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Inactivation of Lassa, Marburg, and Ebola Viruses by Gamma Irradiation

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Because of the cumbersome conditions experienced in a maximum containment laboratory, methods for inactivating highly pathogenic viruses were investigated. The infectivity of Lassa, Marburg, and Ebola viruses was inactivated without altering the immunological activity after radiation with Co_{60} gamma rays. At 4°C, Lassa virus was the most difficult to inactivate with a rate of 5.3×10^{-6} log 50% tissue culture infective dose per rad of Co_{60} radiation, as compared with 6.8×10^{-6} log 50% tissue culture infective dose per rad for Ebola virus and 8.4×10^{-6} log 50% tissue culture infective dose per rad for Marburg virus. Experimental inactivation curves, as well as curves giving the total radiation needed to inactivate a given concentration of any of the three viruses, are presented. We found this method of inactivation to be superior to UV light or β-propiolactone inactivation and now routinely use it for preparation of material for protein-chemistry studies or for preparation of immunological reagents.

The isolation of several hemorrhagic fever viruses that are highly pathogenic for humans led to the construction of a special maximum containment laboratory at the Centers for Disease Control, Atlanta, Ga. All work involving infectious materials from these agents is done under strict containment conditions that are somewhat cumbersome. Much of the work on class-4 viruses does not require the use of infectious material, and so we studied methods of inactivating infectious viruses to remove class-4 viruses from special maximum containment conditions.

In this paper, we report the results of studies on the inactivation of Lassa, Ebola, and Marburg viruses by gamma irradiation. This technique was chosen because it permitted inactivation of the viruses with no detectable change in the biological activity of proteins, allowing us to carry out a wide variety of biochemical and immunological studies with antigenic, noninfectious viruses. In addition, this method facilitated the preparation of noninfectious diagnostic materials which are sent to many laboratories throughout the world for use in the diagnosis and surveillance of viral hemorrhagic fevers.

MATERIALS AND METHODS

Cell cultures. Vero cells (6), constituting a continuous line of green monkey kidney cells, were used to prepare stock virus and for all experimental procedures. The cells were grown in Eagle minimum essential medium containing 1 U of penicillin, 0.5 μg of streptomycin, and 0.02 μg of amphotericin B per ml, with 10% fetal bovine serum, and were maintained in Eagle minimal essential medium with 2% fetal bovine serum.

Virus strains. Three virus strains were used for this study. Lassa fever virus, which is a member of the *Arenavirus* genus, was first described in 1970 (1). It is a single-stranded RNA virus that is enveloped and has a molecular weight of approximately 3.5×10^6 (10). Its shape is spherical or pleomorphic, with a diameter of 50 to 300 nm (mean, 110 to 130 nm). For these studies, a 1976 human isolate of Lassa virus (Vero passage 3) from Sierra Leone, West Africa, was used. The Marburg virus used was a 1967 isolate from autopsy material from Marburg, West Germany (14) (Vero passage 5). The Ebola virus used was a 1977 isolate from Zaire (7) (Vero passage 3). Marburg and Ebola viruses are single-stranded RNA viruses that are structurally nearly identical (13). They are somewhat rhabdovirus like but are different enough to be proposed as a new family, the Filoviridae (13). They are long, filamentous, pleomorphic viruses with an average diameter of 80 nm and extremely variable lengths of 130 to 2,300 nm (the average length for Ebola is 970 nm, and that for Marburg is 790 nm). The approximate molecular weight of each is 4.5×10^6.

To prepare stock virus, confluent monolayers of Vero cells were infected with virus at a multiplicity of infection of approximately 10^{-3} 50% tissue culture infective dose ($TCID_{50}$) per cell. These cultures were incubated for 4 days with Lassa or Ebola virus and for 7 days with Marburg virus, frozen and thawed once to lyse the cells, and stored in 1-ml volumes at −70°C. A sample of each virus was assayed for infectivity by inoculating 10-fold dilutions into Vero cells and incubating at 37°C for 10 days. The presence of viral antigens for Ebola and Marburg viruses was confirmed

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by an indirect fluorescent-antibody test (15) with standardized antigen-specific antisera. The presence of Lassa virus antigen was detected by the reverse passive hemagglutination test (4). The titers of the experimental pools were as follows: Lassa, $10^{6.0}$ $TCID_{50}$/ml; Marburg, $10^{6.8}$ $TCID_{50}$/ml; and Ebola, $10^{6.5}$ $TCID_{50}$/ml.

