Indirect immunofluorescence for the diagnosis of Lassa fever infection *

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The indirect immunofluorescent technique is a rapid method for identification of Lassa virus and Lassa virus antibody. In the study reported here, Lassa virus antigen was detected by this method in Vero cell cultures within 24 hours of their inoculation with an infected human blood specimen. A diagnosis could be made from field-collected specimens within 3 days of their receipt.

Fluorescent antibodies against Lassa virus were detected in human serum as early as 7 to 10 days after onset of illness, and were detected as long as 61 months after infection. Complement fixing antibodies were not as long lasting.

No antigenic differences were noted by the indirect immunofluorescence technique between several Lassa virus strains isolated from Nigeria, Liberia, and Sierra Leone over a 6-year period.

INTRODUCTION

Lassa fever was first recognized in 1969 in a missionary nurse in the small town of Lassa in the North-Eastern State of Nigeria. Her initial symptoms were a high fever, pharyngitis, myalgia, and headache. Later, haemorrhagic manifestations developed, with fatal outcome. Since 1969, several outbreaks of the disease have been described (1, 2), with nosocomial infections and high case-fatality rates. They were centred in two regions of West Africa, namely, the North-Eastern State and the adjacent Benue-Plateau State of Nigeria, and the Eastern and Southern Provinces of Sierra Leone and North-West Liberia. Lassa fever cases seem to occur in these areas every year. In Nigeria, most cases occur during January and February, but in parts of Sierra Leone and Liberia, Lassa fever occurs in all months of the year.

Early recognition of the disease is very important, so that patients can be isolated and further spread of the infection can be prevented. Early diagnosis also permits prompt implementation of strong supportive treatment and administration of human immune serum. The latter therapeutic method, although not yet fully evaluated, has been associated with spectacular recoveries.

Because earlier studies had shown the indirect immunofluorescent antibody (IIFA) test to be applicable to other arenaviruses, namely, lymphocytic choriomeningitis and Machupo viruses (3, 4), it was believed feasible to use this test for Lassa virus. This report describes the application of the IIFA method to Lassa fever diagnosis. This method permitted earlier detection of Lassa fever infection than isolation and confirmation by the complement fixation (CF) test, and it was more sensitive.

MATERIALS AND METHODS

Cell cultures

An African green monkey kidney cell line (Vero) was used for the propagation of Lassa virus. The cells were prepared by the Tissue Culture and Media Section, Center for Disease Control (CDC), and were supplied either as a suspension for Lab-Tek chambers [a] and Leighton tubes, or as monolayers grown in tissue culture tubes. The Vero cells were maintained in our laboratory on Eagle's minimal essential medium (MEM) to which inactivated bovine fetal serum (2 ml/100 ml) was added. The pH was adjusted with sodium bicarbonate to a level of 7.2 to 7.4.

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Table 1. Lassa virus strains examined by the IIFA test

Virus strain	Source of specimen	Date and country of collection	Passage	Origin
L.P.	human serum	25/2/69 Nigeria	MP1 Vero 5	Yale [a]
A.C.	human serum	11/4/72 Liberia	Vero 3	CDC
S.B.	human serum	25/9/72 Sierra Leone	Vero 3	CDC
M-80 T	*Mastomys* tissue pool	1972 Sierra Leone	Vero 2	CDC
B.M.	human blood	9/3/74 Nigeria	Vero 1	CDC
P.K.	human blood	12/1/75 England (Nigeria) [b]	Vero 1	CDC

[a] Dr J. Casals.

[b] Infection was acquired in Nigeria but the specimen was sent to us from England.

Virus strains

All manipulations with Lassa virus were carried out in CDC's Maximum Security Laboratory, a Class III facility for working with highly pathogenic agents. The Lassa virus strains used as antigens for the IIFA test are listed in Table 1.

Each of 4 partitions of a Lab-Tek chamber was inoculated with 0.1 ml of a suspension containing 10^2 to 10^3 TC ID_{50} of the respective virus strain. The chambers were incubated until about 30% of the Vero cells had become infected, as indicated by daily IIFA testing of replicate preparations. The cell sheet was washed with phosphate buffered saline (PBS), pH7.2, and after air-drying was fixed in acetone at room temperature for 10 minutes. The slides were then stored in a mechanical freezer at −70°C until they were used. Slides stored in the freezer were usable for at least 6 months without apparent deterioration in quality.

