Anti-HIV-1 Activity of Chemically Modified Heparins: Correlation between Binding to the V3 Loop of gp120 and Inhibition of Cellular HIV-1 Infection *in Vitro*†

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ABSTRACT: Chemically modified heparins were tested for their activities in (i) inhibiting HIV-1 replication *in vitro* and (ii) inhibiting the binding to recombinant HIV-1 gp120 of monoclonal antibodies specific for the V3 loop. The results reveal that *N*-desulfation reduces activity, although this is largely restored on *N*-acetylation. Selective *O*-desulfation also markedly reduces activity, whereas carboxyl reduction has little effect. Overall these results show that the anti-HIV-1 activity of heparin does not depend simply on negative density, and indicate instead that particular structures, notably *O*-sulfates, are involved. Our studies reveal that for chemically modified heparins and heparin-derived fragments there is a striking correlation between anti-HIV-1 activity *in vitro* and binding to the V3 loop of gp120 in solid phase ELISA. This strongly suggests that the heparin exerts its anti-HIV-1 activity by binding to the V3 loop of gp120.

Human immunodeficiency virus type 1 (HIV-1)[1] and HIV-2 infection of $CD4^{+ve}$ cells *in vitro* is inhibited by the sulfated polysaccharides dextran sulfate and heparin at concentrations on the order of 10 μg/mL (Ueno & Kuno, 1987; Ito et al., 1987; Mitsuya et al., 1988). Indeed, a number of polyanionic compounds, including aurintricarboxylic acid (Balzarini et al., 1986), pentosan polysulfate, stilbene (Baba et al., 1988a), and disulfonic acids (Cardin et al., 1991), are also active anti-HIV agents *in vitro*. Both dextran sulfate and heparin inhibit the absorption of HIV-1 virions to $CD4^{+ve}$ cells (Mitsuya et al., 1988; Baba et al., 1988b). Dextran sulfate also inhibits the formation of HIV-1-induced syncytia *in vitro* (Mitsuya et al., 1988); however, heparin is less active in this latter regard (Baba et al., 1990).

The molecular mechanisms underlying the anti-HIV-1 activity of dextran sulfate and heparin are the subject of some controversy. Lederman et al. (1989) reported that dextran sulfate binds to CD4 and that dextran sulfate and heparin inhibited the high-affinity binding of viral rgp120 to its cellular receptor, CD4. Through the use of anti-CD4 monoclonal antibodies (Mabs) specific for epitopes in the amino-terminal V1 domain of CD4, Parish et al. (1990) located a polyanion binding site in proximity to the CD4 binding site. This latter study showed, however, that heparin binds poorly, relative to dextran sulfate. In previous work (Harrop et al., 1994), we confirmed that dextran sulfate binds to rsCD4 and blocks rgp120 binding, but found that heparin is essentially inactive. Thus, although these two sulfated polysaccharides both exert anti-HIV activity, their modes of action appear to differ.

Another site at which sulfated polysaccharides may exert anti-HIV-1 activity is the third variable loop (V3 loop) of gp120. This sequence, delineated by a disulfide bridge between invariant cysteines-303 and -338, shows considerable sequence conservation, in particular in the hexapeptide GPGRAF occurring at the crown of the loop (LaRosa et al., 1990). Polyclonal antisera arising during the course of HIV-1 infection or on immunization with rgp120 recognize the V3 loop as the principal neutralizing domain [reviewed by Nara et al. (1991)]. Both polyclonal antisera and Mabs specific for the V3 loop neutralize the virus without interfering with gp120–CD4 interactions (Skinner et al., 1988). Moreover, the V3 loop sequence is a primary determinant of T-cell verus monocyte–macrophage tropism (Hwang et al., 1991). Thus, the V3 loop is thought to be involved in events in cellular infection which follow gp120–CD4 binding and are essential for plasma membrane–viral envelope fusion. One possible event is

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[1] Abbreviations: AT-III, antithrombin III; bFGF, basic fibroblast growth factor; CCID, cell culture infectious doses; COSY, correlation spectroscopy; DQFCOSY, double quantum filtered correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation; TOCSY, total correlation spectroscopy; HIV-1, human immunodeficiency virus type 1; Mab, monoclonal antibody; rsCD4, recombinant soluble CD4; rgp120, recombinant gp120.

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proteolytic cleavage by a cell-surface protease within or proximal to the GPGRAF sequence (Clements et al., 1991).

