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Anti-Human Immunodeficiency Virus Effects of Dextran Sulfate Are Strain Dependent and Synergistic or Antagonistic When Dextran Sulfate Is Given in Combination with Dideoxynucleosides

MARIANO E. BUSSO and LIONEL RESNICK*
Department of Retrovirology Research, Mount Sinai Medical Center, Miami Beach, Florida 33140

Received 30 May 1990/Accepted 1 August 1990

The effects of three molecular weight ranges of dextran sulfate on five different human immunodeficiency virus (HIV) isolates (from patients with acquired immunodeficiency syndrome), alone and in combination with dideoxynucleosides, were investigated in vitro. The higher the molecular weight range of dextran sulfate, the more potent the activity as assessed by a quantitative syncytium formation assay. Although all five HIV isolates had similar susceptibilities to the inhibitory effects of dideoxynucleosides, the two clinical isolates of HIV (HIV type 1 [HIV-1] TM and SP) exhibited a pattern of reduced susceptibility to dextran sulfate when compared with the two cloned isolates (HIV-1 WMF and HIV-2 ROD) and a prototype laboratory strain (HIV-1 IIIB). In combination with dideoxynucleosides, the high-molecular-weight range of dextran sulfate (500,000) resulted in an antagonistic response directed against the two clinical isolates of HIV (HIV-1 TM and SP) when the antiviral concentrations of dextran sulfate were in the ineffective range. Additive or synergistic effects were seen with the other three HIV isolates and all five HIV isolates when the low-molecular-weight range of dextran sulfate (8,000) was used. The results of these studies raise issues on the impact of drug-resistant strains on disease progression and the use of dextran sulfate in combination with nucleoside analogs for the clinical management of HIV disease.

Dextran sulfate is a member of a family of sulfated polysaccharides with potent anti-human immunodeficiency virus (HIV) activity in vitro (1, 2, 19, 20, 29). They inhibit the binding of the HIV envelope glycoproteins to host cells and prevent the development of syncytium formation (18). Genotypic variation in the HIV envelope glycoprotein region has been associated with the finding of biological differences among the HIV variant forms (9, 24). These differences could result in HIV strains with reduced susceptibility to drugs that work by interacting with the HIV envelope glycoproteins. In this study, experiments were performed to investigate the effects of different molecular weight ranges of dextran sulfate on different HIV isolates in vitro. The combination of dextran sulfates with dideoxynucleosides (reverse transcriptase inhibitors), compounds that inhibit HIV at different target sites, was also studied (14, 15, 23, 25).

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MATERIALS AND METHODS

Drugs. Dextran sulfates (USHERDEX; sulfur content of approximately 17% according to the specifications of the manufacturer) were provided by Polydex Pharmaceuticals (Toronto, Canada). Their molecular weights had median ranges of 8,000, 40,000, and 500,000. Nonsulfated dextran 480,000 (480,000 molecular weight) was obtained from Sigma Chemical Company (St. Louis, Mo.). 3′-Azido-3′-deoxythymidine (zidovudine; AZT) was purchased from Burroughs Wellcome (Research Triangle Park, N.C.), and 2′,3′-dideoxyadenosine (ddA) and 2′,3′-dideoxycytidine (ddC) were obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). The drugs were dissolved in sterile phosphate-buffered saline and stored in aliquots at −20°C until used.

Virus strains. H9 cells were infected with HIV type 1 (HIV-1) (IIIB [prototype laboratory strain], TM and SP [wild-type clinical strains] [4], and WMF [clone 3D] [24]) or HIV-2 ROD (11). HIV-1 TM and SP represented low-passage (3 to 4 passages) virus strains that were obtained from the culture supernatant of peripheral blood mononuclear cells from two patients with acquired immunodeficiency syndrome. All HIV strains were obtained from patients who had not received dextran sulfate or dideoxynucleosides. At the peak of the cytopathic effects, the culture supernatants were divided into aliquots and kept frozen at −85°C until use.

Cells. Chronically HIV-infected H9 cells were selected from acutely infected cells resistant to cytopathic effects. The human T-cell leukemia virus type 1 immortalized cell line, MT-2, and H9 cells (uninfected or chronically infected with HIV) were maintained in growth medium (Dulbecco modified Eagle medium or RPMI 1640 supplemented with 15% heat-inactivated fetal calf serum, 2 mM glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml).

