JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1981, p. 527-529
0095-1137/81/110527-03$02.00/0

Vol. 14, No. 5

Preparation of Polyvalent Viral Immunofluorescent Intracellular Antigens and Use in Human Serosurveys

K. M. JOHNSON,* L. H. ELLIOTT, AND D. L. HEYMANN

Center for Infectious Diseases, Bureau of Laboratories, Centers for Disease Control, Atlanta, Georgia 30333

Received 2 March 1981/Accepted 2 June 1981

A method is described for preparation of polyvalent antigens for use in rapid screening for immunofluorescent antibodies to Lassa, Marburg, and Ebola viruses. The technique uses mixtures of specifically infected Vero cells placed on Teflon-templated microscope slides. It was found to be as sensitive as the use of monovalent antigens for detection and quantitation of antibodies to these highly hazardous human pathogens.

Indirect immunofluorescence using infected cultured cells, either as preformed monolayers or as suspensions fixed to microscope slides, is widely used in diagnosis and population-based antibody surveys of many viral infections. This method is our current standard for such work with the African class IV zoonotic viruses Lassa (10), Marburg (9), and Ebola (2). Because diagnosis of acute disease caused by these agents often represents a true medical emergency, and because specific antibodies to these viruses may be formed during the acute stage of an infection (10), it is customary to store suitable antigens at low temperature so that tests may be completed within hours after specimens are received.

It occurred to us that mixtures of cells infected with the three respective viruses might serve to speed the diagnostic screening process and to achieve major savings in time and cost in conducting serological surveys for antibodies to these agents in many countries of Africa, where little or nothing is known concerning patterns of human infection. We here describe a method for preparation of such polyvalent antigens as well as results of their initial application in a survey for infection in Central Africa.

MATERIALS AND METHODS

Virus strains. The viruses used were from human blood specimens and were isolated and passaged only in Vero cells. Lassa virus was strain Josiah, Sierra Leone 1976, passage 3. The Ebola virus was strain Mayinga from Zaire 1976, passage 3. Marburg virus was strain Voege 1967, passage 5. All work with infectious virus was carried out in a maximum containment laboratory.

Cell cultures. Vero cells designated pool 76 from a frozen stock prepared at the Middle America Research Unit, Balboa, Canal Zone, in 1966, were used to prepare a new stock of frozen master seed cells designated pool 76-CDC. These Vero cells were originally obtained from Japan and were never cloned or otherwise certified by the American Type Culture Collection, although they were shown in the Canal Zone to have the predominant aneuploid karyology (2N=58) rather than 2N=60 of *Cercopithecus aerthiops* monkeys, from which the line was derived (1). Cells were grown as monolayers in T-150 flasks by using Eagle minimum essential medium containing 10% heat-inactivated fetal bovine serum and an antibiotic mixture designed to provide 1 U of penicillin, 0.5 μg of streptomycin, and 0.02 μg of amphotericin B per ml. Stock cells are tested monthly for mycoplasma by the cytoplasmic deoxyribonucleic acid-fluorescence technique (3). Only mycoplasma-free cells were used. Before inoculation with viruses, growth medium was replaced with a similar formula in which fetal bovine serum was reduced to 2%.

Antisera, antiglobulin conjugates, and microscopy. The indirect immunofluorescent procedure was used throughout. Antisera used for test standardization and positive controls were obtained from humans convalescing from the respective viral diseases or were prepared by single intraperitoneal inoculation of individual viruses into hamsters or guinea pigs. Blood was harvested and serum was prepared 4 weeks after these animals were infected. Antiglobulin conjugates were obtained from commercial sources. Anti-human reagents with mixed, immunoglobulin G, and immunoglobulin M specificity were obtained from Burroughs-Wellcome Ltd., Beckenham, England, and those for hamster and guinea pig immunoglobulins were supplied by Cappell Labs, Cochranville, Pa. Conjugates were used at dilutions of 1:10 to 1:50, and Evans blue dye at a concentration of 1:1,000 was incorporated into all conjugates as a counterstain.

The staining technique was a standard one which included 30-min primary and secondary reaction steps carried out at ambient temperature and vigorous stirred washing steps after each reaction in phosphate-buffered saline at pH 7.2. Slides were then air-dried, mounted with cover slips in glycerine-phosphate-buffered saline (9:1), pH 9.0, and examined in a Leitz microscope equipped with epi-illumination. A 50 W halogen light source was used, together with a BG38

red-absorbing filter and KP-500 primary and K510 secondary filters. We used 6.3× oculars and 25- and 50-power water-immersion objectives designed to maximize fluorescent intensity at the wavelengths delivered by this system.

