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Novel, Negatively Charged, Human Serum Albumins Display Potent and Selective *in Vitro* Anti-Human Immunodeficiency Virus Type 1 Activity

ROBERT W. JANSEN,[1] DOMINIQUE SCHOLS, RUDI PAUWELS, ERIC DE CLERCQ, and D. K. F. MEIJER

Department of Pharmacology and Therapeutics, University Centre for Pharmacy, Groningen State University, Groningen, The Netherlands (R.W.J., D.K.F.M.), and Rega Institute for Medical Research, Katholieke Universiteit Leuven, Belgium (D.S., R.P., E.D.C.)

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SUMMARY

We prepared a series of modified proteins and peptides by derivatizing the positively charged ϵ-amino groups of the lysine amino acids through reaction with anhydrides of succinic acid (Suc) and aconitic acid (Aco). Human serum albumin (HSA) was modified by introduction of a single carboxylic group (Suc-HSA) or two carboxylic groups (Aco-HSA) per amine function, yielding strongly negatively charged compounds. The *in vitro* anti-human immunodeficiency virus (HIV)-1 IC_{50} of Suc-HSA was about 1 μg/ml, and the most polyanionic modified albumin of the series (Aco-HSA) exhibited an IC_{50} as low as 0.02 μg/ml. Similar derivatization of the plasma protein orosomucoid or the synthetic polypeptide polylysine did not produce compounds with significant anti-HIV-1 activity, indicating an HSA-specific effect. The mechanism of action of Suc-HSA was reported to be the inhibition of a post-binding virus-cell fusion event, probably due to interference with the gp41-mediated fusion process. In the present study we demonstrate that the more potent Aco-HSA also interferes with this fusion process but, additionally, this compound inhibits (i) the binding of soluble CD4 to HIV-infected cells, (ii) the binding of HIV particles to MT-4 cells, and (iii) the binding of anti-gp120 monoclonal antibody to the gp120 molecule. This indicates that Aco-HSA, apart from post-binding fusion, also inhibits virus-cell binding by shielding viral gp120. The simultaneous inhibition of binding and fusion may lead to a synergistic effect, explaining the extreme potency of Aco-HSA. The polyanionic HSAs are significantly less active against HIV-2 and do not interfere with the replication of feline immunodeficiency virus or 12 other DNA or RNA viruses, indicating a HIV-1-specific effect. In contrast, another polyanionic compound, the sulfated polysaccharide dextran sulfate, inhibits the replication of various viruses in a more nonspecific way, as a general polyanion. Dextran sulfate also exhibits strong anticoagulant activity, whereas Suc-HSA and Aco-HSA do not show this unwanted side effect.

HIV, the causative agent of the acquired immune deficiency syndrome (1, 2), specifically infects $CD4^+$ cells (3–5). The reason for this cell tropism is the high affinity binding of the viral envelope glycoprotein gp120 to the CD4 molecule (5, 6). After viral attachment to the target cells, HIV enters these cells by a pH-independent fusion of viral and cell membranes (7, 8). The transmembrane viral envelope glycoprotein gp41 is thought to play a pivotal role in this fusion process (7, 9). A second way in which HIV can infect $CD4^+$ cells is by the fusion of HIV-infected cells with uninfected $CD4^+$ cells, leading to the formation of multinucleated giant cells (syncytia) (10). The latter process, which is also caused by gp120-CD4 binding followed by gp41-mediated fusion, largely contributes to the pathological decrease in T4 lymphocyte counts. The ability of polyanions to inhibit viral replication was described by De Somer *et al.* (11) as early as 1968. De Clercq (12) suggested that polyanions like dextran sulfate and heparin may have anti-HIV activity. Ito *et al.* (13) and Ueno and Kuno (14) provided experimental data that confirmed this hypothesis. The polyanionic compounds can be divided into at least three classes based on their mechanism of action, namely (i) the inhibition of HIV binding (and syncytium formation) through interference with ("shielding of") gp120, with examples being the sulfated polysaccharides like dextran sulfate (13, 15, 16), (ii) the inhibition of HIV binding (and syncytium formation) through interference with the CD4 molecule, by, for instance, aurin tricarboxylic acid (17), and (iii) the inhibition of the fusion process, as was recently described by us for negatively charged modified HSAs, e.g., Suc-HSA (18). In this novel category of anti-HIV compounds, we introduced a single carboxylic group on the ϵ-amino groups of the lysine residue. A