Callahan et al. (1991) have shown that a low molecular weight preparation of dextran sulfate (5K) with anti-HIV-1 activity *in vitro* inhibits the binding of V3 loop-specific Mabs to rgp120, although it is unable to block the binding of rgp120 to rsCD4. These authors also point out that the V3 loop shows the highest density of basic amino acids in the sequence of gp120, although a second cluster of basic amino acids occurs near the carboxy terminus (Hounsell et al., unpublished results). Batinic and Robey (1992) showed that [^{3}H]heparin binds to synthetic peptides modeled on the V3 loop of several HIV-1 isolates. In our previous studies of the anti-HIV-1 activity of heparin and dextran sulfate (Harrop et al., 1994), we found that heparin selectively inhibited the binding of V3 loop-specific Mabs to rgp120. Furthermore, we found that preincubation of rgp120 with excess concentrations of rsCD4 markedly increased the ability to inhibit the binding of these Mabs. This latter phenomenon is entirely consistent with the increased exposure of the V3 loop following CD4 binding, as indicated by its increased susceptibility to thrombin cleavage and by the increase in V3 loop Mabs to HIV-1 virions preincubated with recombinant soluble CD4 (rsCD4) (Sattentau & Moore, 1991).

In this paper, we exploit a series of chemically modified heparins in order to investigate the structural requirements for anti-HIV-1 activity. We also provide further evidence that the V3 loop of gp120 is the site of the anti-HIV-1 activity of heparin.

MATERIALS AND METHODS

Reagents. Two series of chemically modified heparins were synthesized from bovine lung heparin obtained from Sigma Chemical Co., St. Louis, MO. Series A consisted of (a) *N*-desulfated, (b) *N*-desulfated and subsequently *N*-acetylated, (c) totally (i.e., both *O*- and *N*-) desulfated, (d) totally desulfated and subsequently *N*-acetoacetylated (*N*-3-ketobutanoylated), and (e) decarboxylated heparins, and their synthesis has recently been fully described (Belford et al., 1992). The sulfate content by weight of these preparations was determined to be as follows: unmodified, 32.1%; *N*-desulfated, 23.4%; acetylated *N*-desulfated, 19.8%; totally desulfated, 0.2%; carboxyl reduced, 31.5%. Series B consisted of (a) *N*-desulfated, (b) *N*-desulfated and subsequently *N*-acetylated, (c) totally desulfated and subsequently re-*N*-sulfated, and (d) selectively 6-*O*- and *N*-desulfated, and subsequently re-*N*-sulfated. The synthesis of this series is described elsewhere (Mulloy et al., 1994). The sulfate content by weight of these preparations was calculated to be the following: unmodified, 35%; (a) 26%; (b) 26%; (c) 17%, and (d) 24%. Porcine intestinal mucosal heparin and its *N*-desulfated derivative were obtained from Sigma Chemical Co. None of the chemical modifications employed resulted in appreciable cleavage, except carboxyl reduction which resulted in a small amount of fragmentation detectable by gel electrophoresis (Hampson & Gallagher, 1984).

The low M_r (4–5K) heparin enoxaparin, derived by cleavage of pig mucosal heparin by benzoylation and alkaline elimination, was purchased from Rhone Poulenc Rorer, Dagenham, Essex, U.K. A low M_r heparin of similar size range, SPL-D, was donated by Scientific Protein Labs Inc., Waunakee, WI. The ultralow M_r heparin (2.5K) CY222 was donated by Sanofi Recherche, Gentilly, France. Nitrous acid cleavage of heparin was performed essentially by the method of Shively and Conrad (1976). Briefly, 400 mg of crude unbleached heparin (Sigma Chemical Co.) was dialyzed extensively against 1 M NaCl followed by two changes of distilled water and freeze-dried. After dissolving in 2 mL of 1.8 M acetic acid, pH 2, depolymerization was initiated by the addition of 28 mg of $NaNO_3$. After 30 min at room temperature, the reaction was stopped by the addition of ammonium sulfamate at a final concentration of 1 M. The pH of the mixture was raised to 5.5 prior to desalting on a 40-mL column of Biogel P4 eluted with 2% (w/v) ammonium bicarbonate, pH 7.5. The oligosaccharide peak was then freeze-dried and subject to gel filtration on a 200-mL Biogel P6 column. The sizes of the resulting oligosaccharide peaks were assessed by gel electrophoresis (Hampson & Gallagher, 1984), and where further resolution was required, the gel filtration chromatography was repeated. Individual peaks were freeze-dried and stored at −20 °C.

Recombinant baculovirus-expressed gp120 (rgp120) from the HTLV-IIIB isolate of HIV-1 produced by American Biotechnologies Inc., Cambridge, MA, was supplied by the UK Medical Research Council AIDS Directed Programme. Monoclonal antibody (Mab) 9284 specific for the V3 loop of HIV-1 gp120 was purchased from Du Pont UK Ltd., Stevenage, Herts, U.K. Mab D7324 which recognizes an epitope within 16 amino acids of the carboxy terminus of HIV-1 gp120 was obtained from Aalto Bioreagents, Dublin, Eire. Rabbit anti-mouse immunoglobulin conjugated to alkaline phosphatase and AMPAK ELISA amplification reagents were purchased from Dako Patts, High Wycombe, Bucks, U.K. Skimmed milk powder (Marvel) was obtained from Cadbury Ltd., Birmingham, U.K.

