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We sought to validate an in vitro system which could predict the minimal effect dose of antiretroviral agents. Mixtures of uninfected CEM cells and CEM cells chronically infected with human immunodeficiency virus (HIV) type 1 MN were exposed to 2',3'-didehydro-3'-deoxythymidine (D4T) in vitro in a hollow-fiber model which simulates the plasma concentration-time profile of D4T in patients. Drug concentration was adjusted to simulate continuous intravenous infusion, or an intravenous bolus administered twice daily. The effect of the dosing regimen was measured with viral infectivity, p24 antigen, and reverse transcriptase or PCR for unintegrated HIV DNA. Dose deescalation studies on a twice-daily dosing schedule predicted a minimum effect dose of 0.5 mg/kg of body weight per day which correlated with the results of a clinical trial. Antiviral effect was demonstrated to be independent of schedule for every 12-h dosing versus continuous infusion. Finally, at or near the minimal effect dose, efficacy appeared to depend on the viral load. The ability of this in vitro pharmacodynamic model to assess the response of HIV-infected cells to different doses and schedules of antiviral agents may be useful in the design of optimal dosing regimens for clinical trials but requires validation with other types of antiretroviral agents.

In vitro models have been used to evaluate the pharmacodynamics of antibacterial and anticancer agents for nearly two decades. Several types of in vitro systems have been developed in which cells or bacterial pathogens are grown in a suitable medium and exposed to fluctuating concentrations of drugs which are adjusted to mimic concentrations in serum obtained in humans following single or multiple doses. The results of studies with antibacterial compounds alone or in combination have been consistent with observations in established animal models of infection and have proven useful in the design of subsequent clinical investigations (3, 6, 7, 9).

Hollow-fiber (HF) bioreactors (13) have been used to cultivate hybridomas and other animal and human cell types. These systems have also been employed to grow human immunodeficiency virus (HIV) to high density and to harvest p24 antigen (10). We have developed a perfusion system in which either HIV-infected or uninfected human T-cell lines can be propagated in the extracapillary chamber of multiple HF bioreactor units. Nutrients are continuously pumped through this system to support growth of HIV-infected cells; antiviral agents can be added and removed at a rate which simulates human pharmacokinetic patterns for the agent. In these studies, we have used this system to analyze drug exposure-efficacy relationships for the nucleoside antiretroviral agent D4T (2',3'-didehydro-3'-deoxythymidine). This system permits continuous cell growth to densities of 2×10^9 to $3 \times$ 10^9 cells per unit as documented in preliminary experiments performed with CEM and other lymphoid cells (2).

MATERIALS AND METHODS

Cells and viruses. The human T-lymphoblastoid cell line CEM and the H9 cell line infected with HIV type 1 (HIV-1) strain MN (HIV-1$_{MN}$) (H9/HTLV-III$_{MN}$ NIH 1984) were obtained from the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. CEM cells adapted to growth in HF bioreactors were infected with cell-free supernatant from HIV-1$_{MN}$-infected H9 cells, and a CEM cell line which continuously produced HIV-1$_{MN}$ (CEM-MN) was established. All cell lines do not require exogenous interleukin 2 and were cultivated in RPMI 1640 medium with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (90%) and 10% fetal calf serum (Paragon, Baltimore, Md.). The percentage of infected cells was determined by flow cytometry with the anti-p24 monoclonal antibody KC-57 as previously described (14). Reverse transcriptase activity present in cell-free supernatants from HF cultures was determined as described by M. Popovic et al. (18).

In vitro pharmacokinetic model system. A schematic diagram of our perfusion system which utilizes commercially available HF bioreactor units is shown in Fig. 1. Human T-lymphocyte cell lines were propagated on and surrounding a network of HF artificial capillaries enclosed within the extracapillary chamber of a plastic bioreactor cartridge (Mini-B cellulose, 10,000-Da mean pore size; Cellco Inc., Germantown, Md.). Experiments to monitor virus spread were initiated with 10^7 CEM cells and 10^4 CEM-MN cells (1:1,000 infected/

