Journal of Drug Targeting, August 2003 VOL. 11 (7), pp. 443–448

Taylor & Francis
healthsciences

Optimisation of the Degree of Sulfation of a Polymer Based Construct to Block the Entry of HIV-1 into Cells

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(Received 26 January 2004)

Blocking the entry of HIV-1 into CD4+ cells is an important new therapeutic target for the development of novel vaginal microbicides. In this study, sulfated derivatives of the linear polysaccharide dextrin were synthesised whose percentage sulphation increased incrementally from 7.4 to 48.3%. Their anti-HIV-1 activity in C8166 cells was first seen when percentage sulfation reached 33.2%, but it was only seen in peripheral blood mononuclear cells when it reached 36.3%. It did not increase further when sulfation reached 40.2%. Primary viruses with a V3 loop charge of greater than +5 were blocked by 80 μg/ml of dextrin 2 sulfate but primary viruses with a V3 loop charge of less than +3 required 1,600 μg/ml to block viral entry effectively. Our results identify the relative contribution of the percentage sulfation of a polymer based construct for optimising its anti-HIV-1 activity whilst minimising its toxicity. A better understanding of these structure-function relationships will inform the design and development of novel vaginal microbicides to effectively block the sexual transmission of all primary viral isolates of HIV-1.

Keywords: HIV-1; Sulfated polymers; Vaginal virucides; CD4+ cells

BACKGROUND

The entry of HIV-1 into lymphocytes and macrophages is initiated by binding of the viral envelope glycoprotein gp120 to cell surface CD4. As three gp120 molecules associate non-covalently with the gp41 trimer to form the viral envelope oligomer, multiple CD4 molecules are able to bind to the same envelope oligomer with high affinity. This interaction leads to the exposure of a binding site on gp120 for the chemokine co-receptors CCR5 and CXCR4 (Deng *et al.*, 1996; Feng *et al.*, 1996; Salzwedel and Berger, 2000; Kuhmann *et al.*, 2000). They are the physiologically relevant chemokine co-receptors used by HIV-1 *in vivo* with the former mediating the entry of HIV-1.R5 isolates during the sexual transmission of the virus. The latter mediates the entry of HIV-1.X4 isolates in patients with AIDS.

The sulfated amino terminal of these chemokine co-receptors has now been shown to bind to a sterically restricted surface on the virus that has a basic charge. The electrostatic potential of this region is strongly influenced by the overall charge rather than the precise structure of the third variable (V3) loop of gp120 with a small additional contribution from the V2 loop of gp120 (Cormier *et al.* 2000; Genoud *et al.* 1999; Kwong *et al.*, 2000). The overall charge of the V3 loop ranges from +2 to +5 for an HIV-1.R5 isolate and from +7 to +10 for an HIV-1.X4 isolate (Briggs *et al.*, 2000). Although previous enzyme immunoassay based studies have shown that sulfated polysaccharides bind to the V3 loop of gp120, this analysis of gp120-polyanion interactions has been limited to monomeric gp120 derived from T-cell line adapted isolates of HIV-1 that used CXCR4 as their chemokine co-receptor (Moulard *et al.*, 2000). Primary viral isolate derived soluble gp120 has not been studied because it has not been available. Recent crystallographic, mutational analysis and molecular modelling studies using the sulfated polysaccharides heparin and dextran sulfate now suggest that the chemokine co-receptor binding site on gp120 has a conserved and a variable component. The conserved component is a basic region within the V3 loop of gp 120 whose overall charge is the crucial determinant (Moulard *et al.*, 2000; Cormier and Dragic, 2002). This highly conserved co-receptor binding region within the CD4/gp120 complex is thought to be the binding site for sulfated polysaccharides.

In this study, we set out to define the role of the percentage sulfation and of the position of sulfated of

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ISSN 1061-186X print/ISSN 1029-2330 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1061186042000203574

a polymer based construct for optimising its anti-HIV-1 activity and minimising its toxicity.

