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QUARTERLY

The V3 region of gp120 is responsible for anti-HIV-1 activity of heparin sulphate[o]

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The effect of heparin sulphate on the infection of $CD4^+$ lymphocytes by recombinant HIV-1 clones pIIIB and by pIIIB/V3-BaL was investigated. It was demonstrated that heparin sulphate decreased the infectivity of $CD4^+$ lymphocytes by the pIIIB virus stronger than by the pIIIB/V3-BaL clone, and that the effect of heparin was concentration-dependent. This was accompanied by an inhibition of binding of the monoclonal antibodies 447-52-D to the V3 region and G45-60 to the C4 region of oligomeric glycoprotein 120 (gp120). It has been concluded that the inhibitory effect of heparin sulphate on the infection of $CD4^+$ lymphocytes by recombinant HIV-1 clones is mediated mainly by the V3 region of gp120. However, the C4 region contributes to the inhibitory effect of heparin sulphate.

HIV-1 has been recognized as the principle causative agent of acquired immunodeficiency syndrome (AIDS). Major target of the HIV-1 virus are the $CD4^+$ lymphocytes and the entry of HIV-1 into the target cells involves binding to specific surface receptors, followed by fusion with the plasma membrane. The essential role in the two processes is played by envelope glycoproteins gp120 and gp41 [1, 2]. The oligomeric form of gp120 consists of five constant (C) and five variable (V) regions whose function has been thoroughly investigated [3]. One of the constant regions, designated as C4, is responsible for the binding of the virus to the CD4 receptor, while the V3 region is responsible for the fusion of the virus with the target cells, and the V2 region probably influences the tropism of the virus [3, 4]. The V2, V3 region, as well as the C4 region, belong to the neutralizing domains of gp120 [3].

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Abbreviations: mAb, monoclonal antibodies; FITC, isothiocyanate; PBS, phosphate-buffered saline; FCS, fetal calf serum; gp120, glycoprotein 120.

Numerous attempts have been made to inhibit the binding of the virus and its fusion with the target cells. It has been recently demonstrated that sulphated polysaccharides affect the binding and inhibit the fusion of the virus with its target cells [5]. It seems that the negative charge of these compounds facilitates their binding to the CD4 receptor, or gp120, and that this might prevent the binding and/or fusion of the virus with the target cells [6, 7]. However, the mechanism(s) underlying the anti-HIV-1 properties of sulphated polysaccharides has not been fully understood. Among the different polysaccharides investigated so far, heparin sulphate exhibits an appreciable anti-HIV-1 activity [5]. However, the mode of action of heparin sulphate has not been extensively studied.

To investigate anti-HIV-1 properties of heparin sulphate we have used two well characterized recombinant viral clones: pIIIB and pIIIB/V3-BaL. The recombinant pIIIB virus is lymphotropic and exhibits strongly positive charge of the V3 region, whereas the pIIIB/V3-BaL virus is monotropic and shows much less positive charge of the same region [3]. To evaluate the effects of heparin sulphate on the infection of the $CD4^+$ lymphocytes by these recombinant viruses, flow cytometry and specific monoclonal antibodies directed against the V3, C4 and V2 regions of gp 120 were employed.

MATERIALS AND METHODS

Viruses and cell line. The HIV-1 recombinant clones pIIIB and pIIIB/V3-BaL were donated by Dr. B. Cullen (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC, U.S.A.). The pIIIB/V3-BaL virus comprises a backbone, originating from a lymphotropic pIIIB virus, to which the V3 domain of the pBaL strain was substituted [3].

The COS-1 cell line was obtained from Dr. J. Sodroski (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, U.S.A.) and was maintained in monolayer culture in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD, U.S.A.). The COS-1 cells were transfected with recombinant viruses by the DEAE-dextran method [8]. At 72 h after transfection, the COS-1 cells were transferred into a medium containing phytohaemagglutinin-activated peripheral blood mononuclear cells at the density of 10^6 cells/ml. These cells were cocultured with the transfected COS-1 cells for 3 days, then washed by aspiration, and maintained in expanded culture for 4 more days. On day 7 after infection, the supernatants were removed, filtered through a 0.45 μm filter and the viral stocks were stored at -70°C.

