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Dextran Sulfate as an Inhibitor against the Human Immunodeficiency Virus (42811)

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Abstract. Dextran sulfate (DS) is a potent inhibitor of the growth of human immunodeficiency virus type 1 (HIV-1) in the H9 cell. Its minimal inhibitory concentration is about 1 μg/ml. Its therapeutic index is ⩾200 which is higher than that of 38 for zidovudine. At the ID_{100} range, DS blocks the synthesis of HIV-1 antigens completely for at least 21 days; zidovudine at the subtoxic concentration of 3 μg/ml is incapable of achieving such a complete blockage. DS is still active when added to H9 cell cultures 4 hr after the addition of HIV-1. DS does not inactivate extracellular HIV-1 and is incapable of inducing interferons. It interferes partially with the infection of the H9 cells by the HIV-1. It inhibits the activity of HIV-1 reverse transcriptase. These activities may account, at least in part, for the inhibitory activity of dextran sulfate against the HIV-1. DS has a narrow antiviral spectrum; it is noninhibitory to the herpes simplex, vesicular stomatitis, polio, or adeno viruses. Dextran is not inhibitory to HIV-1. After sulfonation, the sulfonated dextran is highly inhibitory. Therefore, the sulfate group in the DS molecule appears to be essential for its anti-HIV-1 activity. The molecular weights of DS within the range 4000 to 12,000 do not appear to influence its anti-HIV potency.

Dextrans are a class of polysaccharides synthesized from sucrose by bacterial enzymes (1). They were used formerly as an anticoagulant and plasma expander (2). Heating dextrans in sulfuric acid followed by esterification with chlorosulfonic acid (1, 3) results in the formation of dextran sulfate. DS with an average mol wt 7000 and up to two or three sulfate groups per sugar residue has anticoagulant properties and inhibitory activity against attenuated polio virus (2, 4). In two recent brief communications (5, 6), DS was described as a potent inhibitor of the growth of human immunodeficiency virus (HIV) *in vitro*. Since we have a long-standing interest in the antiviral activity of polysaccharides, we initiated a study to evaluate and expand on the two brief reports. Our goals are to evaluate its anti-HIV activity under different sets of experimental conditions, to attempt to elucidate its mode of anti-HIV action, to determine its specificity for the HIV, to determine the functional group in the molecule which is responsible for anti-HIV activity, and to relate the average molecular weight to anti-HIV activity.

Materials and Methods. *Dextran sulfate.* DS with average mol wt 4000, 5000, 7000, 8000, 12,000 and 500,000 were purchased from commercial sources. All had sulfur contents of 17.3 to 17.7%. They were dissolved in phosphate-buffered saline (pH 7.2) and filtered through 0.45-μm filters. The filtrates were stored at 0°C for subsequent use. Unless otherwise specified, DS (D6001), purchased from Sigma Chemical Co., was used in all experiments.

Sulfonation of dextran. Dextran (average mol wt 9400) was sulfonated with chlorosulfonic acid in pyridine under a nitrogen atmosphere. The procedure was similar to that of Ricketts (7) except that the sulfonated dextran was purified on Sephadex G75 column chromatography in place of dialysis.

Human immunodeficiency virus type 1 (HIV-1). The HIV-1 was a gift from Dr. Robert E. Gallo. A pool was prepared by infecting the H9 cell culture (8). Fourteen days after infection, the culture was centrifuged at 600*g* for 10 min and the supernatant was collected and distributed into small portions.

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The portions were stored below −70°C until use. The pool used in experiments described in this report had a $TCID_{50}$ of $10^{5.3}$/ml.

Human T helper cells. The H9 cell (8), a line of human T helper cells, was also a gift from Dr. Gallo. It was grown in a medium consisting of 10% inactivated fetal calf serum in RPMI 1640 which contained 20 μg of gentamycin/ml. For use in experiments, sufficient H9 cells were pelleted and the cell pellet was suspended in medium to achieve 10^5 cells/ml. All cultures were incubated in a humidified 5% CO_2 incubator at 36°C.

