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Charge Modification of Plasma and Milk Proteins Results in Antiviral Active Compounds

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Abstract: Previous studies have shown that acylated plasma and milk proteins with increased negative charge, derived from various animal and human sources, are potent anti-HIV compounds. The antiviral effects seemed to correlate positively with the number of negative charges introduced into the various polypeptides: proteins with a high content of basic amino acids in which all of the available εNH_2 groups were anionized yielded the most potent anti-HIV compounds. It remained unclear however whether the total net negative charge of the various derivatized proteins, or rather the charge density on the protein backbone, is essential for the observed anti-HIV activity. Earlier studies have shown that acylated albumins preferentially block the process of HIV/cell fusion through binding to the HIV envelope proteins gp120 and gp41 as well as to the cell surface of the HIV target cells. Some of these polyanionic proteins have been shown to interfere also with the gp120–CD4 mediated virus/cell binding. The relative contribution of these effects to the anti-HIV activity may depend both on the total negative charge introduced as well as the hydrophobicity of the acylating reagent added to the particular proteins. In this study we show that the higher the charge density of the derivatized proteins, the more potent their HIV replication inhibiting effects are. In contrast, the addition of positive charge to the studied plasma and milk proteins through amination resulted in a reduced anti-HIV activity but a clearly increased anti-HCMV activity, with IC_{50} values in the low micromolar concentration range. Interestingly, native lactoferrin (Lf) was antivirally active against both HIV and HCMV. Acylation or amination of Lf increased the anti-HIV and anti-HCMV activity, respectively. The *N*-terminal portion of Lf appeared essential for its anti-HCMV effect: *N*-terminal deletion variants of human Lf were less active against HCMV. Circular dichroism of the modified proteins showed that the

Abbreviations: Aco-HSA, aconitylated-HSA; AZT, zidovudine; BSA, bovine serum albumin; Cat-HSA, cationized-HSA; CC_{50}, 50% cytotoxic concentration; CSA, chicken serum albumin; DTPA, diethylenetriaminepentaacetic anhydride; EDA, ethylene diamine; EDCI, 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide; FLF, foetal lung fibroblasts; HCMV, human cytomegalo virus; HIV, human immunodeficiency virus; HSA, human serum albumin; IC_{50}, 50% inhibitory concentration; Lf, lactoferrin; RSA, rat serum albumin; SIV, simian immunodeficiency virus; SSA, simian serum albumin; Suc-HSA, succinylated-HSA.

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secondary structure of the tested proteins was only moderately influenced by acylation and/or covalent attachment of drugs, making these (derivatized) proteins useful candidates as antiviral agents and/or intrinsically active drug carriers. The relatively simple chemical derivatization as well as the abundant sources of blood plasma and milk proteins provides attractive opportunities for the preparation of potent and relatively cheap antiviral agents for systemic or local applications.

Keywords: HIV; HCMV; polyanions; polycations; circular dichroism

INTRODUCTION

The field of anti-HIV chemotherapy has evolved rapidly in the past decade. Numerous reverse transcriptase and protease inhibitors have been forwarded as potential antiviral agents. Some of these compounds, like the reverse transcriptase inhibitors zidovudine (AZT (3′-azido-2′,3′-dideoxythymidine)), lamivudine (3TC (2′,3′-dideoxy-3′-thiacytidine)), stavudine (d4T (2′,3′-dideoxythymidine) and the protease inhibitors among others saquinavir (*N*-*tert*-butyl-decahydro-2-[2(*R*)-hydroxy-4-phenyl-s(*S*)-[[*N*-(2-quinolylcarbonyl)-L-asparaginyl]butyl]-(4aS,8aS)-isoquinoline-3(*S*)-carboxamide) and indinavir (*N*-(2(*R*)-hydroxy-1(*S*)-indanyl)-2(*R*)-(phenylmethyl)-4(*S*)-hydroxy-5-[1-[4-(3-pyridylmethyl) - 2(*S*) - (*N* - *tert* - butylcarbamoyl)-piperazinyl]]pentanamide) [1–3] were approved for the treatment of HIV infection. However, chronic use of such antivirals may not only cause toxicity problems, but also can lead to drug-resistant virus strains [4–7]. Therefore, combinations of different anti-retroviral drugs have been extensively screened *in vitro* and *in vivo* for improvement of the current therapies. Synergistic antiviral activity was reported among various combinations of drugs [8–10]. Triple therapies showed drastic reductions in viral load, often to undetectable levels [11,12]. Unfortunately, the required long treatment with these combinations is very costly and drug resistance has been recently reported.