Preparation of antigens. Vero cell monolayers grown in plastic T-150 flasks were rinsed with phosphate-buffered saline and inoculated with 10^4 to 10^6 50% tissue culture infective doses of the respective viruses. After several trials it became apparent that a standard inoculum from a given virus pool produced 20 to 40% infected cells in a given number of days: 3 for Lassa and Ebola viruses, and 6 for Marburg. Accordingly, flasks were inoculated so that all cells could be harvested on a given day. Flasks were rinsed once in 0.05% trypsin with 0.002% ethylenediaminetetraacetic acid, and cells were removed by incubation for a few minutes at 37°C in 5 ml of the trypsin-ethylenediaminetetraacetic acid solution, which was removed just before cells began to detach from the flasks. A 10-ml volume of isotonic saline containing 5% fetal bovine serum (serum was necessary to prevent spontaneous clumping of cells) was added to each flask, and the cells were vigorously pipetted mechanically to break up clumps.

Suspensions of cells were next placed in 10-cm-diameter plastic petri dishes in volumes of 5 ml and subjected to ultraviolet irradiation with a Mineralight Lamp, model R52 (UV Products, Inc., San Gabriel, Calif.). With an intraviolet meter (Blak-Ray, model J225, UV Products, Inc.), lamp distance was adjusted to deliver 1,200 to 2,000 $\mu W/cm^2$, and the suspensions were exposed for 20 min to inactivate viral infectivity. Suspensions were then diluted to contain about 120,000 cells per ml and pooled. About 0.015 ml was then placed on each "spot" of Teflon-templated microscope slides (Cel-line Assoc., made to indirect fluorescent-antibody specifications, 12 spots per slide) by using plastic microtiter droppers. Cell suspensions were stirred during this procedure to ensure nearly uniform distribution of cells on each spot. The concentrations and volumes used resulted in a loose mat giving good cell separation and few clumps. The mixing procedure ensured that not more than 10% of cells were infected with a given virus, ensuring that a majority of cells were in fact a control "blank" for each viral antigen.

Slides were air-dried, fixed in acetone for 10 min, placed in small plastic slide boxes, removed from the maximum containment laboratory, and subjected to gamma irradiation (cobalt-63 source, 200,000 rads) to destroy residual infectivity in cells infected with Marburg and Ebola viruses and to decontaminate the surfaces of the slides and the interior of the slide boxes. Slides were than stored at −70°C until needed for antibody tests, which were done outside the maximum containment laboratory.

RESULTS

Preparation of polyvalent antigens. Slides prepared according to the described procedure displayed bright fluorescence when individual wells were reacted with antisera specific for Lassa, Marburg, or Ebola viruses. The percentage of stained cells ranged from about 5 to 15%. By careful examination of cytoplasmic granules with the 50-power objective, Lassa reactions (very fine granules) could be distinguished from Marburg or Ebola reactions, which gave large condensed inclusions. Antibody titers to reference sera were similar whether monovalent or polyvalent antigens were used (Table 1). Negative control sera were included in all tests.

Use of polyvalent antigens for diagnosis of infection and serological surveys. Sera from nine patients known to have been infected with one of the three viruses were titrated using polyvalent slides. Results were not different from those originally obtained with monovalent antigens, and each was reconfirmed with a battery of such antigens.

A total of 1,143 sera from indigenous humans living in Cameroon and the Central African Republic was screened by using the polyvalent antigens. Sera were tested initially at 1:4 dilution, and all those scored as even weakly positive were then titrated by using individual monovalent antigens. We found 90 sera positive for Ebola antibodies at a dilution of ≥1:16, 9 sera reactive to Marburg antigen, and none positive to Lassa antigen. Moreover, all sera scored at least 2+ (1+ to 3+ scale) on the screen proved to have titers of at least 1:4 (the range was 1:4 to 1:64) when tested with monovalent antigens. A few sera weakly reactive in the screen were judged not to meet our criteria for positive reaction in the follow-up test.

