Development of an Immunofluorescence Focus Assay for Ebola Virus

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A 48-h indirect immunofluorescence focus assay for the quantitation of Ebola virus was developed, utilizing HeLa-229 cell monolayers. The dose dependency and the sensitivity of this assay as compared with conventional assays are reported. This indirect immunofluorescence focus assay can be used as a rapid, quantitative test for the detection of Ebola virus, an agent from Africa known to cause hemorrhagic fever.

The recently discovered (1976) Ebola virus is responsible for one type of human hemorrhagic fever on the African continent (10). The virus is highly pathogenic, and no vaccine is available; therefore, it is classified as a class 4 viral agent (2). We initiated a program to investigate the ecology, biology, immunology, and chemistry of this virus and its relatives, and have recently reported on the RNA and protein composition of purified Ebola virions (9, 14). These studies and others have indicated that this virus possesses unique properties which may qualify it for consideration as a member of a new family of animal viruses. To facilitate further studies, we developed a rapid, quantitative test to measure viral infectivity and antibodies.

Several assays have been described for negative-strand viruses (1, 3, 4, 6–8, 12, 16) and were considered for the needs described above. These include hemagglutination, plaque formation, noncytopathic hemadsorption plaques, and immunoperoxidase staining, among others. Although the plaque assay recently reported for Ebola virus (11) is quantitative, we were unable to obtain consistent results. In addition, it requires at least 1 week to obtain results with this assay.

Immunofluorescence assay techniques for viruses have been widely used. An immunofluorescence focus assay for adenoviruses 1, 2, and 12, adeno-associated virus type 1, and reovirus type 1 used human amnion cells (17). Fluorescent cell counting has been used as an assay for respiratory syncytial virus (15). In addition, extensive studies using immunofluorescence focus assay techniques have been carried out with persistent mumps virus infections and other agents (18, 19). We describe here a rapid, quantitative test, the immunofluorescence focus unit (IFU) assay, which we adapted for use with Ebola virus. We also demonstrate that the test can be used for the quantitation of Lassa virus, a class 4 arenavirus.

MATERIALS AND METHODS

Cells. HeLa-229 cells were obtained from Paul Price of the Cell Culture Laboratory, Centers for Disease Control, Atlanta, Ga., and were grown at 35°C in 5% CO2 in Eagle minimal essential medium with Hanks salts, 10% heat-inactivated fetal bovine serum, 0.1% NaHCO3, and 100 U of penicillin G potassium and 100 μg of streptomycin sulfate per ml. The cells were tested and found to be free of Mycoplasma spp. by staining with 4′,6-diamidino-2-phenylindole (Bioassay Systems Corp., Cambridge, Mass.).

Viruses and antisera. The Ebola virus used in this study was isolated from the blood of a patient infected during the 1976 outbreak in Zaire and has been used in several other studies (9, 14). This strain was originally isolated in Vero cells, and the viral stock used in this study was from the third passage in Vero cells. The Ebola antiserum used to detect the foci of virus-infected cells was human convalescent-phase serum. Lassa virus and its corresponding antiserum used in these experiments have been described previously (21). Lassa virus (from Sierra Leone) was also isolated in Vero cells and was low-cell-passage material. Anti-human immunoglobulin (sheep) was obtained from Burroughs-Wellcome Laboratories, Research Triangle Park, N.C. No heterologous (i.e., Lassa, Ebola, Marburg, Congo, Rift Valley fever, or Korean Hemorrhagic fever virus) staining was observed with either Lassa or Ebola antiserum (20, 21), and positive and negative control sera were tested and found to have expected reactivities. Because of the pathogenicity of the Ebola and Lassa viruses, all work was performed in the Centers for Disease Control maximum containment laboratory (Atlanta, Ga.).

Solutions. Phosphate-buffered saline (PBS) (0.01 M, pH 7.2) was used as the diluent for antisera and as the rinsing agent during the immunofluorescent staining.
procedure. Buffered glycerol (9.5 parts glycerol to 0.5 part PBS) was applied to the specimens for microscopic examination. Evans blue (1.0% stock solution) was used at a 1:1,000 dilution in the fluorescein-labeled conjugate.

Microscopy and photography. Specimens were observed on a Leitz Orthomat epillumination fluorescence microscope. Photomicrographs were taken with a Leitz Orthoplan camera and Kodak daylight Ektachrome (ASA 64). The optimal exposure ranged from 4 to 7 s. Before specimens were photographed, petri dishes were sealed in plastic, surface decontaminated, removed from the maximum containment facility, and inactivated by gamma irradiation for 72 min (9.4 × 10^5 rads) (5). An examination of the effect of gamma irradiation on the maintenance of immunofluorescence in methanol-fixed tissue culture cells demonstrated that there was no significant difference in fluorescence with 0, 36, or 72 min of gamma irradiation (Truant, unpublished data).