Preparation of $CD4^+$ lymphocytes. Peripheral mononuclear cells were isolated by Ficol density gradient centrifugation and were then stimulated by phytohaemagglutinin. To separate the lymphocytes from the monocytes-macrophages, peripheral mononuclear cells were incubated for 1 h at 37°C in a polystyrene flask. Under these conditions the monocytes-macrophages adsorb to the surface of the flask, while the peripheral blood lymphocytes remain in the supernatant. Peripheral blood lymphocytes were then coated with mouse antibody OKT-4 directed against CD4 antigen (Organon Teknica Co., West Chester, PA, U.S.A.), during incubation at 4°C for 1 h followed by 1 h incubation on ice, in a polystyrene plate, coated with rabbit antibodies directed against mouse IgG (Organon Teknica Co., West Chester, PA, U.S.A.). Under these conditions the $CD4^+$ lymphocytes were bound to the plate, while the remaining cells were removed by washing. The $CD4^+$ lymphocytes were finally recovered by scraping and maintained in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 50 U of interleukin-2 (Gibco BRL, Gaithersburg, MD, U.S.A.), 10% FCS and antibiotics. The $CD4^+$ lymphocytes were infected with 100 ng of virus antigen p24 and maintained in RPMI-1640 medium.

Monoclonal antibodies. Anti-gp120 mAbs included: G3-4, a mouse mAb which recognizes a discontinuous conformation-dependent epitope in the V2 region [9], G45-60 a mouse mAb, directed against an epitope located in the C4 region [10], and a human mAb 447-52-D, which recognizes the "crown" of the V3 loop in a relatively broad range of HIV-1 isolates [11]. The G3-4 and G45-60 mAbs were obtained from Dr. M.S. Fung (Tanox Biosystems Inc., Houston, TX, U.S.A.), and the 447-52-D mAb was donated by Dr. M.K. Górny (Department of Pathology and Center for AIDS Research, New York University Medical School, New York, NY, U.S.A.).

Infection of $CD4^+$ lymphocytes with the recombinant viruses. The $CD4^+$ lymphocytes were incubated without or with 3, 10, 30 and 100 μg/ml of low molecular mass heparin sulphate (Sigma Chemical Co., St. Louis, MO, U.S.A.), before and after the addition of recombinant viruses pIIIB and pIIIB/V3-BaL, in a concentration of 5 ng of p24 antigen. After 2 h the cells were washed and cultured in RPMI 1640 medium for 10 days without or with the indicated concentrations of heparin sulphate, and the medium was changed twice a week. Detection of the viral strains was based on the estimation of p24 antigen production, determined by immunocapture assay (Du Pont Company, Wilmington, DE, U.S.A.). In all cultures the cells were resuspended to a density of 10^6 cells/ml and the supernatants were harvested after 24 h to monitor the p24 antigen levels.

Indirect flow cytometry and blocking asssay. The $CD4^+$ lymphocytes that were infected with either recombinant virus pIIIB or pIIIB/V3-BaL for 10 days, were preincubated, for 30 min at 4°C, with anti-gp120 mAb, G3-4, G45-60 or 447-52-D (1 μg/ml) in PBS containing 2% FCS and 0.1% sodium azide (PBS/FCS) in the absence or presence of 3, 10, 30 and 100 μg/ml heparin sulphate. The preincubation was followed by incubation with a second, FITC-conjugated, antibody. The cells were washed three times in PBS/FCS, then stained with a 1:40 diluted fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of goat anti-mouse or anti-human IgG (Organon Technica Co., West Chester, PA, U.S.A.) and fixed by overnight incubation in 1% paraformaldehyde at 4°C. The cells were then washed in PBS/FCS and their fluorescence was determined in PROFILE II Coulter Cytofluorograf System (Coulter Carpenteria, CA, U.S.A.). For the analysis of the heparin-mediated inhibition of binding of the anti-gp120 mAbs to gp120, the infected cells were incubated and analysed by flow cytometry.

