JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1988, p. 231–235
0095-1137/88/020231-05$02.00/0
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Vol. 26, No. 2

Evaluation of Antiviral Drugs and Neutralizing Antibodies to Human Immunodeficiency Virus by a Rapid and Sensitive Microtiter Infection Assay

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Received 3 August 1987/Accepted 28 October 1987

A 96-well microtiter infection assay for the human immunodeficiency virus (HIV) is described. The assay utilizes human T-cell lymphotropic virus type I-immortalized MT-2 cells as targets for infection and requires only 4 to 5 days for completion. Cytolysis was quantitated by vital dye uptake of poly-L-lysine-adhered cells as an endpoint for infection. The assay's efficacy was proven by the sensitive and accurate assessment of several known anti-HIV agents including two inhibitors of reverse transcription (3′-azido-3′-deoxythymidine and 2′,3′-dideoxycytidine), three biological response modifiers (recombinant interferons α and β and mismatched double-stranded RNA), a direct inactivator of HIV virions (amphotericin B), and neutralizing antibodies from two HIV-positive human subjects. Evaluation of data was facilitated by computer-assisted analysis. This assay provides a means for rapid, sensitive, and inexpensive large-scale in vitro testing of potential anti-HIV therapeutic regimens and quantitation of HIV-neutralizing antibody titers.

Since the identification of human immunodeficiency virus (HIV) as the retrovirus responsible for acquired immunodeficiency syndrome (AIDS), several sources of human cells have been found applicable for in vitro infection assays with this virus (1, 6, 14, 16). Most of these cell systems have utilized T-helper lymphocytes expressing OKT4 surface antigen molecules which act as essential components of the receptor for this virus (3, 10). As an endpoint for virus infection, one or more of a variety of parameters may be examined. These parameters include immunofluorescence, immunoprecipitation, antigen capture, and Western blotting (immunoblotting) for viral antigen expression, reverse transcriptase activity in conditioned culture fluids for virus production, and syncytium formation or viable cell counts for cytopathic effect. Factors limiting the efficient in vitro testing of anti-HIV agents are the number of man-hours required for testing large numbers of samples and the high cost of immunological reagents and radioisotopes required. To circumvent these obstacles, we have developed, and describe herein, a 96-well microtiter infection assay for HIV which is rapid and sensitive and requires no expensive immunological reagents or radioisotopes. The broad applicability of this assay was demonstrated using known anti-HIV drugs including 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddCyd) as inhibitors of reverse transcription (12, 13), interferon (IFN) and mismatched double-stranded RNA (dsRNA, Ampligen) as biological response modifiers (9, 15), and amphotericin B as a direct inactivator of virions (17). The application of this method to quantitating HIV-neutralizing antibody titers is also described.

MATERIALS AND METHODS

Cell and virus culture. A clone of the human T-cell lymphotropic virus type I (HTLV-I)-transformed T4$^+$ T-lymphoblastoid cell line MT-2 was used as the target for microtiter infection assays. This clone was derived by John Riggs and Michael Ascher at the California State Department of Health, Berkeley (T. Haertle, C. J. Carrera, J. S. McDougal, L. C. Sowers, D. D. Richman, and D. A. Carson, J. Biol. Chem., in press), and is well suited for infection assays due to its short HIV expression time after virus challenge and subsequent 100% promotion of cytolysis (6; Haertle et al., in press). High-titer HIV preparations were obtained from H9/HTLV-III$_B$ cultures. Conditioned culture fluids were clarified of cells by low-speed centrifugation and filtration (0.45-μm-pore-size cellulose acetate filters). Viral titers are defined in this study by 50% tissue culture infectious dose values obtained by endpoint titration on MT-2 cells, using the microtiter infection assay described in this report. All cultures were grown and maintained in RPMI 1640 containing 16% fetal calf serum and 50 μg of gentamicin (Sigma) per ml.

