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Early detection of antigen and estimation of virus yield in specimens from patients with Marburg virus disease

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Autopsy specimens from patients with Marburg disease having at least $10^{4.5}$ $TCID_{50}$ of virus per gram of tissue were found to contain sufficient fluorescent antigen-positive cells to make a specific diagnosis possible in less than 3 h. Liver, heart, spleen, and kidney tissues were found to contain significant amounts of virus. Tissue suspensions, as well as blood or serum samples, inoculated into Vero cell cultures produced virus-specific immunofluorescence within 2–5 days. At least one specimen of all virus-positive persons yielded Marburg virus-specific antigen on day 2 or 3 after inoculation. Furthermore, tissues with at least $10^{5.5}$ $TCID_{50}$ of virus/g had Marburg antigen of sufficient titre to be used in complement fixation tests.

Marburg virus (*1*, *2*), which causes a severe systemic disease with haemorrhagic manifestations, was first described in 1967 (*3*). The illness was transmitted by African green monkeys (*Cercopithecus aethiops*) transported from Uganda to the Federal Republic of Germany and Yugoslavia. Since that time, only one additional outbreak is known to have occurred: in 1975, 3 cases were recognized in South Africa (*4*).

Because the fatality rate is high (25–33%) and virus spread from person to person has been observed in both outbreaks, it is very important for a laboratory diagnosis to be made as early as possible. No specific treatment for the disease is known, but early diagnosis permits supportive treatment to be initiated promptly (*2*, *4*) and strict isolation procedures to be enforced.

In 1967, the virus was isolated in guinea-pigs inoculated intraperitoneally with acute phase blood from patients. In 1975, the preliminary clinical diagnosis for the index case was Lassa fever. Subsequently, however, blood and liver suspension were inoculated into Vero tissue cultures, and 4 days later the causative agent was identified as Marburg virus by the indirect fluorescent antibody (IFA) test.

We have re-examined various autopsy materials, blood samples, and sera from seven Marburg virus patients (four infected in 1967 and three in 1975) to evaluate methods for the early detection of virus-specific antigen. We have also determined the amount of virus in each specimen. Serological diagnosis of Marburg and other haemorrhagic virus infections will be described in a separate report.

MATERIALS AND METHODS

Virus propagation in tissue cultures

Vero cell cultures in passage 140–150 were used for virus propagation. The cultures were maintained on Eagle's minimum essential medium to which bovine fetal serum had been added (2 ml/100 ml).

All possibly infectious material was processed and evaluated in the Maximum Containment Laboratory of the Center for Disease Control, an installation for handling highly pathogenic agents.

In 1975, a 10% suspension was made of tissues collected at autopsy from four Marburg virus patients who became ill in 1967. As diluent, 0.75% bovine serum albumin in phosphate buffered saline

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was used. Suspensions of autopsy materials as well as serum and blood, the latter collected just before death, were also available from one person who contracted the disease in 1975 (patient 5). The tissue suspensions were clarified by centrifugation (2000 *g* for 15 min), and the supernates were inoculated and titrated in tubes of Vero tissue culture. Blood samples and sera from two persons who recovered from the disease in 1975 were also included. Three culture tubes were used for each dilution step; in addition, a number of tubes were inoculated with undiluted specimens for early evaluation of virus growth.

Virus identification by immunofluorescence

Evaluation of original specimens. A small volume of each tissue suspension was dropped before clarification on Teflon-coated slides with 8 or 12 wells. The volume of each drop was between 0.015 ml and 0.020 ml. Touch impressions from original tissues were also made, as well as smears from the blood samples. The slides were air dried, fixed in acetone at room temperature for 10 min, and stained. Technical details of the staining procedure have been described (*5*). As a source of antibody, a guinea-pig immune serum prepared against the S.P. strain of Marburg virus was used. This serum had a homologous IFA titre of 1 : 1280; for staining purposes, it was diluted 1 : 64. As a second overlay, a commercially prepared rabbit anti-guinea-pig immunoglobulin G conjugated with fluorescein isothiocyanate was used.[a] Normal guinea-pig serum was included in each test as a control.

Evaluation of infected Vero cells. Early determination of Marburg virus-specific antigen in inoculated Vero cell cultures was made by harvesting oen infected culture each day. The cells were scraped into one-tenth of the original volume of medium, and the suspension was dropped on Teflon-coated slides. Further handling of the slides was as described above. Normal Vero cells were processed in the same manner as the controls. Fourteen days after inoculation, all tissue cultures used for the titrations were evaluated by the IFA method.