METHODS

Complete details of the chemical synthesis and the characterisation of sulfated dextrins have been published elsewhere (Shaunak *et al.*, 1994). In brief, sulfation of dextrin to afford dextrin 2 sulfate was achieved using the reagent sulfur trioxide–trimethylamine complex. The degree of sulfonation obtained was directly related to the stoichiometry of this reagent in the reaction mixture. Dextrin 2 sulfate products were reliably characterised by elemental and spectroscopic analysis. Sulfation was confirmed by infra-red spectroscopy; the products showed characteristic sulfate bands at 1220 cm^{-1} and between 820 and 840 cm^{-1}. These two bands were not present in the unmodified dextrin. The degree of sulfation was determined from combustion analysis. A sulfate content of 37% relates to one sulfate per glucan unit. Sulfation was distinguished between the $2'$, $3'$ and $6'$ positions of the glucan unit using ^{13}C NMR. Dextrin 3 sulfate and dextrin 6 sulfate were compared with dextrin 2 sulfate to assign the signals with each dextrin derivative giving rise to a unique spectrum. For example, the major C-1 peak for dextrin 2 sulfate moves up-field to 99.8 ppm (from 100.3 ppm for dextrin) due to the 2-O-sulfation.

Virus Isolation and Characterisation

Low passage primary viral isolates were obtained from HIV-1+ patients by culturing 2 million peripheral blood mononuclear cells (PBMCs) with 2×10^6 phytohemagglutinin (PHA)-activated PBMCs from seronegative donors (Alfano *et al.*, 1999; Von Briesen *et al.*, 1999). The titre of infectious virus [50% tissue culture infectious dose ($TCID_{50}$)] used for the infectivity experiments was determined using a limiting dilution assay that was performed with PHA-activated PBMCs. No primary viral isolate was passaged more than twice for any of the experiments described. These virus isolation techniques have been shown to preserve the *in vivo* phenotype of primary viral isolates (Alfano *et al.*, 1999).

Each viral isolate was evaluated for its use of the chemokine receptors CCR1, CCR2b, CCR3, CXCR4 and CCR5 using U97-CD4-coreceptor transfected cells (Veryard *et al.*, 2000). The V2 and V3 loop charge distribution was determined by polymerase chain reaction (PCR) amplification of HIV-1 cDNA using a nested PCR. The primer pairs for the V2 loop were AATTAACCCCACTCTGTGTTAGTTTA and GCTCTCCCTGGTCCCCTCTGG (first round), and AATTAACCCCACTCTGTGTTAGTTTA and TGATACTACTGGCCTGATTCCA (second round). The purified PCR amplified products were sequenced using an Applied BioSystems dye terminator sequencing kit and an Applied Biosystems 373A Automated DNA Sequencer. Alignments were performed against the HIV-1 isolates JR-CSF, JR-FL and HXB-2 using the Clustal programme. At least 3 sequences from both DNA strands were obtained from each virus.

Determination of the 90% Inhibitory Concentration of Sulfated Dextrins against HIV

C8166 cells (Salahuddin *et al.*, 1983) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 250 IU/ml penicillin and 250 μg/ml streptomycin. They were incubated with doubling dilutions of each compound for 1 h at 37°C and then infected with a $TCID_{50}$ of $10^{1.2-2.4}$/ml of the HIV-1 isolates MN, IIIb and RF. Syncytial assays and measurement of p24 antigen (EIA, Coulter, Luton, UK) were used to determine the anti-HIV-1 activity of each compound.

PHA-activated PBMCs (2×10^6) were incubated for 1 h at 37°C with doubling dilutions of each test compound prior to the addition of the primary viral isolate. Twenty-four hours later, the cells were centrifuged, washed with PBS/2% FCS, centrifuged, and re-suspended in lymphocyte growth medium (RPMI 1640, 20 mM L-glutamine, 250 IU/ml penicillin, 250 μg/ml streptomycin and 15% FCS) containing 20 IU/ml IL-2. The test compound was then re-added at each concentration. Four days later, cell free culture supernatants were harvested for p24 antigen.