RESULTS

Effect of heparin sulphate on the infection of $CD4^+$ lymphocytes with recombinant viral clones pIIIB and pIIIB/V3-BaL

The effects of different concentrations of heparin sulphate on the replication of the recombinant viruses pIIIB and pIIIB/V3-BaL were studied in the infected cells. The replication of the viruses was determined by the analysis of p24 antigen levels in the culture supernatants from the $CD4^+$ lymphocytes, harvested 3, 6, 9 and 12 days after infection. The results revealed that heparin sulphate decreases the infectivity of both the recombinant strains in a dose-dependent manner. The inhibitory effect of heparin sulphate on the replication of recombinant virus pIIIB (Fig. 1A) was stronger than on the pIIIB/V3-BaL clones (Fig. 1B).

Influence of amino-acid sequence of V3 region on the interaction of heparin sulphate with V2 and C4 regions of gp120 derived from recombinant clones pIIIB and pIIIB/V3-pBaL

To explain the stronger inhibition of infection of the $CD4^+$ lymphocytes by the pIIIB virus than by the pIIIB/V3-pBaL clone, we ex-

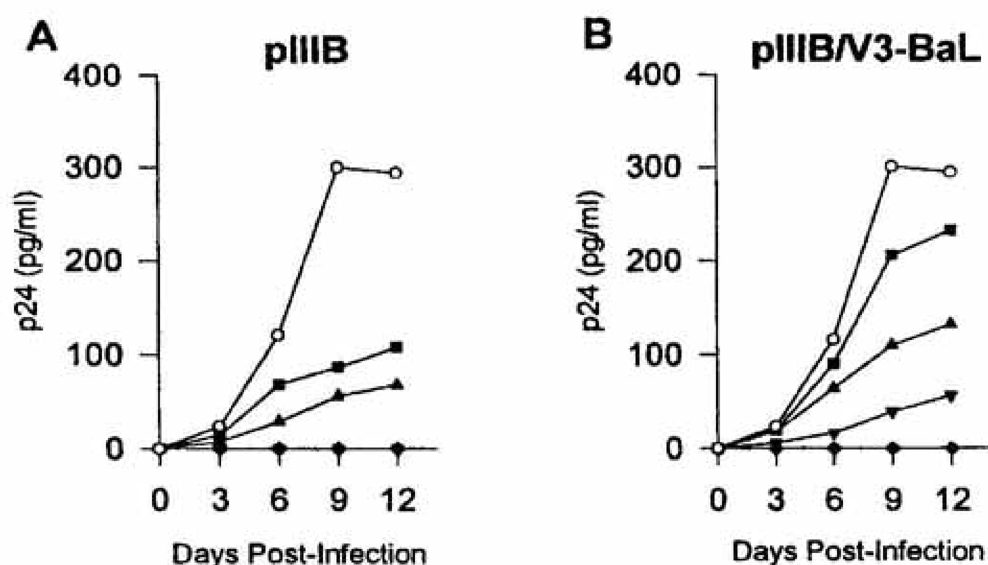

Figure 1. Effect of heparin sulphate on replication of proviral clones pIIIB (panel A) and pIIIB/V3-BaL (panel B).

The $CD4^+$ lymphocytes were infected by pIIIB or pIIIB/V3-BaL proviral clones in the absence (○) or presence of heparin sulphate in concentrations of 3 (■); 10 (▲); 30 (▼) and 100 (◆) μg/ml. The cells were then washed and cultured in the absence or presence of the same concentrations of heparin sulphate. At 3, 6 , 9 and 12 days after infection the culture supernatants were harvested to determine the p24 antigen concentration. The results represent the means of triplicate assays with ±S.D. < 15% from three separate experiments.

amined the effect of two different amino-acid sequences of the V3 region on the interaction of heparin sulphate with the C4 and the V2 regions of the oligomeric gp120. We used the $CD4^+$ lymphocytes infected by the two recombinant viruses pIIIB and pIIIB/V3-BaL. The