Complement fixing (CF) antibody determination. The CF test was performed according to the method described by Casey (*6*). Clarified tissue suspensions were used as antigens and were diluted for the test in twofold steps, starting with a dilution of 1 : 2. A guinea-pig immune serum prepared against the S.P. strain of Marburg virus was used as the source of antibody. The serum was also diluted in twofold steps, starting with a dilution of 1 : 16. A normal guinea-pig serum was incorporated in the test as a control. Each antigen dilution was tested against each dilution of normal and anti-Marburg guinea-pig serum.

[a] The coniugate was obtained from Miles Laboratories, Kanakee, IL, USA.

RESULTS

Marburg virus-specific antigen formed aggregates that stained brightly. The aggregates varied in shape and size from small, sand-like particles to large, odd-shaped clumps up to 10 μm in diameter. Extracellular antigen was quite often noted in frozen tissues (Fig. 1 and 2). Intracytoplasmic inclusions were seen in Vero cells. On primary passage of Marburg virus, antigen very often spilled over to adjacent cells; it was also found lying on top of the nucleus (Fig. 3).

As shown in Table 1, Marburg virus-specific antigen could be rapidly identified by the IFA test in autopsy specimens having a virus titre of at least $10^{4.5}$ $TCID_{50}$ per gram of tissue. Organs with a high virus content were the liver, the heart and, occasionally, the spleen. We evaluated how much of a given surface of a tissue fragment was showing brightly stained clumps of antigen. The percentages given in Table 1 were rough estimates, but a diagnosis was possible when at least 1% of the tissue fragments in the microscope field showed aggregates of antigen under low magnification. Tissue suspensions gave better results than touch impressions. Virus-specific antigen could not be detected with certainty in any of the blood smears made from frozen material.

In addition to evaluating original tissues for virus-specific antigen, we used the IFA test to identify Marburg virus in Vero cells that had been inoculated with autopsy materials, blood samples, and sera from patients 2–5 days previously (Tables 1 and 2). In all, specimens from seven Marburg virus-infected patients were investigated; at least one specimen from each of four patients was positive for Marburg virus on day 2 after inoculation, and at least one specimen from two more patients showed Marburg virus-specific antigen on day 3. Virus could not be isolated from the seventh person, but only lymph node and spleen tissue from this patient were available for testing. It should be noted that the virus content of blood was considerably higher than that of serum collected on the same day.

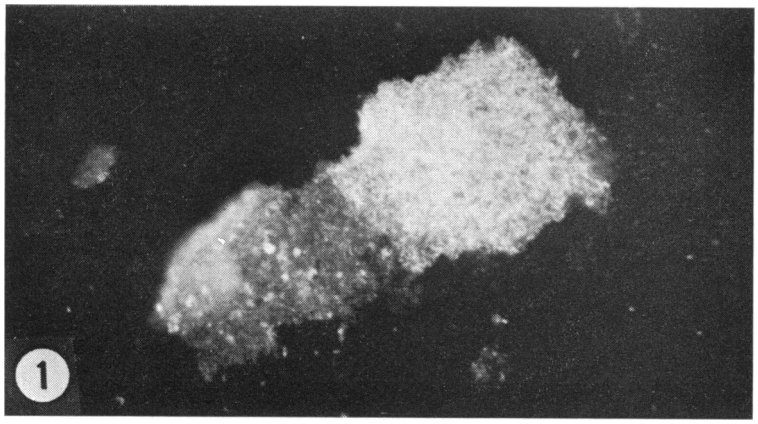

Fig. 1. Immunofluorescence of viral aggregates in human liver cells from patient 5. Evaluation of tissue suspension. Some antigen is located extracellularly. × 520

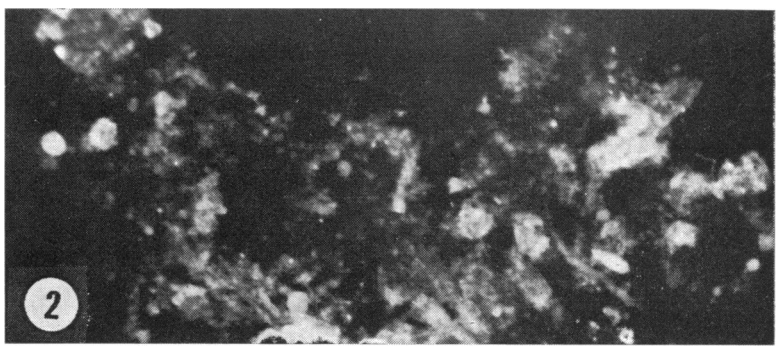

Fig. 2. Immunofluorescence of viral aggregate in human spleen cells from patient 3. Evaluation of tissue suspension. Some antigen is located extracellularly. × 520

