JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1984, p. 239–244
0095-1137/84/080239-06$02.00/0

Vol. 20, No. 2

Detection of Lassa Virus Antigens and Lassa Virus-Specific Immunoglobulins G and M by Enzyme-Linked Immunosorbent Assay

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Received 20 February 1984/Accepted 14 May 1984

Rapid diagnosis of Lassa fever is desirable for the timely therapeutic intervention and implementation of strict quarantine procedures both in West Africa field hospitals where the disease is endemic and at international crossroads. An enzyme-linked immunosorbent assay (ELISA) to measure Lassa virus antigens in viremic sera was developed in which experimentally infected monkeys were used as a model for the human disease. In this test, Lassa virus antigens in test sera were captured in wells of microtiter plates by monkey anti-Lassa virus immunoglobulin. Guinea pig anti-Lassa virus immunoglobulin was then added, and binding of specific immunoglobulin was quantitated by the addition of rabbit anti-guinea pig immunoglobulin followed by alkaline phosphatase-labeled anti-rabbit immunoglobulin. This test detected viremia titers as low as 2.1 $\log_{10}$ PFU/ml in experimentally infected monkey sera, a titer often exceeded in patients with Lassa fever. Inactivation of infectious virus by β-propiolactone or γ-irradiation did not diminish reactivity. Antigen-ELISA concentrations increased with infectivity for the first 10 days after infection but then declined while infectivity titers remained high, suggesting that the presence of humoral antibody in viremic sera diminishes the sensitivity of the antigen ELISA. Lassa virus-specific immunoglobulin M (IgM) titers measured in an IgM capture ELISA were detectable within 10 days of infection and peaked after 36 days but remained detectable for 1.5 years. The Lassa virus-specific IgG ELISA response was slightly delayed, peaking on day 73 but declining only slightly thereafter. These studies in a realistic primate model suggest that the antigen detection ELISA or the IgM capture ELISA described, in which β-propiolactone-inactivated sera are used, should be useful for the rapid diagnosis of human Lassa fever.

Lassa fever is a severe, often fatal human disease now recognized to be of considerable public health importance in regions of West Africa. Lassa virus, the etiological agent, was originally associated with explosive hospital outbreaks of Lassa fever in Nigeria (2, 7). However, virulent Lassa virus activity is now generally recognized to be endemic to certain areas of Sierra Leone (12), Liberia and Guinea (13), and perhaps Nigeria, where the impact of this disease is considerable and the incidence of human patients is believed to number in the thousands to tens of thousands per annum. Development of a method for the definitive diagnosis of Lassa fever in patients soon after their admission to the hospital would be desirable, since early recognition would permit the timely therapeutic intervention with immune plasma or antiviral drugs (e.g., ribavirin) thought to be beneficial when initiated early in the course of the disease (9). Early recognition of Lassa fever would also alert attending personnel to isolate the patient and handle blood and secretions with extreme care to minimize spread of the infection to other patients and staff.

Most Lassa fever patients develop specific immunoglobulin M (IgM) antibodies which can be measured by the indirect fluorescent-antibody (IFA) test within several days of admission (17). Although the presence of specific IgM is generaly considered diagnostic, definitive diagnosis could be made even more rapidly if an assay system were available to detect Lassa virus antigens in sera obtained immediately after hospital admission. Although almost all patients with Lassa fever are viremic upon admission, assays to determine Lassa virus infectivity require 2 to 7 days, tissue culture facilities, and a high biological containment (P-4) laboratory. Thus, viremia determinations are usually available only retrospectively. We report here the development of an enzyme-linked immunosorbent assay (ELISA) for quantitating Lassa virus antigens in viremic sera obtained from rhesus monkeys experimentally infected as a model for human Lassa fever. We also report the application of ELISA for detection of Lassa virus-specific IgM and IgG in the same model. All tests can be performed with sera inactivated by β-propiolactone or other methods. Results suggest that antigen and antibody ELISAs used in combination could provide definitive diagnosis of Lassa fever within several hours for patients confined under field hospital conditions.