Gamma radiation. The gamma radiation source was a gamma-cell (model 220; Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada) model 220 gamma-cell containing Co_{60} with an activity of 1.96×10^6 rads/h in October 1977. To ensure that samples received adequate irradiation, calculations were made on a monthly basis to account for the decay of the Co_{60}.

Experimental procedures. One milliliter volumes of each virus were placed in heat-sealed plastic ampules (Qualicum Scientific Ltd., Toronto, Canada) which were surface decontaminated with sodium hypochlorite, transferred to secondary screw-capped Teflon containers which were also decontaminated, and brought out of the special maximum containment laboratory virus for irradiation. The samples were thawed and then kept cool with crushed ice at 4°C or kept frozen on dry ice at −60°C during irradiation. Each sample was irradiated with a different quantity of radiation and then returned to the special maximum containment laboratory for assay of remaining virus activity. Two different diluents were evaluated: phosphate-buffered saline at pH 7.2 plus 0.75% bovine serum albumin and undiluted normal human serum. Each virus was titrated in Vero cells, and the presence of viral antigen was confirmed with the indirect fluorescent-antibody test or the reverse passive hemagglutination test.

To investigate structural alterations after irradiation, we used samples of a virus preparation containing 8 logs of Ebola virus which was inactivated by 1% glutaraldehyde alone, 1.2×10^6 rads of gamma irradiation at 4°C and then 1% glutaraldehyde, or 2.0×10^6 rads of gamma irradiation at −60°C and then 1% glutaraldehyde.

The relationship between total radiation exposure and reduction in infectious virus was measured by generating a regression line with the equation $\log V_r = \log V_0 + KR$, where $\log V_r$ is the virus concentration remaining after a given amount of radiation R, $\log V_0$ is the beginning concentrations of virus, K is a constant, the dimensions of which are log $TCID_{50}$ of virus (inactivated) per rad of gamma irradiation, and R is the total rads of radiation applied to the specimen.

RESULTS

Several different standard equations were used to plot the experimental data, and correlation coefficients were calculated. Based on the highest correlation coefficients, inactivation curves were constructed from experimental data with the linear equation $\log V_r = \log V_0 + KR$. The value of K was then derived for each virus. The derived values of K for each virus were as follows: ^{K}Lassa $= -5.3 \times 10^{-6}$ log $TCID_{50}$/rad (4°C) and -3.2×10^{-6} (−60°C), ^{K}Ebola $= -6.8 \times 10^{-6}$ log $TCID_{50}$/rad (4°C) and -4.6×10^{-6} (−60°C), and ^{K}Marburg $= -8.4 \times 10^{-6}$ log $TCID_{50}$/rad (4°C) and -4.7×10^{-6} (−60°C).

The inactivation curves for each virus are shown in Fig. 1 to 3.

We also derived an expression for calculating the total radiation (in rads) necessary to inactivate a given concentration of virus with the constants resulting from our experimental data. The results are shown in Fig. 4 and 5. The general equation is total rads = rads V_0 − [(log V − log V_0)/K] where log V_0 is the initial virus titer determined from the experimental data, rads V_0 is the amount of radiation required to inactivate log V_0, K is the inactivation constant, and log V is the concentration of virus to be inactivated. For Ebola virus, total rads are calculated as $967{,}510 - [(\log V - 6.53)/(-6.75 \times 10^{-6})]$. For Ebola virus, total rads = 0.15×10^6 log V (4°C) and 0.22×10^6 log V (−60°C). For Lassa virus, total rads = 0.19×10^6 log V (4°C) and 0.31×10^6 log V (−60°C). For Marburg virus, total rads = 0.12×10^6 log V (4°C) and 0.21×10^6 log V (−60°C).