Cytotoxicity studies. MT-2 cells (2×10^5 cells per ml) in exponential growth phase were exposed to various drug concentrations either alone or in combination. After 4 days, the cell viability was assessed by the trypan blue exclusion method and compared with controls without drug.

Quantification of virus titer. The cell-free virus titer was determined by an endpoint titration method using MT-2 cells (2×10^5 cells per ml) in 96-well microtiter plates. The titrations were performed in sextuplicate, and the virus titer was calculated by the method of Reed and Muench (7). The virus titer was adjusted to a reverse transcriptase activity of 50,000 cpm/ml and represented a multiplicity of infection of approximately 0.001 for the HIV infectivity assays. For all virus strains, this virus input reproducibly led to 50 to 100 syncytium-forming units (SFU) per MT-2 cell culture without drug on day 4 after infection. At this virus input, all HIV

* Corresponding author.

TABLE 1. Effects of dextran sulfates and dideoxynucleosides on the cell-free infection of different HIV isolates[a]

Treatment	ED_{50} (μM) for:				
	HIV-1				HIV-2 ROD
	IIIB	TM	SP	WMF	
DxS 8,000	2.75 ± 0.26	45.00 ± 1.38	48.75 ± 1.53	7.75 ± 0.45	5.63 ± 0.39
DxS 40,000	0.28 ± 0.02	3.35 ± 0.21	3.13 ± 0.17	0.73 ± 0.05	0.58 ± 0.05
DxS 500,000	0.01 ± 0.0004	0.11 ± 0.007	0.15 ± 0.009	0.036 ± 0.004	0.034 ± 0.003
AZT	0.05 ± 0.002	0.04 ± 0.003	0.06 ± 0.09	0.05 ± 0.008	0.04 ± 0.002
ddA	0.12 ± 0.009	0.11 ± 0.003	0.10 ± 0.008	0.08 ± 0.004	0.15 ± 0.008
ddC	0.04 ± 0.004	0.06 ± 0.004	0.10 ± 0.003	0.08 ± 0.004	0.10 ± 0.005

[a] MT-2 cells were infected with different HIV strains (multiplicity of infection ~ 0.001) and exposed to multiple concentrations (range, 0.001 to 125 μM; tested at half-log dilutions) of dextran sulfate 8,000, 40,000, and 500,000 and dideoxynucleosides. On day 4, the number of SFU in the control culture (infected cells without drug) plateaued (68 ± 8 SFU per culture) and was compared with the number of SFU in the infected cultures with drug (21). A dose-response curve was obtained, and the ED_{50} was determined by linear regression analysis. Results represent the mean ± standard deviation of three independent experiments performed in triplicate assays. DxS, Dextran sulfate.

isolates had similar susceptibilities to the inhibitory effects of dideoxynucleoside analogs.

Infectivity assays using cell-free HIV. Target MT-2 cells were exposed to DEAE dextran (25 μg/ml; Sigma) for 20 min, washed, and incubated with HIV at 37°C for 1 h. MT-2 cells (2×10^5/ml) were placed in growth medium and transferred to a 96-well microtiter plate, immediately followed by the addition of drug. The cells were maintained at 37°C in humidified air containing 5% CO_2 for 6 days. Uninfected and infected MT-2 cells without exposure to drugs were used as controls. Additional controls consisted of infected MT-2 cells in the presence of different concentrations of dideoxynucleosides (AZT, ddA, and ddC).

HIV-induced cytopathology was assessed on day 4 after infection (peak of SFU formation) by SFU and viable cell determinations (4, 21). The detection of intracellular HIV-1 p24 antigen was measured by indirect immunofluorescence using a monoclonal antibody on day 6 as previously described (21). HIV-2-infected cells were detected by indirect immunofluorescence using an HIV-2 immune human serum.

Reverse transcriptase assays. The presence of reverse transcriptase activity from disrupted virions in cell supernatants was detected as previously reported (22). The HIV-1 reverse transcriptase (recombinant enzyme provided by Upjohn Pharmaceuticals, Kalamazoo, Mich.) and mammalian alpha DNA polymerase (purified calf thymus enzyme provided by A. So, University of Miami, Fla.) assays were performed as previously described (13, 28). When HIV reverse transcriptase-containing supernatants (~100,000 cpm/ml) were incubated with therapeutic anti-HIV concentrations of the dextran sulfates (30 min at 37°C), no reverse transcriptase activity was detected. Since the dextran sulfates interfered with the reverse transcriptase determination, this parameter of HIV expression was not utilized for the antiviral evaluations (26).