Anti-HIV drugs. AZT, ddCyd, and human recombinant alpha A IFN (rIFN-α_A) (>10^8 IU/mg) were obtained from Hoffmann-LaRoche. Human rIFN-β(Ser-17, 10^8 IU/mg) was obtained from Triton Biosciences, Inc. IFNs were calibrated on WISH cells challenged with vesicular stomatitis virus and assayed for viral cytopathic effect as previously described (5). Reference standards were obtained from the World Health Organization (human IFN-α, WHO standard B, 69/19; human IFN-β, WHO no. G-023-902-527). Amphotericin B (Fungizone) was purchased from GIBCO. Mismatched dsRNA (Ampligen) was provided as a lyophilized powder in a salt buffer by HEM Research, Inc., Rockville, Md. A 2.5-mg/ml stock solution of mismatched dsRNA was prepared as described by the supplier and stored in aliquots at −70°C until used.

PLL-coated plates. Flat-bottom, 96-well microdilution plates were coated with poly-L-lysine (PLL; molecular weight, 90,000; Sigma) by incubating 100 μl of a 50-μg/ml solution made in phosphate-buffered saline (PBS) per well for 1 h at room temperature. The solution was then aspirated, and the wells were washed twice with 150 μl of PBS. After the final removal of PBS, the PLL-coated plates were stored at 4°C until used.

Assay of cell protection. MT-2 cells were agitated to

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disperse clumps, and 100 μl was added at a density of 3×10^4 to 4×10^4 cells per well to 100 μl of growth medium containing or lacking twofold dilutions of effectors in triplicate in flat-bottom 96-well microdilution plates. Plates were incubated in an atmosphere of 5% CO_2 in air at 100% humidity and 36°C for 4 h and then challenged with HTLV-III_B (50 μl containing 5×10^4 to 25×10^4 infectious particles). Incubation was continued for 3 to 5 days with the plates sealed in a modular incubator chamber (Billups-Rothenberg, Del Mar, Calif.) flushed with 5% CO_2 in air. Under these conditions, MT-2 cells had a generation time of 36 to 40 h. Cells were suspended by micropipette action, and 100 μl from each well was transferred to the corresponding well of a PLL-coated, 96-well plate containing 100 μl of 0.014% Finter neutral red (4) in growth medium per well. The volume of cell suspension removed was then replaced with an equal volume (100 μl) of fresh growth medium. Cells in the PLL-coated plate were permitted to settle, adhere, and stain for 1 h at 36°C. The medium was then removed, the adhered cells were washed twice with 150 μl of PBS, and the dye was extracted into 100 μl of acidified alcohol (50% ethanol in 1% acetic acid). The extracted dye solution was quantitated colorimetrically at 540 nm with a Flow Titertek colorimeter. For the standard assay procedure, each plate contained eight cell control wells (no virus or effectors) and eight virus control wells (virus, no effectors), which were averaged. The difference in absorption between these controls defined the range for percent protection in test wells. Variability in the eight cell control wells acted as a quantitative measurement of the uniformity of cells transferred. Data reduction was performed with Lotus 1-2-3 spreadsheet software installed on an IBM-PC-AT computer. The latter was interfaced to a Titertek type 3100 microdensitometer by a module type 312B, both from Flow Laboratories. The A_{540} range for all infection assays in this report was 0.242 to 0.463.

Assay of HIV inactivation. Twofold serial dilutions of amphotericin B were made in a total of 100 μl of growth medium per well. Fifty microliters of virus (HTLV-III_B, 5×10^4 to 25×10^4 infectious particles) was added, and the plates were incubated for 4 h at 36°C in an atmosphere of 5% CO_2 (100% humidity) while sealed in a modular incubator chamber. Dispersed MT-2 cells were then added at a density of 3×10^4 to 4×10^4 cells per well in 100 μl of growth medium. The plates, each containing eight cell control wells and eight virus control wells, were incubated and processed for determination of remaining viable cells as described above.

Quantitation of HIV-neutralizing antibody titers. Eight twofold serial dilutions of HIV-positive or -negative human sera were made in triplicate in a total of 50 μl of growth medium per well for a dilution range of 1:4 to 1:512 after the addition of 50 μl of virus (5×10^4 to 25×10^4 infectious particles). The plates were incubated for 1 h at 36°C in an atmosphere of 5% CO_2 (100% humidity) while sealed in a modular incubator chamber. Dispersed MT-2 cells were then added at a density of 3×10^4 cells per well in 100 μl of growth medium, and the incubation was continued for 4 days. Plates were then processed for quantitation of neutral red uptake as described above. Each assay plate contained uninfected and infected cell controls. HIV-positive sera were from individuals with AIDS-related complex as confirmed by enzyme-linked immunosorbent assay, coculture, and RNA hybridization methods (2).