MATERIALS AND METHODS

ELISA procedures. (i) Lassa virus antigen detection. A triple-antibody (sandwich) ELISA was used routinely to quantitate Lassa virus antigens. Rhesus monkey anti-Lassa virus immunoglobulin (see below) was diluted 1:200 in coating buffer (0.05 M sodium carbonate, pH 9.5 to 9.7) and added in 100-μl volumes to 60 of the 96 wells (excluding the outer rows) of polystyrene Microtiter plates (Cooke M 29 AR; Dynatech Laboratories, Inc., Alexandria, Va.). After 1 h of incubation at 37°C, the plates were washed four times in rinsing solution (0.9% saline with 0.05% Tween 20). The test sample (100 μl), undiluted or diluted in ELISA buffer (phosphate-buffered saline, without Mg or Ca, plus 0.05% Tween 20 and 0.5% bovine serum albumin), was then added to the well.

After another hour of incubation at 37°C and washing four times in rinsing solution, 100 μl of guinea pig anti-Lassa virus immunoglobulin (see below), diluted 1:200 in ELISA

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buffer, was added as a second antibody and the plate was again incubated for 1 h at 37°C. After being washed four times in rinsing solution, 100 μl of rabbit anti-guinea pig IgG (Miles Laboratories, Elkhart, Ind.), diluted 1:400 in ELISA buffer, was added and incubated for 1 h at 37°C. After being washed four times in rinsing solution, 100 μl of alkaline phosphatase-labeled swine anti-rabbit IgG (Orion Diagnostica, Helsinki, Finland), diluted 1:200 in ELISA buffer, was added. After another incubation (1 h at 37°C) and washing, 100 μl of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) as substrate, diluted in 1 M diethanolamine buffer (pH 9.8) plus 0.5 mM $MgCl_2$, was added. The reaction was read after 20 min at room temperature by use of a spectrophotometer at 405 nm. A sample was considered positive when the optical density (OD) was higher than the mean background when 30 negative samples (plus 2 standard deviations) were tested. All samples were tested in duplicate. For detection of nonspecific binding, each serum sample was also added to one well and treated as above, except normal (nonimmune) guinea pig immunoglobulin diluted 1:200 in ELISA buffer was substituted for Lassa virus-immune guinea pig immunoglobulin. No nonspecific binding was observed for any of the samples tested in the present study.

In preliminary testing, a double-antibody ELISA was also compared with the triple-antibody procedure described above. In the double-antibody procedure, the use of rabbit anti-guinea pig immunoglobulin was omitted. After incubation of plates with guinea pig anti-Lassa virus immunoglobulin and washing, alkaline phophatase-labeled goat anti-guinea pig IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was added and plates were incubated for 1 h at 37°C. Substrate was added and read by the following procedure described above.

(ii) Detection of Lassa virus IgG antibodies. Guinea pig anti-Lassa virus immunoglobulin was diluted 1:200 in coating buffer, and 100 μl was added to 60 of the 96 wells of the microtiter plates. After incubation for 1 h at 37°C, the plates were washed four times in rinsing solution, and 100 μl of undiluted cobalt-inactivated (γ-irradiated) Lassa virus antigen (see below) was then added and incubated for 1 h at 37°C. After four washes, 100 μl of the monkey test serum (diluted 1:4,000 in ELISA buffer containing 1% normal guinea pig serum) was added to duplicate wells. After 1 h of inoculation at 37°C, followed by four washes, 100 μl of alkaline phosphatase-labeled swine anti-human IgG (Orion Diagnostica), diluted 1:200 in ELISA buffer, was added and incubated for another 1 h at 37°C. After four washes, 100 μl of *p*-nitrophenyl phosphate as substrate, diluted in diethanolamine buffer, was added. The reaction was read after 20 min at room temperature by use of a spectrophotometer at 405 nm.

