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Short Communication

Susceptibility of human immunodeficiency virus to antiviral agents measured by infectious virus yield reduction

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Summary

Under single growth cycle conditions in C8166 lymphoblastoid cells human immunodeficiency virus shows a replication curve which is completed at 24 h post-infection. At lower multiplicity of infection virus yield peaks at approximately 72 h post-infection but in both cases the titer of the virus released in the medium is negligible with respect to that which remains cell-associated. A method based on back-titration of virus in cryolysates of C8166 cells infected with HIV and treated with antiviral compounds has been used to evaluate HIV sensitivity to such agents. Under single growth cycle conditions dose response curves appear linear and permit rapid and accurate determination of the endpoint activity. Under multiple growth cycle conditions the inhibitory activity may be measured during the exponential growth phase, at 48 h post-infection. This method, which directly measures production of infectious virus rather than indirect probes of viral replication such as reverse transcriptase or antigen production, offers the advantage of a precise determination of the degree of activity of antivirals also acting on viral assembly or release.

HIV; Susceptibility to antiviral agents

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Introduction

In a previous paper (Dianzani et al., 1988) we have shown that C8166 cells, a CD4 positive lymphoblastoid line infected with human immunodeficiency virus (HIV) under single growth cycle conditions, show a HIV replication curve which is completed within 24 hour postinfection. This is different from most cell lines susceptible to HIV infection, namely MOLT-4, H9, U937, HUT-78, MT-4 and ATH8, in which viral replication takes at least 4–5 days to be completed (our own observation and Baba et al., 1988; Ezekowitz et al., 1989; Mitsuya et al., 1985; Vogt et al., 1987).

Specifically in the C8166 system, detectable infectious virus is released in the extracellular fluid starting from the 16th hour postinfection, peaks 4 h later and then slowly declines as the cells start to die. Conversely, the titer of cell-associated virus is high after adsorption, declines during the eclipse phase (8–12 h), rises again to 10^5 $TCID_{50}$ between the 12th and the 20th hour, stays relatively stable for about 24 h, and then rapidly falls to undetectable levels thereafter. At any time of the replication cycle, with the exception of the peak of the eclipse period, the titer of cell-associated virus is approximately two log higher than virus released in the medium suggesting that, in contrast with murine retroviruses (Wong and MacLeod, 1975), HIV matures substantially at internal membrane sites. In theory, this experimental model could be useful for determination of antiviral activity of chemotherapeutic agents. Anti-HIV measurements are often based on the measurement of reverse transcriptase (RT) activity and antigen production, both indirect probes of viral growth and not necessary expression of actual viral replication. The model that we propose here is based on the production of infectious virus particles and provides a method for direct evaluation of inhibition of HIV replication.

Materials and Methods

Cells

A CD4 positive T cell line, C8166, containing the HTLV-1 genome and expressing only the tat gene (Sodroski et al., 1984) was obtained through the courtesy of Dr. M. Cloyd, UTMB, Galveston, Texas and it was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Flow Lab. Inc., Irvine, U.K.) and gentamycin (0.5 μg per ml). HIV induces an easily detectable cytopathic effect (CPE) in these cells (Clapham et al., 1987).

Antiviral agents

3′-Azido-2′,3′-dideoxythymidine (AZT), 2′,3′-dideoxycytidine (ddCyd) and methylisoprinosine (IP) were kindly provided by Wellcome, Roche and Sigma-Tau, respectively. The compounds were dissolved in absolute ethanol as mother solutions, divided in aliquots and kept at −80°C until used. Further dilutions were made in tissue culture medium.

Virus

The HTLV-III_B strain of HIV, originally isolated by R.C. Gallo (Popovic et al., 1984) and kindly provided as chronically HIV-infected H9 cells by Dr. M. Cloyd, was used in the study. Virus stock yielded in C8166 cells a titer of approximately $10^{5.5}$ $TCID_{50}$/ml. To perform experiments under single growth cycle conditions virus was concentrated as follows: 200 ml of clarified supernatants from H9/HIV cells were pelleted in a Beckman L8-70M ultracentrifuge at 4°C (SW 28 rotor at 25 000 rpm for 90 min). The pellet was overlaid with 2 ml (1/100 of the original volume) of RPMI supplemented with 2% FCS, stored overnight at 4°C, and then resuspended by gentle stirring. The titer of these concentrated preparations ranged between $10^{7.0}$ and $10^{7.5}$ $TCID_{50}$/ml. Titration was performed in C8166 cells by standard limiting dilution method (0.5 log ratio, 4 replicates per dilution) in 96-well microtiter plates (Falcon). The infectious titer was determined by scoring syncytia under the light microscope after four days of culture. A standard virus preparation was included in each titration and the infectious titer variations did not exceed 0.4 log. CPE readings were performed under blind conditions and infectious titer was calculated by the Reed and Muench method.