Measurement of cytotoxicity. The toxicity of each drug on MT-2 cells in microdilution plates was measured essentially as described for the assay of cell protection except that virus was omitted. Here, the range was defined by the difference in absorption between the averages of eight cell control wells and eight blank wells.

RESULTS

Standardization of conditions. MT-2 cells were first examined to determine their ability to adhere to PLL-coated 96-well microdilution plates as well as to ascertain the incubation period required for maximum uptake of neutral red dye. When 1.6×10^5 cells in 0.2 ml of growth medium were allowed to settle in PLL-coated wells for 1 h at 36°C, less than 7% of cells remained free-floating, as determined by hemacytometer counts. Cells adhered to PLL-coated wells in this way were stained for various lengths of time. It was found that maximum dye uptake requires 50 to 60 min of incubation. We then confirmed by dye uptake that 1 h of incubation further permits 93% of cells to become PLL adherent and that the remaining small percentage of cells do not adhere even after longer incubation periods. From these results, we chose 1 h as our standard combined PLL adhesion and dye uptake incubation period. Under these standard conditions, twofold serial dilutions of MT-2 cells in the range of 2×10^4 to 24×10^4 cells per 0.2 ml per well demonstrated a linear relationship with neutral red dye uptake (Fig. 1).

Anti-HIV activities of various drugs. Concentration-dependent anti-HIV activities of various known antiretroviral drugs were analyzed by 96-well microtiter infection assay (Fig. 2). In all cases, greater than 90% cytolysis occurred in virus control wells 3 days post virus challenge. One hundred percent protection was provided at all concentrations of each drug at this time. Virus breakthrough, as evidenced by measurable cytolysis, occurred first for the IFNs. Here,

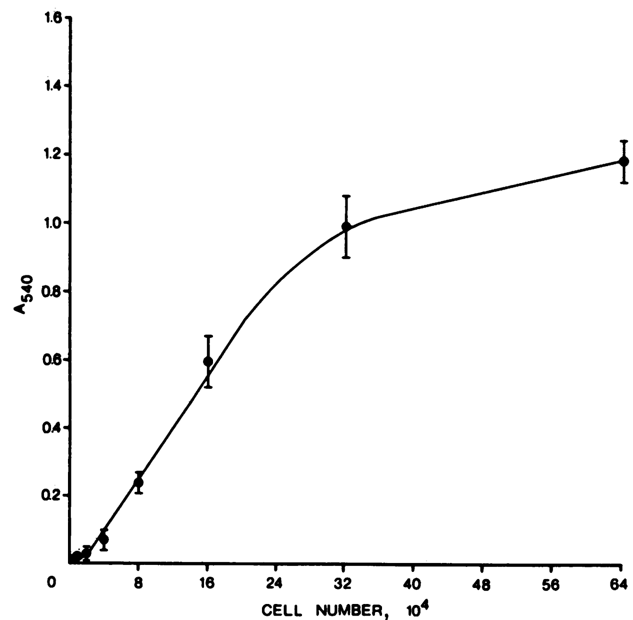

FIG. 1. Neutral red dye uptake by MT-2 lymphoblastoid cells. Twofold serial dilutions of cells ranging from 0.5×10^4 to 64×10^4 cells per well were adhered to and stained for 1 h in PLL-coated, 96-well microdilution plates. Dye was extracted from washed adhered cells and quantitated in a Flow Titertek colorimeter. Values represent an average of three determinations ± standard deviation.