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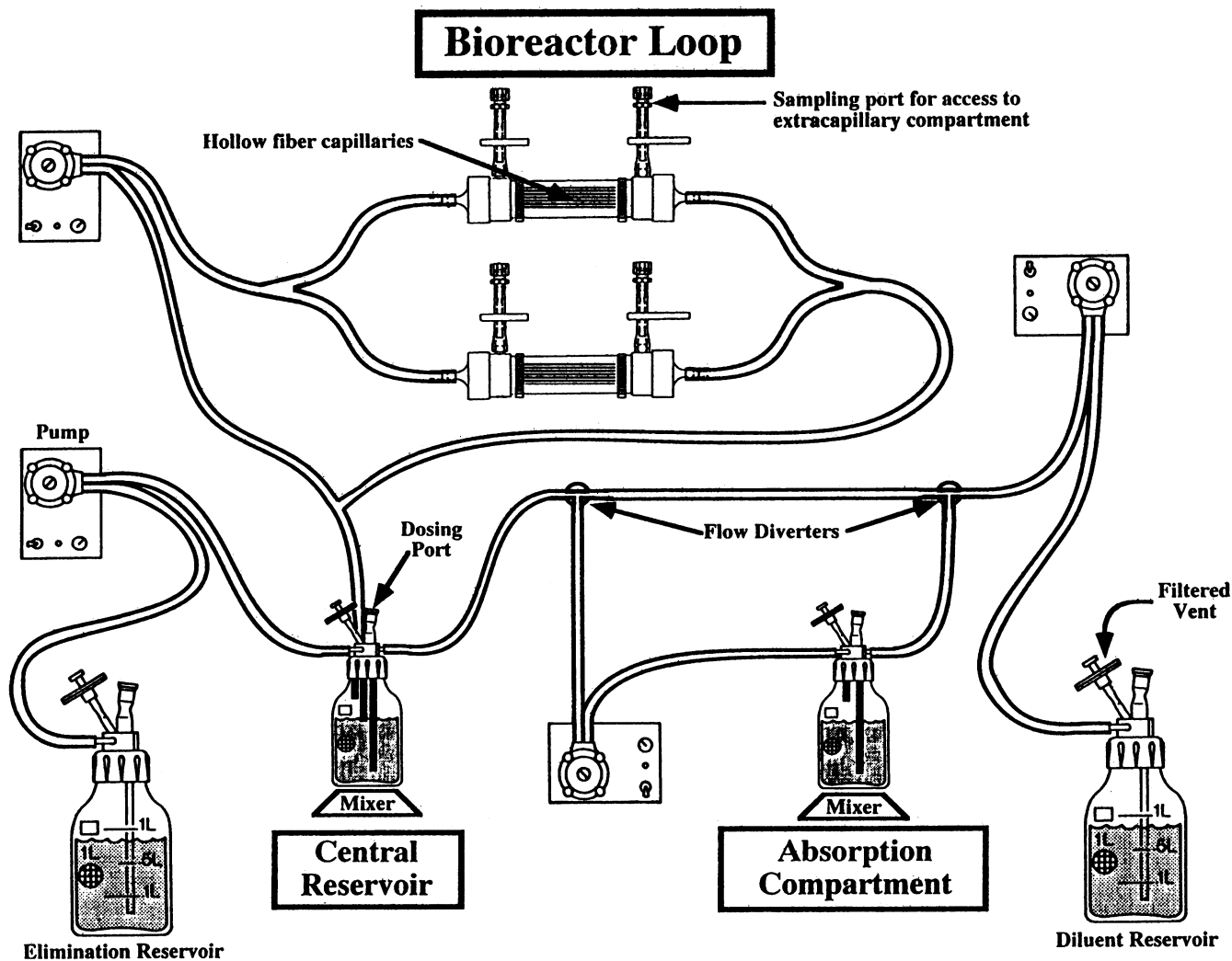
FIG. 1. Schematic diagram of the in vitro pharmacodynamic model system. One of the two systems enclosed within a single incubator housed in a biological safety cabinet is shown. Cells are grown, and samples are removed from the extracapillary compartment of HF bioreactors. Constant infusion, oral, or intravenous bolus doses are introduced through the dosing ports in the diluent reservoir, absorption compartment, or central reservoir, respectively. Exposure of cells to fluctuating concentrations of D4T is affected by programmed dilution of drug within the central reservoir, while the volume of the central compartment is maintained as a constant by elimination. The mean pore diameter of the HF capillaries (10 kDa) would prevent HIV or HIV-infected cells from exiting the bioreactor and from circulating through the tubing. (Further information is available upon request.)

