

Anti-AIDS Virus Activity *in Vitro* of Dextran Sulfates Obtained by Sulfation of Synthetic and Natural Dextrans

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ABSTRACT: Anti-AIDS virus activity of synthetic and natural dextran sulfates was assayed *in vitro* by using a MT-4 cell line. These dextran sulfates with the number average molecular weight (\bar{M}_n) of 1.8×10^4 — 10.7×10^4 showed potent anti-AIDS virus activity in the concentration as low as 3.3 or 10 $\mu\text{g ml}^{-1}$, respectively. This high activity was almost the same as that of curdlan sulfates which was reported previously. The degree of sulfation per glucose residue in polymer backbone was 1.0—1.9 (the highest theoretical value is 3.0) which was calculated from the elemental analysis. The relationship between the structure and anti-AIDS virus activity is discussed based on high resolution NMR spectroscopy.

KEY WORDS Ring-Opening Polymerization / Sulfated Polysaccharide /
Synthetic Dextran / Anti-AIDS Virus Activity / Anticoagulant Activity /

Dextran is a naturally occurring polysaccharide consisting of 1,6- α linked poly-D-glucose and having branches depending on the strain. Dextran sulfate¹ is a sulfated polysaccharide with a high negative charge which binds strongly to thrombin and shows anticoagulant activity.² Since dextran sulfate has also lipemea-clearing activity, it has been clinically used for the treatment of high blood-lipid levels in Japan.

We successively synthesized stereoregular polysaccharides with biological activities by the ring-opening polymerization of anhydro sugars.³ Synthetic dextran by the ring-opening polymerization of 1,6-anhydro- α -D-glucose derivatives has only a straight-chained structure.^{4,5} We also reported the sulfation of synthetic dextrans with 3-*O*-(β -D-glucopyranosyl) or 3-

O-(α -D-mannopyranosyl) branches.^{6,7} Naturally occurring N4 dextran elaborated by *L. mesenteroides* strain N4 has been shown to have immunological specificity of 1,6- α -D-glucosidic linkages. We reported that the synthetic mannose-branched dextrans reacted with N4 dextran-concanavalin A conjugate to form antibodies in rabbit.⁸

It was found that dextran sulfate was effective for protection against scrapie virus *in vivo* experiment using mice⁹ and has an inhibitory effect on retroviruses¹⁰ and AIDS (acquired immunodeficiency syndrome) virus (HIV) infection *in vitro*.^{11–18} In this study, we report that synthetic and naturally occurring dextrans were sulfated with piperidine *N*-sulfonic acid in DMSO to give dextran sulfates which showed highly inhibitory effects on

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AIDS virus infection *in vitro*. The structural analysis of dextran sulfates using high resolution NMR experiments was made to correlate in detail the structures of linear and branched dextran sulfates with the anti-AIDS activity.

EXPERIMENTAL

General Methods

270 MHz ^1H and 67.8 MHz ^{13}C NMR spectra were recorded with a JEOL GX-270 spectrometer for solutions in CDCl_3 or D_2O . A solution of the free and sulfated dextrans in D_2O was freeze-dried several times and a sample solution was made as 10–20% solution in D_2O containing DSS (4,4-dimethyl-4-silapentane-1-sulfonate) as the internal reference (0.015 ppm for ^1H and 0.00 ppm for ^{13}C spectra). Specific rotation was measured for solution in CHCl_3 or H_2O by a Perkin-Elmer 241 polarimeter. Molecular weight was determined by GPC and calculated with polystyrene and dextran standards as references for THF soluble and water soluble samples, respectively.

Compounds

1,6-Anhydro-2,3,4-tri-*O*-benzyl- β -D-glucopyranose (LGTBE) and 1,6-anhydro-2,4-di-*O*-benzyl-3-*O*-*tert*-butyldimethylsilyl- β -D-glucopyranose (LGDBS) were prepared by the protection of 1,6-anhydro- β -D-glucopyranose according to the previous methods.^{5,6}

Polymerization

Polymerization was performed under high vacuum in sealed glass ampoules as described in the previous paper.¹⁹ Polymerization conditions are shown in Table I. Polymers were purified by reprecipitation using chloroform-methanol system and then freeze-dried from benzene.