This calculation is simply the reciprocal of the K for each virus with the sign reversed, multiplied by the titer (in log $TCID_{50}$) of virus which is to be inactivated. All of these constants are free of volume considerations since the radiation field in our gamma-cell is uniform.

We established that the kinetics of this reaction were independent of initial virus concentration and were not significantly different when agents were suspended in buffered saline or whole serum. As expected, greater energy was required to inactivate a given amount of infectivity in the frozen state than in the liquid state. When concentrated, purified virus preparations containing 8 to 10 logs of virus were irradiated with this method, safety tests showed that the virus was completely inactivated. We generally treat the material for 10% longer than our data suggest as an added safety measure. Although the results of this method have been very consistent in our day-to-day operations, we still test our materials to leave no doubt about their inactivation because of the dangerous nature of these viruses.

We did not observe any destruction of the morphology of the viruses by the radiation when we examined them by electron microscopy (photographs not shown). This is consistent with our finding that only extreme quantities of radiation (12 times that necessary for inactivation) change the fluorescence titer of the antigen against standard antisera.

DISCUSSION

Gamma irradiation produced reproducible single-order inactivation of Lassa, Marburg, and Ebola viruses. It was of interest that more energy was required to inactivate the *Arenavirus*, Lassa, than the rhabdovirus-like Marburg

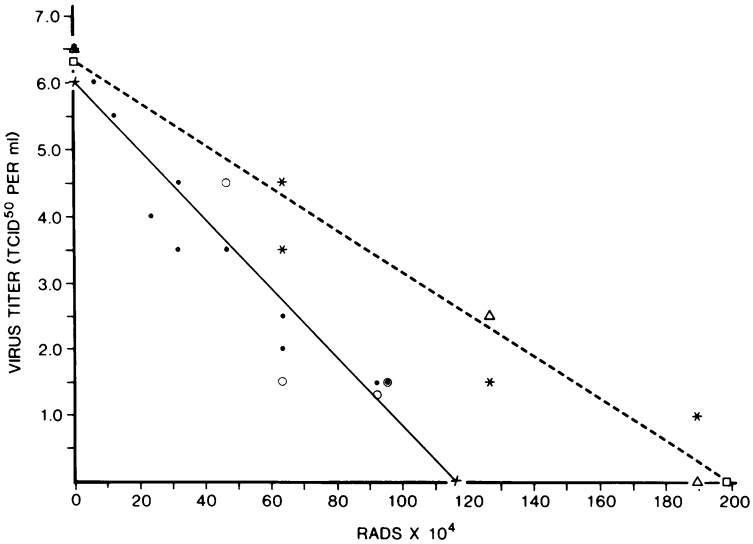

FIG. 1. Inactivation of Lassa virus. Solid line, 4°C; broken line, −60°C. Symbols: ● and *, phosphate-buffered saline; △ and ○, 100% undiluted normal human serum.

and Ebola agents. These results are in accord with the known sizes of the respective viral RNA genomes (11, 12). They are also consonant with the finding that, despite the fact that Pichinde, another *Arenavirus*, was found to have two genomic segments, UV light inactivation kinetics were first-order, indicating that only a single ionizing hit was necessary to destroy infectivity (2). The value we measured for Ebola virus inactivation was not markedly different from that previously reported (9), although that study was done with a less sensitive plaque assay for infectivity, did not specify the temperature of the reaction, and reported results in time rather than in rads required.