[1] Present address: Department of Clinical Chemistry, Medical Spectrum Twente, P.O. Box 50000, 7500 KA Enschede, The Netherlands.

ABBREVIATIONS: HIV, human immunodeficiency virus; HSA, human serum albumin; Suc, succinic acid; Aco, aconitic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; APTT, activated partial thromboplastin time; FPLC, fast protein liquid chromatography; PBS, phosphate-buffered saline; FIV, feline immunodeficiency virus; CC_{50}, 50% cytotoxic concentration; FITC, fluorescein isothiocyanate.

clear positive correlation between the net negative charge and the anti-HIV-1 potency was demonstrated (18).

In the present study, we coupled two carboxylic groups per lysine residue of HSA, using *cis*-aconitic anhydride (yielding Aco-HSA), and compared the *in vitro* anti-HIV activity with that of Suc-HSA. Furthermore, we studied the mechanism of action. We also report on the antiviral activity of these negatively charged HSAs with several DNA and RNA viruses. Finally, we investigated whether these compounds show any anticoagulant activity, one of the major drawbacks of the polyanions from class I such as dextran sulfate.

Materials and Methods

Chemicals. HSA (fraction V), dextran sulfate (M_r 5000), and *cis*-aconitic anhydride were obtained from Sigma Chemical Co. (St. Louis, MO). Succinic anhydride was obtained from Janssen Chimica (Beerse, Belgium). All other chemicals were of analytical grade or the best grade available and were used without further purification.

Preparation of Suc-HSA and Aco-HSA. For derivatization of HSA wih succinic anhydride or *cis*-aconitic anhydride, HSA (500 mg) was dissolved in 50 ml of 0.2 M K_2HPO_4, pH 8.0. Solid succinic (or *cis*-aconitic) anhydride (500 mg) was added and the solution was stirred until all anhydride was dissolved. The pH was kept between 8.0 and 8.5 with sodium hydroxide (6 M). For purification, the solution was filtered through a 0.2-μm filter to remove insoluble material, purified on a Sephadex G25 column, washed with distilled water on a PM10 membrane in an Amicon stirred cell concentrator, and finally lyophilized.

The amount of protein was determined both according to the method of Lowry *et al.* (19) and using the Bio-Rad protein assay kit based on the method of Bradford (20). Estimation of the free ε-lysine groups of the derivatized HSAs was performed according to the method of Habeeb (21).

FPLC. For charge estimation, the relative net negative charge of the modified HSAs was determined on a FPLC system (Pharmacia, Woerden, The Netherlands) equipped with a Mono-Q anion exchange column (Pharmacia), as described previously (22). Buffer A was Tris·HCl buffer (0.02 M), pH 7.4, and buffer B consisted of buffer A plus 2 M NaCl. Elution was performed at a flow rate of 0.25 ml/min with a gradient from 100% buffer A to 100% buffer B in 30 min. Samples were dissolved at 1 mg/ml in buffer A, and 100 μl were injected into the FPLC system.

For molecular weight estimation, the percentage of monomers, dimers, and polymers was determined with a FPLC system, using a Superose-12 column (Pharmacia), as described previously (23). The eluting buffer was PBS, pH 7.4, and the flow rate was 0.5 ml/min.