(iii) Detection of Lassa virus IgM antibodies. An IgM capture ELISA (4) was employed. Goat anti-human IgM (μ-chain specific; Cappel Laboratories, Cochranville, Pa.) was diluted 1:200 in coating buffer and added in 100-μl volumes to each of the 60 wells of the microtiter plates. After incubation for 2 h at 37°C and washing four times in rinsing solution, test sera, diluted 1:400 in ELISA buffer, were added (100 μl per well). After 1 h of incubation at 37°C, 100 μl of undiluted, cobalt-inactivated Lassa virus antigen was added, and plates were reincubated for 1 h at 37°C. Four washes were followed by guinea pig anti-Lassa virus immunoglobulin, diluted 1:200 in ELISA buffer (100 μl), and incubation for 1 h at 37°C. After another four washes, goat anti-guinea pig IgG conjugated with alkaline phosphatase (Kirkegaard & Perry Laboratories), diluted 1:400 in ELISA buffer, was added. The plates were incubated again for 1 h at 37°C and washed four times, and 100 μl of *p*-nitrophenyl phosphate was added. The reaction was read after 15 min at room temperature by use of a spectrophotometer at 405 nm. For detection and control of nonspecific binding, all samples (both IgG and IgM) were run in duplicate wells, on plates with Lassa virus antigens and on plates with uninfected Vero cell culture fluids substituted for Lassa antigens. A sample was considered positive when the OD was higher than the mean (plus 2 standard deviations) of the OD of 20 sera known to be negative (6). Nonspecific binding did not occur for the series of samples tested.

Preparation of Lassa virus-immune immunoglobulin. Guinea pig and rhesus monkey sera were collected and pooled from animals 6 to 8 months after infection with the Josiah strain of Lassa virus. Neutralizing antibody titers, expressed as a $\log_{10}$ neutralization index (10), were 4.7 and 5.1, and IFA titers (see below) were 1:5,120 and 1:10,240 for the guinea pig and monkey serum pools, respectively. Immunoglobulin preparations of the guinea pig and monkey Lassa virus antisera were made by use of ammonium sulfate fractionation (3). To decrease the background activity of the ELISA, interspecies-reactive anti-immunoglobulin was eliminated by affinity chromatography (11). Cyanogen-bromide-activated Sepharose 4B gel (1) was used to prepare three different columns; one was coupled to normal guinea pig serum, one to normal monkey serum, and one to normal rabbit serum. A 1-g amount of gel was coupled to 1 ml of serum for each species separately. Monkey anti-Lassa virus serum was absorbed on the columns coupled with normal rabbit and normal guinea pig sera. Guinea pig anti-Lassa virus and rabbit anti-guinea pig sera were absorbed with a column coupled with normal monkey serum. The serum to be absorbed was passed through the column and incubated for 30 min at room temperature. The immunoglobulins were then removed from the gel with phosphate-buffered saline and concentrated back to the original volume with a Minicon concentrator. The unwanted interspecies-reactive immunoglobulins were eluted from the columns with 0.2 M glycine (pH 2.8), and the pH was normalized with phosphate-buffered saline before the gel was used again.

Virus infectivity assays and antigen preparation. Lassa virus, strain Josiah, was isolated from the serum of a human patient in Sierra Leone (18). For preparation of stock virus suspensions, this virus was passaged at a low multiplicity in monolayer cultures of Vero cells (an African green monkey kidney cell line) grown to confluency in 75-cm^2 flasks. Used at the fourth passage, this virus stock contained 2.5×10^7 PFU/ml and had been used in previous studies of Lassa virus pathogenesis in rhesus monkeys (9) and guinea pigs (10). Infectious virus was quantitated by counting numbers of PFU on Vero cell monolayers as described previously (9). To prepare Lassa virus antigen for IgG and IgM ELISA determinations, stock Josiah strain Lassa virus was passaged one additional time in Vero cells. Supernatant tissue culture fluid, containing 6.5×10^6 PFU/ml, was inactivated by γ-irradiation (2×10^7 rads) at −60°C with a Gammacell irradiator (model 220; Atomic Energy of Canada, Ltd., Ottawa, Ontario) (5). Irradiated antigen was tested and found free of residual infectious virus before being used outside the maximum-containment (P-4) system.