uninfected cell [i/u] ratio). In some experiments, bioreactors were started at a 1:100 i/u ratio (10^7 CEM cells and 10^5 CEM-MN cells). The system was perfused with modified RPMI 1640 medium with 25 mM HEPES buffer and 50 µg of gentamicin per ml (90%; Paragon Biotech), 10% fetal calf serum, and Nutridoma-Hu (Boehringer, Indianapolis, Ind.). Glucose utilization was monitored with glucose test strips, and the percentage of viable cells was measured with trypan blue exclusion. D4T was added to either the diluent reservoir (continuous infusion) or the central reservoir (intravenous bolus). Details of the simulation of human plasma pharmacokinetics of antiviral agents and biological response modifiers by programmed dilution of drug introduced into the central reservoir will be published elsewhere (1a). Plasma D4T concentrations simulated in the model were based on published pharmacokinetic parameters from patients (8). The diluent pump rate (i.e., the system clearance) was determined from the

product of the desired elimination rate constant (based on a 1-h half-life) and the total volume of the in vitro model system (100 ml). Drug doses for the model were calculated to yield the same peak and area under the curve values that would be obtained in vivo for a 70-kg human receiving the milligram per kilogram of body weight dose level tested.

ELISA for HIV p24. Aliquots of cells and tissue culture media were removed through the extracapillary access ports on the bioreactors at the indicated time points and centrifuged for 5 min at 1,500 × g. HIV gag p24 protein in cell-free culture supernatants was measured by the Coulter p24 enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's guidelines (Coulter Immunology, Hialeah, Fla.). Absorbance was measured and data were analyzed with a computer-supported microplate reader (Molecular Devices, Menlo Park, Calif.). Baseline (day 0, time zero) p24 was subtracted from values obtained on later days. Mean ± standard deviation of

TABLE 1. Effect of D4T on the spread of HIV-1$_{MN}$[a]

Treatment	i/u Ratio[b]	No. of unintegrated HIV copies per μg of DNA	RT[c] activity (cpm/10 μl)	p24 (pg/ml)
Constant infusion				
None	CEM-MN	<100	57,175	120,500
None	CEM	<100	1,142	Not done
None	1:100	145,000	75,200	102,000
None	1:1,000	22,000	19,100	47,600
1 mg/kg/day	1:100	<100	10,750	6,730
1 mg/kg/day	1:1,000	<100	9,240	1,590
Bolus				
None	1:100	87,000	49,200	123,000
None	1:1,000	19,000	11,700	20,300
0.5 mg/kg/BID	1:100	<100	Not done	14,800
0.5 mg/kg/BID	1:1,000	<100	Not done	3,000

[a] Comparison of the efficacy of 1 mg of D4T per kg/day administered as a constant infusion and as a 0.5 mg/kg twice daily. Mixtures of CEM cells chronically infected with HIV-1$_{MN}$ (CEM-MN) and uninfected CEM cells were grown in HF bioreactors. Cells were exposed to D4T either as a constant infusion or as a twice-daily dose administered as a simulated intravenous bolus with levels of D4T in the HF bioreactor mimicking typical human serum pharmacokinetics. At day 9, samples of cells and tissue culture fluid from the extracapillary space were taken at a trough point for the analysis of HIV-1 DNA, p24, and reverse transcriptase activity.

[b] The initial ratio of CEM-MN cells to uninfected CEM cells used to initiate HF bioreactors.

[c] RT, reverse transcriptase.

duplicate samples was used for calculation of p24 level. The coefficient of variation is approximately 5% for p24 assays performed on different days.