Toxicity to H9 cells. DS was diluted twofold serially in medium. To 0.2 ml of diluted DS in duplicate, 0.8 ml of a H9 cell suspension was added. A control consisting of 0.8 ml of the H9 cell suspension and 0.2 ml medium in quadruplicate was included in every assay. After 3 days of incubation, the number of viable cells in each culture was counted by dye exclusion. If the average viable count of cultures containing a certain concentration of DS was two or more standard deviations below the mean of the control, DS was considered cytotoxic at that concentration. Subtoxic concentration was the highest concentration that was nontoxic to the cell.

Anti-HIV activity. DS was diluted twofold serially in medium. To 0.2 ml diluted DS in duplicate, 0.7 ml H9 cell suspension and 0.1 ml HIV preparation were added. A virus control in quadruplicate consisting of the same number of H9 cells and the same amount of HIV in the same total volume was included in every assay. After 3 days of incubation, cells in each culture were smeared, fixed, and stained for HIV antigens by immunofluorescence (9), using as antibody a pool of sera from 18 homosexual males suffering from the lymphadenopathy syndrome (10). Percentage inhibition was calculated as follows: 100 times (percentage positive cells in virus control minus percentage positive cells in treated culture) divided by percentage positive cells in virus control. The minimal inhibitory concentration (MIC) was the lowest concentration that reduced the percentage of HIV-positive cells by three standard deviations or by 50% (whichever was the larger reduction). ID_{100} was the minimal concentration that blocked the synthesis of HIV antigens completely. This assay was described in detail elsewhere and shown to relate to the amount of infectious HIV released (11).

Quantifying infectious HIV. Cultures to be tested were centrifuged at 3000*g* for 3 min in a Fisher centrifuge. The supernatant fluid was titrated for HIV by terminal dilution using the H9 cell as indicator cells. The results were expressed as $TCID_{50}$ according to the formula of Reed and Meunch (12); a difference of one or more log $TCID_{50}$ between two preparations is statistically significant (13).

Inactivation of extracellular virions. To 0.1 ml of HIV, 0.7 ml of medium and 0.2 ml of appropriately diluted DS were added. For control, 0.9 ml of medium was added to 0.1 ml of HIV. Both were incubated in a 37°C water bath for 60 min and then titrated for residual HIV infectivity. A similar procedure was used for the testing of inactivation of other viruses.

Interference of HIV infection. H9 cells (10^5) were suspended in 1 ml of HIV ($TCID_{50}$ $10^{5.3}$) with and without DS (10 μg/ml). The cell suspensions were incubated at 37°C for 2 hr with agitation once every 15 min. The cells were washed six times with medium to remove unadsorbed virus and the number of infected cells was titrated by terminal dilution.

Interferon induction. The 50% plaque reduction test was used (14). Briefly, 2 ml medium containing DS at a specified concentration was added to a confluent culture of human fibroblasts in a 3.5-cm-diameter petri dish. After 20 hr at 36°C, the culture was tested for susceptibility to vesicular stomatitis virus by the plaque assay. Positive (poly I:C) and negative (medium) controls were included in each assay.

Inhibition of reverse transcriptase. Reverse transcriptase from HIV was prepared and assayed by the procedure by Rey *et al.* (15). For assay of inhibition, equal volumes of DS and reverse transcriptase were mixed, kept at 0°C for 30 min and 37°C for 10 min, and then assayed for reverse transcriptase activity. Appropriate controls were included in each assay.

Inhibition against viruses other than HIV. The following viruses were included: herpes simplex type 2 (HSV), vesicular stomatitis (VSV), respiratory syncytial (RSV), polio type 1 (PV), and adeno type 7 (AV). For viruses that formed plaques readily (HSV, VSV, PV), the 50% plaque reduction test was used (16). For AV, a suspension of virus was titrated by terminal dilution in HeLa cell culture in medium with and without DS (10 μg/ml).

Results. *Cytotoxicity.* DS was nontoxic to the H9 cell at concentrations to 200 μg/ml. In contrast, zidovudine was toxic at a concentration of about 6 μg/ml.