The anti-HIV-1 activity of sulfated dextrins was expressed as the concentration that blocked infection by 90% (IC_{90}); this is a more useful measure of the ability of a molecule to block viral entry than the IC_{50} value because studies with recombinant soluble CD4 have shown that its inhibitory effect on plasma viraemia correlates better with the *in vitro* IC_{90} than with the *in vitro* IC_{50} (Schacker *et al.*, 1994; Javan *et al.*, 1997). The lowest concentration of dextrin 2 sulfate (40.2% sulfation) that was used for the detailed virological studies was 80 μg/ml because our previous studies had shown that the IC_{90} for a small number of low passage primary viral isolates of HIV-1 was 69 ± 6 μg/ml (Javan *et al.*, 1997).

U87-CD4-CCR5 and U87-CD4-CXCR4 cells were also incubated with each compound for 1 h prior to the addition of the primary viral isolate at a $TCID_{50}$ of $10^{1.2-2.4}$/ml. Forty-eight hours later, the number of giant cells positive for p24 antigen by immunohistochemistry were counted (Shaunak *et al.*, 2001). An MTT assay was used to determine the cellular toxicity of each compound.

Anticoagulation Assays

Several assays are used in conjunction to determine whether a compound interferes with the ability of blood to coagulate. The prothrombin time (PT) utilises the extrinsic coagulation system. It is the time taken for citrated plasma to clot when a tissue factor (brain extract) and calcium are added. Therefore, in a coagulation screen, it is sensitive to deficiencies of Factors V, VII, X, prothrombin

and fibrinogen. The partial thromboplastin time (APTT) utilises the intrinsic coagulation system. It is the time taken for citrated plasma to clot when kaolin (which activates Factor XII), platelet substitute and calcium are added. Therefore, in a coagulation screen, it is sensitive to deficiencies of Factors V, VIII to XII, prothrombin and fibrinogen.

Conversion of fibrinogen to fibrin takes place when thrombin is generated by the activation of these coagulation pathways. This leads to the depletion of fibrinogen. The Factor Xa assay is a very sensitive measure of the presence of heparin-like activity.

All results are expressed as the mean ± SEM. All comparisons were made using a 2 tail Mann-Whitney *U*-test.

RESULTS

Anti-HIV-1 activity was first seen in C8166 cells when percentage sulfation in the 2′ position reached 33.2% with an IC_{90} of $31 \pm 4\,\mu g/ml$. Increasing the percentage sulfation to 39.4% led to a further improvement in the IC_{90} to $4 \pm 1\,\mu g/ml$ (Table I). In contrast, in PBMCs, anti-HIV-1 activity was first seen when the percentage sulfation reached 36.3% with an IC_{90} of $73 \pm 16\,\mu g/ml$, with little further change as the percentage sulfation was increased to 48.3%. Studies of the anticoagulant properties of these sulfated dextrins showed that there was a progressive increase in their anticoagulant-like activity as measured using the prothrombin time and the kaolin partial thromboplastin time. This shows that dextrin 2 sulfate interferes with the extrinsic and the intrinsic coagulation systems. The negative Factor Xa assay demonstrates that this is not due to heparin-like activity. In view of this, further studies were undertaken with a dextrin 2 sulfate construct whose percentage sulfation was 40.2% in order to maximise the anti-HIV-1 activity and minimise the anticoagulant activity.