Depending on the purpose of inactivation, several methods for inactivating viruses are available (3). Gamma irradiation has replaced UV light and β-propiolactone as our standard method for inactivation of all highly hazardous RNA viruses. Short-wave UV light inactivated Lassa virus successfully after 20-min of exposure to a Mineralight Lamp (model R52; UV Products, Inc., San Gabriel, Calif.) adjusted to

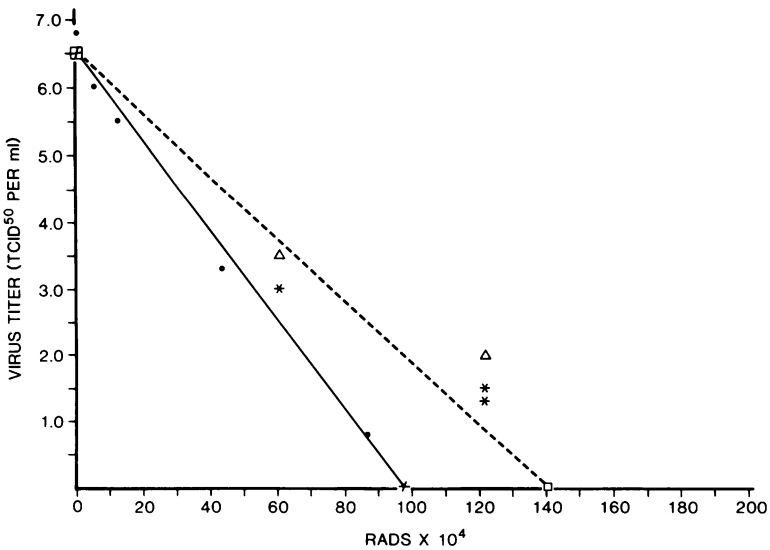

FIG. 2. Inactivation of Ebola virus. See legend to Fig. 1 for definitions.

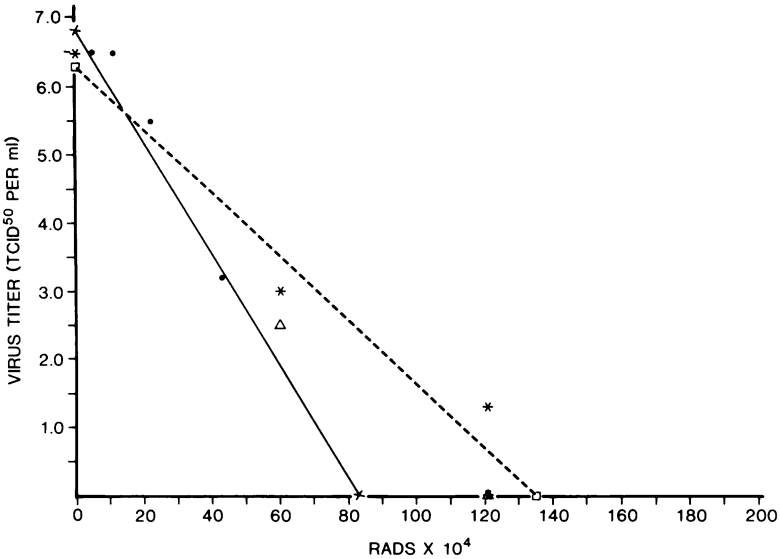

FIG. 3. Inactivation of Marburg virus. See legend to Fig. 1 for definitions.

deliver 1,200 to 2,000 W/cm^2. However, the same treatment of Marburg and Ebola viruses left residual infectivity, and longer treatment diminished antigenicity (L. H. Elliott and K. M. Johnson, unpublished data). Successful complement fixation antigens were made for Lassa virus with β-propiolactone, as described by Buckley and Casals (1). However, this method produced lower-titered antigens in our experiments with Marburg and Ebola viruses and, in addition, the known carcinogenic beta-hazard of β-propiolactone (8) prompted us to discard this method.