Deprotection

Desilylation²⁰ of polymers was carried out

with tetrabutylammonium fluoride in THF under reflux for 1 h to give partially benzylated dextrans. The debenylation of fully-benzylated linear dextrans was performed with sodium in liquid ammonia.¹⁹

Branching of Partially Benzylated Dextrans

To a partially benzylated dextran (0.2 g) solution in benzene (40 ml) was added 3,4,6-tri-*O*-acetyl- α -D-mannose 1,2-(methyl orthoacetate) (0.25 g). After the reaction mixture was heated at the boiling temperature ($\sim 80^\circ\text{C}$) for 15 min, the solvent was partially distilled to remove a small amount of water as benzene azeotrope and then 2,6-lutidinium perchlorate (1.5 mg) was added. The mixture was stirred under reflux for 20 min, and then dried up by evaporation. The product was purified by reprecipitation of the chloroform solution with methanol, followed by freeze-drying from benzene; yield, 0.30 g, $[\alpha]_{\text{D}}^{25} = +134.8^\circ$ ($c = 1$, CH_3Cl), $\bar{M}_n = 3.7 \times 10^4$.

Sulfation of Free Dextrans

To a linear dextran (0.10 g) solution in 15 ml of dry DMSO was added piperidine *N*-sulfonic acid (0.51 g). The reaction mixture was stirred at 85°C for 1 h. After cooling with an ice-bath, it was neutralized with saturated sodium bicarbonate solution, and then acetone was added to the mixture until a precipitate was appeared. The precipitate was collected by centrifugation, washed with acetone three times, redissolved in water (*ca.* 60 ml), and then dialyzed against deionic water overnight. The dialyzate was concentrated to *ca.* 20 ml solution and freeze-dried to give dextran sulfate. Yield was 0.19 g. The sulfur content and number average molecular weight of the dextran sulfates are shown in Table III.

Anti-AIDS Virus Activity

Anti-AIDS virus activity of the compounds against human immunodeficiency virus HIV-1 was determined by the inhibition of virus-induced cytopathic effects on MT-4 cells and

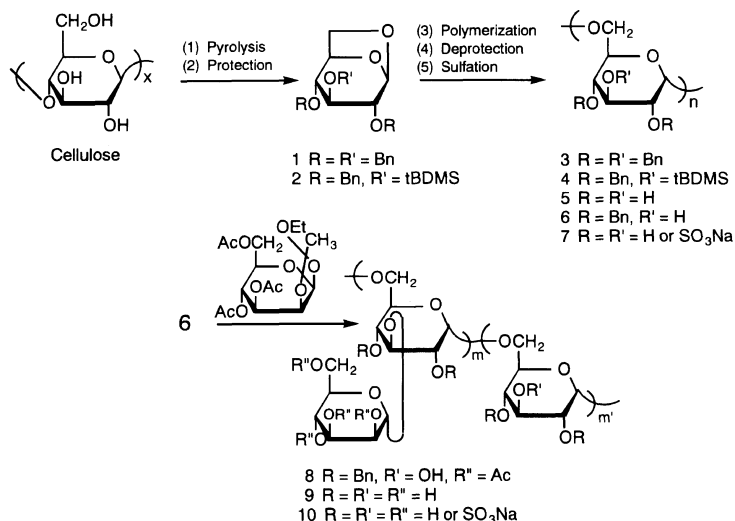
virus-specific antigen detected on MT-4 cell surface.¹⁵ In the 3- and 6-day cultures of MT-4 cells after HIV infection, the number of viable cells and percentage of virus antigen-positive cells were measured by the trypan blue dye exclusion method and indirect immunofluorescence (IF), respectively.

RESULTS

Synthesis of Free Dextran

A stereoregular dextran was prepared by cationic ring-opening polymerization of tri-*O*-benzylated 1,6-anhydro-glucopyranose LGTBE at -60°C with phosphorus penta-

fluoride as catalyst and subsequent debenzylation. The ring-opening polymerization of di-*O*-benzylated and 3-*O*-silylated monomer LGDBS was carried out by the same procedure as above to give the 3-*O*-silylated dextran derivative. Table I shows the results of the polymerizations. The polymers showed large and positive specific rotations, suggesting the polymers to have α -stereoregularity. The number average molecular weight was 13.2×10^4 for the tri-*O*-benzylated polymer and 3.7×10^4 for the 3-*O*-silylated polymer, respectively. It was found that the ^{13}C NMR spectra of the polymers showed high stereoregularity because of the appearance of a single absorption at



Scheme 1. Synthesis of dextran sulfates.