Cells and virus. MT-4, a T4 lymphocyte cell line carrying Human T-lymphotropic virus type I (24), was used for the anti-HIV-1 assay and was kindly provided by Dr. N. Yamamoto (Yamaguchi University, Yamaguchi, Japan). The MT-4 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 20 μg/ml gentamycin. MOLT-4 cells (clone 8) (25) were used for the syncytium formation assay. The cells were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. Every 3–4 days, cells were centrifuged and seeded at 2×10^5 cells/ml in new culture flasks. At regular time intervals, the cells were analyzed for the presence of *Mycoplasma* and were consistently found to be *Mycoplasma* free.

HIV-1 (strain Human T-lymphotropic virus type III_B) was obtained from the culture supernatant of persistently HIV-1-infected HUT-78 cells. The virus titer of the supernatant was determined in MT-4 cells. The virus stock was stored at −70° until used.

Antiviral assay. Anti-HIV activity of the test compounds was assessed by measuring their inhibitory effects on virus-induced cytopathogenicity in MT-4 cells and was monitored by the MTT method, as described previously (26). Cytotoxicity was also monitored by the MTT assay. The anti-influenza activity of the compounds was assessed by measuring the fusion of the virus with erythrocyte gosts. All other antiviral assays used standard procedures.

Virus adsorption assay. The procedure for the detection of binding of HIV-1 particles to the cell surface has been described previously (27). Briefly, MT-4 cells were exposed to HIV-1 virions in the absence or presence of the test compounds. After incubation at 37° for 30 min, the cells were washed to remove unbound virus particles. The cells were then stained for indirect fluorescence using a polyclonal antibody to HIV-1 and were analyzed for HIV-1 particles bound to the cells by laser flow cytofluorography.

CD4 immunofluorescence assay. CD4 expression was determined by FACStar (Becton-Dickinson) analysis, as described previously (17). Briefly, MT-4 cells were incubated for various times at room temperature in PBS with or without test compound. The cells were then stained with optimal concentrations of the monoclonal antibodies FITC-OKT4A (Ortho Diagnostics) or phycoerythrin-Leu-3a and the Simultest immune monitoring with control kit (FITC-labeled IgG1 and phycoerythrin-labeled IgG2) (Becton Dickinson) for 20 min at 4°, washed once with PBS, and fixed in 0.5 ml of 0.5% paraformaldehyde in PBS.

Glycoprotein gp120 immunofluorescence assay. Persistently HIV-1-infected HUT-78 cells (200,000 cells) in 100 μl of RPMI 1640 medium were washed twice with RPMI 1640 medium, incubated with the compounds at various concentrations at 20° for 15–20 min, washed twice with RPMI 1640 medium to remove residual compound, stained with anti-gp120 monoclonal antibody (9284; DuPont de Nemours, Brussel, Belgium) for 45 min at 37°, washed twice with RPMI 1640 medium, incubated with FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin antibody (Prosan, Ghent, Belgium) for 45 min at 37°, washed twice with PBS, resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS, and analyzed by flow cytometry, as described previously (15).

Anticoagulant activity assay. Different amounts of the compounds were dissolved in 30 μl of PBS, pH 7.2, and added to 270 μl of plasma, to the final concentrations indicated in Fig. 4. The APTT was measured by standard procedures. Heparin and PBS were used as positive and negative controls, respectively.

Results

Syntheses and characterization. Fig. 1 shows the reaction of HSA with succinic or *cis*-aconitic anhydride. All 61 amino groups of lysine were derivatized, as shown with the free-amino assay of Habeeb (21). The reaction and purification procedures did not cause major polymerization of the protein. The percentage of monomers decreased only from 92% for the parent HSA to 89% and 90.5% for Suc-HSA and Aco-HSA, respectively. The percentage of dimers for HSA, Suc-HSA, and Aco-HSA was 8%, 11%, and 9.5%, respectively.

The chromatographic behavior of the compounds on a strong anion exchange column is depicted in Fig. 2. The retention time of HSA was about 20 min, whereas Suc-HSA had a retention time of 29.8 min and Aco-HSA was eluted only after 32.4 min. This indicates an increase in net negative charge due to the introduction of one (Suc-HSA) or two (Aco-HSA) carboxylic groups on every lysine in the HSA molecule.