ELISA of V3 Loop Binding. The ability of heparin derivatives to block the binding of the V3 loop-specific Mab 9284 to immobilized rgp120 was measured essentially as previously described (Harrop et al., 1994). D7324, 10 μg/mL in 30 mM $NaHCO_3$, pH 8.5, was absorbed onto the surface of Nunc Maxisorb (GIBCO BRL, Paisley, Scotland) plate wells by incubation overnight at 4 °C. After being washed 3 times with 25 mM Tris-HCl buffer, pH 7.5, the wells were blocked with Tris buffer containing 2% (w/v) skimmed milk powder, which was used for all subsequent steps. Rgp120, 0.2 μg/mL, was bound by incubation for 2 h, and following washing, sulfated polysaccharide (50 μL/well) was added. After 15 min, Mab 9284, 5 ng/50 μL, was added, and following 2-h incubation, the plate was washed and developed with the AMPAK reagents. The concentration of 9284 was chosen to give 80% maximal binding under these conditions, and titration curves of 9284 binding were obtained with each set of assays performed to confirm this.

In Vitro Cell Infection Assay. The ability of heparin derivatives to inhibit the infection of CD4^{+ve} C8166 human T lymphoblastoid cells with HIV-1_{IIIB} was examined as described previously (Mahmood et al., 1993). Briefly, aliquots of 4×10^4 cells per microtiter plate well were mixed with 5-fold dilutions of compound 2–3 min prior to addition of 10 $CCID_{50}$ units of virus. Formation of syncytia was examined from 2 days postinfection, supernatant was collected at 5–6 days postinfection, and gp120 antigen production was measured by ELISA, using the lectin GNA (from *Galanthus nivalis*) to capture gp120 and human anti-HIV serum for detection (Mahmood & Hay, 1992). Cell viability was determined by the MTT–formazan method (Pauwels et al., 1988).

NMR Spectroscopy. Samples evaporated 3 times from D_2O were dissolved in 500 μL of D_2O (Sigma Chemical Co.) containing 1 μL of acetone, and transferred to 5-mm NMR

tubes (Wilmad Glass Co. Ltd., Buena, NJ). One-dimensional spectra were obtained at 315 and 295 K using a Bruker AM500 500-MHz NMR spectrometer. Two-dimensional homonuclear phase sensitive double quantum filtered correlated spectroscopy (DQFCOSY) was carried out at 295 K using a Bruker AM400 400-MHz NMR spectrometer. Total correlation spectroscopy (TOCSY) and heteronuclear multiple quantum correlation (HMQC) experiments were carried out at 295 K on a Varian Unity 600-MHz spectrometer. TOCSY experiments (Braunschweiler & Ernst, 1993) were acquired using an MLEV-17 (Bax & Davies, 1985) mixing sequence of duration 100 ms and field strength approximately 7 kHz. HMQC spectra were acquired with GARP decoupling during acquisition (Shaka et al., 1985) and were optimized for a coupling constant of 165 Hz. Suppression of the water signal was achieved by selective presaturation in all of the NMR experiments. The chemical shift scale is derived from an internal reference signal of acetone which is set to resonate at 2.225 ppm at 295 K.

RESULTS

V3 Loop-Blocking Activities of Chemically Modified Heparins. We have previously shown by a solid phase ELISA that heparin inhibits the binding of three different V3 loop-specific Mabs to rgp120 of HIV-1, with EC_{50} values on the order of 10 μg/mL (Harrop et al., 1994). In order to examine the structural requirements for this interaction, chemically modified heparins were examined in this assay. As shown in Figure 1A, the activities of the modified variants of bovine lung heparin (series A) vary greatly depending on the particular chemical modification performed. The selective removal of the sulfate groups from the amino groups of glucosamines reduces activity, giving an EC_{50} of 100 μg/mL compared to an EC_{50} of 20 μg/mL for the unmodified preparation. *N*-Acetylation of this de-*N*-sulfated heparin preparation largely restores activity. However, total *N*- and *O*-desulfation removes all activity (data not shown), and furthermore, the activity is not improved by subsequent *N*-acetoacetylation (Figure 1A). By contrast, reduction of the carboxyl groups on uronic acid residues only marginally affects activity.

A second series, series B, of chemically modified bovine lung heparins was also examined by this method. For series B, *N*-desulfation completely abolishes activity (Figure 1B), compared to the partial loss of activity seen with this same modification for series A (Figure 1A). However, as seen with series A, *N*-acetylation of the series B *N*-desulfated material restores activity (Figure 1B). The effects of *O*-desulfation of bovine lung heparin, series B, are shown in Figure 1B. Total *O*-desulfation essentially inactivates this material whereas selective removal of 6-*O*-sulfates results in only partial reduction of activity.

Pig mucosal heparin is as active as the unmodified bovine lung preparation (Figure 1C). However, in this case, selective *N*-desulfation also completely abolishes activity, as found for series B (Figure 1B). A commercial low molecular weight preparation of pig mucosal heparin, enoxaparin (M_r 4–6K), is somewhat less active than the full-length heparin chains (Figure 1C).