Table I. Ring-opening polymerization of 1,6-anhydro-2,3,4-tri-*O*-benzyl- β -D-glucopyranose (LGTBE) and 1,6-anhydro-2,4-di-*O*-benzyl-3-*O*-*t*-butylmethylsilyl- β -D-glucopyranose (LGDBS)

No. ^a	Monomer	Catalyst		Time		Yield	$[\alpha]_D^{25}$ ^b	\bar{M}_n ^c	Stereoregularity	
			mol%		h				%	deg
1	TBLGE	PF ₅	3	0.5	86.0	+115.9	13.2	(1→6)- α	100	
2	LGDBS	PF ₅	3	20	90.0	+124.1	3.7	(1→6)- α	100	

^a Monomer, 0.5 g; temp, -60°C ; solvent, CH₂Cl₂, 1 ml.

^b Measured in CHCl₃ ($c = 1$).

^c Determined by GPC.

^d Calculated by ^{13}C NMR spectrum.

97.0 ppm due to the C1 peak having α -stereoregularity (Figure 1B). Desilylation of poly(LGDBS) with tetrabutylammonium fluoride in tetrahydrofuran (THF) afforded 2,4-di-*O*-benzyl-(1 \rightarrow 6)- α -D-glucopyranan, which was reacted with 3,4,6-tri-*O*-acetyl- α -D-mannose-1,2-(methyl orthoacetate) in benzene to give a mannose-branched dextran derivative. In the anomeric region of the mannose-

branched polymer (Figure 1A), two peaks appeared at 97.0 and 96.0 ppm. A new broad peak at 96.0 ppm should be ascribed to the C1 absorption of the mannose-branches. By comparison with the integration value of C1 and C6 absorptions of the two units, respectively, the degree of mannose-branching was estimated to be 40%, which was the highest of all the branched synthetic polysaccharides.³

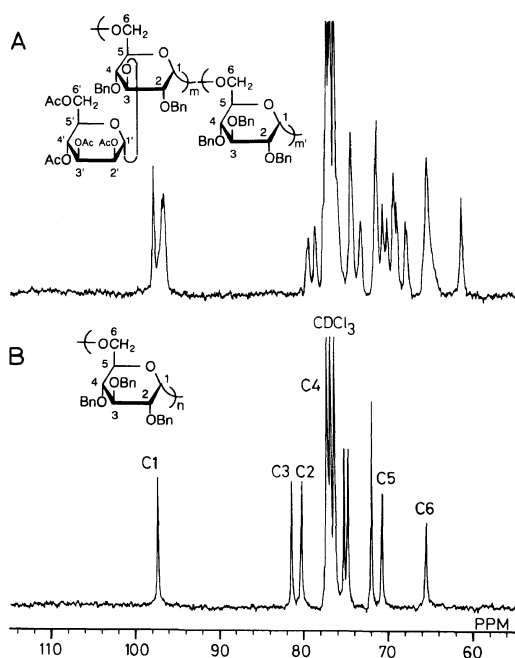


Figure 1. 67.8 MHz ^{13}C NMR spectra of (A) benzylated dextran with mannose-branches and (B) benzylated linear dextrans (CDCl_3 as solvent).

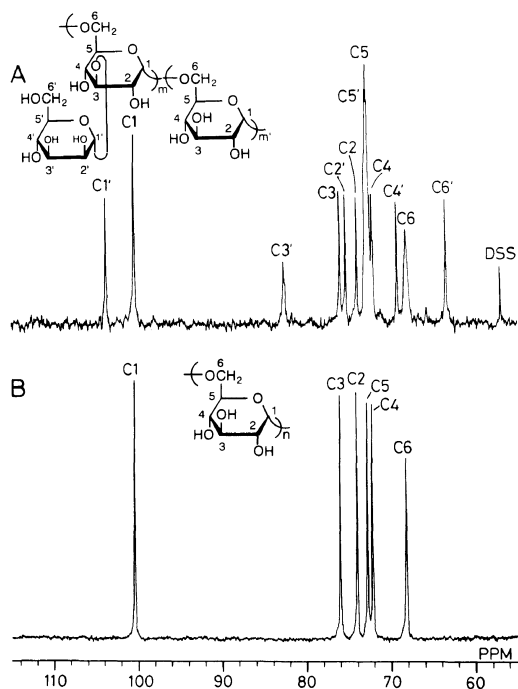


Figure 2. 67.8 MHz ^{13}C NMR spectra of (A) mannose-branched and (B) linear dextrans (D_2O as solvent at 37°C).