Quantitation of inhibition of cell fusion by HIV-infected cells. This assay measures the development of cell fusion with few, if any, rounds of viral infection and replication. H9 cells (uninfected or greater than 95% infected with HIV) were cocultivated with MT-2 cells (10^6 cells per ml, ratio 1:1) in the presence of drugs. When no drug was added, the mixture of H9 HIV-infected cells and MT-2 cells was completely fused within 8 h, with less than 10% viable cells persisting. The percent inhibition of HIV-induced cell fusion was determined by the following formula: [(viable cell number in HIV-infected culture exposed to drug − viable cell number in HIV-infected culture without drug)/(viable cell number in uninfected culture with drug − viable cell number in HIV-infected culture without drug)] × 100. Uninfected cells in the presence of drug served as toxicity controls.

Evaluation of combined drug effects. Drug interactions were evaluated by the isobologram method (8). The effects of combined compounds on HIV expression were studied by determining the 50% effective dose (ED_{50}; dose required to reduce HIV expression to 50% of control) by linear regression analysis for each compound individually and in the presence of different concentrations of the other compound. The fractional inhibitory concentration was calculated by dividing the concentration of the compound needed to achieve 50% inhibition in the combination by the amount of the drug required to give the same degree of inhibition by itself. When the sum of the fractional inhibitory concentration for each compound is less than or equal to 0.5, the combination has a synergistic effect; between 0.5 and 1, it is subsynergistic; equal to 1, it is additive; between 1 and 1.5, it is subantagonistic; and greater than or equal to 1.5, it is antagonistic.

RESULTS

Anti-HIV effects of different molecular weight ranges of dextran sulfates. All five HIV isolates were susceptible to the inhibitory effects of the dextran sulfates as assessed by the MT-2 syncytium-forming assay. The three different molecular weight ranges of dextran sulfate exhibited similar anti-HIV efficacy, but differed in their potency profiles (Table 1). The highest molecular weight range of dextran sulfate (500,000) yielded the most potent HIV inhibitory effect. The ED_{50}s of dextran sulfate 8,000, 40,000, and 500,000 directed against HIV-1 (TM) were 360, 134, and 56 μg/ml, respectively. Although it has been reported that cell toxicity is dependent on the molecular weight ranges of dextran sulfate, in agreement with previous reports, no dextran sulfate achieved a 50% cytotoxic dose at 1 mg/ml (20). Nonsulfated dextran had no antiviral effects.

Anti-HIV effects of dextran sulfates on different HIV isolates. The HIV isolates exhibited different susceptibilities to the dextran sulfates as determined by quantitation of HIV-induced syncytium formation in acutely infected MT-2 cells (Table 1). The ED_{50}s ranged over 20-fold when dextran sulfate 8,000 was tested among the different HIV isolates. In addition, the HIV isolates displayed a greater than 10-fold difference in their susceptibilities to the higher-molecular-weight dextran sulfates (40,000 and 500,000). The most susceptible HIV isolate was the prototype laboratory strain IIIB, and the least susceptible were the wild-type clinical

TABLE 2. Inhibition of HIV-induced cell fusion among different HIV isolates by dextran sulfates[a]

Treatment	ED_{50} (μM) for:				
	HIV-1				HIV-2 ROD
	IIIB	TM	SP	WMF	
DxS 8,000	7.63 ± 0.90	122.75 ± 11.38	92.30 ± 7.60	40.50 ± 3.82	22.32 ± 2.00
DxS 40,000	1.10 ± 0.07	14.32 ± 0.90	12.62 ± 1.02	0.98 ± 0.04	1.06 ± 0.06
DxS 500,000	0.02 ± 0.004	0.61 ± 0.03	0.50 ± 0.03	0.11 ± 0.008	0.06 ± 0.009