Dextrin 2 sulfate blocked infection of U87-CD4-CXCR4 cells by all of the monotropic X4 primary viral isolates studied at a concentration of 80 μg/ml (Fig. 1). The percentage block achieved at this concentration (91 ± 2%) did not increase when the concentration of dextrin 2 sulfate was increased five-fold to 400 μg/ml (94 ± 1%; $p = 0.3$). A higher concentration of dextrin 2 sulfate was required to block infection by all of the monotropic R5 viruses studied when they were tested using U87-CD4-CCR5 cells. At a concentration of 80 μg/ml, a percentage block of only 59 ± 4% was achieved. Increasing the concentration of dextrin 2 sulfate five-fold to 400 μg/ml increased the percentage block to 71 ± 2% ($p = 0.02$). Only by increasing it a further five-fold to 1,600 μg/ml was it possible to achieve complete inhibition of viral entry.

Multitropic (i.e. R5X4 or R5X4R3) primary viral isolates were also studied using U87-CD4-CXCR4 cells and U87-CD4-CCR5 cells. Dextrin 2 sulfate blocked infection of U87-CD4-CXCR4 cells by multitropic viruses at a concentration of 80 μg/ml (96 ± 2%) with no increase

TABLE I Correlation of percentage sulfation with the anti-HIV-1 activity of sulfated dextrins in C8166 cells and in PHA-activated PBMCs

		IC_{90}		Prothrombin time (s)	APTT (s)	Fibrinogen (g/l)	Factor Xa activity (V/ml)
Compound	% Sulfation (±0.2%)	In C8166 cells (μg/ml)	In PBMCs (μg/ml)	control = 17	control = 40	control = 1.31	control = 0
Dextrin 2 sulfate	7.4	>125	>250				
	14.3	>125	>250				
	19.2	>125	>250	15	56	1.45	0
	23.8	>125	>250				
	25.8	>125	>250				
	27.3	>125	>250	15	51	1.33	0
	29.4	>125	>250				
	32.4	>125	>250				
	33.2	31 ± 4	>250				
	34.9	16 ± 3	>250	16	127	1.29	0
	35.3	12 ± 2.5	>250				
	36.3	8 ± 2	73 ± 16				
	37.0	8 ± 2	62 ± 12				
	39.4	4 ± 1	55 ± 11				
	40.2	4 ± 1	50 ± 12	14	160	0.89	0
	47.2	4 ± 1	48 ± 3	14	>180	0.57	0
	48.3	4 ± 1	42 ± 4				
Sulfated glucose	40.1	>125	>250	17	40	1.32	0

The primary position of the sulfate group on the glucan ring is the 2′ position. The anticoagulant effect of each sulfated polymer construct was determined at 100 μg/ml using the prothrombin time (PT in seconds), kaolin partial thromboplastin time (APTT in seconds), fibrinogen and Factor Xa activity in the Diagnostic Hematology laboratory of Hammersmith Hospital. The anti-HIV-1 activity is expressed as the concentration that blocked infection by 90% (IC_{90}).