Table II. Deprotection of benzylated dextrans

No.	Benzylated dextran		Yield	Free dextran		
	$[\alpha]_D^{25}$ deg	\bar{M}_n $\times 10^4$		$[\alpha]_D^{25a}$ deg	\bar{M}_n^b $\times 10^4$	Stereoregularity ^c %
1 (Linear)	+115.9	13.2	89	+176.8	10.8	(1 \rightarrow 6)- α 100
2 (50% Mannose-branched)	+134.9	5.5	85	+155.6	1.8	(1 \rightarrow 6)- α 100

^a Measured in water ($c=1$).

^b Determined by GPC.

^c Calculated by ^{13}C NMR spectrum.

The deprotection of the linear and branched polymers with sodium in liquid ammonia gave free dextrans in 89 and 85% yields, respectively, as shown in Table II. The specific rotation of the free dextrans exhibited large positive values, $+176.8^\circ$ and $+155.6^\circ$, and the C1 absorption appeared at 100.6 ppm as a single peak in the ^{13}C NMR spectra (Figure 2), suggesting that the free dextrans had complete 1,6- α -stereoregularity. Assignments of the ^{13}C peaks (Figure 2B) have been established by Gagnaire and Vignon.²¹ In Figure 2A, the absorption of the mannose-branches appeared clearly at 104.0 ppm (C1'), 82.7 9 (C3'), 75.4 (C2'), 73.4–72.2 (C5'), 69.3 (C4'), and 63.4 ppm (C6').

Sulfation of Synthetic and Natural Dextrans

Table III summarizes the results of sulfation. The free synthetic dextrans were sulfated with piperidine *N*-sulfonic acid in dimethyl sulfoxide (DMSO) to give linear dextran sulfates and mannose-branched dextran sulfate, respectively. Natural branched dextrans, NRRL B-742 (No. 5 and 6) and B-512F (No. 7), were

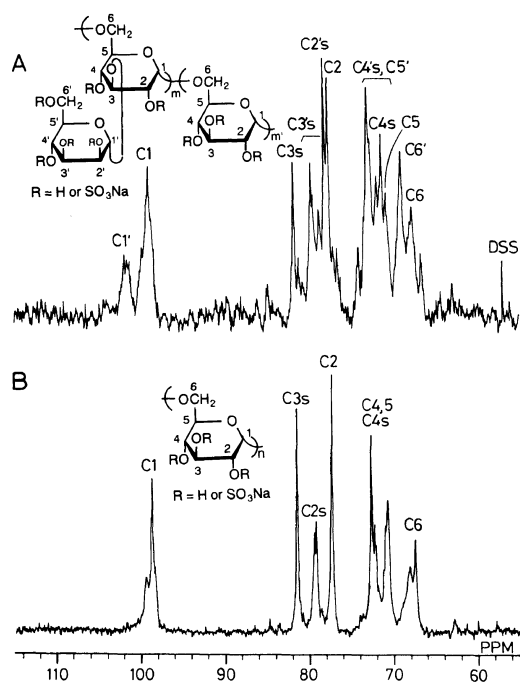


Figure 3. 67.8 MHz ^{13}C NMR spectra of (A) mannose-branched dextran sulfate (No. 4 in Table III) and (B) linear dextran sulfate (No. 3 in Table III) (D_2O as solvent at 37°C). s, sulfate substituted carbon.

Table III. Sulfation of synthetic and natural dextrans

Polymer No.	g	DMSO ml	PSA ^a g	Yield g	$[\alpha]_D^{25b}$ deg	\bar{M}_n^c $\times 10^4$	Elemental analysis			DS^d
							C %	H %	S %	
Synthetic										
1 SDS-1	0.10	15	0.51	0.17	+108.5	4.0	18.77	3.04	12.4	1.0
2 SDS-2	0.13	20	0.30	0.21	+127.3	4.2	23.06	3.85	12.4	1.2
3 SDS-3	0.10	15	0.51	0.19	+105.2	1.8	18.58	3.08	13.5	1.7
4 SDS-4 ^e	0.40	30	0.92	0.14	+ 80.6	2.4	18.00	3.01	15.2	1.9
Natural										
5 NDS-1 ^f	0.50	40	1.16	0.57	+146.2	7.6	28.71	4.33	9.0	0.7
6 NDS-2 ^f	0.50	40	1.45	0.80	+121.7	6.6	21.97	3.73	13.5	1.4
7 NDS-3 ^g	0.50	40	1.35	0.72	+114.7	10.7	23.27	4.11	14.0	1.4

^a Piperidine *N*-sulfonic acid.