Assay of antiviral activity

A total of 10^6 C8166 cells in 0.2 ml of medium were incubated with HIV at a multiplicity of infection (MOI) of 5 $TCID_{50}$ per cell, for single growth cycle conditions (SGC), or at MOI of 0.1 for multiple growth cycle (MGC) conditions. At the same time dilutions of the proper antiviral agent were added to each tube. One hour later cells were washed three times, resuspended in 1 ml of medium containing the antiviral agent at the appropriate concentrations, and incubated at 37°C. With this procedure no residual virus was detectable in the supernatants. Twenty-four or 48 h later, depending on growth under SGC or MGC conditions, the cells were subjected to three cycles of freeze-thawing, cell debris was removed by low speed centrifugation and the supernatants were titrated as described in the previous section. Exploratory experiments showed that this procedure caused no loss of HIV infectivity.

This experimental approach was chosen since, as already stated, at any time of the growth curve, with the exception of the peak of the eclipse phase, the titer of cell-associated virus was approximately 2 log higher than the titer of the virus released in the medium (Dianzani et al., 1988).

Cell viability

Toxicity of the compounds was tested by trypan blue exclusion, [methyl-^{3}H]-dThd incorporation and cell replication (Dianzani et al., 1988). The doses applied in the experiments allowed cell viability $\geq$ 90%, and did not significantly impair dThd incorporation and cell growth as compared to untreated controls.

Results

Assay of the antiviral activity of AZT under SGC or MGC conditions

Fig. 1 shows in log scale a typical dose response curve obtained by testing serial dilutions of this drug in C8166 cells infected at high MOI. The inhibition curve is rather linear. In this system a 0.5 log yield reduction is statistically significant. Calculation of the endpoint activity by extrapolation is both easy and correct (0.04 μM in the experiment reported in Fig. 1). Repeated experiments also showed that this finding is highly reproducible. However, SGC conditions, which requires preparation and concentration of relatively large amounts of virus, may be unpractical. Therefore we explored the possibility of applying the same experimental plan to cell cultures infected at lower MOI, easily obtained by using crude supernatants from H9/HIV cells. For this purpose we used C8166 cells to establish the kinetics of infectious HIV production after infection at an MOI of 0.1. A typical HIV growth curve under these conditions is shown in Fig. 2. Each point represents the mean value for two separate experiments run in quadruplicate, standard deviation never exceeding 0.4 log. Both extracellular and cell-associated virus titers peak at 72 h post infection, and during most of the growth curve the titer of cell-associated virus is much higher than the virus released in the medium. Additionally, at 48 h cell-associated virus grows in a rather exponential fashion. Therefore we chose this time point to test antiviral activity under MGC conditions of AZT, ddCyd and IP. The mean results of 4 separate experiments are shown in Fig. 3. Both AZT and ddCyd reduced virus yield by 99% at a concentration higher than 0.2 μM and 0.5 μM, respectively. IP did not show inhibition at any of the con-

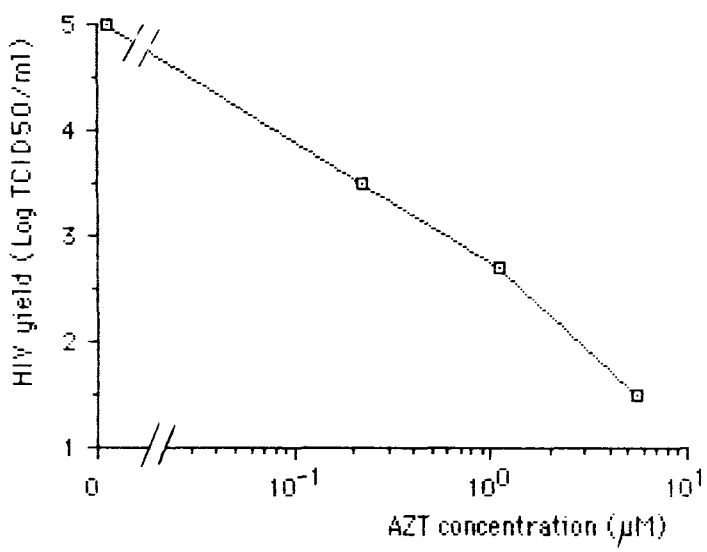

Fig. 1. Effect of AZT on HIV replication in C8166 cells under single growth cycle conditions.

