Sensitive and Specific Monoclonal Immunoassay for Detecting Yellow Fever Virus in Laboratory and Clinical Specimens

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A solid-phase radioimmunoassay (RIA) was developed for the detection of yellow fever (YF) virus in infected cell culture supernatant fluid and clinical samples. The test employed a flavivirus group-reactive monoclonal antibody attached to a polystyrene bead support and a radiolabeled type-specific antibody probe in a simultaneous sandwich RIA format. Optimal assay conditions specified a 16-h incubation at high temperature (45°C). Monoclonal antibody to tetanus toxoid was added to the radiolabeled probe to inhibit nonspecific binding. The sensitivity of the assay for cell culture-propagated virus was 2.0 log₁₀ 50% mosquito infectious doses per 100 µl or 100 pg of gradient-purified virion protein per 100 µl. Specificity, assessed with human sera from 512 patients with liver diseases other than YF, including acute viral hepatitis, showed a false-positive rate of 0.0 to 0.6%. Sera from experimentally infected rhesus macaques containing >3.0 log₁₀ units/100 µl of YF virus were positive by RIA. Sera and liver tissue from human patients were found to be positive.

Yellow fever (YF) is an acute flaviviral infection of humans and some species of lower primates, characterized in its severe form by hepatic and renal failure, hemorrhage, and shock. One of the great scourges of mankind between the late 17th and early 20th centuries, it remains today an important public health problem in tropical South America and Africa. A high priority has been placed by international health authorities on surveillance based upon the development and application of early, rapid diagnostic tests (21). At the present time, diagnosis relies almost exclusively on histopathological examination of postmortem liver specimens, except in a few centers in which viral isolation and serologic tests are available. The latter methods are not well suited to rapid and early diagnosis.

Since human YF infections are characterized by a phase of viremia of sufficient magnitude to infect vector mosquitoes, lasting several days to a week or more, it should be possible to directly detect viral antigen in blood by immunoassay. In a preliminary report, Monath and Nystrom (13) described an enzyme immunoassay (EIA) for detection of YF virus in cell culture fluid and sera from experimentally infected monkeys. This technique proved useful for rapid diagnosis of human infections but was less sensitive than virus isolation in mosquito cell cultures or intrathoracically inoculated *Toxorhynchites* mosquitoes (15).

In an attempt to improve the sensitivity of the YF immunoassay, we have investigated various parameters of the test, including selection of monoclonal antibodies for antigen capture and detection and determination of optimal reaction conditions. A solid-phase radioimmunoassay (RIA) procedure was employed, but the test is adaptable to EIA conditions. Sensitivity and specificity were determined; cell cul-

ture-grown virus, infected-monkey sera, and blood and liver specimens from human YF patients were examined.

MATERIALS AND METHODS

Viruses and clinical samples. YF 17D vaccine (Connaught Laboratories, Inc., Swiftwater, Pa.) was passed twice in monolayer cultures of Vero cells. Cell culture supernatant containing 20% fetal bovine serum (FBS) was frozen at -70°C in multiple aliquots for subsequent infectivity titrations and RIAs. Purified YF 17D virus was prepared by polyethylene glycol precipitation and glycerol-potassium tartrate gradient centrifugation as previously described (12). Protein concentration of purified YF virus was determined by the method of Lowry et al. (10).

Experimental infections of rhesus macaques were conducted at the Division of Vector-Borne Viral Diseases, Centers for Disease Control (CDC), Ft. Collins, Colo. Monkeys were inoculated subcutaneously with 500 to 5,000 Vero cell PFU of a virulent, wild YF viral strain (Dak H 1279). Sera were stored at -70°C for periods of 1 month to 3 years prior to testing. Some monkey sera containing virulent YF virus were inactivated before RIA by beta-propiolactone (BPL) as described by Clarke (3).

Blood and liver specimens were obtained from human cases during a YF epidemic in Burkina Faso in 1983. Blood specimens were frozen and thawed several times before RIA testing, whereas livers were continuously frozen at -70°C at the Institut Pasteur, Dakar, Senegal, until shipment on dry ice to the CDC, Ft. Collins. Suspensions (10% [wt/vol]) of liver in FBS were prepared in Ten Broeck grinders and tested by RIA without centrifugation. To evaluate specificity of the RIA, we screened patients at Massachusetts General Hospital with liver disease other than YF, including acute viral hepatitis. Sera were tested undiluted in the YF RIA.