Anti-HIV activity. Table I shows the results of two assays. The MIC were 0.8 and 1.6 μg/ml; the ID_{100} were 6.2 μg/ml; and the inhibitions were dose-dependent. In four other assays the MIC varied from 0.4 to 1.6 μg/ml and the ID_{100}, 6.2 to 12.5 μg/ml. DS (10 μg/ml) was still inhibitory to the HIV even when added to H9 cell cultures 24 hr after infection by the HIV; see Table II. By extending the assay to the 21st day, DS at the ID_{100} of 10 μg/ml continued to block the synthesis of HIV antigens completely. On the 21st day, DS-treated cultures contained no HIV-positive cells and no extracellular HIV; in the control cultures not treated with DS, most of the cells were positive for HIV antigen and the culture fluid contained 10^5 to 10^6 $TCID_{50}$ of HIV/ml. In comparison, the MIC for zidovudine in our assay varied from 0.02 to 0.16 μg/ml (average 0.08 μg/ml) which is close to that ($ID_{90} \leq 0.13$ μg/ml) reported by the manufacturer. In the 21-day assay, zidovudine at the subtoxic dose of 3 μg/ml failed to significantly reduce the percentage of HIV-positive cells.

Anti-HIV activity of dextran and DS with varying molecular weights. Dextran was noninhibitory even at 200 μg/ml. After sulfonation, the sulfonated dextran was inhibitory to the HIV with a MIC of about 0.8 μg/ml. The anti-HIV activities of DS with mol wt 4000, 5000, 7000, 8000, 12,000, and 500,000 were also compared. Their MIC were 0.4 μg/ml except for DS 500,000 which had an MIC of 1.6 μg/ml.

Inhibition of HIV reverse transcriptase. Results of a typical assay are presented in Table III; the 50% inhibitory concentrations were between 1.6 and 3.2 μg/ml. Similar results were obtained in three other assays.

Interference with HIV infection. H9 cells (10^5) were suspended in 1 ml of HIV ($TCID_{50}$ $10^{5.3}$) with and without DS (10 μg/ml). After 2 hr at 37°C, the cells were washed six times and the number of HIV-infected cells titrated by terminal dilution. In both experiments, treating H9 cells with HIV in the absence of DS resulted in the infection of virtually all cells ($10^{5.3}$ and $10^{4.8}$), while in the presence of DS only 10% of the cells were infected (10^4 and 10^4). Treating the H9 cells with DS first for 2 hr, washing the DS-treated cells by centrifugation, and then infecting the cells with HIV did not result in a reduction of the number of infected cells.

Interferon induction. DS at concentrations of 10 and 100 μg/ml failed to induce interferons in human fibroblasts.

Inactivation of extracellular virions. DS

TABLE I. REDUCTION IN PERCENTAGES OF HIV ANTIGEN-POSITIVE CELLS BY DS

Concn. of DS (μg/ml)	% HIV-pos cell		% Inhibition	
	Expt 1	Expt 2	Expt 1	Expt 2
12.5	0	0	100	100
6.2	0	0.1	100	100
3.1	0.3	1.1	99	97
1.6	3.1	1.7	89	95
0.8	11.0	21.5	62	37
0.4	32.4	45.9	0	0
None	28.9 ± 5.8	34.3 ± 4.3		

TABLE II. PERCENTAGE INHIBITION OF HIV BY DS (10 μg/ml) ADDED TO THE H9 CELL AT INTERVALS AFTER THE VIRUS

Hrs after infection	% HIV-pos cell	Percentage inhibition
2	0	100
4	0	100
6	0.3	99
24	1.6	95
48	11.2	67
Control (no DS)	34.3 ± 4.3	

(100 μg/ml) did not reduce the infectivity titers of suspensions of HIV, VSV, RSV, PV, or AV.

Inhibition against viruses other than HIV. DS at 10 μg/ml did not inhibit HSV, VSV, or PV in the 50% plaque reduction test or the AV in the infectivity titration.

Discussion. We have confirmed the recent brief reports (5, 6) that dextran sulfate is a potent inhibitor of HIV *in vitro.* The minimal inhibitory concentration of DS against the HIV varied from 0.4 to 1.6 μg/ml (average 1 μg/ml) which was lower than the reported values of 4 to 16 μg/ml. This difference is probably due to the higher sensitivity of our assay procedure. In our assay, the MIC for zidovudine against the HIV varied from 0.02 to 0.16 μg/ml (average 0.08 μg/ml) which is close to the value ($ID_{90} \leq 0.13$ μg/ml) reported by the manufacturer. In addition, we have shown that, at the ID_{100} of 10 μg/ml, DS shuts off the synthesis of HIV completely for a duration of at least 21 days. Under similar conditions, zidovudine at the subtoxic concentration (3 μg/ml) failed to significantly reduce the number of HIV-positive cells on the 21st day. Despite a delay of 4 hr in treating infected cultures, DS still shuts off HIV synthesis completely. The therapeutic index (subtoxic dose/MIC) for DS was ≥200 which is much larger than the value of 38 for azidothymidine as determined in our assays.