Unintegrated DNA assay. Samples of 1×10^6 to 3×10^6 cells were used for isolation of high- and low-molecular-weight DNA with an extraction procedure modified from that described by Hirt (11). Cells from bioreactors with extensive unintegrated HIV DNA synthesis were often diluted 10- and 100-fold in uninfected CEM cells prior to Hirt DNA preparation. Hot-start PCR was performed with the SK38/39 *gag* primers and probe (SK19) for the HIV-1 *gag* region with reagents from and conditions recommended by Perkin-Elmer Cetus (Norwalk, Conn.). Amplification products were analyzed on 1% NuSieve agarose gels, transferred to nitrocellulose (BIOS, New Haven, Conn.), and fixed by exposure to UV. The blots were hybridized with an SK19 probe labeled with digoxigenin-dUTP by using terminal deoxynucleotide transferase. Hybridization conditions and detection with antidigoxigenin Fab fragments conjugated with alkaline phosphatase (Genius System; Boehringer Mannheim) were according to manufacturer's instructions. The numbers of copies of HIV proviral DNA detectable in high- and low-molecular-weight DNA preparations were estimated by extrapolation to a standard curve performed with known copy numbers of HIV-1 plasmid DNA.

RESULTS

Inhibition of HIV replication by continuous infusion of D4T. By initiating a series of HF bioreactors with a mixture of infected and uninfected cells, we have shown the ability of D4T (a pyrimidine analog and inhibitor of reverse transcriptase) to prevent the spread of HIV-1$_{MN}$ to uninfected CEM cells in continuous-infusion regimens. Table 1 shows that D4T, when administered as a simulated continuous intravenous infusion of a dose of 1 mg/kg/day, inhibited the spread of HIV to
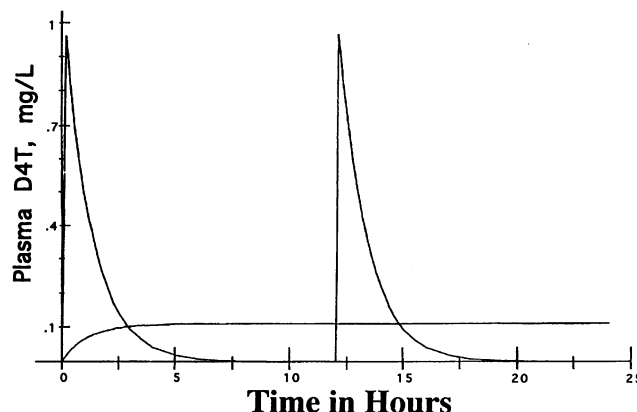

FIG. 2. Simulated plasma concentration curve for D4T (1 mg/kg/day) given as a continuous infusion or divided into two doses administered at 12-h intervals. The elimination half-life of D4T was set according to human studies (8).

uninfected cells. Inhibition of de novo replication was demonstrable by measurement of reverse transcriptase or p24 in the cell-free media or by quantitative analysis of integrated and unintegrated species of HIV-1 DNA by PCR. High levels of unintegrated DNA are associated with active HIV DNA synthesis (12, 17). The lower copy numbers of unintegrated HIV DNA in D4T-treated HF cultures, relative to untreated controls, are similar to the decrease in unintegrated HIV DNA in lymphocytes from AIDS patients after initiation of antiretroviral therapy (5).

In vitro simulation of human pharmacokinetics. The same two-compartment in vitro system was used to simulate both continuous infusion and an intravenous bolus of the antiviral agent. In experiments in which drug was removed from the central compartment at a rate simulating the pharmacokinetic pattern of D4T (5), the 1-mg/kg/day D4T dose was studied in an every-12-h dosing schedule (Fig. 2). Figure 3a depicts the effect of D4T at a dose of 0.5 mg/kg administered at 12-h intervals. The daily levels of p24 antigen in the HF units inoculated with HIV-1$_{MN}$-infected CEM cells at 1:1,000 and 1:100 i/u ratios are shown. While p24 levels increased in the untreated bioreactor culture (open squares), there was a constant level of release of viral p24 into the extracapillary chamber of bioreactors exposed to D4T on a twice-daily (BID) schedule. This constant level of p24 expression represents virus release from the chronically infected cells which were used to initiate infection of the bioreactors. Analysis of HIV-1 DNA showed that the numbers of unintegrated copies of HIV-1 DNA increased markedly over a 7-day interval in untreated HF units (Fig. 3b). In marked contrast, <100 copies of unintegrated HIV DNA were amplified from D4T-treated cultures, indicative of effective viral suppression by the dose employed (Table 1). Flow cytometric analysis of the number of cells expressing intracellular p24 indicated that D4T inhibited cell-to-cell spread of HIV since the percentage of infected cells in the D4T-exposed cultures approximated the input i/u ratio, while untreated cultures had >70% p24-positive cells.