Toxicity problems in antiviral therapy might also be, at least partially, overcome by site-specific delivery of the particular drug. In this concept, therapeutic agents are covalently attached to or included in macromolecular carriers that are then selectively recognized and taken up by the target cells. That this drug-targeting concept is valid was previously shown for the nucleoside analogue adenine arabinoside monophosphate (ara-AMP) that was linked to lactosaminated albumin, in a clinical study on the treatment of hepatitis B [13]. In our laboratory, AZT-monophosphate derivatives of (neo)glycoproteins were prepared [14] that exhibit extensive binding to the cell surface of T-lymphocytes and macrophages. In the latter cell type, scavenger receptors are instruments in the internalization of these conjugates. In T-lymphocytes an AZT effect of the AZT-glycoprotein conjugate could be demonstrated, indicating a partial uncoupling of the nucleoside analogue, AZT [15].

We showed that, among various sugar-modified albumins, mannosylated albumin exhibited an intrinsic anti-HIV activity [16,17]. This antiviral effect was explained by the extra negative charge introduced in the molecule through the chemical reaction chosen for sugar coupling. We and others recently described a pronounced anti-HIV-1 and anti-HIV-2 effect of albumins following acylation using anhydrides of succinic acid, *cis*-aconitic acid [18–21], maleic acid [22] or hydroxy-phthalic acid [23,24]. The negatively charged albumins such as succinylated-HSA (Suc-HSA) and aconitylated-HSA (Aco-HSA) are able to inhibit replication of various HIV strains *in vitro* including clinical isolates, in the nanomolar concentration range [19].

Like other polyanionic compounds, such as dextran sulfate and heparins, the negatively charged albumins act in the early phase of the viral replication and presumably at a post-adsorption event [19,25]. This was established by epitope mapping of the viral glycoproteins gp120 and gp41. It was shown that the negatively charged albumins interact with specific domains of these glycoproteins [26–28].

Very little is known about the relation between the overall negative charge of the proteins and the antiviral potency of various proteins. Earlier studies showed that the addition of extra positive charge to the polypeptides might result in potent anti-cytomegalo virus proteins. Yet little information became available in the relation between the overall charge and the antiviral potency. Therefore, in the present study the anti-HIV and anti-HCMV activity of a series of blood plasma and milk proteins was studied. The present study represents a first attempt to establish a structure-activity relationship of proteins with added negative or positive charge with regard to their anti-HIV and anti-HCMV potency.

MATERIALS AND METHODS

Chemicals

Human serum albumin (HSA) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, Netherlands). Rat serum albumin (RSA) and simian serum albumin (SSA) were isolated and purified at our laboratory by ethanol fractionation techniques. Bovine milk lactoferrin (bLf), casein and conalbumin with a purity of minimal 95% were obtained from DMV, Leeuwarden, Netherlands. Human Lf (hLf) and murine Lf (mLf) were obtained from Pharming, Leiden, Netherlands. *N*-terminal amino acid deletion variants of hLf, Lf $^{-2N}$ and Lf^{-3N}, missing two or three *N*-terminal amino acids respectively, were also obtained from Pharming. α-Lactalbumin, β-lactoglobulin-A, β-lactoglobulin-B, isolated from bovine milk were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Lysozyme isolated from chicken egg, bovine serum transferrin, asialofetuin isolated from foetal calf serum, human serum α_1-acid glycoprotein, γ-globulins, bovine serum albumin (BSA), chicken serum albumin (CSA) and poly-l-lysine were also purchased from Sigma. The AZTMP modified HSAs and Lfs were synthesized as described [14]. *cis*-Aconitic anhydride and succinic anhydride were obtained from Janssen Chimica (Beerse, Belgium). All other chemicals were analytical reagent grade and purchased from Sigma.