Guinea-pig immune serum

Antisera against the various Lassa virus strains were prepared in young adult guinea-pigs (Hartley strain) by several different methods. Table 2 gives the immunization schedules followed.

Human immune serum

Acute and/or convalescent sera used in this study were from Lassa fever patients who had acquired their infections at different times in various areas.

Table 2. Immunization schedule for preparation of Lassa virus antisera in guinea-pigs

Virus strain	Dosage [a] ($TCID_{50}$/ml)	Booster dosage	Booster week	Final bleeding (week)
L.P.	3×10^4	3×10^4	5	8
A.C.	5×10^6	5×10^6	4	8
S.B.	1×10^7	1×10^7	4	8
M-80 T	3×10^4	None		7
P.K.	1.5×10^3	None		7

[a] Intraperitoneal inoculation.

One person (L. P.) contracted the disease in 1969 in Nigeria, and another (B. M.) in 1974 in Nigeria. One person (S. K.) was infected in 1975 in Sierra Leone. The others acquired the disease in 1972, either in Liberia or in Sierra Leone.

Staining procedures

All tests were performed in duplicate. The cell sheet was overlayed with the appropriate serum dilution and allowed to incubate for 30 minutes in a moist chamber. Afterwards, the cell sheet was washed once briefly with PBS, and then twice with PBS for 10 minutes each time. The slides were air-dried and overlayed with a dilution of either rabbit anti-guinea-pig immunoglobulin G conjugated with fluorescein isothiocyanate (FITC) [a] or goat anti-human immunoglobulin conjugated with FITC. The latter was kindly supplied by Dr Charles B. Reimer, Immunological Products Branch, CDC. Each slide overlayed with conjugate was incubated at room temperature for 30 minutes, then rinsed 3 times with PBS to remove the unbound conjugate. The slides were allowed to dry in air, mounted in buffered glycerol (pH 8.5), and read with a Leitz Dialux microscope equipped with an incident light fluorescence vertical illuminator. A 16× objective was used; occasionally, a 25× or 40× objective was used to verify the end-point. Both the number of cells that showed specific fluorescence and the degree of fluorescence decreased with increasing dilution of the Lassa virus antiserum. The IIFA end-point was defined as the serum dilution that definitely stained the majority of the infected cells but showed slightly diminished brightness. Brightness was considerably

[a] Conjugate was obtained from Miles Laboratories, Inc., Kankakee, IL, USA.

diminished or indistinguishable at one dilution below this end-point.

Early detection of Lassa virus in cultures

Specimens from suspected Lassa fever patients were inoculated into Vero cell culture tubes and Lab-Tek chambers or Leighton tubes. The medium was changed after 24 hours if a toxic effect was noted. Each day, one inoculated slide or coverslip was fixed and stained, and the percentage of fluorescing cells was recorded. Occasionally, smears were prepared; the cell monolayer was scraped off the glass, sedimented, spread on a slide, fixed, and stained as described. The cultures were also read for cytopathic effect.

Lassa immune serum prepared in guinea-pigs against the L. P. strain of Lassa virus was used as source of antibody. Normal guinea-pig serum was used as control.

RESULTS

Specific immunofluorescence manifested itself by a bright yellow-green staining of aggregates in the cytoplasm of the infected cells. Staining could be detected in 1% of the cells within 24 hours of inoculation with a blood specimen from patient P. K. (Table 3). This specimen had been sent on dry ice from Europe. Vero cell cultures inoculated with specimens from Africa, which had travelled several days longer and were shipped unfrozen, showed immunofluorescence in 10–40% of the Vero cells on day 3 after inoculation. A specific Lassa virus cytopathic effect did not develop until 2–3 days after diagnosis by the IIFA method could be made.

Examples of the fluorescence obtained 2–4 days after inoculation of the cell cultures are shown in Fig. 1–4.

Fluorescent antibodies (FAs) in Lassa fever patients were found to develop 7–10 days after the onset of illness (Table 4). It is interesting to note that FAs could be detected during the viraemic phase of the illness. The serum from V.K., which was collected on day 7 after the onset of illness, had a low FA titre of 1 : 4; the serum from S. K., collected on day 10, had a titre of 1 : 32; and the serum from A. M., collected on day 11, had a titre of 1 : 64.