In Vitro Anti-HIV-1 Activity of Chemically Modified Heparins. The chemically modified heparins and their parent preparations were tested for their ability to inhibit HIV-1 cell replication *in vitro*. The antiviral activity was measured by three criteria: inhibition of syncytial formation, inhibition of gp120 expression, and inhibition of the cytopathic effect of the virus. As may be seen in Table 1, for all the preparations

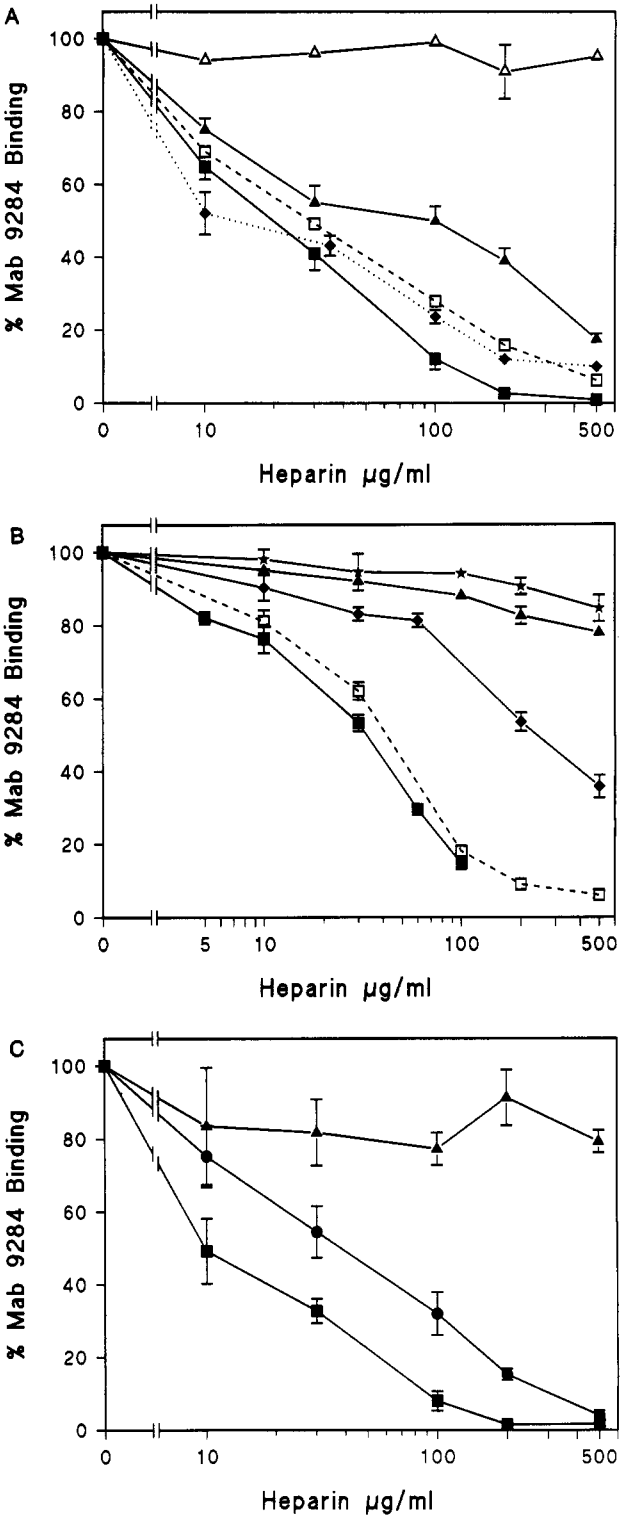

FIGURE 1: Inhibition of the binding of V3 loop-specific anti-gp120 Mab to immobilized rgp120 by heparin derivatives. (A) Bovine lung heparin derivatives, series A: unmodified (■), *N*-desulfated (▲), acetylated and *N*-desulfated (□, dashed line), acetoacetylated and totally desulfated (△), and carboxyl reduced (♦, dotted line). (B) Bovine lung heparin derivatives, series B: unmodified (■), *N*-desulfated (▲), acetylated and *N*-desulfated (□, dashed line), selectively 6-*O*-desulfated (♦), and totally *O*-desulfated (★). (C) Porcine intestinal mucosal derivatives, series C: unmodified (■), *N*-desulfated (▲), and enoxaparin (●). Data are expressed as a percentage of A_{492nm} obtained in the absence of heparin. For (A), the results shown are the means of values from four independent experiments, each with triplicate determinations. For (B) and (C), the data shown are of a single representative experiment. Error bars indicate the SEM where this is larger than the symbol size for 12 replicates for (A) and 3 or 4 replicates for (B) and (C).