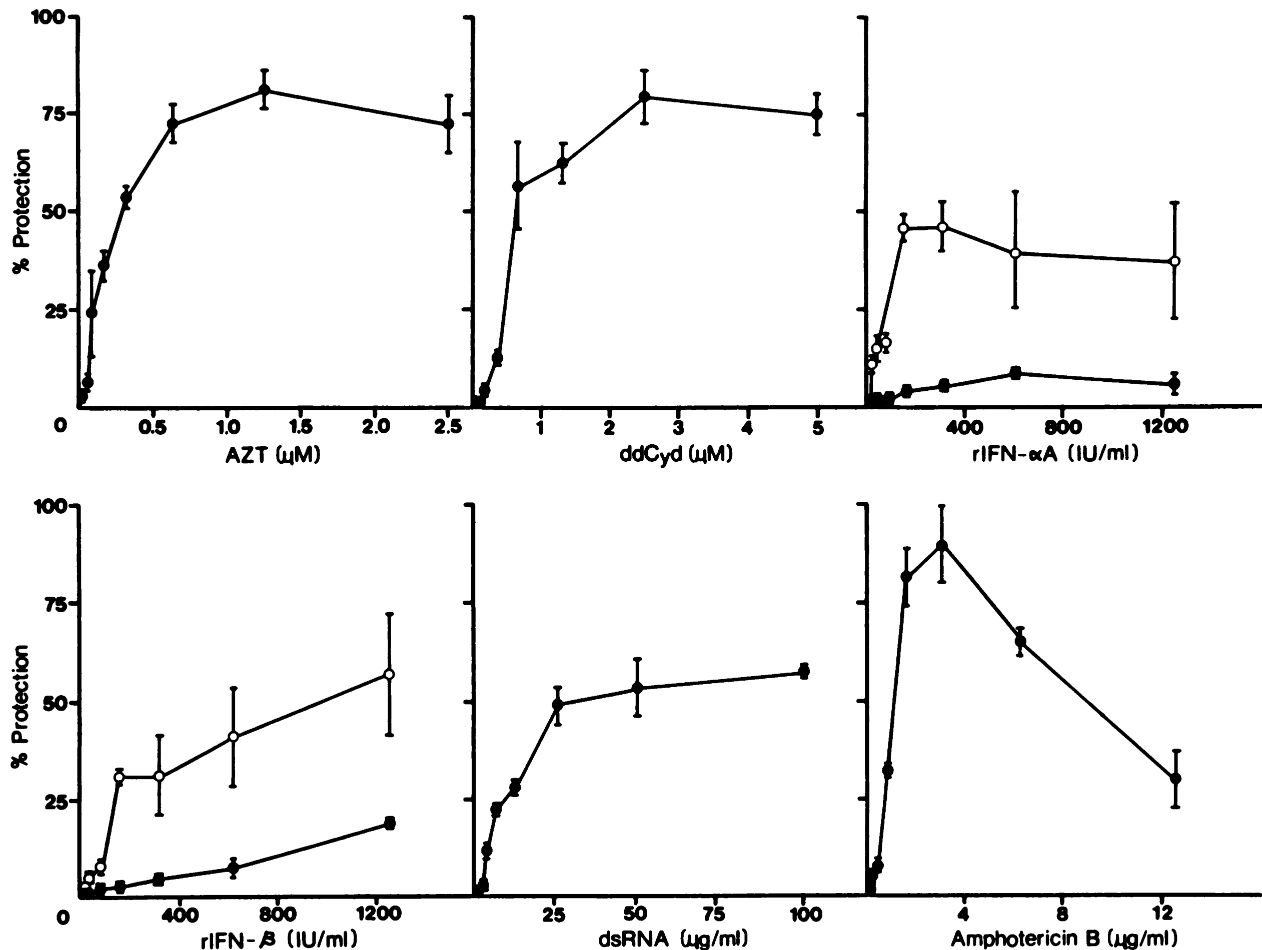

FIG. 2. Microtiter infection assay of anti-HIV drug activities. MT-2 cells were preincubated in the presence of twofold serial dilutions of AZT, ddCyd, rIFN-α_A, rIFN-β, and mismatched dsRNA for 4 h before the addition of virus. In the case of amphotericin B, virus was preincubated in twofold serial dilutions of the drug for 4 h before the addition of cells. Plates were processed for analysis of percent protection by vital dye uptake on days 3, 4, and 5. Represented are the data on days 4 (○) and 5 (●). Values represent an average of three determinations ± percent standard deviation.