IFU assay. The protocol that we describe follows most closely that described by Truant and Hallum (18). Monolayers of HeLa-229 cells were grown to confluence in plastic tissue culture dishes (10 by 35 mm) (Falcon Plastics, Oxnard, Calif.). At cell confluence (18 to 24 h) the medium was aspirated and the monolayer was rinsed with PBS. Viral dilutions were adsorbed to the cell monolayers for 0.5 h, with rocking at 15-min intervals. After the adsorption period, 2 ml of minimal essential medium containing 0.25% Bacto-Agar (Difco Laboratories, Detroit, Mich.), 2% heat-inactivated fetal bovine serum, 0.1% NaHCO_3, and 100 μg of gentamicin per ml was added to each plate. After incubation for 48 h at 35°C in a 5% CO_2 atmosphere, the agar was gently decanted. Monolayers were washed with PBS to remove residual agar and fixed for 5 min with 95% methanol. To rehydrate the monolayers, they were then rinsed with PBS. The fixed cells were then tested for the presence of viral antigen by previously described indirect immunofluorescence methods (21). Briefly, the cells were stained with convalescent-phase serum for 0.5 h, rinsed thoroughly three times with PBS, and stained for an additional 0.5 h with fluorescein-conjugated anti-human immunoglobulin. After being rinsed, the cells were stained with Evans blue. For quantitation of viral foci, the number of microscopic fields per petri dish was determined with an ocular micrometer.

Conventional cytopathology and immunofluorescence assays. Conventional assays (9, 14, 21) were performed in parallel with the IFU assay. For the cytopathic effect (CPE) assay, Vero cells were grown to confluence in screw-capped tubes, the medium was replaced with fresh medium, 10-fold dilutions of viral inoculum were added, and the cultures were incubated for 7 days. Any cultures demonstrating CPE were designated as positive. Titers were calculated by the method of Reed and Muench (13).

For the fluorescent antibody (FA) assay, cells in those cultures lacking evidence of microscopic CPE were scraped off, placed on a glass slide, and allowed to air dry. The cells were fixed for 5 min in acetone and stained with antiserum as described above. Any tubes demonstrating specific immunofluorescence were designated as positive, and titers were calculated by the method of Reed and Muench (13).

RESULTS AND DISCUSSION

When infected cells were examined at 48 h postinfection, foci similar to that shown in Fig. 1

FIG. 1. Immunofluorescent staining of a typical Ebola focus (one fluorescent focus unit) in HeLa-229 cells. Cells were stained by the indirect immunofluorescence method at 48 h postinfection. Foci were observed and photographed with a Leitz Orthomat fluorescence microscope. Magnification, ×1,120.
were observed. The foci were easily detectable and involved few cells. Background staining was virtually absent. Because of the ease of determining the number of infected cells per focus, this method may be useful in estimating the viral growth rate (i.e., each concentric ring of infected cells should approximate one complete replicative cycle).

To determine whether the dose-response curve for this assay was linear, cells were infected with 10-fold dilutions of Ebola and Lassa viruses and assayed as described above. The dose-response curve was indeed linear at the viral concentrations tested (Fig. 2).

The IFU assay was compared with two conventional assays (FA and CPE) used to titrate Ebola virus. The comparison of IFU, FA, and CPE assays represents the titers of one representative Ebola virus sample, with titers determined from 1:10 to extinction: the quadruplicate samples for each assay type were taken from the same titration, and all three assays were done simultaneously. The Ebola virus titers (average of quadruplicate samples) obtained with the IFU, FA, and CPE assays were $5.8 \times 10^5$, $2.0 \times 10^6$, and $3.2 \times 10^5$ per ml, respectively. Other replicated titrations were carried out, and the results agreed with those described in this analysis. These results indicated that the IFU assay was at least as sensitive as tube titration using immunofluorescence and 10-fold more sensitive than tube titration using CPE to determine the endpoint. When the IFU assay was used to quantitate Lassa virus infection, its sensitivity was similar to that of the plaque assay (data not shown). Previous reports have documented the specificity of the antisera used to detect both Lassa and Ebola virus antigens (20, 21).

Although we have described indirect immunofluorescent staining throughout this report, the assay can also be performed by using a direct immunofluorescence technique. We have also determined that the IFU assay works well when Vero cells are used in place of HeLa-229 cells (data not shown).

The results presented here demonstrate that the IFU assay is a sensitive method for titration of Ebola and Lassa viruses. The advantages of this assay over other assays are its speed (2 days versus 5 days or longer) and applicability to a variety of cell lines and viruses. The latter advantage is due to the fact that the assay requires only antigen production and not cell destruction. A potential disadvantage is that the assay might be time consuming when one examines large numbers of samples, especially samples that have low titers (e.g., $<10^5$ per ml).

This assay has been used in experiments designed to determine the minimum length of the Ebola virion (Regner et al., manuscript in preparation), in RNA infectivity studies (14), in neutralization experiments, and for determining titers of a variety of viral preparations. The sensitivity and specificity of the assay should be improved by the use of high-titered monoclonal antibodies to Ebola virus that we have recently produced.

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LITERATURE CITED