Table 1. Effect of heparin sulphate on interaction of anti-gp120 mAb with V3 and C4 regions of oligomeric gp120

Proviral clones	Heparin sulphate (μg/ml)	Inhibition of antibody binding (%)	
		447-52-D	G45-60
pIIIB	3	30 ± 2	27 ± 3
pIIIB/V3-BaL		13 ± 1	14 ± 2
pIIIB	10	60 ± 5	55 ± 5
pIIIB/V3-BaL		30 ± 4	37 ± 3
pIIIB	30	76 ± 8	59 ± 6
pIIIB/V3-BaL		50 ± 6	51 ± 5
pIIIB	100	92 ± 8	62 ± 7
PIIIB/V3-BaL		60 ± 7	55 ± 5

$CD4^+$ lymphocytes were infected with proviral clones pIIIB or pIIIB/V3-BaL. After 10 days of culture in the presence or absence of heparin sulphate the cells were stained with antibodies directed against either the V3 (447-52-D) or the C4 (G45-60) region of gp120.

Percentage of heparin sulphate-mediated inhibition of binding of the mAbs to gp120 was calculated as 100 − (MFx−MFo)/(MFc−MFo) × 100, where MF is mean fluorescence intensity of infected cells, stained with anti-gp120 antibody in the presence (MFx) or absence (MFc) of heparin sulphate. Controls represent fluorescence of labelled antibodies alone (MFo). Results represent the means ± S.D. from three separate experiments.

results revealed that, in the presence of heparin sulphate, the binding of the anti-V3 mAb to gp120 of the the pIIIB clone was inhibited much stronger than to the pIIIB/V3-BaL (Table 1) and was accompanied by attenuation of binding to the C4 region of gp120 of the same virus. However, heparin sulphate did not influence binding to the V2 regions of gp120 of both recombinant viruses (not shown).

DISCUSSION

In this study we demonstrated that, in the presence of heparin sulphate, the inhibition of infection of the $CD4^{+}$ lymphocytes by the pIIIB virus was stronger than by the pIIIB/V3-BaL clone. This was accompanied by attenuation of binding of the mAbs to the V3 and the C4 regions of the oligomeric gp120. The substitution of the V3 region of gp120 originating from pBaL clone in IIIB virus, which results in chimeric virus pIIIB/V3-BaL, has changed the susceptibility of the $CD4^{+}$ lymphocytes to the anti-infective effect of heparin sulphate. In comparison to pIIIB, the pIIIB/V3-BaL clone contains two additional lysine and two arginines in the immediate vicinity of a semi-conserved "crown" sequence -Gly-Phe-Gly-Arg- [3, 11]. The diverse effect of heparin sulphate on the two recombinant clones can be explained by the higher density of positive charges in the V3 region of the pIIIB clone than in the same region of the pIIIB/V3-BaL virus. It was shown that sulphated polysaccharides react with positively charged residues of the amino acids lysine and arginine [4, 12, 13]. Moreover, Callahan and coworkers [6] demonstrated that the higher density of positive charges in the V3 region of gp120 correlates with the binding of sulphated polysaccharides, other than heparin sulphate, to this region. It seems that the stronger inhibition, by heparin sulphate, of infection of $CD4^{+}$ lymphocytes by pIIIB virus, than by chimeric pIIIB/V3-BaL clone, correlates with an increased binding of this polysaccharide to the V3 region of gp120. These results are consistent with our earlier observations that β-glucan sulphate (curdlan sulphate) inhibited more strongly the infectivity of the pIIIB virus than of the chimeric pIIIB/V3-BaL strain [4].

It was inferred from this study that, like other sulphated polysaccharides, heparin sulphate inhibits infectivity of the recombinant HIV-1: pIIIB and pIIIB/V3-BaL. The negative charge of the V3 loop plays an important role in the infection process of the $CD4^{+}$ lymphocytes, and the C4 region contributes to the inhibitory effect of heparin sulphate. It is postulated that the V3 loop is of critical importance in the process of infection of target cells by HIV-1.

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