Infectivity titrations. Cell culture-propagated YF 17D virus and sera from experimentally inoculated monkeys were titrated by plaque assay in Vero cell cultures grown in

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TABLE 1. Characteristics of monoclonal antibodies used to construct RIAs for YF virus^a

Immunizing virus and group ^b	Antibody	Isotype	Flavivirus specificity	Competitive binding results	
				Inhibited	Augmented
YF 17D					
B	2E10	IgG2a	YF type	2E10, 4G2	5H3
C	3A3	IgG1	YF type	2E10	
C	2D12	IgG2a	YF type		
D	5E6	IgG2b	Group	2E10, 4G2, 5H3	
D	5H3	IgG2a	Group	4G2, 5H3	2E10
Dengue 2	4G2	IgG2a	Group	2E10, 4G2, 5H3	

^a The method used to develop the monoclonal antibodies was adapted from methods of Schlesinger et al. (16) and Gentry et al. (6).

^b The group was based on biologic and serologic characteristics (11).

six-well plastic panels (11). This virus seed was also titrated by intrathoracic inoculation of *Aedes aegypti* mosquitoes by C. J. Mitchell, CDC, Ft. Collins. Titrations of human blood and liver were performed at the Institut Pasteur, Dakar, in fluid cultures of AP 61 *Aedes pseudoscutellaris* cells; cultures were held for up to 7 days and examined for cytopathic effect and immunofluorescent antigen. Titers were expressed as log₁₀ units/100 µl.

Antibodies. Monoclonal antibodies to YF 17D and dengue type 2 viruses used to construct RIAs were developed by Schlesinger et al. (16) and Gentry et al. (6), respectively. Selected characteristics of these antibodies are shown in Table 1. Antibodies were purified by ammonium sulfate precipitation and affinity chromatography with protein A-Sepharose (4). Antibodies were labeled with ¹²⁵I to a specific activity of 8 to 12 µCi/µg by the Iodogen method (5). Radiolabeled antibodies were stored at 4°C in FBS for up to 6 weeks before use.

RIAs. The RIA technique has been previously described for detection of hepatitis B surface antigen (19). Polystyrene beads (outer diameter, 0.64 cm; Precision Plastic Ball, Chicago, Ill.) were coated with monoclonal ascitic fluid diluted in phosphate-buffered saline (pH 7.2) for 16 h at 22°C with continuous agitation and then washed extensively with distilled water. Virus-containing samples diluted in FBS were placed in 100-µl volumes in duplicate wells of 25-well plastic reaction trays (Centocor Inc., Malvern, Pa.), after which antibody-coated, washed beads were added. For "simultaneous sandwich" RIAs, 100 µl of radiolabeled detecting antibody containing 150,000 cpm was immediately added to each well. Trays were incubated for various times at specified temperatures. Beads were then washed with a mechanical apparatus (Pentawash; Abbott Laboratories, North Chicago, Ill.), and bound radioactivity was determined in a gamma well counter. In some assays, a "forward sandwich" format was used. Virus samples (200 µl) were incubated with coated beads which were then washed, incubated with radiolabeled second antibody (150,000 cpm in 200 µl), washed again, and counted.

Results were expressed as the ratio of the mean counts per minute bound in test versus control samples (S/N, signal-to-noise ratio). Controls were FBS (in tests on cell culture-propagated virus) or normal monkey or normal human sera.

Blocking antibodies. To reduce nonspecific binding of radiolabeled YF monoclonal antibody, a mixture of anti-tetanus toxoid monoclonal antibodies (B₂TT) was added at a

concentration of 200 µg/ml. Development of these antibodies, which were isotypes immunoglobulin G1 (IgG1) and IgG2A, has been previously described (V. R. Zurawski, Jr., J. G. R. Hurrell, W. C. Latham, P. H. Black, and E. Haber, Fed. Proc. 39:4922, 1980).

Serum enzymes. Alanine aminotransferase levels in sera from experimentally infected rhesus monkeys were determined with Ames diagnostic kits (Ames Co., Elkhart, Ind.).