DISCUSSION

This method represents a simple modification of a technique which has long been used to measure antibodies to a wide variety of micro-

TABLE 1. *Antibody titers in sera selected for specificity to Lassa, Marburg, and Ebola viruses*

Serum and antigen	Reciprocal antibody titer by method[a]:	
	Monospecific	Polyvalent
Lassa		
Human	1,024	1,024
Guinea pig	512	256
Marburg		
Human	128	128
Guinea pig	256	256
Ebola		
Human	512	1,024
Guinea pig	512	512

[a] Specific (Monospecific) and polyvalent immunofluorescent antigens.

bial agents. Its application appears to be appropriate under the following conditions: (i) agents to be employed should not show extensive antibody reactions with related agents, (ii) prevalence of antibodies to a given agent should not be so high as to force titration of a majority of the sera screened, and (iii) immunofluorescent antibodies to the agents in question should appear rapidly (diagnostic use) and persist for long intervals (serosurveys). Lassa, Marburg, and Ebola viruses all meet these criteria (4, 4a, 6). The reactions observed appear to be specific. In the case of Lassa virus, potential non-specificity due to rheumatoid factor has been studied on a limited basis and has been discounted. However, more definitive methods for specific antibody measurement for each of these viruses need to be developed. We plan to add antigens for Congo-Crimean hemorrhagic fever and Rift Valley fever viruses, which are known to cause acute hemorrhagic fever in Africa (5, 7), in order to constitute a screen for agents associated with that clinical syndrome on the African continent.

An alternative approach to this conceptual problem was proposed by Wang (8), who prepared multivalent "spots" of geometrically positioned cells infected with different chlamydial agents. This approach has great merit where the overall prevalence of antibodies to one or more agents in the screening panel is likely to be relatively high. However, where overall prevalence of antibody to any agent in the polyvalent screen is likely to be low, we believe that our method is more cost effective.

We debated adding yellow fever virus antigen to this mixture, but decided against it in light of the extensive cross-reactions to be expected from related flaviviruses found in Africa. Further work with other viruses, particularly those which cause zoonotic human infection, in which neutralizing and immunofluorescent antibody patterns are compared may well indicate other polyvalent antigen mixtures useful for diagnostic or epidemiologic work.

LITERATURE CITED

1. **American Type Culture Collection.** 1975. Catalogue of Strains-II, p. 74–75. American Type Culture Collection, Rockville, Md.
2. **Anonymous.** 1978. Ebola haemorrhagic fever in Zaire, 1976. Report of an international commission. Bull. W.H.O. **56**:271–293.
3. **DelGuidice, R. A., and H. E. Hopps.** 1977. Mycoplasma infection in cell cultures, p. 57–69. Plenum Press, New York.
4. **Heymann, D. L., J. S. Weisfeld, P. A. Webb, K. M. Johnson, T. Cairns, and H. Berquist.** 1980. Ebola hemorrhagic fever: Tandala, Zaire, 1977–1978. J. Infect. Dis. **142**:372–376.

4a. **Saluzzo, J. F., J. P. Gonzalez, A. J. Georges, and K. M. Johnson.** 1981. Mise en évidence d'anticorps vis à vis de virus Marburg parmi les populations humaines du Sud-Est de la République Centrafricaine. C. R. Acad. Sci. **292**:29–31.

5. **Simpson, D. I. H., E. M. Knight, G. Courtois, M. C. Williams, M. P. Weinbien, and J. W. Kibukamusoke.** 1967. Congo virus: a hitherto undescribed virus occurring in Africa. Part I. Human isolations—clinical notes. East Afr. Med. J. **44**:87–92.
6. **van derGroen, G., K. M. Johnson, P. A. Webb, H. Wulff, and J. V. Lange.** 1978. Results of Ebola antibody surveys in various population groups, p. 203–208. *In* S. Pattyn (ed.), Ebola virus haemorrhagic fever. Elsevier/North-Holland Biomedical Press, New York.
7. **VanVelden, D. J. J., J. D. Meyer, J. Olivier, J. H. S. Gear, and B. Mcintosh.** 1977. Rift Valley fever affecting humans in South Africa. A clinico-pathological study. S. Afr. Med. J. **51**:867–871.
8. **Wang, S.-P.** 1971. Trachoma and related disorders, p. 273–288. Exerpta Medica, London.
9. **Wulff, H., and K. M. Johnson.** 1979. Immunoglobulin M and G responses measured by immunofluorescence in patients with Lassa or Marburg virus infection. Bull. W.H.O. **57**:631–635.
10. **Wulff, H., and J. V. Lange.** 1975. Immunofluorescence for the diagnosis of Lassa fever infection. Bull. W.H.O. **52**:429–436.