[a] H9 cells chronically infected with different HIV strains were cocultivated with MT-2 cells (ratio 1:1) and exposed to dextran sulfate 8,000, 40,000, and 500,000 or dideoxynucleosides as described in Materials and Methods. After 8 h of incubation, the percent inhibition of cell fusion was determined. In cultures not exposed to dextran sulfates, complete cell fusion developed with less than 10% viable cells remaining. The ED_{50} was determined by linear regression analysis after a dose-response curve was generated by using at least four different concentrations of each compound. The dideoxynucleosides (AZT, ddA, and ddC) had no inhibitory effects. Results represent the mean ± standard deviation of three sets of experiments performed in triplicate. DxS, Dextran sulfate.

strains, TM and SP ($P < 0.001$ by *t*-test analysis). The two cloned isolates of HIV-1 and HIV-2 exhibited susceptibilities similar to that of IIIB. All the HIV isolates had similar susceptibilities to the inhibitory effects of dideoxynucleoside analogs. Although the absolute values were different, a similar pattern of drug susceptibility was seen among the HIV isolates when the human promonocytic cell line, U937, was used as a target cell (data not shown).

To determine if dextran sulfate had an inhibitory effect on the cell-to-cell transmission of HIV, chronically infected H9 cells were cocultivated for 8 h with MT-2 cells in the presence of dextran sulfate (Table 2). The high- and low-molecular-weight ranges of dextran sulfate were effective in inhibiting the development of cell fusion between HIV-infected and uninfected cells in a dose-response fashion. Since a higher input dose of HIV was necessary for the rapid production of syncytia, the ED_{50}s were higher when compared with cell-free HIV infection of MT-2 cells. The susceptibility profile among the HIV strains was similar to that observed with cell-free infection, the IIIB isolate being greater than 10-fold more susceptible than TM and SP ($P < 0.001$ by *t*-test analysis). AZT and the other dideoxynucleosides did not prevent the development of syncytia by the infected H9 cell line.

Effects of the combination of dextran sulfates and dideoxynucleosides on the replication of HIV. The dextran sulfates were tested in combination with dideoxynucleosides to determine their anti-HIV effects. Combinations of the high-molecular-weight range of dextran sulfate (500,000) and AZT revealed an antagonistic response when evaluating HIV-1 TM and SP (Table 3 and Fig. 1). At ineffective antiviral concentrations of dextran sulfate (0.002 to 0.06 μM), the combination with AZT yielded antagonistic effects. Similar antagonistic effects were seen when dextran sulfate (500,000) was combined with ddA or ddC. At these ineffective concentrations, dextran sulfate did not antagonize the cytotoxic effects of AZT or the other dideoxynucleosides (Table 4). The antagonistic effect was not detected with any HIV isolate by using the low-molecular-weight range of dextran sulfate (8,000) or with the HIV IIIB, WMF, and ROD isolates by using the high-molecular-weight range of dextran sulfate (500,000). In these cases, dextran sulfate in combination with dideoxynucleosides resulted in additive or synergistic effects. The cytotoxicity of AZT was enhanced at the highest concentrations of dextran sulfate that were tested (Table 4). Nonsulfated dextran did not shift the dose-response curve for AZT or the other dideoxynucleosides.

TABLE 3. Concentration of the combination of AZT and dextran sulfate (500,000) required to inhibit HIV-1-induced syncytium formation by 50% in MT-2 cultures[a]

Concentration (μM) at the ED_{50} for:			
HIV-1 TM		HIV-1 IIIB	
AZT	DxS 500,000	AZT	DxS 500,000
0.07	0.00 (1)	0.08	0.00 (1)
0.72	0.002 (10.22)[b]	0.09	0.00006 (1.14)
0.94	0.006 (13.45)[b]	0.02	0.002 (0.45)[c]
1.47	0.02 (21.18)[b]	0.01	0.006 (0.73)
0.00	0.11 (1)	0.00	0.01 (1)

[a] Results represent the means of at least three separate experiments performed in triplicate. DxS, Dextran sulfate. Results in parentheses show the fractional inhibitory concentration index.

[b] The combination is antagonistic.

[c] The combination is synergistic.

DISCUSSION

The results of these studies indicate that the antiviral effects of the dextran sulfates vary among different HIV isolates. These findings were not unexpected since strain-specific antiviral effects of dextran sulfate have been reported with other viruses such as poliovirus (27). Two distinct patterns of strain-specific differences in susceptibility to the dextran sulfates occurred. The clinical isolates of HIV-1 (TM and SP) exhibited a pattern of drug resistance when compared with the cloned isolates of HIV-1 (WMF) and HIV-2 (ROD) and with the prototype laboratory strain of HIV-1 (IIIB), which had the most susceptible phenotype. These findings were not specific to the in vitro system that was employed since the use of a different biologic assay that utilized a different cell line (U937) and viral endpoint (Abbott p24 antigen enzyme immunoassay) resulted in a similar pattern of drug susceptibility among the HIV isolates (personal observation). Whether these biological variations are the results of differences in the pathogenic potential among strains or whether isolates from asymptomatic patients would be more susceptible to dextran sulfate is a topic of further investigation.