RESULTS

Selection of monoclonal antibodies. Antibody pairs were evaluated in simultaneous sandwich RIAs for detection of YF 17D virus grown in Vero cells. This virus preparation contained 6.7 log₁₀ 50% mosquito infectious doses (MID₅₀) per ml and 6.0 log₁₀ Vero cell PFU/ml. RIAs were performed with beads coated with a 1:1,000 dilution of ascitic fluid, 10-fold dilutions of virus, and incubation for 4 h at 22°C.

The relative efficiencies of various antibody combinations for binding of a high concentration (5.4 log₁₀ MID₅₀/100 µl) of YF virus are shown in Table 2. Efficiency is expressed as the percentage of the S/N ratio of an arbitrarily selected antibody pair showing moderately high binding (2D12 and 4G2). Optimum binding was achieved by use of 5H3 antibody on the solid phase and radiolabeled 2E10 antibody. In further experiments, combinations of two or three antibodies were used for both capture and detection of YF virus; no combination proved superior to the 5H3-2E10 pairing (data not shown).

Optimal concentration of capture antibody. Beads coated with 5H3 ascitic fluid diluted 1:500, 1:800, 1:1,000, and 1:2,000 were tested against a range of concentrations of YF 17D virus. Binding was maximal at the 1:500 dilution, which was therefore used in all subsequent assays.

Time and temperature of incubation. At high concentrations of YF 17D virus (5.4 log₁₀ MID₅₀/100 µl), peak binding occurred after 2 h of incubation and did not change appreciably over 16 h (Fig. 1). Binding was higher at 45°C than at 4, 22, or 37°C. At lower virus concentrations, binding at all temperatures increased with time, reaching peak levels at 16 h, when the assays were terminated (Fig. 2). On the basis of these data, RIAs were performed for 16 h at 45°C.

Sensitivity of the RIA. In six replicate RIAs with infected cell culture fluid, sensitivity was found to be approximately 2.0 log₁₀ MID₅₀/100 µl, or 1.3 log₁₀ Vero cell PFU/100 µl (Fig. 3). Cell culture supernatants may contain both intact virions and noninfectious structural and nonstructural viral proteins released from infected cells. Sensitivity of the RIA was therefore also assessed with gradient-purified virions and was found to be approximately 100 pg/100 µl (Fig. 4).

Comparison of simultaneous and forward sandwich RIAs.

TABLE 2. Efficiency of various combinations of monoclonal antibodies for binding YF 17D virus in RIA

Capture antibody	Efficiency of binding with ¹²⁵ I-labeled detecting antibody (%) ^a					
	4G2	2D12	5H3	3A3	2E10	5E6
4G2	14	73	18	25	233	48
2D12	100	11	167	18	58	23
5H3	16	29	6	121	417	38
3A3	25	30	89	15	22	30
2E10	23	38	233	14	218	49
5E6	15	82	18	52	236	66

^a Efficiency of binding is expressed as a percentage relative to the antibody pairing of 2D12 (capture antibody) and 4G2 (¹²⁵I-labeled antibody).

Sensitivity of the simultaneous sandwich assay (incubated at 45°C for 16 h) was compared with that of the forward sandwich format, with sequential incubations of 16 and 4 h at 45°C. S/N values in the simultaneous sandwich format were 33% higher than those in the alternate design.

Specificity. A total of 512 human sera from disease controls at Massachusetts General Hospital were tested. Three (0.6%) sera initially gave false-positive reactions by YF RIA, with S/N values of 2.5, 10.3, and 11.4. The clinical features and histories of these three individuals were not different from others in the control group. These three sera were retested twice and found negative. A blocking test was developed, in which 20 μ l of YF hyperimmune rabbit serum was incubated with 200 μ l of test sample for 30 min prior to testing by RIA. This procedure reduced binding of YF virus by $\geq 95\%$. Since no false-positive sera were available, we were unable to confirm that the blocking test would distinguish true from false-positive reactions.