FAs apparently persist for several years. A serum sample that was drawn 5 years after Lassa fever infection had an FA titre of 1 : 16 (patient L. P.). None of the sera tested reacted with the noninfected Vero cell controls.

For comparison with the IIFA test results, some CF antibody titres are presented in Table 4. CF antibodies were detected 10–14 days after the appearance of FAs. The CF antibody titres were considerably lower than the FA titres, at least in the small number of human sera that were tested.

Homologous and heterologous FA responses were also determined with guinea-pig Lassa-immune sera prepared against various strains and selected Lassa

Table 3. Development of fluorescent cells and cytopathic changes in Vero cells inoculated with specimens from Lassa fever patients

Patient	Specimen	Days after inoculation [a]											
		1		2		3		4		5		6	
		FAC (%)	CPE [b]	FAC (%)	CPE	FAC (%)	CPE	FAC (%)	CPE	FAC (%)	CPE	FAC (%)	CPE
P.K.	blood	1	–	20	–	70	±	95	1–2+	98	3+	100	4+
B.M.	blood	0	–	0	–	10	–	50	–	90	–	95	1+
E.S.	pleural fluid	0	–	0	–	10	–	30	–	Culture contaminated			
T.K.	serum	ND	–	ND	–	40	–	ND	±	ND	1+	ND	3–4+

[a] FAC = percentage of cells showing fluorescent antibody staining; ND = not done.

[b] Degree of CPE:

– = no CPE noted
± = 1 or 2 very small foci per culture
1+ = 25 % of the cells destroyed
2+ = 50 % of the cells destroyed
3+ = 75 % of the cells destroyed
4+ = cells sheet completely destroyed

Table 4. Period of viraemia and development of antibody in Lassa fever patients

Patient	Time after onset of illness	Virus isolated from serum	Antibody titre[a] IIFA	CFA
	days			
S.B.	3	+[b]	<4[c]	ND[d]
A.M.	5	+	<4	ND
V.K.	7	+	4	ND
M.Q.	7	+	<4	ND
A.M.	8	+	<8	ND
S.K.	10	+	32	ND
A.M.	11	+	64	ND
V.K.	13	−	64	ND
S.B.	14	−	256	<4
A.M.	15	−	128	ND
P.H.	20	−	128	4
J.Z.	22	−	128	<4
B.M.	23	−	64	4
B.M.	39	ND	128	16
	months			
B.M.	3	ND	128	16
L.P.	5½	ND	64	32
L.P.	61	ND	16	<4

[a] IIFA, indirect immunofluorescent antibody; CFA, complement fixing antibodies.
[b] + = virus isolated; − = virus not isolated.
[c] Titres are given as reciprocal of serum dilution.
[d] ND = not done.

Table 5. Homologous and heterologous fluorescent antibody titres of Lassa virus guinea-pig antisera

Source of Lassa antigen	Reaction with guinea-pig Lassa antiserum[a] L.P.	A.C.	S.B.	M-80 T	P.K.
L.P. 1969 Nigeria	128	256	256	1024	2048
A.C. 1972 Liberia	256	256	256	1024	2048
S.B. 1972 Sierra Leone	256	512	512	1024	2048
M-80 T 1972 Sierra Leone	128	512	512	1024	2048
P.K. 1975 Nigeria	256	512	512	1024	2048

[a] Titres are given as reciprocal of serum dilution.

Table 6. Fluorescent antibody titres of human Lassa convalescent sera with various Lassa virus strains

Source of Lassa antigen	Reaction with human Lassa convalescent serum[a] A.M. 1972 (15) Sierra Leone	P.H. 1972 (20) Liberia	J.Z. 1972 (22) Liberia	B.M. 1974 (99) Nigeria
L.P. 1969 Nigeria	64	128	128	32
A.C. 1972 Liberia	128	128	128	64
S.B. 1972 Sierra Leone	128	128	128	64
M-80 T 1972 Sierra Leone	128	128	128	64
B.M. 1974 Nigeria				128
P.K. 1975 Nigeria	64	128	128	64

[a] Titres are given as reciprocal of serum dilution; the figures in parentheses following the year indicate the number of days after the onset of illness.

virus antigens (Table 5); and human convalescent sera were tested against a variety of Lassa virus antigens (Table 6). No significant differences in titres were detected, regardless of the virus strain used to produce antigen or antibody; thus, by the IIFA test, the strains were essentially indistinguishable.