Unlike HIV resistance to AZT, in which isolates with reduced susceptibility to drug occur only after prolonged exposure in vivo (16), these results document the existence of natural HIV variants with reduced susceptibilities to the dextran sulfates. Since individual isolates of HIV in vivo are composed of populations of genetically and biologically distinct variants (quasispecies), the chronic administration of dextran sulfate could select for the predominant growth of resistant strains in vivo or prevent the development of more pathogenic strains and the worsening of clinical status (17).

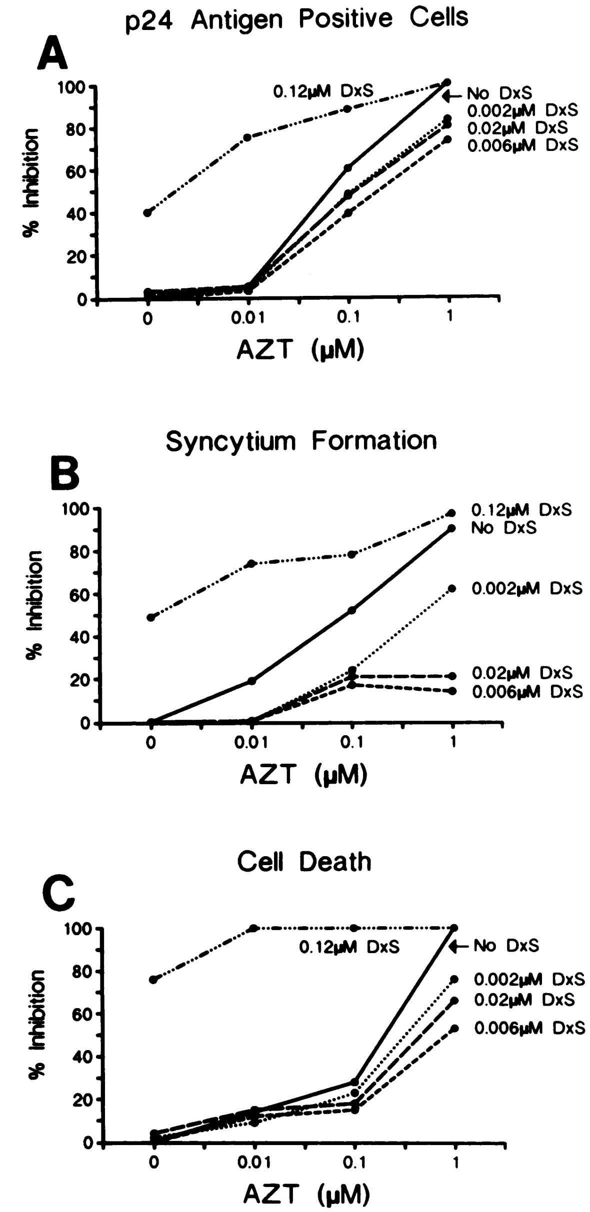

FIG. 1. Anti-HIV-1 (TM) effects of the combination of dextran sulfate 500,000 and AZT. MT-2 cells were infected with HIV-1 (TM) and exposed to multiple-drug combinations. On day 4, infected cultures without drug exposure had 57 ± 4 SFU per culture, and on day 6, 69 ± 7% of the cells had staining for intracellular HIV-1 p24 antigen by indirect immunofluorescence (greater than 300 cells per slide were counted). Uninfected controls had no SFU and less than 1% cellular staining. After four days, infected cultures exposed to AZT (3 μM) had $1.9 \times 10^6 \pm 0.3 \times 10^6$ cells per ml and uninfected cultures without drugs had $2.1 \times 10^6 \pm 0.3 \times 10^6$ cells per ml. Results are representative of triplicate determinations from seven sets of experiments. Panels represent inhibition of HIV expression by AZT in the presence of different concentrations of dextran sulfate as assessed by intracellular p24 antigen (A), syncytium formation (B), and cell viability (C).