Anti-HIV activity. Table 1 shows the antiviral activity of the compounds with HIV-1, HIV-2, and FIV. The IC_{50} of Suc-HSA was 0.9 μg/ml, indicating that Suc-HSA was about equipotent with dextran sulfate. Aco-HSA was 30 times more potent (IC_{50}, 0.023 μg/ml). The corresponding IC_{90} values for Suc-HSA and Aco-HSA were 4 and 0.7 μg/ml. Typical dose-effect curves for Suc-HSA and Aco-HSA are shown in Fig. 3.

None of the compounds was cytotoxic to MT-4, Vero, or HeLa cells, freshly isolated rat hepatocytes, human lympho-

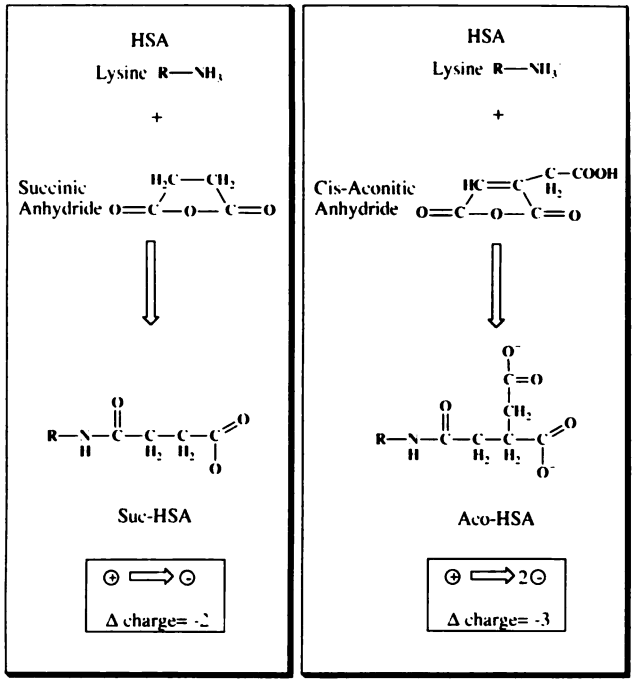

Fig. 1. Scheme for reaction of succinic anhydride (A) and *cis*-aconitic anhydride (B) with HSA.

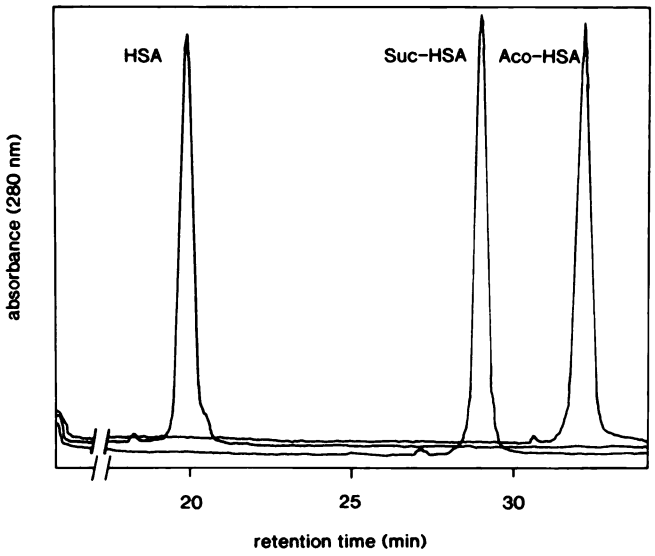

Fig. 2. Typical chromatograms for HSA, Suc-HSA, and Aco-HSA using a strong anion exchange column. The retention time is related to the net negative charge.

cytes, or fibroblasts, at concentrations up to 1 mg/ml (Table 1). This resulted in a selectivity index (CC_{50}/IC_{50}) for Aco-HSA well above 43,000 (Table 1).