rIFN-α_A and rIFN-β remained active at days 4 and 5, where partial protection was provided at concentrations from 19.5 to 1,250 IU of IFN per ml. However, distinctly less activity remained on day 5 than on day 4. The remaining drugs, which included AZT from 0.019 to 2.5 µm, ddCyd from 0.038 to 5 µM, mismatched dsRNA from 0.78 to 100 µg/ml, and amphotericin B from 0.1 to 12.5 µg/ml, did not permit cytolysis until 5 days post virus challenge. At this time, however, clear patterns of concentration-dependent anti-HIV activities had emerged. The optimum concentrations of the two reverse transcription inhibitors, AZT and ddCyd, proved to be 1.25 and 2.5 µM, respectively. Mismatched dsRNA demonstrated optimal anti-HIV activity at concentrations from 25 to 100 µg/ml. The sharp decrease in percent protection provided by amphotericin B at concentrations greater than 4 µg/ml were the result of cell toxicity.

HIV neutralizing antibody titers. Relative titers of HIV neutralizing antibodies from two HIV-positive pre-AIDS individuals, one with a high titer and one with a low titer, are shown in Fig. 3. At the time of harvest (4 days), the high-titer serum provided 100% protection at 1:4 to 1:16 dilutions. This protection decreased at subsequent dilutions to a minimum of 24% protection provided by a 1:128 dilution. In contrast, the low-titer serum provided 100% protection limited to 1:4 and 1:8 dilutions and minimum partial protection (7%) limited to a 1:32 dilution. A negative human control serum gave no protection at any of the dilutions tested (data not shown).

Toxicities. All drugs and sera were evaluated with respect to their toxicity to MT-2 cells in microtiter plates. The concentrations of each drug reported in this study were without toxicity with the exception of amphotericin B, which was toxic at concentrations grater than 4 µg/ml. Toxicities of the remaining drugs began at concentrations greater than 100 µg/ml for dsRNA, 10 µM for AZT and ddCyd, and 1,200 IU/ml for the IFNs. The two sera used in this study showed no toxicity at the dilutions used (1:4 to 1:512). However, we have occasionally observed cellular toxicities in other serum samples at the lower dilutions.

DISCUSSION

The development of a microtiter infection assay for HIV is crucial to the processing of large numbers of samples. The search for new anti-HIV drugs will require massive screening efforts, as will the investigation of the levels at which these new drugs affect HIV (i.e., infectivity of virus or infectability of target cells). Furthermore, various combinations of anti-HIV drugs may prove more beneficial than

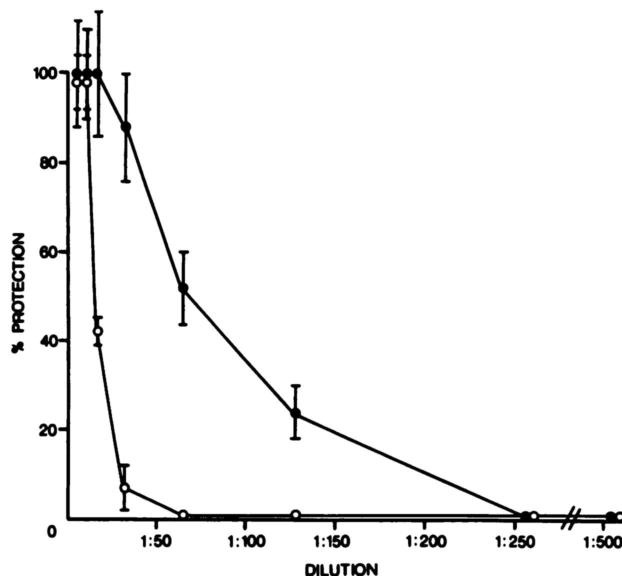

FIG. 3. Microtiter infection assay of HIV neutralizing antibody titers. Virus was preincubated in the presence of twofold serial dilutions of sera from two HIV-positive individuals for 1 h before the addition of MT-2 cells. Plates were processed for analysis of percent protection by vital dye uptake on day 4. Values represent an average of three determinations ± percent standard deviation.