Table 1: Anti-HIV-1 Activity *in Vitro* of Chemically Modified Heparins[a]

sample	IC_{50} syncytia formation[b] (μg/mL)	IC_{50} gp120 expression[c] (μg/mL)	EC_{50} growth of infected cells[d] (μg/mL)
series A: bovine lung heparin			
unmodified	0.32	0.32	0.32
N-desulfated	40	40	40
acetylated, *N*-desulfated	1.6	1.6	1.6
totally desulfated	200	200	200
acetoacetylated, totally desulfated	100	500	100–500
carboxyl reduced	8	1.6	1.6
series B: bovine lung heparin			
unmodified	0.32–1.6	0.32–1.6	0.32–1.6
6-*O*-desulfated	200	200	200
totally *O*-desulfated	>1000	>1000	>1000
N-desulfated	200	200	200
acetylated, *N*-desulfated	1.6	0.32–1.6	0.32–1.6
series C: pig intestinal mucosal heparin			
unmodified	0.064	0.064–0.32	0.064
N-desulfated	>1000	>1000	>1000

[a] C8166 cells were added to a 5-fold dilution series of each compound 2–3 min prior to virus infection. Anti-HIV-1 activity was assessed by [b]inhibition of syncytia formation, [c]concentration inhibition of gp120 antigen expression, and [d]inhibition of virus-induced cytotoxicity of infected cells. Single concentration values are those closest to giving 50% inhibition, but where 50% inhibition lies midway between two concentrations, both are given. No heparin-induced toxicity of uninfected cells was observed at concentrations up to 1 mg/mL. All results are representative of at least duplicate independent determinations.

tested, these three activities gave similar or identical end points. The unmodified bovine lung and pig mucosal heparins are active at around 1 μg/mL or less (Table 1). Selective *N*-desulfation of the bovine lung preparations reduces the estimated EC_{50} by 100-fold or greater (Table 1, series A and B). As with the V3 loop binding assay, subsequent *N*-acetylation of the series A material almost completely restores activity. Totally desulfated bovine lung heparin is some 500-fold less active than the unmodified material, and subsequent acetoacetylation does not restore activity. Carboxyl reduction lowers activity some 4-fold. *O*-Desulfated (i.e., totally desulfated, re-*N*-sulfated) bovine lung heparin is completely inactive (series B). However, the selectively 6-*O*-desulfated preparation retains a low level of activity. With pig mucosal heparin (series C), selective *N*-desulfation completely abolishes activity, in contrast to the bovine lung heparin (series A and B) where this modification results in some retention of activity.

Correlation of V3 Loop Binding with Inhibition of Cell Infectivity in Vitro. Comparison of the anti-HIV-1 activities (Table 1) and V3 loop binding abilities (Figure 1) of these chemically modified heparin preparations reveals a complete correlation (Figure 2). Indeed, as may be seen in Figure 2, this correlation also holds for a wide range of heparin derivatives, including low molecular weight heparins and heparin fragments. All those heparin preparations and derivatives tested which were effective in blocking V3 loop Mab binding also had high anti-HIV-1 activity *in vitro*, and *vice versa*. Those preparations with low V3 loop-blocking activity also had low anti-HIV-1 activity. The correlation is nonlinear, but this is probably because the V3 loop binding ELISA is unable to discriminate between samples of high activity.

NMR Analysis of Chemically Modified Heparins. Since the specific chemical modifications of heparin result in marked changes of activity in both of the above assays, we sought to establish the nature of the derivatives produced. Of special interest was a structural comparison between the *N*-desulfated bovine lung heparins, which have intermediate activity in both assay systems, and the *N*-desulfated porcine intestinal mucosal preparation, which is virtually inactive in the V3 blocking ELISA (Figure 1B), and inactive in the cellular infectivity assay (Table 1).

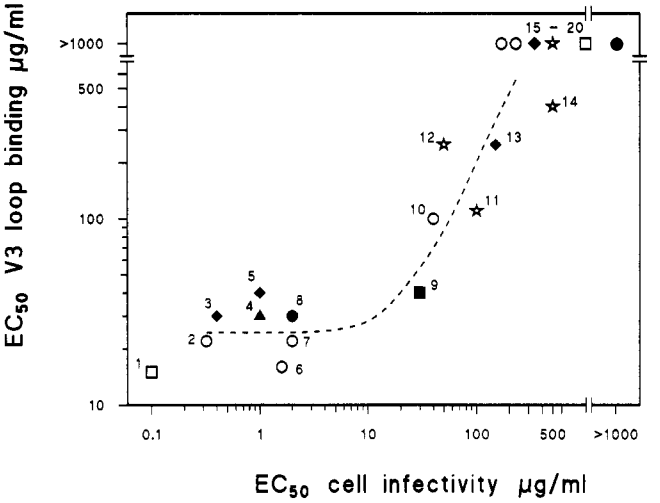

FIGURE 2: Correlation between the EC_{50} for the HIV-1 cell infection assay and the EC_{50} for the V3 loop ELISA for modified and size-fractionated heparin samples. The identities of the individual preparations are as follows, samples 15–20 being numbered left to right. Bovine lung heparin and its derivatives, series A (○): 2, unmodified; 6, carboxyl-reduced; 7, acetylated and *N*-desulfated; 10, *N*-desulfated; 15, totally desulfated; 16, acetoacetylated and totally desulfated. Bovine lung heparin, series B (♦): 3, unmodified; 5, acetylated and *N*-desulfated; 13, 6-*O*-desulfated; 17, *N*-desulfated; 20, totally *O*-desulfated. Porcine intestinal mucosal heparin, series C (□): 1, unmodified; 19, *N*-desulfated. Heparin oligosaccharides (☆): 11, dodecamer; 12, decamer; 14, octamer; 18, hexamer and the low molecular weight heparins enoxaparin (●, 8), SPL-D (▲, 4), and CY222 (■, 9). All values are the mean of the EC_{50} values derived from at least two independent experiments. The dashed line is drawn freehand.