The anti-HIV-2 activity of the modified HSAs was significantly weaker than the anti-HIV-1 activity. In contrast, the polyanion dextran sulfate was more potent with HIV-2 than with HIV-1. No compounds interfered with the replication of FIV, at concentrations up to 250 μg/ml.

After aconitic anhydride treatment of two other compounds, the plasma protein orosomucoid (α1-acid glycoprotein) and the synthetic polypeptide polylysine, their net negative charges increased significantly. Table 2 shows, however, that in contrast to Aco-HSA these compounds did not become antivirally active.

Mechanism of anti-HIV-1 action. When Aco-HSA was added 10 min after HIV-1 addition to the MT-4 cells, the antiviral activity was decreased by >500-fold (IC_{50}, 16 μg/ml), indicating that the compound interferes with an early step (virus binding or fusion) in the HIV-1 replication cycle. This is in agreement with our previous results showing that Suc-HSA exerts its activity at an extracellular level. Suc-HSA does not bind to or enter T lymphocytes in appreciable quantities and seems to interfere with the fusion process (18). Data from different assays regarding the mechanism of action of Aco-HSA are listed in Table 3. Table 3, column B, shows that Aco-HSA inhibited the binding of HIV-1 particles to MT-4 cells. The binding of solule CD4 to gp120-expressing cells was also inhibited in a dose-dependent manner (Table 3, column C). We investigated whether the interference with CD4-gp120 binding is due to interaction with the CD4 receptor or with the gp120 molecule. Table 3, columns D and E, clearly indicates that Aco-HSA inhibited the binding of an anti-gp120 monoclonal antibody (directed against the CD4-binding epitope of gp120) to gp120, whereas it did not interfere with the CD4 receptor.

If we compare the combined data from Table 3, columns B-E, with the activity of Aco-HSA in the antiviral assay, it is obvious that the concentrations neccessary to give 50% inhibition of virus binding (CD4-gp120 interaction) were 100–1000 times higher than the IC_{50} in the antiviral assay (Table 3, column A). Although the assays were performed under slightly different conditions, this large concentration discrepancy indicated that inhibition of virus binding by shielding of gp120 cannot be the sole mechanism of action of Aco-HSA.

Antiviral activity with DNA and RNA viruses. The modified HSAs did not inhibit the replication of the DNA viruses tested, in contrast to the polyanion dextran sulfate (Table 4). The effect on RNA viruses is also shown in Table 4. Dextran sulfate inhibited the replication of vesicular stomatitis virus and Sindbis virus, whereas Aco-HSA inhibited only the fusion of influenza virus with erythrocytes.

Anticoagulant activity. Fig. 4 shows the anticoagulant activity of the modified HSAs, measured as APTT. Heparin was used as a positive control. At concentrations of 5 μg/ml and higher, dextran sulfate exhibited an anticoagulant effect. In contrast, Suc-HSA and Aco-HSA did not show anticoagulant activities at concentrations up to 100 and 50 μg/ml, respectively.

Discussion

We recently reported that HSAs can be modified to become polyanions with potent *in vitro* anti-HIV activity (18). Substitution of the positively charged ε-amino groups of lysine with a negatively charged carboxylic group yielded the most potent compound, Suc-HSA, with an IC_{50} of about 1 μg/ml. We also showed that the net negative charge of the modified HSAs and their *in vitro* anti-HIV-1 potency are strongly correlated (18). Therefore, we reasoned that the introduction of two carboxylic groups per lysine residue (as in Aco-HSA) would render HSA even more negatively charged and should provide more potent compounds. In the present study we show that the anti-HIV activity was indeed further increased. The IC_{50} of Aco-HSA was 0.023 μg/ml (0.27 nM), which makes this HSA derivative one of the most potent *in vitro* anti-HIV-1 agents described to date. No cytotoxicity for several cell types was observed at

TABLE 1

Inhibitory effects of the compounds on HIV-1, HIV-2, and FIV

IC_{50} is the concentration needed for 50% inhibition of virus-induced cytotoxicity. CC_{50} is the concentration needed for 50% reduction of cell viability due to the compounds. SI is the selectivity index (CC_{50}/IC_{50}). Data are mean values for at least two separate experiments.