In vitro dose deescalation studies. Figure 4a depicts the effect of a dose deescalation to 0.5 mg/kg/day administered on a every-12-h schedule. As was seen previously, the level of p24 gradually increased in the non-drug-exposed chambers inoculated with HIV-1$_{MN}$-infected cells at a ratio of 1:1,000 (open squares). Viral infection of previously uninfected cells was
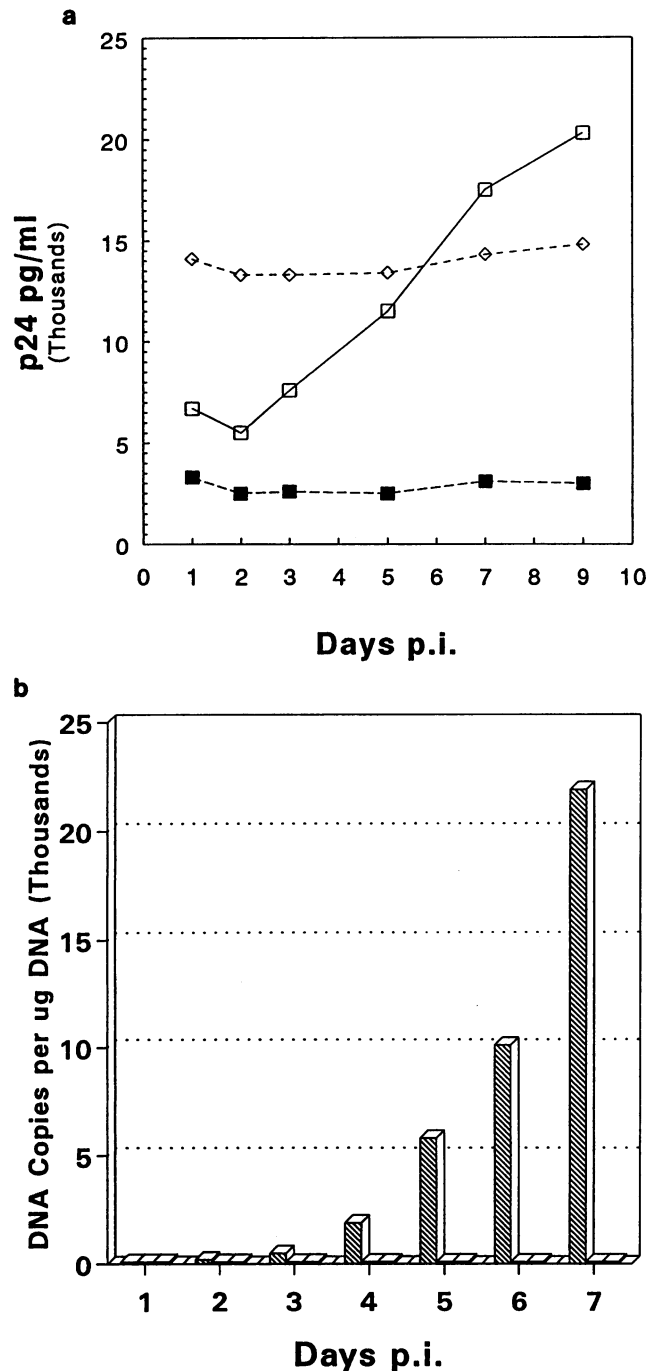
FIG. 3. (a) Effect of D4T (0.5 mg/kg, BID) on the spread of HIV-1$_{MN}$. Two bioreactors were set with 10^7 CEM cells and 10^4 CEM-MN cells (1:1,000 i/u ratio). A third bioreactor was started at a 1:100 i/u ratio. A 1:100 and a 1:1,000 bioreactor were exposed to D4T (0.5 mg/kg) at 12-h intervals, and the pharmacokinetics of D4T was simulated by dilution of the circulating drug. Postdistributional peak concentrations were set at 0.5092 μg/ml (2.27 μM). D4T levels were below the limit of detection 3 to 4 h after dosing. The D4T-treated bioreactors were set in parallel while the untreated unit was continuously exposed to medium without drug. Samples were taken at trough points 12 h after dosing, and p24 was measured as indicated. The results shown are from a single experiment. p.i., postinfection. Symbols: □, no treatment (1:1,000 i/u); ◇, D4T (1:100 i/u); ■, D4T (1:1,000 i/u). (b) PCR was performed on high- and low-molecular-weight fractions of DNA from CEM cells cocultivated with 1:100 or