^b Measured in H_2O ($c = 1$).

^c Determined by GPC.

^d Degree of sulfation.

^e Having 40% mannose-branches.

^f Branches: 1→4, 13%; 1→3, 18%.

^g Branches: 1→3, 4%

sulfated by the same procedure to give the sulfates. The degree of sulfation (DS) was 1.0–1.9 for synthetic dextran sulfates and 0.7–1.4 for sulfated natural dextrans by elemental analysis. The number average molecular weight (\bar{M}_n) was in the range of 1.8×10^4 to 10.7×10^4 and the specific rotation ($[\alpha]_D^{25}$) was higher than $+100^\circ$, except for mannose-branched dextran sulfate ($+80.6^\circ$).

Structure of Dextran Sulfates

The ^{13}C NMR spectra of synthetic linear and mannose-branched dextran sulfates are shown in Figure 3 (A, No.4; B, No. 3 in Table III). The C2 and C3 peaks of the linear dextran sulfate (Figure 3B) shifted downfield by 4 ppm in comparison with those of free dextran (Figure 2B), suggesting that the sulfate group was mainly introduced at C2 and C3 positions of the glucose unit. The mannose-branched dextran sulfate gave a complicated ^{13}C spectrum, as shown in Figure 3A. The absorptions of the mannose-branches, C3', C2', and C4' (seen in Figure 2A), almost disappeared and several new peaks appeared, as shown in Figure 3A. The C2 peak of the glucose unit in the main chain also shifted downfield by 4 ppm after sulfation, indicating that the sulfate group was largely put into the branching mannose units and C2 position of the main chain.

The degree of substitution was determined by the ^1H NMR spectrum of the linear dextran sulfate ($S=13.5\%$, $DS=1.7$) as shown in Figure 4. From the H–H COSY measurement, the H2 proton shifted downfield by 4.4 ppm after sulfation, suggesting that the hydroxyl group at C2 position was sulfated. The chemical shift of the sulfate-substituted proton generally shifted downfield due to the negative charge of the sulfate groups. The H3 proton appeared as two peaks around 4.6 and 4.1 ppm, indicating that lower field absorption should be due to the C3 sulfate-substituted unit and the higher field to the non-substituted one. It may be assumed that the sulfate group was

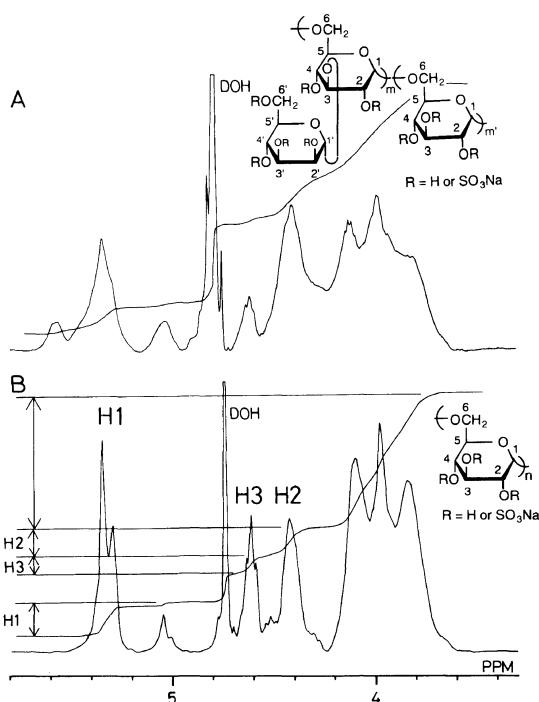


Figure 4. 400 MHz ^1H NMR spectra of (A) mannose-branched dextran sulfate (No. 4 in Table III) and (B) linear dextran sulfate (No. 3 in Table III) (D_2O as solvent at 37°C).

introduced into the H2 position in the ratio of approximately 100% and the H3 about 50%, which was calculated from the intensity of the respective H2 and H3 protons in Figure 4B (No. 3 in Table III). It was also speculated that in the linear dextran sulfate, the H4 position was substituted sulfate groups in the ratio of about 20%, taking into account $DS=1.7$. In Figure 4A (No. 4 in Table III), the ^1H spectrum of the mannose-branched dextran sulfate was too complicated to analyze the structure. One mannose unit was attached to the glucose unit at C3 position of the main chain in the ratio of one branch in roughly two glucose units. Since the branches should be lower sterical less hindered than the main chain glucose unit, the sulfate group might have been easily introduced into the branching mannose unit.