	HIV-1				HIV-2, IC_{50}	FIV, IC_{50}
	IC_{50}		CC_{50}	SI		
	μg/ml	nM	μg/ml		μg/ml	μg/ml
Suc-HSA	0.9	11.2	>1,000	>1,100	78	>250
Aco-HSA	0.023	0.27	>1,000	>43,000	5.9	>250
Dextran sulfate	0.8	160	>1,000	>1,250	0.08	>250

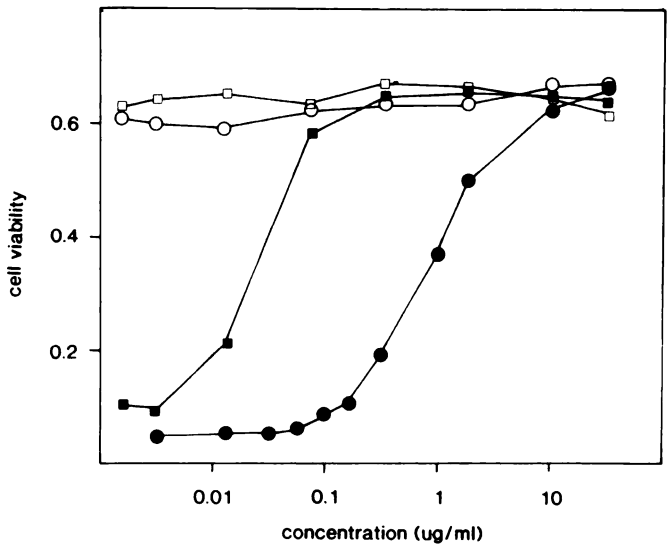

Fig. 3. Typical anti-HIV-1 dose-effect curves for Aco-HSA (■) and Suc-HSA (●) and cell viability in the presence of different concentrations of Aco-HSA (□) and Suc-HSA (○).

TABLE 2

Inhibitory effects of the compounds on HIV-induced cytopathogenicity

Compound	CC_{50}	IC_{50}
	μg/ml	μg/ml
Aco-HSA	>250	0.023
Aco-orosomucoid	>250	>250
Aco-polylysine	>250	>250

concentrations of up to 1 mg/ml, resulting in a selectivity index of >43,000. Furthermore, Aco-HSA did not show anticoagulant activity (see Fig. 4). After intravenous injection into rats of 100 mg of Aco-HSA/100 g of body weight, no sign of acute toxicity was observed. Therefore, we infer that the therapeutic index of Aco-HSA in this species is quite promising.

The mechanism of action of Aco-HSA is somewhat complex. Suc-HSA has a predominant effect on the fusion of virus and cell membrane and on the fusion of infected cells with noninfected cells and has hardly any effect on virus-cell binding. In addition to a predominant effect on the aforementioned fusion processes, Aco-HSA also seems to interfere with virus-cell binding at the level of gp120. The 40-fold higher potency of Aco-HSA, compared with Suc-HSA, is likely to be caused by the combined effects on these subsequent steps in the virus replication cycle. There is strong evidence for synergistic effects of compounds that interfere with different steps of the replication cycle of HIV (28, 29).

TABLE 3

Effect of Aco-HSA on HIV-1-induced cytopathogenicity (column A) and on the binding of HIV-1 particles to MT-4 cells (column B)

Columns C, D, and E represent the inhibition indices (II) for soluble CD4 binding to expressing cells, anti-gp120 monoclonal antibody binding to gp120-expressing cells, and for anti-CD4 monoclonal antibody binding to MT-4 cells, respectively. The gp120-expressing cells were persistently infected HUT-78 cells. Data are the mean values of at least two separate experiments.