Chemical shift data obtained for series A and C preparations by two-dimensional DQFCOSY, TOCSY and HMQC (data now shown) were assigned by comparison with the literature (Perlin et al., 1971; Mulloy & Johnson, 1987; Liu & Perlin, 1992; Horne & Gettins, 1992). From these data, it may be deduced that the major product obtained with each derivatization performed is as expected. Comparison of the unmodified bovine lung (series A) and porcine intestinal mucosal (series C) heparins (Figure 3a,b) shows that there are several structural differences. Importantly, the ratio of protons from the acetamido CH_3 signal of glucosamine (δ 2.05) to the H2 of glucosamine sulfate (δ 3.27) is 0.15 in

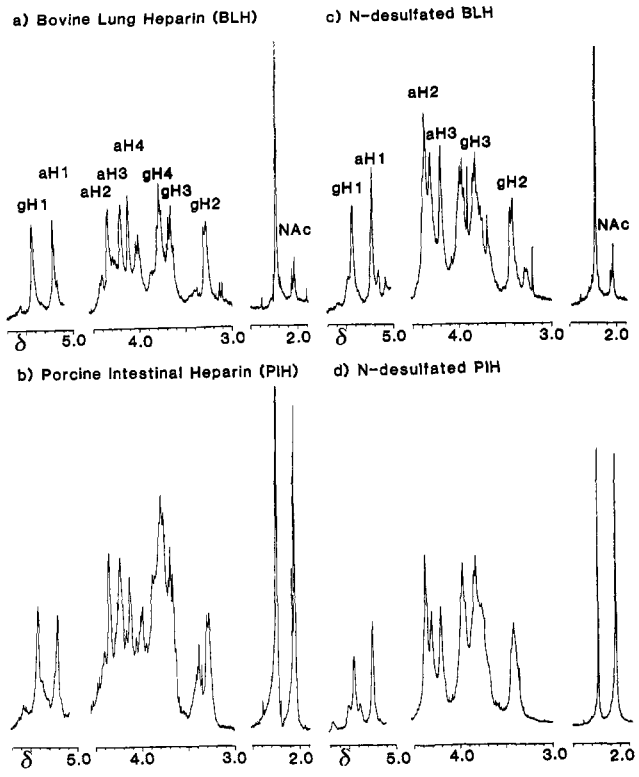

FIGURE 3: ^{1}H NMR spectra of heparins and *N*-desulfated heparins at 500 MHz. (a) and (c), bovine lung heparin, series A; (b) and (d), porcine intestinal mucosal heparin, series C; g, glucosamine; a, iduronate.

series A and 0.81 in series C, indicating that the porcine heparin is considerably enriched in *N*-acetyl groups compared to the bovine heparin sample.

N-Desulfation of the two samples results in spectra which more closely resemble each other (Figure 3c,d) than is the case for the unmodified preparations (Figure 3a,b), although the marked difference in *N*-acetylation is conserved. On *N*-desulfation of series A (Figure 3c), approximately 20% of the signal for H2 of glucosamine sulfate remains (ratio of signals at δ 3.27 and 3.30), whereas in series C (Figure 3d) only glucosamine and *N*-acetylglucosamine are present (absence of signals at δ 3.27, presence of signals at δ 3.30 and 2.05), indicating complete *N*-desulfation.

On *N*-acetylation of the series A *N*-desulfated preparation (data not shown), the chemical shifts were assigned for H1–H3 of glucosamine at δ 5.16, 3.89, and 3.75, respectively, and of iduronate at δ 5.19, 4.30, and 4.10. Some residual nonacetylated NH_2 remains, which may explain why restoration of activity is incomplete. In the totally desulfated material, the chemical shift for H1 of glucosamine was δ 5.5 which shifted upfield to δ 5.19 after *N*-acetoacetylation. Reduction of the carboxyl group of the native heparin (series A) gave significant downfield shifts for the H2 and H3 of iduronate compared to the native heparin.