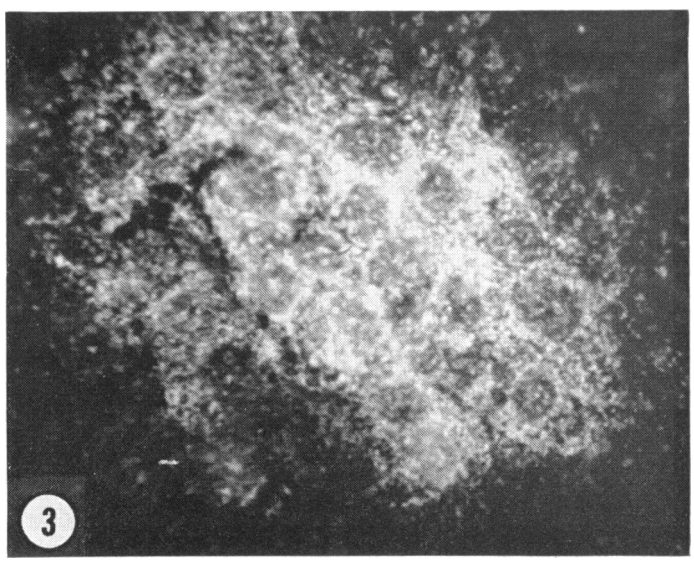

Fig. 3. Immunofluorescence in Vero cells inoculated 2 days previously with human blood specimens from patient 5. Many viral aggregates are located extracellularly. × 520.

Table 1. Results of identification of Marburg virus from autopsy tissues

Specimen	Patient	Percentage of tissue surface with fluorescent antigen after preparation of:		Virus growth in tissue cultures (days after inoculation)	TCID50/g of tissue	CF titre[a]
		tissue suspension	touch impression			
Liver	5	20	ND[b]	2	5.8[c]	128/16
	1	5	1	3	5.5	⩾256/4
Heart	2	5	1	2	6.5	⩾256/4
Spleen	3	20	5	2	7.2	⩾256/8
	5	2	ND	4	4.5	ND
	1	<1[d]	<1	5	3.5	Neg[e]
	4	0	0		<0.8[f]	
Kidney	1	2	1	3	4.7	Neg
	3	1	0	4	4.5	Neg
	5	0	ND		<0.8	
Brain	1	<1	0	5	3.5	Neg
	2	0	0	5	3.5	Neg
	5	0	ND		<0.8	
Lymph nodes	4	0	0		<0.8	

[a] Given as serum dilution/antigen dilution.
[b] Not done.
[c] Titre given on log10 basis.
[d] 2–3 clumps of antigen in whole preparation.
[e] <16/<4.
[f] No virus growth on day 14 after inoculation.

Table 2. Data on isolation of Marburg virus from whole blood and sera, and virus yield of specimens as measured by immunofluorescence

Specimen	Patient	Days after onset of illness	Virus growth in tissue cultures	
			Days after inoculation	TCID50/ml
Blood	5	6 (day of death)	2	5.8[a]
Serum		6 (day of death)	3	4.5
Blood	6	5	3	4.2
Serum		5	5	2.2
Serum		7	5	2.7
Serum		14		0.5[b]
Serum	7	2	2	⩾4.5
Serum		7	5	2.5
Serum		12		<0.5
Serum		26		<0.5

To evaluate an additional serological method for the identification of Marburg virus, we used the tissue suspensions as antigen in the CF test. The four suspensions with titres of $10^{5.5}$ to $10^{7.2}$ $TCID_{50}$/g of tissue yielded sufficient antigen for identification. The titre of the guinea-pig immune serum was verified with an antigen prepared in Vero tissue cultures (7). Normal tissue culture suspension and normal guinea-pig serum gave negative results.

[a] Titre given on log10 basis.
[b] No virus growth in undiluted serum.

DISCUSSION

The data presented showed that laboratory detection of Marburg virus-specific antigen by the IFA test was possible within 3 h when human liver, heart, spleen, and kidney were available for testing. Our results with spleen and kidney specimens were inconsistent; with the limited number of tests run, the liver seemed to be the most important organ for immediate diagnosis. At least one tissue suspension from each of four virus-positive persons showed brightly stained aggregates of specific antigen, which demonstrates that the virus actively multiplied in some organs. We could make a quick diagnosis when at least 1% of the tissue surface was stained. All specimens with a virus titre of at least $10^{4.5}$ $TCID_{50}$/g of tissue could be evaluated directly, without Vero cell passage. Most of the specimens tested had been kept at −65°C for a period of 8–9 years before they were evaluated by the IFA method and titrated in tissue cultures; even after such a long period of time, the virus yield was excellent.