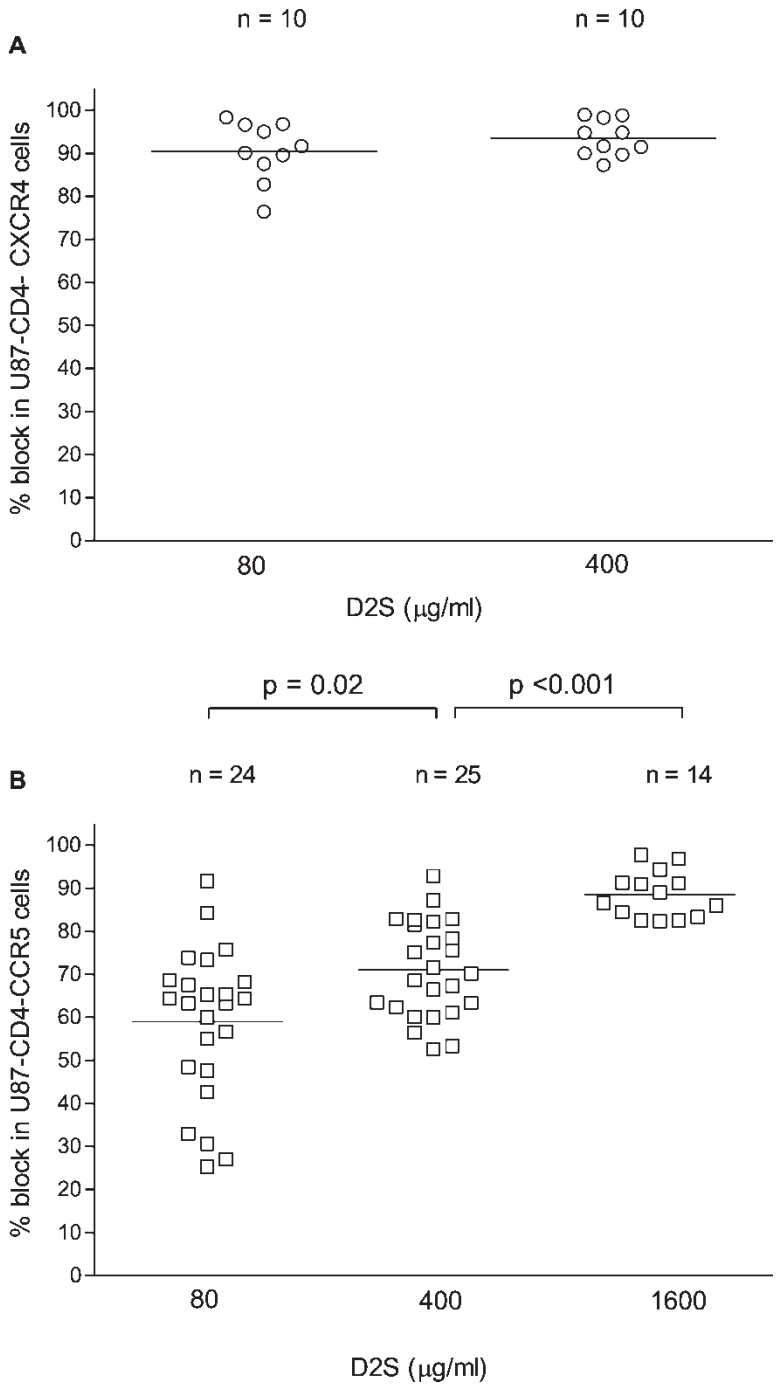

FIGURE 1 Determination of the ability of dextrin 2 sulfate (D2S) to block infection of U87-CD4-chemokine co-receptor transfected cells by low passage, *monotropic* primary viral isolates of HIV-1. (A) U87-CD4-CXCR4 cells and monotropic X4 primary viral isolates were tested at two concentrations of D2S. (B) U87-CD4-CCR5 cells and low passage monotropic R5 primary viral isolates were tested at 3 concentrations of D2S. n = number of viral isolates tested.

when the concentration was increased to 400 μg/ml ($96 \pm 2\%$; $p = 0.5$) (Fig. 2). When these viral isolates were used to infect U87-CD4-CCR5 cells, an $85 \pm 4\%$ block was achieved at a concentration of 80 μg/ml with no further increase in the percentage block at 400 μg/ml ($88 \pm 3\%$; $p = 0.9$).

As the overall charge distribution of the V2 and V3 loops of gp120 that was derived from laboratory adapted viruses has been shown to influence gp120-polyanion interactions in enzyme immunoassay based studies, we determined the V2, V3 and the V2/V3 loop charge distributions of 6 monotropic R5 viruses, 6 monotropic X4 viruses and 6 dualtropic R5X4 viruses by sequence analysis. These results were correlated with the IC_{90} values for dextrin 2 sulfate as determined using chemokine co-receptor transfected cells. Only the overall charge distribution of the V3 loop was found to correlate with the IC_{90} (Fig. 3). Viruses with a V3 loop charge