The chemical shift data for the series B heparins (native, *N*-acetylated with and without *O*-sulfate, *O*-desulfated, and specifically 6-*O*-desulfated) have been reported elsewhere (Mulloy et al., 1994). Comparison of the ^{1}H NMR spectra of these modified heparins at 500 MHz with those of the corresponding monosaccharides (Mulloy et al., 1994), and with the literature chemical shifts for bovine lung heparin (Gatti et al., 1979), indicates that the data are entirely consistent with the chemical modifications applied. In each case, except the 6-*O*-desulfated heparin, the described structure accounted for 90% or more of the integrated intensity in the NMR spectrum. The 6-*O*-desulfated material contained about 30% totally desulfated material, 6-*O*-desulfation was essentially complete, and in addition some 2-*O*-sulfate had been lost from iduronate residues.

DISCUSSION

A number of polyanionic compounds inhibit HIV-1 replication *in vitro*. Of these, a particularly well-studied example is dextran sulfate which has been shown to block HIV-1 virion binding to CD4^{+ve} cells (Mitsuya et al., 1988). Dextran sulfate was also found to inhibit the binding of rCD4 to rgp120 in one study (Lederman et al., 1989) but not in another (Callahan et al., 1991). Like several other sulfated polysaccharides, dextran sulfate is able to occupy a polyanion binding site on CD4 which through the use of monoclonal antibodies has been mapped close to the gp120 binding site (Parish et al., 1990).

However, dextran sulfate and other anti-HIV-1 polyanions, including aurintricarboxylic acid and heparin, have also been shown to bind to the V3 loop of gp120 expressed on the surface of chronically HIV-1 infected cells (Schols et al., 1990). Previous work has shown that heparin does not inhibit the binding of gp120 to CD4, but instead binds to the V3 loop of gp120 (Harrop et al., 1994). The selective binding of heparin to this particular region of gp120 has also been demonstrated by others (Batinic & Robey, 1992). In the current study, we reveal a strong correlation between V3 loop binding activity and anti-HIV-1 activity *in vitro*. Although we cannot formally exclude the possibility that this striking correlation is coincidental, this finding strongly supports the view that heparin exerts its anti-HIV-1 activity primarily through binding to the V3 loop.

The V3 loop of gp120 is a cysteine disulfide bridge-enclosed amino acid sequence of some 35 residues, depending on the viral isolate. Mutations within the V3 loop have major effects on viral infectivity, viral replication kinetics, syncytium formation, and viral tropism (Hwang et al., 1991; Chesebro et al., 1992; Fouchier et al., 1992; Grimaila et al., 1992). Therefore, although it is not directly involved in the initial rgp120–CD4 binding interaction, the V3 loop appears to have an essential role in subsequent virion–plasma membrane fusion events [reviewed by Nara et al., 1991)]. V3 loop-specific monoclonal antibodies show viral type-specific neutralizing activity. Presumably their occupancy of the V3 loop blocks its functional activity. We therefore propose that heparin by binding to the same structure on gp120 exerts its anti-HIV-1 activity in a similar way.

The V3 loop has the highest positive charge density of any region of gp120, containing five to nine basic residues, depending on the viral isolate (Callahan et al., 1991). Moreover, as seen in Figure 4, analysis of the V3 sequences of 245 viral isolates (LaRosa et al., 1990) shows that the distribution of the basic residues, arginine and lysine, in the V3 loop follows a conserved pattern. Positions 3, 11, 12, 22, and 36 show a very strong tendency (95% or greater) to be occupied by a basic residue. By contrast, of the 38 positions lying between the delineating cysteines, 25 show a basic residue occupancy in 2.5% or less of the sequences. Very few positions show basic residue frequencies of around 10%, a value expected from random selection of the 2 basic residues from the possible 20 amino acids. Thus, although the V3 loop is considered to be a hypervariable portion of the HIV-1 gp120 sequence, the distribution pattern of basic charges within it is seen to be essentially conserved across a high proportion of isolates,

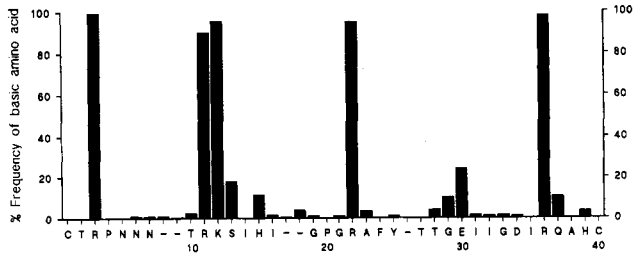

FIGURE 4: Conservation of the distribution of basic residues in the V3 loop of HIV-1 gp120. The V3 loop consensus sequence is displayed along the abscissa with the amino acids represented by the one-letter code. The ordinate shows the frequency with which the basic residues, lysine and arginine, occupy each position. The consensus sequence, sequence data for 245 HIV-1 isolates, and breaks to accommodate alignment are all taken from LaRosa et al. (1990).

strongly implying that they subserve an indispensable functional role.