The only person whose autopsy specimens did not yield Marburg virus died on day 16 after the onset of the illness (patient 4). According to the records, the patient died of kidney failure, but we are not sure whether Marburg virus was the only cause of the kidney damage. Viraemia was demonstrated in this patient on day 5 after the onset of

the illness. All other autopsy specimens were from patients who died between day 6 and day 13 after the onset of the illness.

Virus-specific antigen has been demonstrated by immunofluorescence in experimentally infected guinea-pigs and monkeys; in guinea-pig liver, spleen, testes, and lung (*8*); and in monkey liver, spleen, lymph nodes, and lung (*9*, *10*). Marburg virus-specific antigen was detected in a liver biopsy specimen of a monkey 4 days after inoculation and 3 days before death (*9*). By direct staining, extracellularly located antigen was found in a human liver examined 9 days after the onset of illness and in a semen specimen collected approximately 12 weeks after the onset of illness (*11*), but human tissues were not systematically evaluated. Virus was detected by the same workers in fresh blood from one patient, evident as small " granules " of antigen located extracellularly. We had only frozen blood samples available for testing, which apparently were not suitable for immediate diagnosis.

Direct demonstration of virus from serum by electron microscopy after centrifugation on a carrier as proposed by Siegert & Slenczka (*12*) was not tried because the proper equipment was not available at the time of this investigation.

Although Marburg virus has been propagated successfully in a number of different tissue culture systems with the demonstration of viral antigen by immunofluorescence (*1*), we have exclusively used Vero cells. Previous experience (*7*, *13*) indicated to us that Vero cells were well suited for virus growth. For diagnostic purposes, specimens should be inoculated into Vero cells at the same time that original specimens are evaluated for virus-specific antigen. Growth of Marburg virus in Vero cells could be demonstrated by the IFA method on day 2 or day 3 after inoculation in at least one specimen of all virus-positive persons. A parallel study of Marburg virus isolation in Vero cells and guinea-pigs was not done. Our virus titres equalled and perhaps even surpassed those published for guinea-pig blood (*11*).

Serial serum samples were available from only two patients who had recuperated from the disease. The virus content of the serum was highest soon after the onset of clinical symptoms (patient 7). Good correlation was found between viraemia and fever phases. The virus titre was much higher in the blood than in the serum in specimens collected on the same day; this suggests that the virus was associated with the cells.

The clarified tissue suspensions served as effective antigens in the CF test. When the virus titre in a given specimen was at least $10^{5.5}$/g of tissue, the virus suspension yielded sufficient antigen for identification. This test method is only an additional tool for a quick diagnosis; the IFA test is definitely the method of choice for rapid identification of Marburg virus-specific antigen because results can be obtained much faster and the test is more sensitive than the CF test.

RÉSUMÉ

DÉTECTION PRÉCOCE DE LA PRÉSENCE D'ANTIGÈNE ET DÉTERMINATION DE LA CHARGE VIRALE DANS DES SPÉCIMENS PROVENANT DE PERSONNES ATTEINTES DE LA MALADIE DE MARBURG

Des spécimens prélevés à l'autopsie, ainsi que des échantillons de sang et de sérum provenant de personnes ayant succombé ou survécu à la maladie de Marburg, ont été examinés pour apprécier la possibilité de déceler un antigène spécifique du virus au moyen de la technique des anticorps fluorescents. Des spécimens nécropsiques avec une charge virale d'au moins $10^{4.5}$ $DICT_{50}$ par gramme de tissu se sont révélés contenir un nombre suffisant de cellules fluorescentes par réaction antigénique pour permettre le diagnostic en moins de 3 heures. Des titres élevés de virus ont été décelés dans le foie, le cœur, la rate et le rein.

Après inoculation de tissus, sang et sérums dans des cellules de Vero, on a obtenu dans l'intervalle de 2 à 5 jours un antigène spécifique. On a également eu recours, pour l'identification de l'antigène spécifique du virus de Marburg, à l'immunofluorescence. Pour 6 des 7 personnes frappées, on a pu disposer d'au moins un spécimen avec une charge virale suffisante pour poser le diagnostic le deuxième ou le troisième jour après l'inoculation. Le virus de Marburg n'a pu être isolé sur le septième malade, pour lequel on ne disposait que d'échantillons de ganglions lymphatiques et de rate prélevés le sèizième jour après le début de la maladie.

Dans les tissus à charge virale d'au moins $10^{5.5}$ $DICT_{50}$ par gramme, le titre d'antigène était suffisant pour procéder à l'épreuve de fixation du complément.

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