Attempts at the elucidation of the mode of anti-HIV action have revealed that DS, unlike other acidic polysaccharides (17) or anionic polymers (18, 19), did not induce interferons. DS also did not inactivate extracellular virions (HIV, HSV, RSV, VSV, PV, and AV). When the H9 cell was mixed with the HIV at the multiplicity of infection of about two, the percentage of cells infected by the HIV was reduced from about 100 to 10% by DS at 10 μg/ml. This finding indicates that DS interferes (at least partially) with the extracellular phase of viral replication cycle (possibly the binding of HIV to cell receptors). The importance of polysaccharides in the initiation of HIV infection has been suggested by others (20–23). After treatment of the H9 cell with DS, washing of the DS-treated cells, and then infection of the cells with HIV, there was no reduction in the number of infected cells. Therefore, the interference of the extracellular phase of the viral replication cycle is not likely to be due to the alteration of the cell surface by DS. In addition to this interference with the extracellular phase of the replication cycle, DS must also interfere with some intracellular phase of HIV replication. This statement is based on our observation that 4 hr after infection by the HIV, the addition of DS still completely inhibited the synthesis of HIV antigens. Active uptake of DS by mammalian cells *in vitro* has been documented by

TABLE III. INHIBITION OF HIV REVERSE TRANSCRIPTASE ACTIVITY BY DS AT VARYING CONCENTRATION

Concn. DS (μg/ml)	RT activity (cpm)	% Inhibition
0	8115[a]	
0.8	6833	16
1.6	5940	27
3.1	2507	69
6.2	762	91
12.5	236	97
25	50	99
50	10	100

[a] The cpm above background; average of duplicate.

cytochemical and cinematographic studies (4, 24). It is possible that this intracellular step involves interference with the reverse transcriptase. In our assays, DS clearly inhibited HIV reverse transcriptase. Others reported DS to be inhibitory to avian myeloblastosis (6) and not to HIV reverse transcriptase even at the concentration of 125 μg/ml (5). This discrepancy needs to be resolved.

Ueno and Kuno (6) suggested (no data presented) that anti-HIV activity of sulfonated polysaccharides was closely related to the number of sulfate groups per monosaccharide unit. Our data that dextran can be converted from an inactive to a highly active anti-HIV compound through sulfonation provide conclusive evidence for the importance of the sulfate group in anti-HIV activity. The sulfate group is also important in other pharmacologic attributes of DS such as anticoagulating and antilipemic activities (1, 7, 26, 27). Just why the sulfate group is so critical to the pharmacologic attributes of DS, including the anti-HIV activity, is not known. The knowledge that sulfate groups are essential for its anti-HIV activity provides an opportunity for chemical manipulation to increase its therapeutic index. It is interesting that several anionic dyes reported to be inhibitory to a mouse retrovirus are also polysulfonated compounds (25).

DS appears to have a narrow antiviral spectrum since it is noninhibitory to the HSV, VSV, AV, and PV. It should be of interest to determine if DS is active against other retroviruses.

DS is a readily available and inexpensive chemical. Much is already known about its pharmacology and human toxicity (1, 2, 26, 27). It has therapeutic potential against HIV infection and this potential needs to be exploited expeditiously.

While this manuscript was under review, an important publication appeared in *Science* (28). Two findings are particularly relevant. These authors found that bovine serum albumin nullified the inhibition of reverse transcriptase by DS and that DS also inactivated mammalian α-DNA polymerase; they concluded that, in view of these results, the capacity of DS to block the infectivity of HIV without cellular toxicity might be difficult to attribute to selective inactivation of viral reverse transcriptase per se. Using virions labeled with radioactive isotope, they showed that DS completely inhibited the binding of HIV to H9 cells. This finding is consistent with our data except that, in our experiments using bioassay, this inhibition of binding by DS was partial rather than complete.

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