were thawed once again and N tests were repeated. Titers were noted to be considerably lower in many sera, particularly among the earlier specimens. For this reason we have used geometric mean titers (GMT) from a previous paper for comparison (6).

Twelve sera from four cases of AHF were provided by Dr. Julio Barrera Oro of the Instituto Nacional de Microbiología, Buenos Aires, Argentina.

Safety test. Slides were flooded with an excess (approx 1 ml) of the same Machupo-infected Vero cell suspension used to prepare the viral antigen slides and dried (4 to 6 hr in the CO_2 incubator at 37°). From some slides the infected material was then eluted in a 0.5 ml volume of 199 with 1% FBS. Other slides were immersed in acetone at −70° for 24 hr before elution. All samples were held at −70° and titrated simultaneously in Vero cell cultures.

Results. Staining characteristics. Specific staining was manifest in all tests by a bright green, granular fluorescence confined to the cytoplasm of infected Vero cells. End points were readily determined as the last dilution which had definite staining of 25 to 50% of the infected cells.

Incubation of sera with viral antigen for 18 hr at 4° or 30 min at 37° resulted in a twofold gain in sensitivity over 30 min at room temperature. Since the increased sensitivity was accompanied by an increase in nonspecific staining, 30 min at room temperature was adopted as standard. Addition of 10% fresh guinea pig serum to two serum specimens failed to change their titer.

A positive control serum and a normal human serum pool were stored in aliquots at −20° and run with each test. The normal serum gave a titer of less than 1:2 in all 19 tests. The positive control serum showed a titer of 1:8 once; 1:16, 15 times; and 1:32, 3 times.

Reactions with viruses of Tacaribe complex. A single BHF convalescent serum (RL No. 109769) was titrated with antigens of a variety of Tacaribe complex viruses. A homologous titer of 1:64 was obtained. Heterologous titers were 1:16 with Junin and Tacaribe antigens, 1:4 with Tamiami and Parana, 1:2 with Pichinde and Latino, 1:3–1:10 with LCM, and $<$ 1:2 with noninfected Vero cell control antigen.

Reactions of BHF sera with selected arenaviruses. Seven patients with maximum Machupo IFAT serum titers between 1:32 and $\geq$ 1:256 were selected. Three specimens from each subject were tested: an acute serum (2 to 10 days after onset); an early convalescent serum (42 to 120 days, median 72 days); and a late convalescent serum (3 to 24 mo, median 11 mo). These sera were test-

TABLE II. Homologus and Heterologous Fluorescent Antibody Responses to Selected Arenaviruses in Sera from Patients with Bolivian Hemorrhagic Fever.

Patient no.	Age and sex	Time since onset of disease	Reciprocal titer versus antigen; Tacaribe complex				
			Machupo	Junin	Tacaribe	Latino	LCM
1.	21 F	42 days	128	16	8	4	<2
		95 days	≥256	32	16	16	<2
2.	52 F	120 days	128	32	16	32	8
		1 yr	128	8	8	8	<2
3.	37 F	72 days	128	32	8	16	8
		2 yr	16	8	2	4	<2
4.	44 F	42 days	64	8	2	<2	<2
		1 yr	16	4	2	2	2
5.	18 F	54 days	64	32	8	2	8
		1 yr	32	4	2	2	2
6.	50 M	74 days	64	32	16	4	4
		2 yr	4	2	2	2	<2
7.	44 M	80 days	32	8	4	16	8
		2 yr	8	4	2	<2	<2

ed against Machupo, Junin, Latino, and Tacaribe viral antigens and a Vero cell control inoculated with noninfected suckling hamster brain, as well as uninoculated and LCM-inoculated Vero monolayers on coverslips. Acute serum specimens did not react with any antigens. No sera reacted with noninfected cell suspensions or coverslip monolayers. IF antibody titers of the convalescent sera are shown in Table II. The geometric mean titer (GMT) is summarized in Fig. 1. Antibody titers measured with Junin, Tacaribe, and Latino antigens in each subject were proportional to the homologous (Machupo) titer and were decreased in proportion to the diminution in GMT for that particular antigen. In the case of LCM, high Machupo titers were not necessarily associated with the most extensive cross-reactivity (Table II, subject 1 versus subject 7).