Inactivation procedures. To test the effect of various standard viral inactivation procedures on Lassa antigen quantitation by ELISA, we treated viremic serum samples by one of the following three methods: (i) undiluted sera

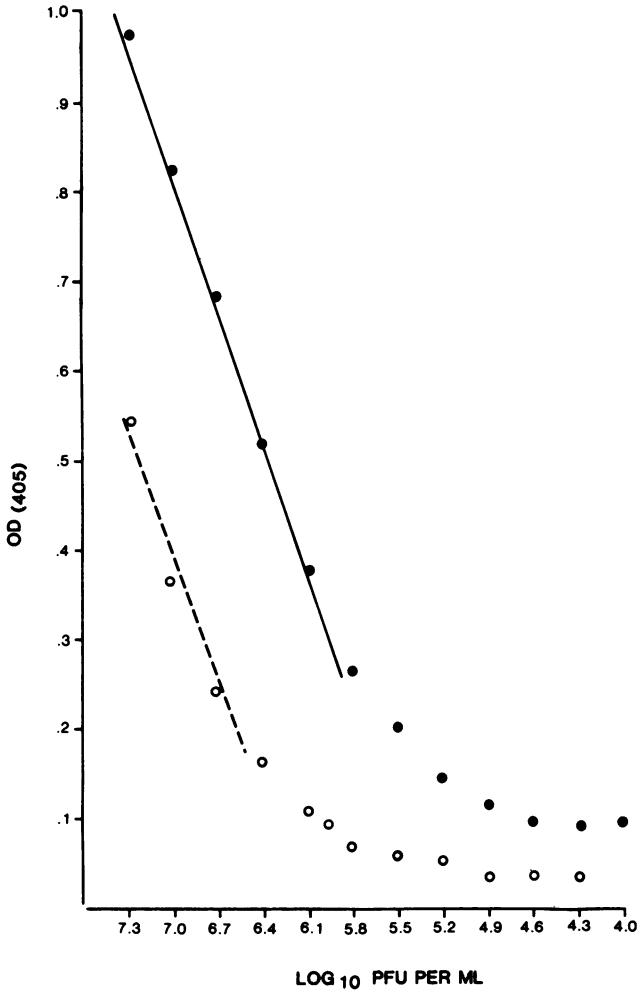

FIG. 1. Comparison of the triple-antibody (●) versus double-antibody (○) ELISAs for detection of Lassa virus antigen in Vero cell culture supernatant fluids. OD points are arithmetic means based on two determinations. Threshold concentrations of detectable antigen, defined as background OD ± 2 standard deviations, were 0.053 for the double-antibody ELISA and 0.104 for the triple-antibody ELISA. OD (405), OD at 405 nm.

were irradiated at −60°C (4.5×10^6 rads) in the Gammacell, as described above; (ii) β-propiolactone (Sigma) was freshly obtained and added in minimal volume to undiluted serum samples to obtain a final concentration of 0.3% and incubated for 2 h at 37°C (14); (iii) formaldehyde, freshly obtained as a 35% solution (Formalin; Fisher Scientific Co., Pittsburgh, Pa.) was similarly added to undiluted serum samples to a final concentration of 0.05% and incubated for 18 h at 37°C. No residual infectivity remained in any treated serum samples.

IFA test. For measurement of Lassa virus-specific IgG and IgM antibodies in convalescent monkey sera, an IFA test was performed in which Vero cells infected with the Josiah strain of Lassa virus were fixed onto circular areas of Teflon-coated slides by immersion in acetone, as previously described (8). After incubation of test serum dilutions with fixed cells, slides were washed and flooded with appropriate dilutions of fluorescein-conjugated goat anti-human IgG or IgM (Miles Laboratories). After reincubation, washing, and mounting, slides were examined with a Leitz Diahex microscope equipped with a 50-W halogen epiluminescence source and evaluated (8).