demonstrated by increased p24 antigen production at day 8 in the D4T-exposed cultures with an i/u ratio of 1:100. This was not apparent for the unit challenged with a ratio of 1:1,000 i/u cells. However, PCR analysis of cells from the 1:1,000 i/u D4T-treated culture showed an increase from <100 (on day 1) to approximately 600 copies of unintegrated DNA per μg on day 10, which suggests a low level of de novo infection of this culture on the 0.25-mg/kg BID regimen. Figure 4b indicates that decreasing the D4T dose to 0.25 mg/kg/day (0.125 mg/kg every 12 h) resulted in levels of viral replication comparable to those of untreated cultures.

DISCUSSION

There were three important findings in our study. The first is that we were able to identify the minimal effect dose of D4T. The phase I study reported by Browne et al. (4) started at 4 mg/kg/day, and the dose was escalated until a daily dose of 12 mg/kg/day was reached. Little extra antiretroviral activity was gained during this dose escalation, but greatly increased drug toxicity became manifest. On an every-12-h dosing schedule, activity was maintained and toxicity was lessened at doses down to 0.5 mg/kg/day. Suboptimal antiviral effects were evident at doses of 0.25 mg/kg/day (1). These clinical findings are in excellent accord with the findings of our in vitro system. It is obvious that a great deal of time in the drug development process can be saved if minimal effect starting doses of antiretroviral agents can be identified before the start of phase I/II investigations. Further, the lack of effect of administration schedule (at least between continuous infusion and BID administration) on antiviral endpoints was demonstrated.

We were also able to demonstrate that the drug effect was, at least to a degree, dependent on the viral load (the absolute challenge to the system). Viral breakthrough occurred one dose deescalation step earlier with the higher (1:100 i/u ratio) challenge. This is a finding which requires clinical correlations. Our ability to demonstrate this in vitro may have been related to the absolute numbers of cells and consequent number of rounds of viral replication that we were able to achieve (the numbers of cells in the HF bioreactors approached 3×10^9).

While other cell types and strains of HIV can be used, this study used CEM cells chronically infected with HIV-1$_{MN}$ to examine the antiviral effect of D4T. These cells were cocultivated with uninfected CEM cells at ratios of 1:100 and 1:1,000 (i/u). These ratios were chosen to represent a formidable challenge 10- to 100-fold above the percentage of HIV-infected CD4$^+$ cells in the lymphoid organs of infected patients (15, 16). Practically, they were also chosen since the results were readily evaluable at the 1:1,000 ratio within 2 weeks under standard conditions in the HF culture unit. In addition, HIV-1$_{MN}$ was chosen because its 50% effective dose for D4T is in the middle of the range for isolates likely to be encountered clinically.

In summary, the in vitro HF system study of D4T pharmacodynamics using simulated human pharmacokinetics predicted the minimal effect dose obtained after extensive and lengthy dose deescalation studies in the clinic. The minimal effect dose was shown to be approximately 0.5 mg/kg/day

1:1,000 CEM-MN cells. Cells in the bioreactors were either untreated or exposed to D4T at 0.5 mg/kg, BID. p.i., postinfection. Symbols: ▨, no treatment; ▨, D4T (1:100 i/u); ▨, D4T (1:1,000 i/u).

administered on an every-12-h schedule either in the HF system or in the clinic. This finding suggests that in vitro HF pharmacodynamic modeling may be an extremely useful tool in the preclinical evaluation process.