AHF sera. Specimens from three AHF patients tested on Junin antigen had titer rises from < 1:2 to > 1:8 and the fourth presented with a titer of 1:16. These same sera tested on Machupo slides demonstrated approximately the same degree of cross-reactivity as BHF sera tested on Junin slides (GMT of positive Junin sera were twofold less with Machupo antigen).

Machupo virus antibodies in serial sera from 40 BHF patients. Acute phase serum and one or more convalescent sera from 40 cases of BHF were tested by IFAT. Thirty-nine patients showed a fourfold or greater rise in titer (35 from < 1:2 to ≥ 1:8), and the 40th patient had an initial titer of

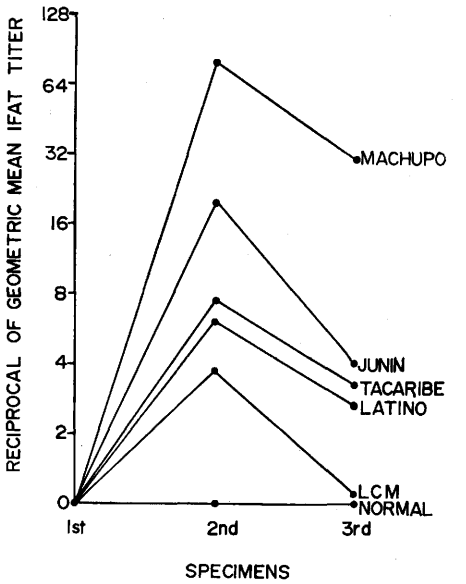

FIG. 1. Arenavirus group cross-reactions of serial sera from 7 BHF patients.

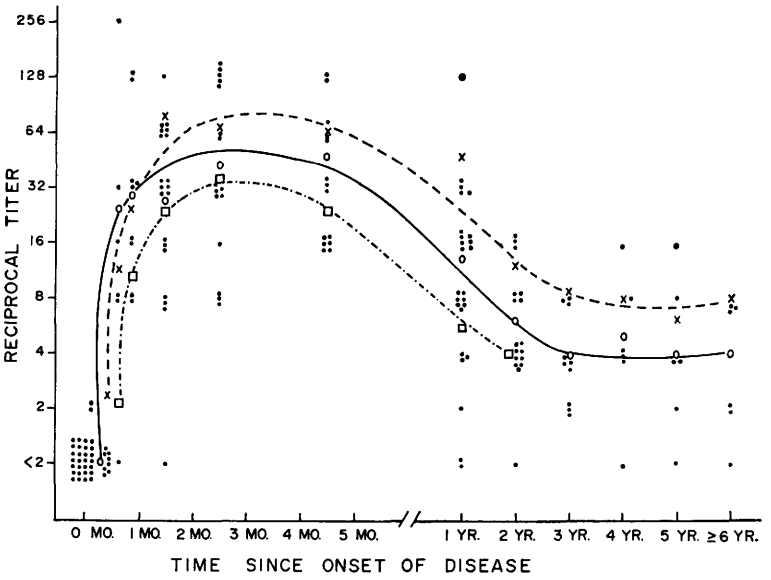

FIG. 2. Comparison of Machupo immunofluorescent, complement fixing and neutralizing antibody response in serial bleedings from 40 human cases of hemorrhagic fever. ●, Individual determination IFAT; ○, geometric mean IFAT; ×, geometric mean N; □, geometric mean CF.

≧1:256 when first sampled 20 days after the onset of his illness. The earliest fourfold conversion was on Day 17 and the latest negative IFAT was on Day 29. Nine patients had their first serum with a titer of ≧ 1:4 during the initial 24 days of disease and nine more had their first positive (≧ 1:4) serum by Day 31.