Aco-HSA	A Protection in antiviral assay	B Inhibition of HIV-1 binding	II Inhibition Index		
			C Soluble CD4	D Anti-gp120 antibody	E Anti-CD4 antibody
μg/ml	%	%			
100.00	100	ND[a]	0.80	0.92	0.0
25.00	100	60	ND	ND	ND
20.00	100	ND	0.77	0.82	0.0
4.000	100	ND	0.73	0.57	0.0
0.800	100	ND	0.48	0.28	0.0
0.160	94	ND	ND	ND	ND
0.032	52	ND	ND	ND	ND
0.016	23	ND	ND	ND	ND

[a] ND, not determined.

TABLE 4

Inhibitory effects of polyanionic compounds on the replication of several viruses

	IC_{50}	
	Aco-HSA	Dextran sulfate
	μg/ml	
DNA virus		
HSV-1	>400	0.7
HSV-2	>400	1.0
Cytomegalovirus	>400	0.2
VZV	>400	2.0
RNA virus		
Coxsackie virus	>400	>400
Polio virus	>400	>400
Vesicular stomatitis virus	>400	7.0
Sindbis virus	>400	2.0
Semliki Forest virus	>400	>400
Reovirus	>400	>400
Parainfluenza virus	>400	>400
Influenza virus	0.8	>400

Apart from potential interactions with gp41 (and to a lesser extent with gp120), a recent report suggests that direct binding to $CD4^+$ cells is also possible through interactions with a scavenger receptor that binds negatively charged proteins (30).

The antiviral activity of the modified HSAs was found to be quite selective for HIV-1 and dramatically decreased from HIV-1 to HIV-2 to FIV. We observed no inhibition by these agents of the replication of 13 other envelope viruses (except for inhibition of fusion of influenza virus).

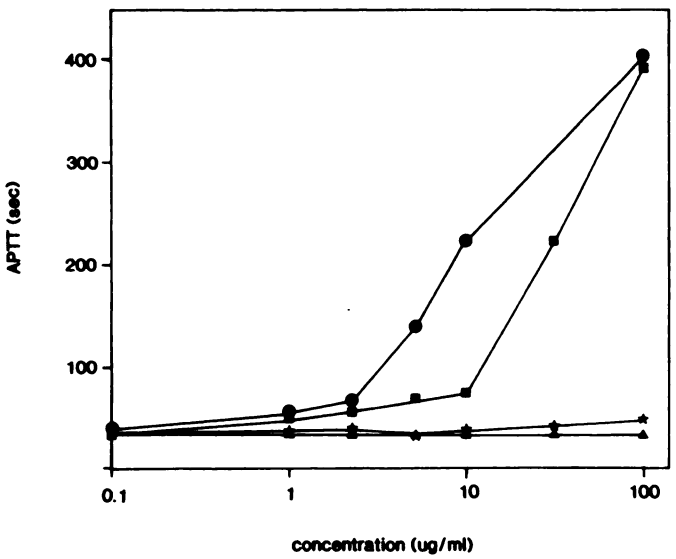

Fig. 4. Anticoagulant activity, measured as APTT, of Aco-HSA (★), Suc-HSA (▲), and dextran sulfate (■). Heparin (●) was used as a positive control. Data are the mean of two separate experiments.

The fact that modification of an endogenous compound like HSA can lead to such potent anti-HIV activity raises the question of whether negatively charged albumins are also formed *in vivo*. Therefore, further studies are in progress to investigate whether negatively charged HSAs can be found in the plasma of healthy volunteers, HIV-infected seropositive patients, or patients with full-blown acquired immune deficiency syndrome.

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Send reprint requests to: Robert W. Jansen, Department of Clinical Chemistry, Medical Spectrum Twente, P.O. Box 50000, 7500 KA Enschede, The Netherlands.