Viremia in experimentally infected monkeys. Of 14 sera from infected monkeys containing virus detectable by plaque assay in Vero cells, 11 were positive by RIA (Fig. 5). Although insufficient viremic sera were available to fully evaluate sensitivity of the assay at low virus concentrations, the limit appeared to be between 2 $\log_{10}$ PFU/100 μ l and 3 $\log_{10}$ PFU/100 μ l. S/N ratios increased with infectivity titer; however, some samples with low plaque titers were strongly positive by RIA. This phenomenon is illustrated by tests on

FIG. 1. Binding of a high concentration of YF 17D virus (5.4 $\log_{10}$ MID₅₀) in monoclonal RIA performed under conditions of varying time and temperature of incubation. Binding at 4°C was lower than that at 22°C (data not shown).

FIG. 2. YF RIAs performed at 45°C showing increasing binding with increased time of incubation at low virus concentrations.

sequential sera obtained from an infected rhesus monkey (Table 3). Infectious virus was first detected on day 3, peaked on day 5, and decreased dramatically during the last 24 h of life. The RIA signal, however, continued to increase in concert with progressive liver necrosis and rising serum alanine aminotransferase levels.

No significant difference in results was observed between RIAs performed with beta-propiolactone-inactivated and fully infectious viremic sera.

Blood and liver specimens from human YF patients. Six samples containing YF virus at titers ranging between 1.3 and 4.2 50% tissue culture infectious doses per 100 μ l were positive by RIA (Table 4). Since the two blood samples had been frozen and thawed several times between infectivity assay and RIA, some antigenic reactivity may have been lost. Liver tissue from patients who had died contained relatively more detectable antigen than infectious virus.

DISCUSSION

Analysis of the YF viral envelope glycoprotein by monoclonal antibodies has defined at least eight epitopes on the basis of competitive binding assays and serological cross-reactivity patterns; an additional epitope has been identified by monoclonal antibodies to dengue type 2 virus (17). We investigated monoclonal antibodies directed against six of these nine antigenic sites in the development of an immunodiagnostic test for YF (Table 2). Comparative assays with various antibody combinations led to the selection of 5H3 for antigen capture and 2E10 for detection as radiola-

FIG. 3. Sensitivity of RIA for detection of YF 17D virus in Vero cell culture fluid. - - -, Lowest positive result.

beled probe. Since 2E10 is a type-specific antibody which displays no cross-reactivity with other flaviviruses (14), the RIA as constructed provides a specific diagnosis.

Of interest is the observation of Schlesinger et al. (17) that antibody 5H3 significantly augments binding of 2E10 to YF virus. Similar synergism between antibodies has been noted in competitive binding assays in other flavivirus systems (7, 8) and has been ascribed to induction of conformational changes in viral antigenic sites. In the simultaneous sandwich RIA constructed for YF antigen detection, antibody interactions with virus may be quite different than in competitive binding assays. Although augmented binding was reciprocally observed with the 5H3-2E10 antibody pair, other antibody combinations known to augment binding in competitive binding assays (17; Table 1) did not similarly

FIG. 4. Sensitivity of monoclonal RIA for detection of purified YF virions. - - -, Lowest positive result.

FIG. 5. Results of YF RIAs and plaque assays with viremic sera from rhesus macaques experimentally inoculated with YF (Dak H 1279) virus. - - -, Lowest positive result.

affect the RIA. In general, however, antibody pairs which showed a high degree of competition in competitive binding assays also showed inhibition of binding in simultaneous sandwich RIA (Tables 1 and 2). Antibodies which compete for the same epitopes would not be expected to perform well in RIAs of this design.

Studies to define the optimal conditions for YF antigen detection illustrated the importance of kinetics and temperature of incubation, especially at low concentrations of virus. Although incubation times as short as 1 h may yield positive results with samples containing high virus concentrations, binding at low concentrations was significantly

TABLE 3. Comparison of alanine aminotransferase levels, infectivity assay, and RIA results in a rhesus macaque experimentally infected with YF virus

Day post-inoculation	ALT ^a (U/liter)	Viremia (log ₁₀ PFU/100 µl)	RIA results	
			Mean cpm	S/N ^b
0	42	0	29	0.9
1	35	0	33	1.0
2	32	0	29	0.9
3	45	2.2	49	1.5
4	45	3.0	139	4.3
5	1,130	7.0	2,106	65.8
6				
a.m.	7,960	3.0	3,988	123.1
p.m. ^c		3.0	3,328	102.7

^a ALT, Alanine aminotransferase.

^b An S/N ratio of >2.0 was considered a positive result.

^c In the evening, the macaque was moribund.