Our data demonstrate that specific chemical modifications of heparin markedly affect its anti-HIV-1 activity; however, in each case, the outcome varies greatly depending on the modification employed. Carboxyl reduction, for example, fails to cause a marked reduction in V3 loop binding and anti-HIV-1 activity *in vitro*. This suggests that the carboxyl groups of the uronic acids have little importance in the interaction of heparin with the V3 loop. In contrast, totally desulfated heparin has very low activity, indicating that at least some of the sulfate moieties are essential. *N*-Desulfation of bovine lung heparin gives preparations with low activities. However, with porcine mucosal heparin, *N*-desulfation completely rather than partially abolishes activity. Comparison of the NMR spectra of this preparation with the bovine lung heparin (series A) reveals structural differences. Among these, the bovine preparation retains a residual amount of *N*-sulfation. Our studies may suggest an important function for these few *N*-sulfates, but we cannot preclude the possibility that the other structural differences may be responsible for the variation in activity of the *N*-desulfated preparations. Interestingly, *N*-acetylation of the *N*-desulfated bovine lung heparin essentially restores full activity. Thus, it appears that *N*-sulfates may be replaced by *N*-acetyl groups with retention of activity. Possibly, the basic charge of the free amino groups of glucosamine residues prevents interaction with gp120 unless masked with either sulfate or acetyl moieties. Alternatively, bulky derivatization of these amino groups may be important in maintaining an appropriate secondary structure in the polysaccharide, although the actual nature of the *N*-derivative, negatively charged (sulfate) or uncharged (acetyl), is unimportant.

Complete *O*-desulfation of porcine heparin abolishes activity in both the V3 loop ELISA and the *in vitro* infectivity assay. Even a selective *O*-desulfation, removing all 6-*O*-sulfates and a low proportion of 2-*O*-sulfates, results in a substantial reduction of activity. These observations indicate an essential role for *O*-sulfates.

In the case of the binding of heparin to antithrombin III (ATIII) and basic fibroblast growth factor (bFGF), specific oligosaccharide sequences with unusual *O*-sulfation structures are required for high-affinity binding. ATIII binding involves a specific pentasaccharide sequence centered on a 3-*O*-sulfated *N*-sulfoglucosaminyl residue (Lindahl et al., 1984), whereas bFGF binds to sequences in which the uronate residues are 2-*O*-sulfated iduronates (Turnbull et al., 1992). It is therefore pertinent to enquire whether the anti-HIV-1 activity of heparin and its ability to bind to the V3 loop of gp120 are interactions which require specific oligosaccharide structures. By comparing the activities of chemically modified heparins obtained in the present study with those reported for other biological assays, it is possible to go some way toward answering this question.

In a study of interactions with acidic FGF (aFGF), it was found that the acetylated *N*-desulfated heparin (series A) had considerably reduced activity in both aFGF binding and supporting aFGF-induced cell proliferation (Belford et al., 1992). By contrast, the same preparation retained high activities in the assays reported here. Furthermore, the *N*-desulfated, acetylated *N*-desulfated, and carboxyl-reduced preparations had enhanced effects compared to the unmodified heparin on the aFGF-induced survival *in vitro* of ciliary neurons (Belford et al., 1992). This increased activity contrasts with the virtually abolished, reduced, or essentially unaltered activities determined respectively for the same preparations here. Moreover, in contrast to the modest effects on the activities studied here, carboxyl reduction of heparin largely abolishes its inhibitory activity on B16 melanoma heparanase (Irimura et al., 1986) and completely abolishes its inhibition of thrombin via heparin cofactor II (Sie et al., 1988). This latter observation is particularly significant since heparin cofactor II activity, unlike ATIII activity and bFGF binding, does not appear to involve a specific heparin oligosaccharide sequence but instead is a nonspecific polyanion interaction depending on charge density (Petitou et al., 1988). Thus, taken overall, chemically modified heparins show quite different spectra of activities in the various biological assays in which they have been tested. In particular, those modified heparins with high activities reported here, namely, carboxyl-reduced and acetylated *N*-desulfated, do not show universally high activities in other assays. This suggests that V3 loop binding and inhibition of cellular infection by HIV-1 *in vitro* involve specific structures which are at most only marginally perturbed by these two modifications. Our data appear to be incompatible with the view that the activities we have measured arise from simple polyanion effects related to charge density. The finding that carboxyl-reduced and acetylated *N*-desulfated heparins have high anti-HIV-1 activity is particularly important since these preparations have low ATIII (Sie et al., 1988; Weiler et al., 1992). Thus, we have demonstrated here that heparin-derived preparations with low anticoagulant activity retain anti-HIV-1 activity.

In conclusion, we show here that for heparin derivatives there is a strong correlation between the inhibition of binding of V3 loop-specific Mabs to rgp120 and the inhibition of HIV-1 replication *in vitro*. This observation lends further weight to the view that the V3 loop of gp120 is involved in an essential postbinding step in the HIV-1 infection of $CD4^{+ve}$ cells. Moreover, our findings suggest that investigation of polyanionic compounds able to bind with high affinity and specificity to the V3 loop of gp120 is a fruitful area in the search for novel anti-HIV-1 agents which act independently of reverse transcriptase.

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