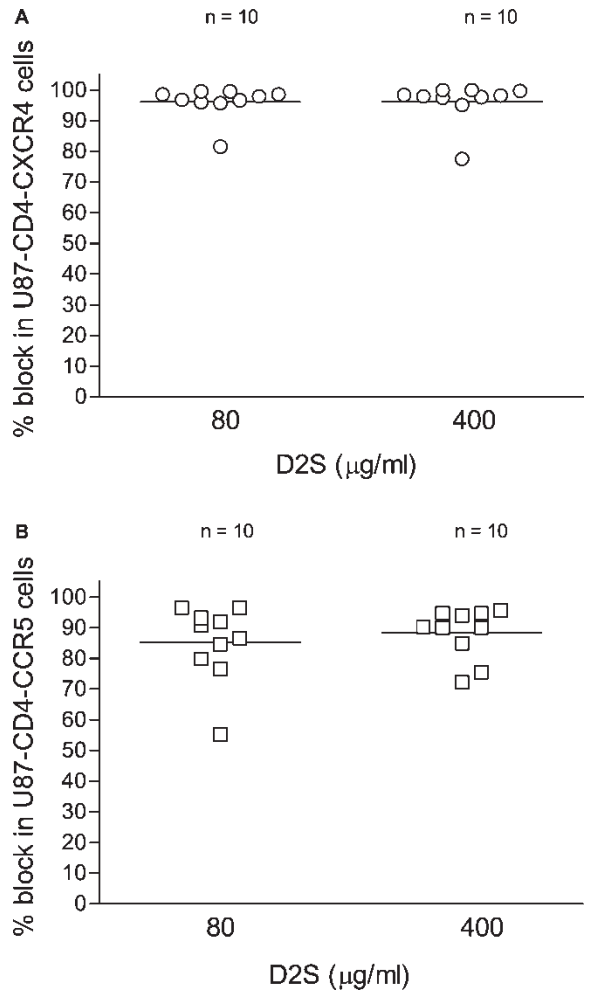

FIGURE 2 Determination of the ability of dextrin 2 sulfate (D2S) to block infection of U87-CD4-chemokine co-receptor transfected cells by low passage, *multitropic* primary viral isolates of HIV-1. (A) U87-CD4-CXCR4 cells and multitropic primary viral isolates were tested at two concentrations of D2S. (B) The same isolates were retested using U87-CD4-CCR5 cells using D2S at the same concentrations.

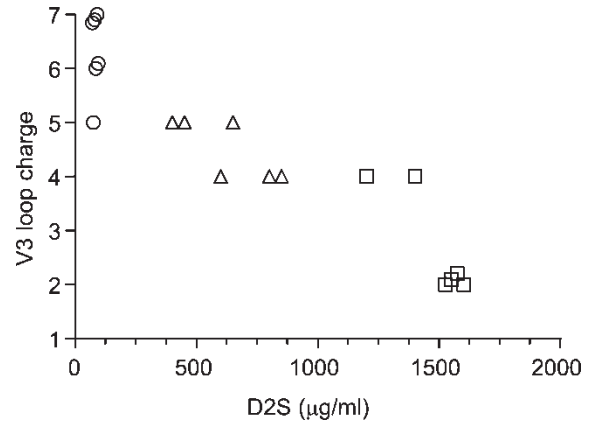

FIGURE 3 Correlation of the overall V3 loop charge of 18 low passage primary viral isolates of HIV-1 with their IC_{90} as determined using U87-CD4-co-receptor transfected cells. ○ = monotropic X4 viruses; □ = monotropic R5 viruses; Δ = multitropic R5X4 viruses.

distribution of more than +5 were blocked by dextrin 2 sulfate at 80 μg/ml, whilst viruses with a V3 loop charge distribution of less than +3 required 1,600 μg/ml to block viral entry.