Inoculation and treatment of monkeys. Fully conditioned male (4.7-kg) rhesus monkeys (*Macaca mulatta*) were caged individually in the maximum containment (P-4) laboratory and inoculated subcutaneously with 0.5 ml of stock Lassa virus diluted to contain 1.2×10^6 PFU per dose. Monkeys were sedated with ketamine hydrochloride (0.1 ml per kg of body weight) before manipulation. Blood was collected by femoral venipuncture with Vacutainer tubes without anticoagulant to obtain serum for virus, antigen, and antibody determinations. Infections were permitted to follow their natural clinical course without therapeutic intervention. Six untreated (control) monkeys, selected from studies reported separately (9; P. B. Jahrling, manuscript in preparation), were tested for development of viremia, antigenemia, and acute antibody responses. For late convalescent antibody determinations, two additional control monkeys from another experiment were included to compensate for the loss of monkeys due to the acute disease. All monkey sera were tested for the presence of rheumatoid factor in a slide agglutination test in which sensitized stabilized sheep erythrocytes were used as supplied in the Rheumaton (Wampole Laboratories, Cranbury, N.J.) test kit.

RESULTS

The relative sensitivities of two ELISA procedures for the detection of Lassa virus antigens in Vero cell culture fluids were compared (Fig. 1). The triple-antibody (sandwich) ELISA was more sensitive than the less time-consuming double-antibody procedure. In the triple-antibody test, the relationship between OD measured and antigen dilution was logarithmic in the range of 5.8 to 7.3 $\log_{10}$ PFU. The minimal concentrations of infectious virus required for detection by ELISA were 5.8 and 4.9 $\log_{10}$ PFU/ml for the double- and triple-antibody procedures, respectively. The triple-antibody ELISA was used for all subsequent tests.

To assess the effect of several viral inactivation procedures on the determination of antigenemia by ELISA, we treated a series of viremic monkey serum samples with γ-irradiation, formaldehyde, or β-propiolactone and then tested them in comparison with untreated sera (Table 1). β-Propiolactone and γ-irradiation did not reduce OD values, whereas formaldehyde treatment did result in a decreased OD relative to values obtained for untreated viremic sera. To

TABLE 1. Lassa viremia and antigenemia in untreated versus inactivated monkey sera

Days after inoculation	Lassa virus antigen by ELISA (OD_{405}) in serum inactivated by[a]:				Infectious virus ($\log_{10}$ PFU/ml)
	γ-Irradiation	Formaldehyde	BPL	No inactivation	
0	0.072	0.056	0.053	0.036	>0.7
4	0.353	0.161	0.286	0.146	2.1
7	0.897	0.480	0.982	0.812	4.2
10	0.501	0.291	0.446	0.461	6.1
14	0.172	0.160	0.146	0.141	5.1
17	0.161	0.144	0.155	0.115	4.7
21	0.115	0.083	0.086	0.077	3.6

[a] Sera were inactivated by γ-irradiation at 4.5×10^6 rads at −60°C, formaldehyde at 0.05% for 18 h at 37°C, or β-propiolactone (BPL) at 0.03% for 2 h at 37°C. Results are expressed as the arithmetic mean OD at 405 nm (OD_{405}) based on two determinations.