Studies with more clinical isolates are necessary to define the issues of strain-specific differences in drug susceptibility.

Among the HIV strains, biologic differences in susceptibility to the dextran sulfates would in part be determined at the level of the viral envelope glycoprotein (24). Although the exact mechanism of inhibition of HIV binding to target cells by dextran sulfates is unknown, differences in (i) virion binding affinity to the CD4 molecule, (ii) virion charge density, (iii) the density of HIV envelope glycoprotein at the cell membrane, (iv) the level of envelope glycoprotein that is shed from the cell surface, and (v) the input dose of virus could account for the observed effects. The reported lack of differences in binding affinities among HIV isolates suggests that anionic charge repulsion or cell surface modifications leading to steric hindrance could play a role (5, 6). Although the HIV envelope glycoproteins contain genetically variable regions, the lack of differences in susceptibilities among the cloned isolates indicate that changes in the primary structure do not necessarily lead to the observed effects. Furthermore, the finding of different strain susceptibilities to dextran sulfate whether infection was performed with cell-free supernatants or HIV-infected cells supports the premise that different levels of expression of the envelope glycoprotein could be occurring among the isolates. If so, this effect could be modulated by host cell or HIV regulatory proteins. To investigate this further, the density of the HIV envelope glycoprotein on the cell surface would need to be determined, but monoclonal antibodies that recognize the clinical strains of HIV-1 (TM or SP) gp120 have not been identified (personal observation).

A major concern in the performance of assays to determine biological profiles is the lack of an accurate standardized method to quantitate the virus input among different HIV strains. A quantitative method should allow for the calculation of the ratio of infectious to total particles since free gp120 envelope glycoprotein in virus stocks would be expected to compete for available CD4 target sites, thereby affecting HIV infectivity. For these experiments, the input dose of HIV was standardized by selecting a concentration of HIV that (i) achieved reproducible cytopathic effects after 4 days in culture and (ii) yielded similar profiles of susceptibility to dideoxynucleosides among the isolates (reverse transcriptase inhibitors). When different virus stocks of the same isolate were standardized and quantified, similar results were generated.

Contrary to a previous report, the anti-HIV activity of dextran sulfate was molecular weight dependent (1). A higher affinity for cell surface acceptor sites (e.g., fibronectin) of the higher-molecular-weight ranges of dextran sulfate may explain this phenomenon (3). Confirming prior reports, the combination of the low-molecular-weight range of dextran sulfate (8,000) with dideoxynucleosides resulted in synergistic effects directed against all five HIV isolates (29). However, the combination of the high-molecular-weight-range of dextran sulfate (500,000) with dideoxynucleosides

TABLE 4. Effects of the combination of dextran sulfate and AZT on cell viability[a]

Dextran sulfate 500,000 (μM)	CD_{50} of AZT (μM)	Dextran sulfate 8,000 (μM)	CD_{50} of AZT (μM)
0.200	75 ± 6.4	125.0	86 ± 4.3
0.060	114 ± 3.1	42.0	121 ± 3.8
0.020	129 ± 2.9	12.5	138 ± 3.7
0.006	137 ± 2.2	4.2	145 ± 3.5
0.000	143 ± 2.0	0.0	143 ± 2.0

[a] Cytotoxicity studies were performed as described in Materials and Methods. The 50% cytotoxic dose (CD_{50}) for the combinations of drugs was determined by linear regression analysis after generating a dose-response curve by using at least six different concentrations of AZT. Cultures not exposed to drugs had $0.96 \times 10^6 \pm 0.05 \times 10^6$ MT-2 cells per ml. The results represent the mean values of two sets of experiments performed in triplicate.

yielded an antagonistic effect directed against the two clinical isolates of HIV-1 (TM and SP). Although the mechanism for this effect is unknown, dextran sulfate 500,000, unlike dextran sulfate 8,000, caused cell aggregation at concentrations below the effective antiviral dose for the two clinical isolates (30). As suggested by Hildreth et al., if cell aggregation facilitates the cell-to-cell transmission of HIV, drugs that act prior to HIV integration, such as reverse transcriptase inhibitors, would be rendered less active (10, 12). These findings emphasize the need to evaluate combinations of singularly effective anti-HIV drugs in vitro and in animal models prior to their clinical use.

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