Homologous IFAT titers were maximum at about 4.5 mo, but by 1 yr began to decline (Fig. 2). In the small number of patients followed beyond 2 yr, IFAT antibodies paralleled those previously determined by N test, though at slightly lower titers of 1:4 to 1:8.

Specificity of Machupo IF antibody. Twenty-five residents of San Joaquin, who had no previous history of BHF and who were not reactive in the Machupo N or CF test, proved nonreactive in the IFAT when tested at a dilution of 1:2.

Safety test. In one trial, approximately 5 × 10^5 plaque-forming units (PFU) of Machupo virus were placed on each slide. Of three dried, and three dried and acetone-fixed slides, none yielded virus at a test level of about 400 PFU/slide. In another test, the slides initially contained 1 × 10^6 PFU. After drying alone, 70 PFU were recovered, but after drying and acetone fixation, no virus was recovered (less than 30 PFU).

Discussion. The Machupo IFAT provides a sensitive test for the diagnosis of Bolivian hemorrhagic fever. Thirty-nine of 40 paired sera from immunologically proven BHF cases demonstrated fourfold titer increases and the other patient had a high initial titer (20-day serum). Although individual patients were not sampled at sufficiently close intervals to provide exact answers, data from 20 subjects whose convalescent serum was obtained during the first month of illness indicated that seroconversion began about 2 wk after onset of illness and was virtually complete by 1 month. Specificity in the particular clinical and epidemiological setting was confirmed by negative results in 35 of 40 acute sera, and all 25 sera from non-BHF subjects living in the same region.

The GMT of IFAT antibodies was slightly higher than that for N antibodies 1 mo after onset of clinical BHF. Thereafter, IFAT titers generally were intermediate between the higher N and the lower CF values.

Since most of the IFAT determinations were done on sera stored for more than 5 yr, it is possible that this technique may prove to be more sensitive when fresh sera are assayed. In this connection it was found that of six N-positive BHF convalescent sera obtained at least 6 yr after disease five were

positive by IFAT. Only one was CF positive.

The IFAT has Tacaribe complex specificity so that antibody to Machupo virus in a BHF case would probably be detected by Junin antigen and vice versa. In most instances it would be possible to make a presumptive identification of the infecting virus on the basis of simultaneous testing with Junin and Machupo antigens, although the N test would be the obvious and reliable method for differentiation. Unfortunately, the antigen sharing is not so extensive that a nonpathogenic member of the Tacaribe complex, such as Tacaribe itself, could be used as an antigen with optimal sensitivity.

Taken together with the results of our safety tests and the fact that three of our nonimmune employees have worked with acetone-fixed Machupo antigens for more than 1 yr without any evidence of infection, the foregoing conclusions recommend the IFAT not only for the diagnosis of recognized arenavirus-caused human disease but also for serological surveys in the search for past human and animal infection with other arenoviruses. The CF test is severely limited for such purposes by the rapid decay of antibodies (7), and the N test, because of its type specificity, cannot detect infection to any agent other than the homologous virus used in the test (6). By adding LCM and one of the newer members of the Tacaribe complex such as Parana or Pichinde, it may be possible to detect old infections by most of the presently recognized arenaviruses and to obtain evidence pointing to the existence of further serotypes.

As with other fluorescent antibody systems, the critical ingredient in the Machupo IFAT was the anti-human conjugate. We found that two different anti-IgG reagents were virus-specific and gave similar titers. Two other commercially obtained anti-IgG conjugates and an anti-human globulin reagent were unsatisfactory because of excessive background staining or low titer. Nevertheless, we plan to try to extend the utility of the IFAT for the study of arenavirus infection. Preliminary tests, for example, suggest that acetone-fixed antigen slides are relatively stable at temperatures up to 10°. These will be tested under field conditions in Bolivia. In addition, an anti-gamma globulin conjugate for the rodent *Calomys callosus* had been prepared and shows good agreement with the N test in detecting Machupo virus antibodies in this species which is the natural reservoir of the virus.

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