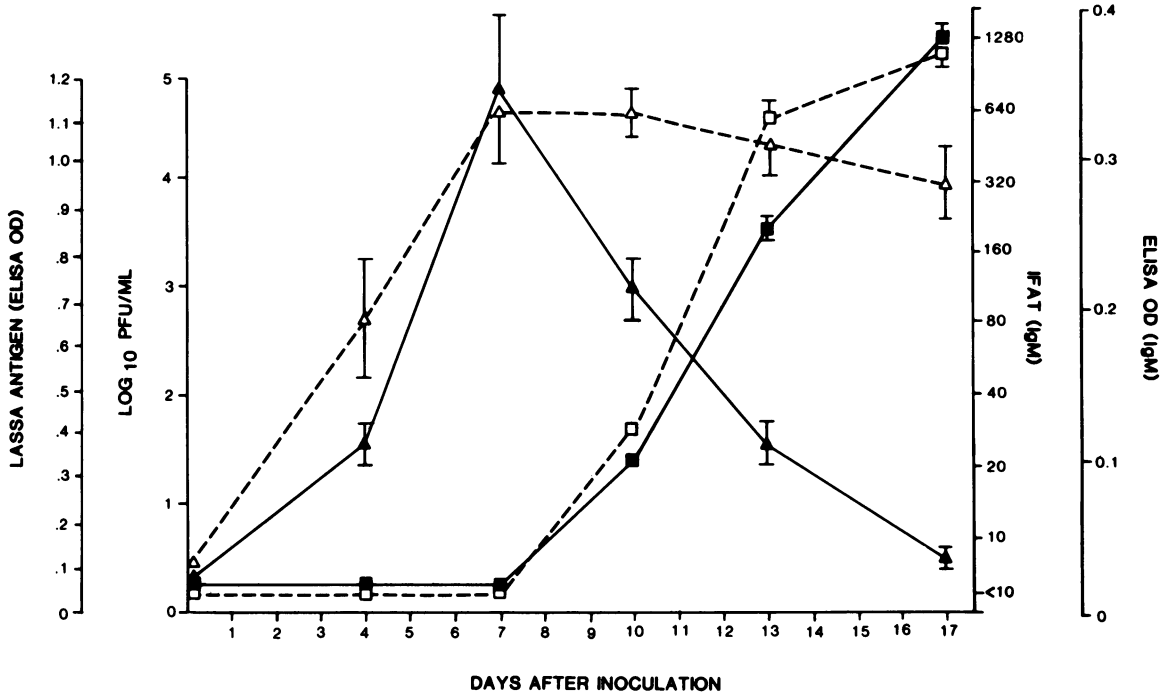

FIG. 2. Viremia (△), antigenemia (▲), and IgM responses measured in the IFA (□) and ELISA (■) tests for six rhesus monkeys inoculated subcutaneously with 1.2×10^6 PFU of Lassa virus. Points are geometric means ± standard error of the mean.

increase safety in handling infectious sera and to eliminate the need of conducting ELISA under P-4 conditions, we γ-irradiated all samples subsequently tested for antigen by ELISA.

A comparison of infectious viremia and antigenemia for the one monkey tested in Table 1 suggested a disparity that required confirmation. A larger series of six monkeys was therefore tested, and similar results were obtained (Fig. 2). Mean viremia titers reached maximum values (4.6 $\log_{10}$ PFU/ml) between days 7 and 10 and then declined gradually to 4.0 $\log_{10}$ PFU/ml on day 17. In contrast, antigen ELISA OD values peaked sharply on day 7. A rapid decline in OD values was observed in the next samples tested, on day 10; this trend continued through days 13 to 17, when antigenemia was barely detectable despite the presence of 4.0 $\log_{10}$ PFU of infectious virus per ml. A plausible explanation for this disparity between viremia and antigenemia comes from examination of the humoral antibody responses (Fig. 2). Lassa virus-specific IgM antibodies, measured in either the IFA or IgM capture ELISA, were undetectable on day 7 (the day of peak antigenemia) but increased to detectable titers by day 10 and continued to increase through day 17, as antigen concentrations declined. Thus, the presence of humoral antibodies in viremic sera may have diminished the sensitivity of the Lassa antigen ELISA or have led to the disappearance of noninfectious antigen.