DISCUSSION

The studies conducted indicate that the IIFA test is an excellent means of making an early diagnosis of Lassa virus infection in man. Specific FA was observed in Vero cell cultures as early as one day after their inoculation with an infected blood specimen. Even diagnostic blood specimens kept at ambient temperature for more than 1 week (as in shipments from Africa) were shown to be infectious; definite diagnosis was made on the third day after inoculation, which is at least 3-5 days earlier than would be possible on the basis of observing cytopathic effect and typing by the CF technique. Furthermore, other studies under way in this laboratory with a variety of arenaviruses (LCM, Pichinde, Tamiami, Tacaribe, Amapari, Latino, Parana, Machupo, Junin) indicate that if the immune serum used in the IIFA test is appropriately diluted (1 : 32 or greater), no cross-reactions are observed.

Fig. 1. Lassa virus immunofluorescence in Vero cells grown in Lab-Tek chambers that had been inoculated with a blood specimen of patient P. K. 2 days previously. Brightly stained aggregates are seen in the cytoplasm of individual cells; stained aggregates lie also on top of surrounding cells.

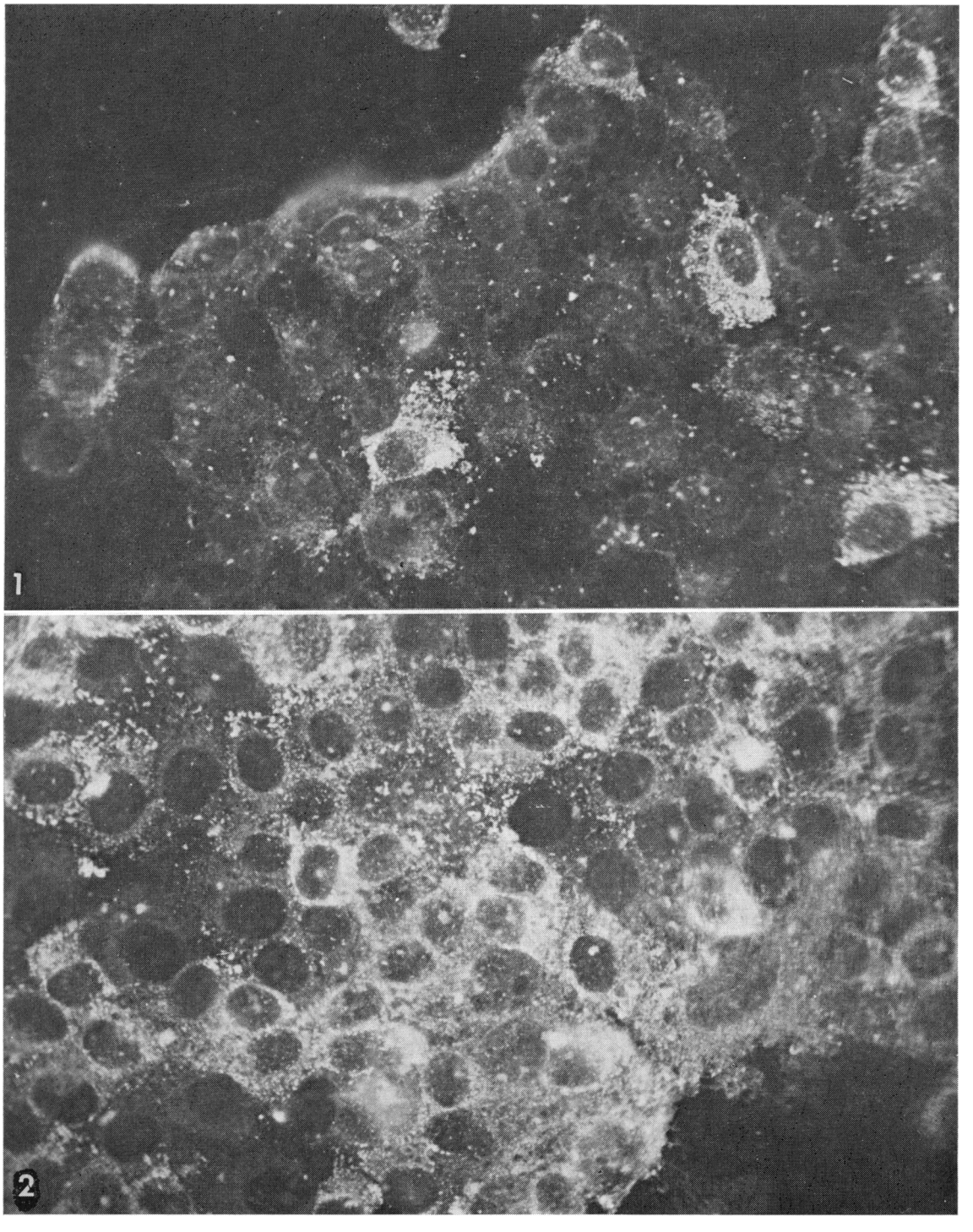
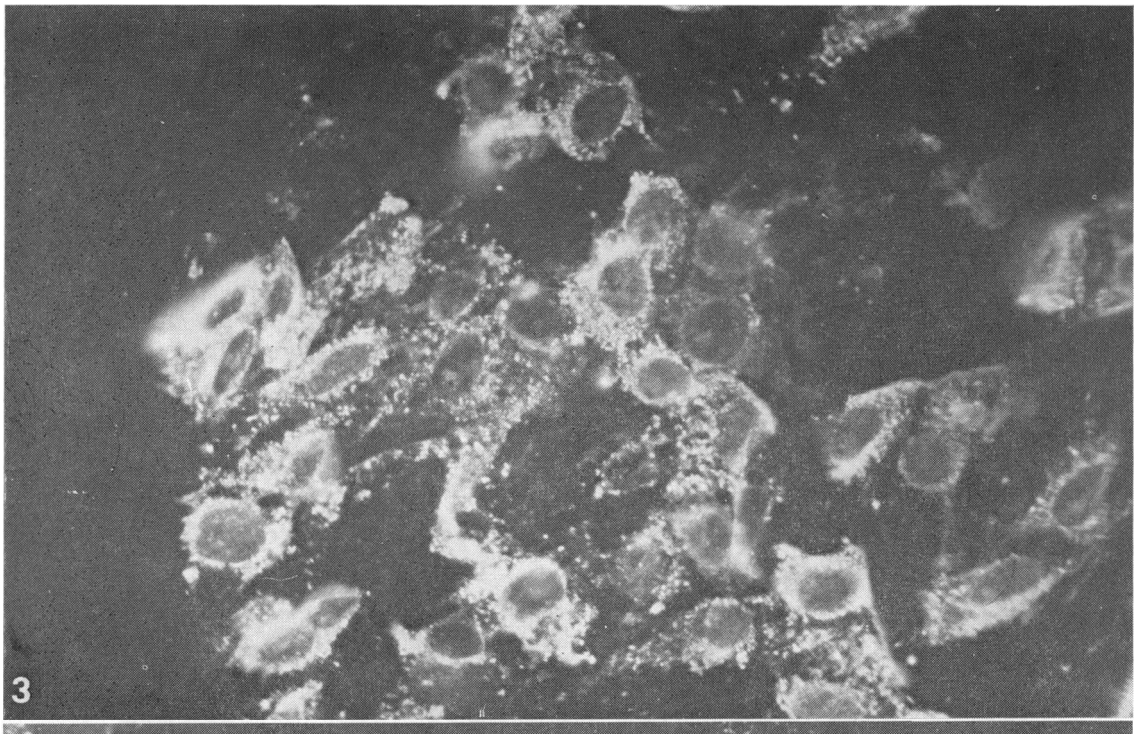


Fig. 2. Same slide as Fig. 1, showing a large focus of brightly stained Lassa virus infected cells.

Fig. 3. Lassa immunofluorescence of a smear prepared from infected Vero cells grown in tubes on day 3 after inoculation of serum from patient T. K.