ACKNOWLEDGMENT

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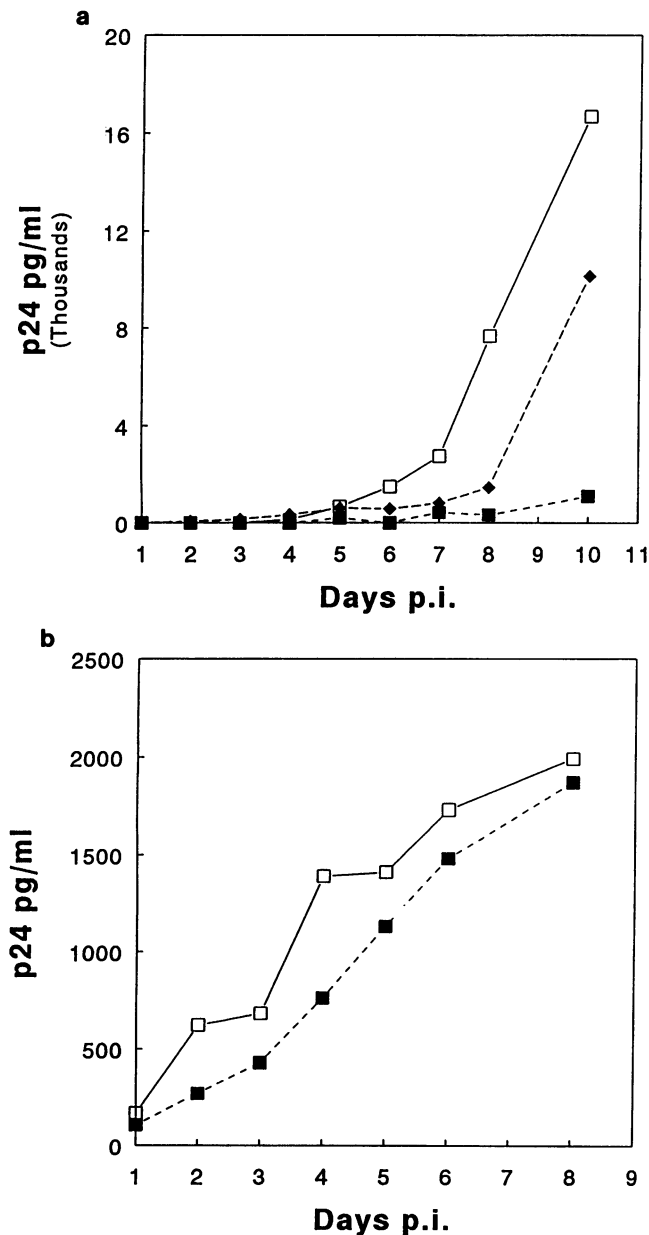16. **Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni,**

FIG. 4. (a) Effect of D4T (0.25 mg/kg, BID) on the spread of HIV-1$_{MN}$. Three bioreactors were initiated with CEM and CEM-MN cells as described in the legend to Fig. 2a. Dosing was at 12-h intervals with 0.25 mg/kg (postdistributional peak concentration, 1.13 μM) administered with simulated human serum kinetics, and samples were taken as described in the text. The results shown are from a single experiment. p.i., postinfection. Symbols: □, no treatment (1:1,000 i/u); ■, D4T (1:1,000 i/u); ◆, D4T (1:100 i/u). (b) Effect of D4T (0.125 mg/kg, BID) on the spread of HIV-1$_{MN}$. Two bioreactors were initiated with 10^7 CEM cells and 10^4 CEM-MN cells (1:1,000 i/u ratio). Dosing was at 12-h intervals with 0.125 mg/kg (postdistributional peak concentration, 0.56 μM) administered with simulated human serum kinetics and samples taken as described in the text. The results shown are from a single experiment. p.i., postinfection. Symbols: □, no treatment; ■, D4T.

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