The humoral antibody response to Lassa virus was examined in more detail. Sera obtained from four surviving rhesus monkeys that had been bled over a longer time period were tested by both IFA and ELISA for Lassa virus-specific IgM and IgG (Fig. 3). When the IFA test was used, IgM antibodies reached maximum geometric mean titers of 1:420 early (i.e., days 13 to 17) in the disease course and then declined sharply to 1:20 or 1:40 by day 60 through day 126. Late in convalescence (days 370 to 532), IgM antibodies measured by the IFA test were undetectable. ELISA IgM antibody titers peaked later on day 36 but were detectable as early as day 10. ELISA IgM titers gradually decreased but, in contrast to IFA IgM titers, remained positive throughout the 532-day observation period. The IgG response measured by the IFA test was accelerated relative to the IgM response and reached higher peak titers of 5,011 by day 36. By ELISA, the initial IgG response was slightly delayed and reached peak titers by day 73. In contrast to IgM titers, IgG titers by either test declined only slightly from their maximum values during the remainder of the observation period.

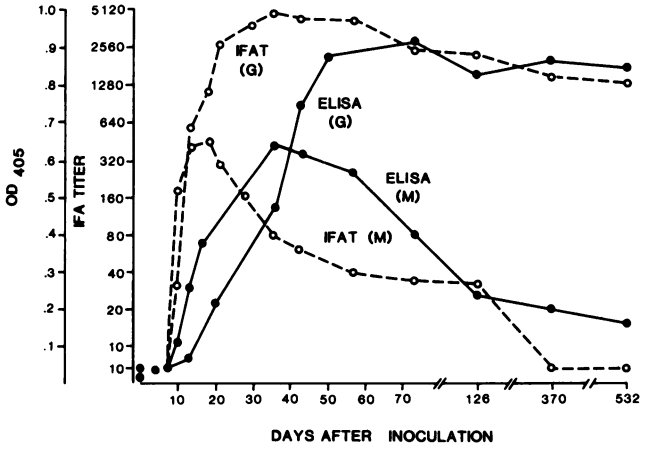

FIG. 3. Humoral antibody responses to Lassa virus infection in four rhesus monkeys, measured by the IFA test (○) for IgG (G) and IgM (M) and by ELISA (●) for IgG and IgM. OD_{405}, OD at 405 nm.

DISCUSSION

We describe an ELISA for detection of Lassa virus antigen in which absorbed polyclonal antibodies and a triple-sandwich technique are used. The test was positive in experimentally infected rhesus monkeys with serum virus titers as low as 2.1 $\log_{10}$ PFU/ml, a titer often exceeded in patients with Lassa fever. Indeed, preliminary testing of sera from patients with Lassa fever has suggested that this test will be useful in diagnosis of human disease (P. B. Jahrling, B. Niklasson, and J. B. McCormick, unpublished observations).

The antigens detected in this test are not known, since the high-titer, late-convalescent sera used as reagents presumably contained antibodies to at least the three major virion polypeptides (G1, G2, and N). The importance of distinguishing between circulating infectious virus and specific antigens is highlighted by our observation that viremia did not correlate with antigenemia during the evolution of Lassa fever in rhesus monkeys. In individual monkeys, the antigen concentration in serum began to fall between days 7 and 10, whereas the virus titer in serum was stable or increasing. Lassa virus-specific IgM was first detected at this time. This temporal correlation may be only coincidental. However, virus antigens may persist in the circulation, but the presence of antibody may interfere with their detection in the ELISA test. Alternatively, the apparent decline in antigenemia could reflect the onset of immune clearance of Lassa virus antigens not associated with infectious virus. Indeed, in recent studies it has been possible to reduce the infectivity of sera obtained during the second week of infection by the addition of IgM antisera, suggesting that infectious virus-antibody complexes circulate late in disease. Circulating antigen-antibody complexes, if they exist, have a potentially deleterious role in disease outcome. Other factors may be involved in this antigen-infectivity disparity, including differential production or differential nonimmune clearance during the course of infection. The situation in Lassa virus-infected monkeys can be contrasted with that in experimental Rift Valley fever, in which detectable antigenemia continues after infectious virus is no longer readily detectable in serum (15).