We needed a method which would not significantly alter the antigenic properties of the viruses. Our criteria for antigenic integrity have been

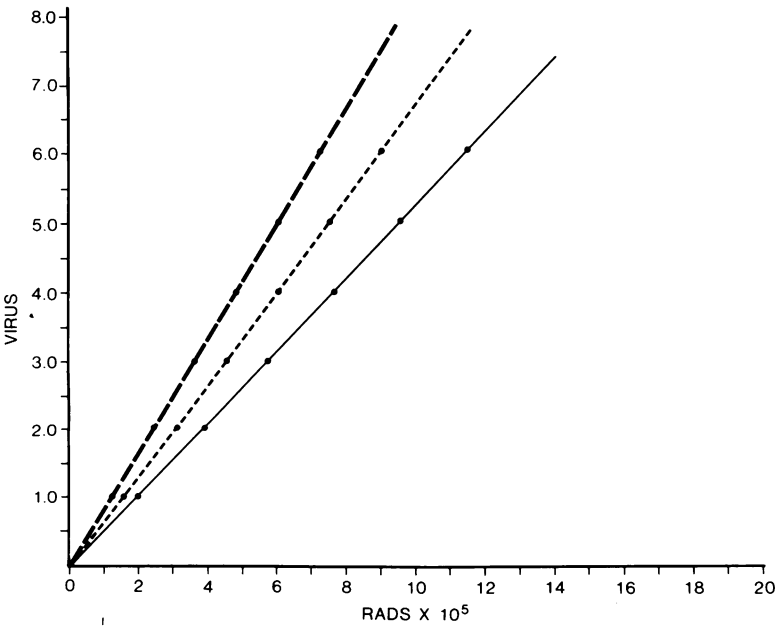

FIG. 4. Total rads required for inactivation of a given amount of virus at 4°C. Symbols: ——, Lassa virus; ——, Marburg virus; – – – –, Ebola virus. Numbers on the vertical axis represent the virus titers (in $TCID_{50}$ per milliliter).

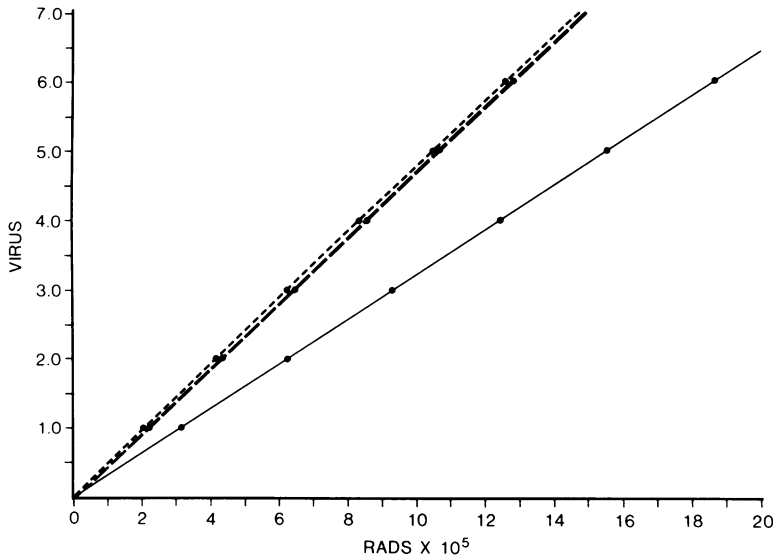

FIG. 5. Total rads required for inactivation of a given amount of virus at −60°C. See legend to Fig. 4 for definitions. Numbers on the vertical axis represent the virus titers (in $TCID_{50}$ per milliliter).

the reproducibility of the indirect fluorescent-antibody titer of standard antisera on infected cells before and after inactivation and the production of high-titered antibodies in animals inoculated with inactivated virus. We found that we could irradiate our immunofluorescent antigen slides (6) for 3 h (3.6×10^6 rads) before any diminution of fluorescence occurred. This is 12 times longer than the time required to inactivate the material. The slides were irradiated without cooling, so that the damage even after 3.6×10^6 rads could have been from heat rather than from the irradiation.

To date we have experienced no difficulty in using gamma-irradiated materials for a variety of purposes: monoclonal antibody production, virion peptide mapping, and production of indirect fluorescent-antibody, enzyme-linked immunosorbent assay, and radioimmunoassay antigens and inactivated reference animal antisera. The technique is clearly the most reliable and convenient of any proposed to date. It also is the most expensive in capital cost ($52,500 in 1977). Psoralen and long-wave UV light may prove to be more suitable for small diagnostic laboratories (5).

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