TABLE 4. Infectivity assays of AP 61 cell cultures and RIA results for blood and liver specimens from human YF patients in Burkina Faso, 1983

Specimen type and no.	Infectivity assay of AP 61 cells (log ₁₀ PFU/100 µl) ^a	RIA results	
		Meanc cpm	S/N ^b
Blood			
F15	2.9	262	3.2
TEN	4.2	165	2.0
Liver			
F9	3.3	10,463	127.6
F10	2.1	295	3.6
F20	2.5	1,714	20.9
F38	1.3	1,550	18.9

^a Titrations in AP 61 cells were performed at the Institut Pasteur, Dakar, Senegal, in 1983.

^b An S/N ratio of >2.0 was considered a positive result.

increased by prolonged (16 h) incubation. Somewhat surprising given the relative temperature instability of flaviviruses (2, 9) was the high optimal temperature (45°C) for incubation. Parallel experiments with a monoclonal RIA for dengue virus (to be reported separately) showed maximal binding at 37°C, indicating the need to define reaction conditions for each virus-antibody system.

The sensitivity of the YF RIA (1.3 to 2.0 log₁₀ units, depending on the assay system used to measure infectious virus) is 100- to 1000-fold more sensitive than a previously described EIA (13) which employed different monoclonal antibodies or human IgM antibody, a forward sandwich design, and overnight incubation of virus at low temperature (4°C). Sensitivity of the RIA for purified YF virions was in the range of 100 pg, similar to that of highly sensitive RIAs developed for detection of hepatitis B surface antigen in blood (19).

In tests with over 500 human sera from non-YF liver disease patients, repeatably false-positive reactions were absent. An irrelevant anti-tetanus monoclonal antibody of the same isotype (IgG2a) was used to block nonspecific binding. Studies to be reported elsewhere have shown that use of B₂TT significantly reduced false-positive reactions in a dengue RIA.

Evaluation of the YF RIA as a diagnostic tool is hampered by absence of quantitative published data on viremia levels in humans and by the difficulty in obtaining specimens from human patients. Now that a sensitive, specific, and rapid diagnostic test is available, intensive efforts should be made to apply it in areas in which YF occurs. Tests on sera from experimentally infected monkeys have indicated the potential usefulness of the YF RIA. However, the course of infection in the rhesus monkey is exceedingly rapid, and death occurs before onset of a detectable immune response. The course of infection in humans is slower, raising the possibility that immune complexes formed during viral clearance may interfere with antigen detection.

All viremic monkey sera with infectivity titers >3.0 log₁₀ PFU/100 µl were positive by RIA. Of interest was the finding that some sera obtained late in the course of infection, when marked liver necrosis was evident, showed a discrepancy between high RIA and low infectivity titers (Table 3). The same observation was made in tests on human liver samples (Table 4). These results probably reflect presence or release of large amounts of noninfectious antigen corresponding to the slowly sedimenting hemagglutinating antigen found in flavivirus-infected mouse brain and serum (1, 18).

Testing of a limited series of human samples from Africa indicated that the RIA could be used for rapid diagnosis of YF. These specimens contained 1.3 to 4.2 log₁₀ units of virus per 100 µl in a highly sensitive infectivity assay. Application of the RIA to postmortem liver specimens eliminates the ambiguities frequently encountered in histopathological examinations. It is even possible that Formalin-fixed liver tissue could be used in RIAs if antigenic sites were reexposed by treatment with proteases.

The assay detected both 17D vaccine and wild YF strains from Senegal and Burkina Faso. Further investigations are required to establish reactivity of the type-specific 2E10 antibody with a wider variety of YF strains from South America and Africa. It is probable that this antibody will provide a generally sensitive assay, since YF type-specific monoclonal antibodies reacted similarly with a variety of YF virus strains by immunofluorescence (14).

Viremia may occur after YF 17D immunization. It will be of interest to determine whether antigen can be detected in the blood of vaccine recipients, although the low level and evanescent nature of the viremia (20) make this unlikely.

For practical application in laboratories engaged in YF diagnosis, the EIA has many advantages over the RIA. Work is in progress to convert the assay described in this report to EIA methodology. From previous experience with conversion of other assays (e.g., for hepatitis B), we expect that this can be accomplished with little loss in sensitivity.

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