These data also represent the first application of ELISA to the measurement of Lassa virus antibodies. Results of conventional IFA tests in rhesus monkeys and humans are apparently similar (9, 16, 17). The IgM IFA used suffers from potential false-positive results (rheumatoid factor) and false-negative results (competition from avid, high-titer IgG antibody). In the rhesus monkeys tested, rheumatoid factor was not detected by means of the erythocyte agglutination test. However, the IgM capture ELISA continued to be positive at 370 and 532 days postinfection, when both human and rhesus monkey sera no longer reacted significantly by IgM IFA. This unexpected persistence of virus-specific IgM in the nonhuman primate model deserves further investigation and should also be evaluated in patients. The finding raises concerns about possible virus persistence and also suggests that the mere finding of virus-specific IgM in a single serum sample tested by ELISA may not imply recent infection.

The IgG ELISA did not become positive until days 13 to 19, although the IgG IFA was usually positive by days 10 to 13. Despite its lack of sensitivity in detecting the early IgG response in this experimental infection, the ELISA has properties which suggest that it may be particularly useful in serological surveys. Reactivity paralleled that of the IgG IFA and showed no decline over a 1.5-year period. Sera collected under field conditions in Africa often show nonspecific reactivity. In the IFA test, these "sticky sera" produce bright-green fluorescence in uninfected control cells, precluding the detection of virus-specific fluorescence of infected cells. High background activities in antibody ELISA tests are detected and quantitated in control antigen wells and thus may permit the development of criteria for positivity in virus antigen-containing wells. Furthermore, quantitation of the ELISA with a spectrophotometer eliminates the inherent subjectivity of visual reading employed in current IFA testing.

In both the antigen and antibody ELISA procedures, intratest variability was minimal; absorbance values for replicate samples varied less than 5%. However, intertest variability was considerable; absorbance values were influenced by timing, temperature, and other variables. Each test was internally controlled with standard, known-positive and -negative sera. To relate absorbance values obtained for different samples in different tests, the absorbance values obtained for the positive controls must be compared and the data normalized on this basis. Although numerous negative controls were routinely included in our tests, threshold values for positive reactions were generally characterized by an OD of >0.01 for the Lassa virus antigen and IgM assays and an OD of >0.05 for the IgG test.

There is considerable need for rapid diagnostic tests for Lassa fever both within Africa and in travelers returning from endemic areas. Patients, once identified as carrying the disease, should be isolated to prevent its nosocomial spread. The antiviral drug ribavirin appears to be useful in therapy, and immune plasma may also have a role in treatment. In addition, reliable exclusion of patients with diseases clinically resembling Lassa fever (e.g., malaria or typhoid) may lead to the initiation of appropriate specific treatment as well as conservation of the limited quantities of Lassa virus-immune plasma. Development of confidence in this test will require the testing of large numbers of serum samples obtained from patients in field hospitals, whose diagnoses are confirmed by independent virological and serological criteria.

These studies, carried out with a realistic primate model of Lassa fever, suggest that the antigen detection or the IgM capture ELISA or both will be positive in patients with Lassa fever. The proposed test requires only hours to perform, and β-propiolactone inactivation does not diminish reactivity. Thus, if evaluation in the human disease supports these findings, the tests could find use in international crossroads, where the differential diagnosis of Lassa fever is a problem, and in endemic areas, where investigational use of expensive and potentially toxic therapeutic regimes is under way. Additional standardization through properly selected monoclonal antibodies and extension of these tests to other body fluids (e.g., urine, throat washes) may provide additional diagnostic information. A second-generation ELISA in which monoclonal antibodies are used may further improve in both sensitivity and specificity for individual virion structural proteins.

ACKNOWLEDGMENTS

This project was supported by the Swedish National Defense Research Institute (FOA-4) and the Swedish Medical Board of the Armed Forces.

We acknowledge the expert and meticulous technical assistance of Shielda B. Smith and Joan B. Rhoderick.

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