CONCLUSIONS

Our results show that the anti-HIV-1 activity of sulfated linear polymer constructs against low passage primary viral isolates is primarily determined by the percentage sulfation of the molecule. It is possible to maximise the anti-HIV-1 activity of these molecules by 40% sulfation whilst minimising their anticoagulant activity.

Our identification of a close correlation between the degree of sulfation of the glucan ring and the anti-HIV-1 activity of these molecules in PBMCs fits with the increasing understanding of the importance of sulfate groups in mediating the interaction of chemokine receptors with the gp120-CD4 complex that forms transiently on the cell surface during infection. Farzan *et al.* have demonstrated that viral entry only requires the amino terminal domain of the CCR5 receptor, and that sulfation of the tyrosine residues in positions 3, 10, 14 and 15 of the amino terminal of CCR5 is the crucial determinant of the efficient binding of a soluble gp120 JR-FL/CD4 complex to CCR5 derived peptides (Farzan *et al.*, 1998; 1999; Cormier *et al.*, 2001). Inhibition of the binding of the gp120/CD4 complex to CCR5 is dependent upon the presence of the sulfate groups and is not simply due to non-specific electrostatic interactions (Jagodzinski *et al.*, 1996; Farzan *et al.*, 2002).

We have previously demonstrated that dextrin 2 sulfate binds to a cell surface protein on T-cell lines and PHA-activated PBMCs with a dissociation constant (K_d) of 82 ± 14 nM. This was greater than the K_d for dextran sulfate, fucoidan, pentosan polysulfate and aurin tricarboxlic acid (Shaunak *et al.*, 1994). The B_{max} of dextrin 2 sulfate binding was 7.2 ± 0.1 pmol/10^6 cells on PHA-activated PBMCs and only 0.18 ± 0.03 pmol/10^6 cells on quiescent PBMCs. On the basis of 1:1 stoichiometry of receptor/ligand binding, this approximated to 2×10^6 binding sites on activated cells and 5×10^4 binding sites on quiescent cells. Although the exact nature of the cell surface receptor to which dextrin 2 sulfate binds on activated CD4+ cells still remains unclear, we now propose that this large B_{max} for activated CD4+ cells is indicative of a high concentration of dextrin 2 sulfate on the surface of these cells. As activated CD4+ T cells make up most of the cells that are susceptible to infection by HIV-1, it is possible that the high local concentration of dextrin 2 sulfate on their surface enables it to compete effectively with the chemokine receptor for the binding site on the gp120-CD4 complex that becomes transiently exposed. When the overall V3 loop charge that is exposed is greater than +5, this competition is highly effective. When the exposed V3 loop charge falls to +3, this competition is poor.

In the first case, the most likely chemokine receptor being used by the virus is CXCR4. As the charge on the V3 loop falls, it becomes increasingly likely that the virus is using CCR5 only. Multitropic viruses often have a high overall positive charge exposed and therefore typically behave like monotropic X4 viruses. These conclusions probably reflect the need of dextrin 2 sulfate to bind sequentially to three or more positively charged molecules by polyvalent and co-operative carbohydrate-protein interactions rather than by simple monovalent binding for it to be an effective competitor.

As there is increasing structural evidence that sulfated polysaccharides block HIV-1 infection by binding to a conserved co-receptor binding site on the gp120/CD4 complex that is transiently exposed during the process of infection, several attempts are now underway to make antagonists for this site. Our study highlights the important determinants that should be taken into account when constructing such molecules. Maximising their binding to activated, HIV-1 susceptible cells and minimising their binding to quiescent cells can result in the synthesis, development and clinical evaluation of new molecules with a good therapeutic index. In the case of dextrin 2 sulfate, it has now progressed to Phase III clinical trials in sub-Saharan Africa as a vaginal virucide (Weber *et al.*, 2001).

Acknowledgements

We are grateful to Laura Barkley and Gary Adamson for technical help. Part of this study was funded by ML Laboratories.

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