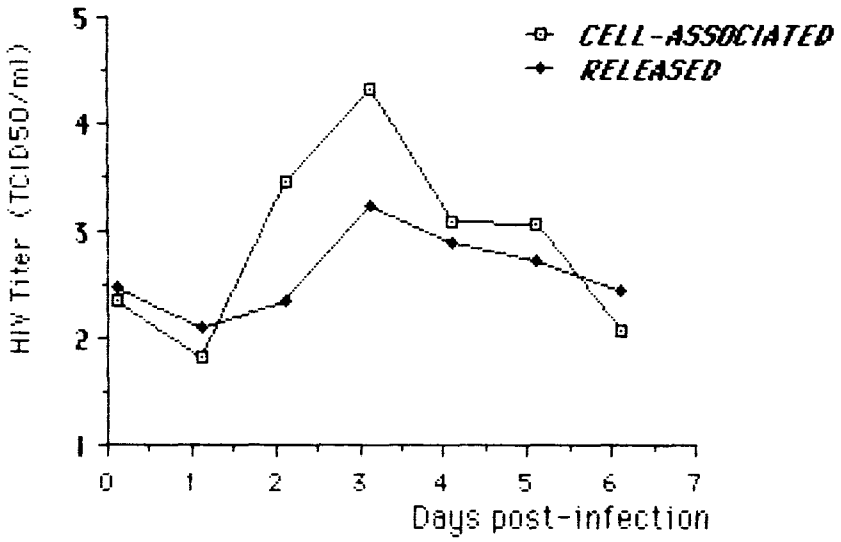

Fig. 2. HIV replication cycle in C8166 under multiple growth cycle conditions. At the indicated times pelleted cells (□) or supernatant fluids (◆) were collected and titrated for virus content.

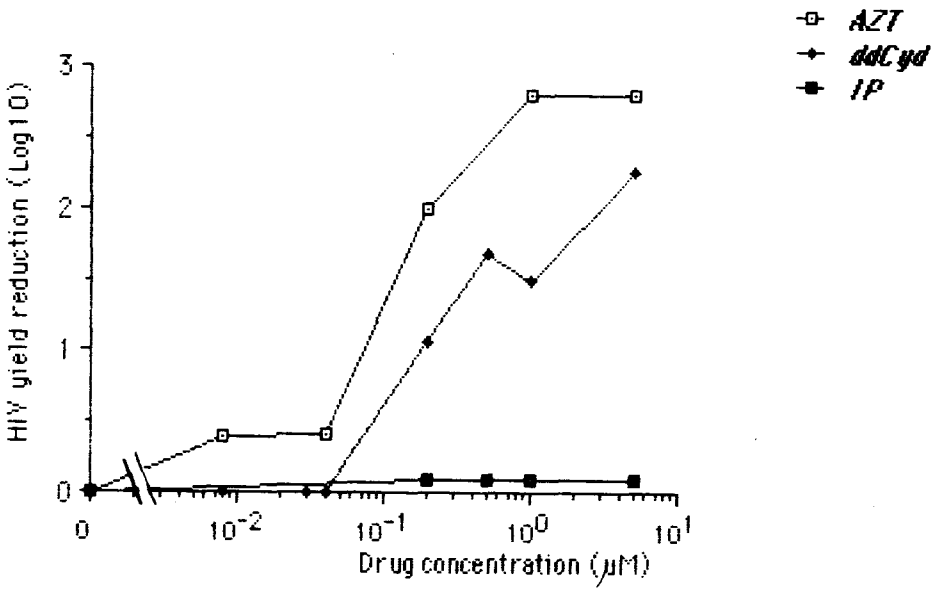

Fig. 3. Effect of AZT, ddCyd and IP on HIV replication in C8166 under multiple growth cycle conditions. (□) AZT; (◆) ddCyd; (■) IP.

centrations used. Basically, the same results were obtained in H9 and HUT-78 cells, although in these cell lines HIV titers peaked at about 4–6 days instead of 1–3 days, and at lower titers (3–4 logs instead of 5–6 logs).

Discussion

The model described here has several advantages as compared to the methods often used to measure in vitro activity of antiviral agents against HIV replication [based on determination of RT activity, viral antigen production, or inhibition of syncytium formation (Ezekowitz et al., 1989; Mitsuya et al., 1985; Vogt et al., 1987)]. The first two techniques measure indirect parameters of viral replication and are only partially quantitative. Although in our hands virus yield inhibition generally parallels reduction of virus antigen and inhibition of RT activity, production of viral proteins may not necessarily reflect the actual production of infectious virus; for instance, the activity of antiviral agents acting on retroviral assembly or release (Friedman et al., 1975; Walker et al., 1987) may not be detected by such indirect tests. Similarly, tests based on inhibition of virus-induced cytopathogenicity usually give reliable dose response curves (Baba et al., 1988; Mitsuya et al., 1985; Walker et al., 1987) but, at least in theory, some agents might be able to inhibit viral cytopathogenicity, or syncytium formation, without inhibiting virus yield. Conversely, the procedure we are proposing accurately measures the extent of infectious virus production, thereby allowing a precise determination of the degree of activity of an antiviral agent at each dose point. The dose response curves tend to be linear and this may permit the study of combination of antivirals at suboptimal doses to explore synergistic or antagonistic effects. Moreover, the possibility to measure the activity on viral replication during the exponential growth phase would offer a more accurate determination due to the higher homogeneity of viral progeny during this phase. Under these conditions the study of acquired drug resistance would be greatly facilitated. Lastly, the method that we propose is relatively simple, requires only routine virological techniques, is not too expensive, and offers a definitive result within 5–6 days.

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