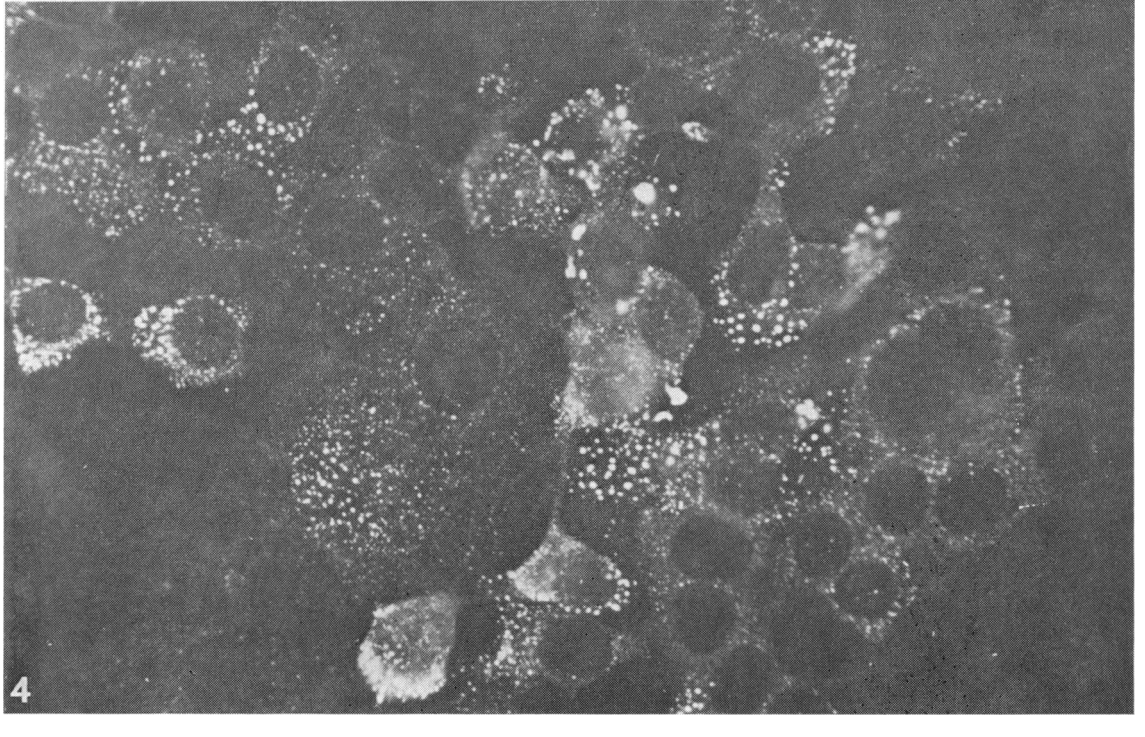


Fig. 4. Lassa immunofluorescence of Vero cells grown in Lab-Tek chamber and inoculated with specimen from patient P. K. 4 days previously. A human convalescent serum from patient B. M. was used as source of antibody.

The outstanding success of the IIFA test in detecting Lassa virus in Vero cell cultures inoculated with test specimens suggests that even further short cuts in diagnosis might be possible. As soon as appropriate specimens are available, we plan to attempt staining of cells in patients' urine samples, buffy coat smears, cells from mucous membrane of the throat, or autopsy material.

The fact that the IIFA test can detect antibodies so early in infection—within 7–10 days—is particularly advantageous for diagnosis. CF antibodies required at least 3 weeks to develop and then were present in only low titre. The long persistence of FAs, at least 5 years, also gives this method a decided advantage over the CF method for area serological surveys.

Our findings correlate well with those obtained with other arenaviruses, like Machupo virus, which causes Bolivian haemorrhagic fever (BHF), and lymphocytic choriomeningitis (LCM) virus (4). After BHF and LCM infections, FAs can be measured for several years. Following BHF disease, FAs appear somewhat later than in Lassa virus infection, and following LCM virus infection, they appear during the first week after initial recognition of signs of CNS involvement. Of 7 sera available to us from Lassa fever patients (obtained between day 7 and day 14 after onset of illness), 5 had fluorescent antibodies. It is of particular interest that of these 5 FA-positive patients, 3 were still viraemic. Possibly the "toxic" appearance of Lassa fever patients during the acute phase of the illness is caused by a cytotoxic interaction of antibody with virus-infected cells.

One additional purpose of our study was to determine whether antigenic differences exist between the various Lassa virus strains. The Lassa virus strains isolated between 1969 and 1975 and evaluated by the IIFA method did not differ.