Anti-AIDS Virus Activity of Dextran Sulfates

Anti-AIDS virus activity of the dextran

Anti-AIDS Virus Activity of Dextran Sulfates

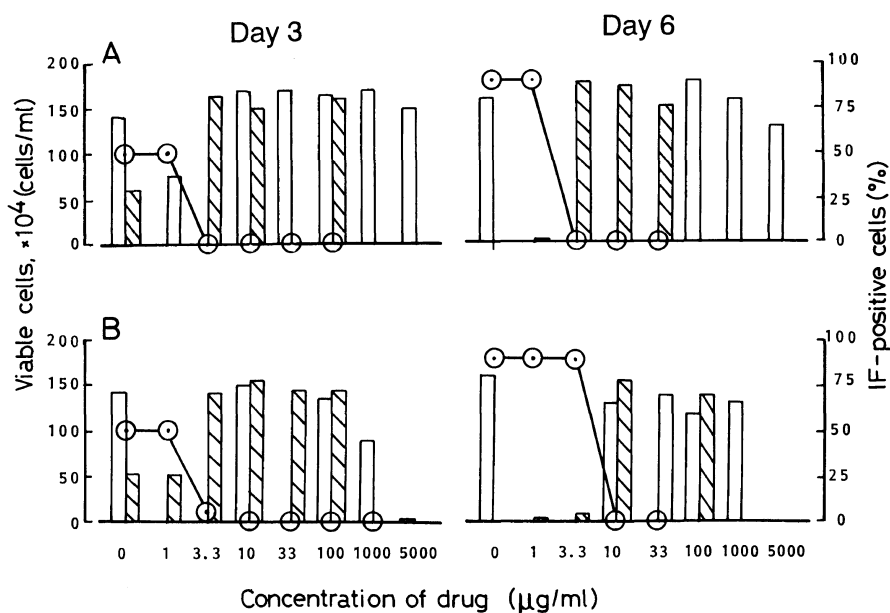


Figure 5. Inhibitory effects of dextran sulfates on cell growth and HIV-induced cytopathic effects. (A) Linear dextran sulfate ($S=13.5\%$, $\bar{M}_n=1.8 \times 10^4$) and (B) mannose-branches dextran sulfate ($S=15.2\%$, $\bar{M}_n=2.4 \times 10^4$). Key: open bars, MT-4 cells; slash bars, MT-4 cells and HIV-infected cells (0.2%); open circles, percentage of IF-positive cells determined by counting the IF-positive cells per approximately 500 cells after 3 and 6 days of infection.

Table IV. Anti-AIDS virus activity of dextran sulfates

Dextran sulfate No.	S content	DS^a	\bar{M}_n^b	EC_{100}^c	AA^d
	%		$\times 10^4$	$\mu\text{g ml}^{-1}$	unit mg^{-1}
Synthetic					
1 SDS-1	12.4	1.0	4.0	3.3	
2 SDS-2	12.4	1.2	4.2	3.3	49
3 SDS-3	13.5	1.7	1.8	3.3	59
4 SDS-4 ^e	15.2	1.9	2.4	10	85
Natural					
5 NDS-1 ^f	9.0	0.7	7.6	> 5000	
6 NDS-2 ^f	13.5	1.4	6.6	10	56
7 NDS-3 ^g	14.0	1.4	10.7	3.3	
Reference dextran sulfate					
NC-1032	18.4		0.7	100	20.6

^a Piperidine *N*-sulfonic acid.

^b Determined by GPC.

^c Minimum concentration for 100% inhibition of antigen expression in 6 day culture.

^d Anticoagulant activity.

^e Having 50% mannose-branches.

^f Branches: 1→4, 13%; 1→3, 18%.