single-agent therapy, such as the synergies observed with combinations of AZT and Ampligen (11), AZT and rIFN-α_A (8), and phosphonoformate and rIFN-α_A (7), and will require large-scale in vitro testing programs for initial identification. Another application of microtiter infection assay will be the evaluation of neutralizing antibody titers in patients' sera. The need for such an assay is relevant to ongoing attempts aimed at correlating neutralizing antibody titers with disease status and determining fluctuations in these titers in patients receiving anti-HIV drugs or vaccines. The microtiter infection assay described in this report is rapid, sensitive, and quantitative and requires no expensive immunological or radiolabeled reagents. The demonstrated applicability of this assay for anti-HIV agents that act as biological response modifiers, inhibitors of reverse transcription, and inactivators of virus particles, in addition to the utility of this assay in quantitating HIV neutralizing antibody titers, establishes its broad application.

When evaluating anti-HIV agents with this infection assay, testing should be performed at viral titers and incubation periods sufficient to allow eventual virus breakthrough (cytolytic response), even at optimal concentrations of the effector, if possible. Only then can true optimum in vitro concentrations of effectors be determined and direct comparisons of relative effectiveness of various effectors be made. It is conceivable that in some cases, if a low enough viral titer is used, a cytolytic response may never occur in the presence of an effector relative to the corresponding virus control. It has been our experience that the use of lower titers (less than a multiplicity of infection of 1) delays the eventual cytolytic response but has no effect on the quantitative outcome with respect to dose-effect curves of drugs or titer-effect curves of neutralizing antibodies. We found here that multiplicities of infection greater than 1 generated suitable responses for rapidity, sensitivity, and quantitation of results. For example, AZT, ddCyd, dsRNA, rIFN-α_A, and rIFN-β at all concentrations tested provided 100% protection 3 days post virus challenge, whereas the virus control wells had greater than 90% cytolysis of cells. It was not until day 4 that the protection provided by all concentrations of the IFNs was overcome, and it was day 5 before the same was true for AZT, ddCyd, and dsRNA (Fig. 2). A true optimum in vitro concentration of effector was identified by this assay when an increased concentration no longer provided an increase in percent protection. We found the optimum concentrations of effectors used in this study to be 1.25 μM AZT, 2.5 μM ddCyd, 156 to 312 IU of rIFN-α_A per ml, 25 to 100 μg of dsRNA per ml, and 1.56 to 3.12 μg of amphotericin B per ml (Fig. 2).

An exception to using a high multiplicity of infection as described above is the application of this assay system to the evaluation of agents which might be expected to attenuate de novo synthesized HIV, such as protease inhibitors or modifiers of glycoprotein processing, as potential anti-HIV agents. Here, a multiplicity of infection much less than 1 should be employed. In this way, progeny virions synthesized in the presence of an effector would be responsible for any quantitative cytolytic response occurring relative to control infection. Lack of a cytolytic response under these circumstances would indicate that an effector had modified the de novo synthesized HIV in such a way as to render it noninfectious.

When applying this assay system to the quantitation of HIV neutralizing antibody titers, a standard, universally accepted definition of titer will be required. As examples, titers could be defined by the greatest dilution providing 100% protection or, alternatively, as the dilution providing a given lesser percent protection, such as 50%. Since these values will be influenced by such variables as cell densities, viral titers, and incubation periods, a predetermined HIV neutralizing serum should always be included as a reference standard. All attempts to standardize cell densities, viral titers, and incubation periods will also facilitate accurate and reproducible assessment of results between laboratories.

Although the microtiter infection assay for HIV described in this report should prove to be a powerful tool to the investigator, it probably falls short of measuring the full potential of at least one class of anti-HIV agents, the biological response modifiers. These agents (i.e., IFNs, dsRNAs) have the potential to elicit pleiotropic responses in vivo beyond those which can be measured by in vitro cell systems. In vitro comparisons of these agents with other anti-HIV drugs should always take this into consideration.

ACKNOWLEDGMENTS

We are indebted to Douglas Richman at the University of California-San Diego VA Medical Center for his generous gift of the MT-2 cell clone used in this study. We are also indebted to Divya Patel for her aid in the preparation of the manuscript.

This work was supported in part by grants from Vanderbilt University (University Research Council) and the DuPont Company.

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