RÉSUMÉ

RECOURS À L'IMMUNOFLUORESCENCE INDIRECTE POUR LE DIAGNOSTIC DE LA FIÈVRE DE LASSA

DISCUSSION

La technique de l'immunofluorescence indirecte (IFI) permet d'identifier rapidement le virus de Lassa et les anticorps dirigés contre lui. Dans l'étude résumée ici, l'antigène du virus de Lassa a été détecté par la méthode IFI dans des cultures cellulaires Vero 24 heures après qu'on leur eut inoculé du sang humain infecté envoyé d'Europe aux Etats-Unis. Les cellules Vero auxquelles on avait inoculé du sang infecté provenant d'Afrique, qui avait voyagé plus longtemps et n'avait pas été conservé dans la glace, se sont révélées immunofluorescentes dans 10 à 40% des cas, le troisième jour après l'inoculation. L'effet cytopathique du virus de Lassa s'est manifesté 2 à 4 jours après la détection du virus par la technique IFI.

Des anticorps IFI contre le virus de Lassa ont été détectés dans des sérums humains déjà 7 à 10 jours après le début de la maladie. On a trouvé occasionnellement à la fois le virus et les anticorps IFI dans les sérums. Le dernier sérum disponible a été prélevé 5 années après l'infection et son titre était de 1 : 16. Les anticorps fixant le complément étaient apparus environ 3 semaines après l'infection, mais ils n'étaient pas aussi persistants.

Les anticorps fluorescents homologues et hétérologues chez le cobaye infecté par le virus de Lassa n'ont pas révélé de différences antigéniques entre les souches de virus de Lassa isolées au Nigéria en 1969, et celles isolées au Libéria et au Sierra Leone en 1972 et au Nigéria en 1975. Du sérum de convalescent prélevé chez des sujets humains au Sierra Leone et au Libéria en 1972 et au Nigéria en 1974 a été éprouvé en présence de divers antigènes du virus de Lassa. On n'a pas décelé de différence de titre significative, quelle qu'ait été la souche de virus utilisée pour produire l'antigène et l'anticorps.

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DISCUSSION

MONATH: Do you know how the development of CF antigen in the infected cell cultures relates to the development of fluorescent staining and cytopathic effect?

WULFF: I have not studied that question.

FABIYI: Since it is difficult to fly specimens from West Africa to the USA because of the stringent regulations, I wonder whether the CF and FA methods could be safely applied under laboratory conditions in Africa. Was infectious virus present in the material on the FA slides that you examined?

WULFF: We have not yet tested the acetone-fixed slides to see if virus can be isolated, but I doubt that this will prove to be the case. If safe, fixed slides could be distributed to laboratories that have the capability to perform fluorescent microscopy, there should not be any special difficulties.

MURPHY: Lassa virus diagnostic procedures require a laboratory where virus isolation can be carried out, not just a serology laboratory. Approximately 3 days is the minimum time required for development of Lassa virus antigen in infected Vero cell cultures inoculated with human specimens. Vero cells are the substrate used by Dr Wulff for rapid indirect FA identification of Lassa virus isolates. To make that procedure safe, biohazard containment is needed in the laboratory. As for the advance preparation and distribution of slides for FA tests, Webb & Johnson have already reported that the acetone fixation step inactivates Machupo virus very effectively. It is anticipated that the same will be true of Lassa virus, and this is at present being verified in the Maximum Security Laboratory at CDC. It is this procedure for the detection of previous infection, not for rapid clinical diagnosis, that may be introduced into conventional virus laboratories.

OLDSTONE: Do you think that the discrepancy between the result of the CF and the immunofluorescent antibody tests are explained by the presence of two different antigens, or is it not more likely to be due to differences in the sensitivity of the techniques you are using?

WULFF: It is quite possibly due to different sensitivities. Dr Casals asked whether it is possible to make a direct diagnosis from field-collected specimens. I have tried this with slides containing cells from urine sediment and from the buffy coat of centrifuged whole blood from Lassa fever patients brought back from Sierra Leone. Very poor immunofluorescent staining could be seen in 1-5% of these cells. Consequently, I was unable to make a firm diagnosis from these materials, which were taken from patients from whom Lassa virus was isolated. Study of this technique should nevertheless be pursued in the future.