^g Branches: 1→3, 4%.

sulfates *in vitro* was assayed by the prevention of HIV-induced cytopathic effects (CPE) and of virus-specific antigen expression using MT-4 cell line. The number of viable cells and ratio of antigen-positive cells were measured by the trypan blue dye exclusion method and indirect immunofluorescence (IF), respectively. The details appear in the previous paper.¹⁵ Since the number of viable cells cultivated in the presence of AIDS viruses was almost the same as that in the absence, synthetic linear and mannose-branched dextran sulfates at concentrations of 3.3 and 10 $\mu\text{g ml}^{-1}$ had potent inhibitory effects against AIDS-virus infection (Figure 5). No IF-positive cells were detected at these concentrations, indicating both dextran sulfates to block completely virus infection after 6 day cultivation *in vitro*. For synthetic and natural dextran sulfates prepared in this experiment, the minimum effective concentration (EC_{100}) for 100% inhibition on HIV infection in 6-day culture is shown in Table IV. Dextran sulfate with low DS of 0.7 (No. 5) showed no anti-AIDS virus activity. It was found that dextran sulfates with more than DS of 1.0 synthesized here had potent activity showing EC_{100} of 3.3 or 10 $\mu\text{g ml}^{-1}$. The anticoagulant activity (AA), an important biological activity intrinsic to sulfated polysaccharides, was measured using bovine plasma according to a modification of the United State Pharmacopoeia.²² Dextran sulfates synthesized in this experiment had higher anticoagulant activities, 49–85 unit mg^{-1} , than that (20.6 unit mg^{-1}) of a commercial dextran sulfate (Meito Sangyo NC-1032). In particular, synthetic dextran with mannose-branches showed the highest anticoagulant activity of 85 unit mg^{-1} . The highly anticoagulant activity may be ascribed to the flexible polymer backbone and high degree of sulfation of the branched dextran sulfate.

DISCUSSION

As shown in Table IV, dextran sulfates with

DS higher than 1.0 and $\bar{M}_n = 1.8 \times 10^4$ had 100% inhibitory effects against HIV infection (EC_{100}) at 3.3 or 10 $\mu\text{g ml}^{-1}$. The No. 5 dextran sulfate with low DS of 0.7 exhibited no anti-AIDS virus activity. Although there were 3 hydroxyl groups in a glucose residue of the main chain, the hydroxyl group at the 2 position may be first substituted by the sulfate group, which was revealed by NMR measurement. Thus, to obtain high anti-AIDS virus activity, the 1,6- α linked dextran sulfate may require the fully sulfate-substituted 2 position. However, dextran sulfate having a sulfate group at C3 or C4 position has not been obtained yet, and a detailed discussion on the sulfate substituents could not be given here. The relationship between the positions of sulfate-substituents and anti-AIDS virus activity is under investigation. For 1,3- β linked curdlan sulfate to acquire high activity, the substitution at 6 position is important.^{23, 24}

Cytotoxicity and anti-AIDS virus activity were measured at the same time using MT-4 cells as shown in Figure 5. Figure 5A shows no cytotoxicity above 5000 $\mu\text{g ml}^{-1}$ of linear dextran sulfate (No. 3 in Table 4). The cytotoxicity of the mannose-branched dextran sulfate was higher than that of the linear one, because former killed almost all cells during 3 days of culture (Figure 5B). Possibly due to too highly sulfate-substituted branches.

These dextran sulfates had high blood anticoagulant activity. The anticoagulant activity would be a side effect for anti-AIDS virus activity, because high anticoagulant dextran sulfate strongly interacted with the blood coagulation proteins in plasma and thus selective interaction to AIDS virus may not occur. Recently, Dextran sulfate has almost no effect as AIDS drug by an oral administration, although no significant side effects were observed.²⁵ Consequently, it was revealed that a possibility of dextran sulfate as an anti-AIDS virus drug *in vivo* may be low. However, it was reported recently that cyclical and low molecular weight polysaccharides such as

sulfated cyclodextrins²⁷ and low molecular weight dextran sulfates ($\bar{M}_w=1000$)¹⁸ show potent anti-AIDS virus activity in some assay systems. Since the anticoagulant activity of the low molecular weight samples was lower than that of higher ones, it may be possible to use the low molecular weight dextran sulfates as anti-AIDS virus drugs by an intravenous administration. It is relatively easy to obtain low molecular weight dextran sulfates by complete chemical synthesis.

Lentian²⁶ and curdlan sulfates^{23,24} hold promise as anti-AIDS virus drugs, because the curdlan sulfate has potent anti-AIDS virus activity but low cytotoxicity and low anti-coagulant activity. For curdlan sulfate, administration by an intravenous injection was started to check its toxicity toward humans.

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