Preparation of Succinic-, *cis*-Aconitic- and Diethylenetriaminepentaacetic Anhydride Treated Proteins

Derivatization of the proteins was done as follows: 10 mg of the protein was dissolved in 10 ml of 0.2 M K_2HPO_4 pH 8.0. Solid succinic anhydride, *cis*-aconitic anhydride or DTPA (0.1 mmol), was added and the solution was stirred until all anhydride was dissolved. The reaction time was 5 min. The pH was kept at 8–8.5 using 3 M NaOH. The modified proteins were desalted by a Sephadex, G-25 filtration. Mixing variable amounts of anhydride with the protein, followed by the above mentioned isolation method did partial acylation of HSA. The total amount of protein was determined according to Lowry *et al.* [29] and the number of free εNH_2 residues of the derivatized products according to the method described by Habeeb [30]. Both the total amount of protein and amount of derivatized εNH_2 groups are the result of a triple determinations with a standard error of the mean of less than 5%. The subscripts in the product names as mentioned in Table 1 represent the number of εNH_2 groups derivatized of the particular protein. If no subscript is given all εNH_2 groups in the protein are derivatized by the respective anhydrides.

Preparation of Cationic Proteins

Proteins were derivatized by the modified method of Purtell *et al.* [31]. In brief: 6.7 ml (0.1 mol) of anhydrous ethylene diamine (EDA) was mixed with 50 ml of distilled water. The solution was cooled to room temperature and adjusted to pH 4.75 with 6.0 N HCl. A total of 200 mg of lyophilized protein and 0.4 μmol of EDCI·HCl were added to the EDA solution. The reaction was continued for 2 h and then stopped by the addition of 3.0 ml of 4.0 M acetate buffer pH 4.75. The products were purified using an Amicon Stirred Cell (Amicon, Danvers, MA, USA) equipped with a Filtron omega membrane (Filtron Technology Corporation, MA, USA) following Sephadex G-25 gel chromatography (Pharmacia, Uppsala, Sweden) through elution with distilled water. The purified product was lyophilized (Lyolab A, LSL Secfroid, Aclens, Switzerland) and stored at -20°C. The total amount of protein was determined according to Lowry *et al.* [29] and the number of free εNH_2 residues of the derivatized products according to the method described by Habeeb [30].

Characterization of the Cationized Proteins

The isoelectric point (IEP) of the cationized proteins was measured by isoelectric focusing on polyacrylamide gel, using Ampholites with a pH range of 3.5–9.5 (Pharmacia-LKB, Uppsala, Sweden).

Linearization of Acylated Proteins

Acylated albumins were linearized by sulphitolyses as has been described by Batra *et al.* [32]. In brief: 10 mg (0.15 μmol) of lyophilized acylated albumin was reacted in 0.2 M phosphate buffer pH 8.0, containing 6 M guanidine and 0.1 mM EDTA with 15 times the molar equivalent of dithiothreitol. After 2 h at room temperature (RT) the sulphydryl groups were irreversibly blocked by a 2 M equivalent (with respect to dithieothreitol) of iodoacetamide. The reduced acylated albumins were desalted by amicon and Sephadex G25 filtration as described.

Fast Protein Liquid Chromatography

The molecular weight estimation and percentage monomers and dimers were determined on a FPLC system, as described by Jansen *et al.* [16].

In brief: molecular weight: we injected 100 μl of 1 $mg \cdot ml^{-1}$ native or modified protein onto the FPLC system, equipped with a Superose 12 column (Pharmacia, Uppsala, Sweden). Elution was performed

Table 1 Anti-HIV Effects (IC_{50} Values) of Native and Acylated Proteins

Compound		mwt (kDa)	δ charge/AA	HIV-1 IIIb (nM)	HIV-2 ROD (nM)	SIVmac251 (nM)	IIvb*
Controls							
1	AZT		–	1.1	1.1	nd	nd
2	ATA		–	nd	nd	nd	1.00
3	DS 5 kDa	5.0	–	4	4	1.8	0.86
4	Succinic acid	0.1	–	>25 000	>25 000	nd	nd
5	Aconitic acid	0.15	–	>25 000	>25 000	nd	nd
6	DTPA		–	Toxic	Toxic	nd	nd
Neo(glyco)proteins							
7	Suc_{36}-HSA	70.6	0.12	3540	>3540	nd	nd
8	Suc_{47}-HSA	71.7	0.16	767	>3510	11.2	0.25
9	Suc_{51}-HSA	72.1	0.17	15.3	1872	nd	0.56
10	Suc_{61}-HSA	73.1	0.20	5.5	773	7.4	0.74
11	Aco_{18}-HSA	69.7	0.09	>3590	>3590	>3590	0.14
12	Aco_{28}-HSA	71.2	0.14	>3510	>3510	>3510	0.26
13	Aco_{37}-HSA	72.6	0.19	12.4	1230	nd	0.50
14	Aco_{51}-HSA	74.6	0.26	5.4	60.3	9.7	0.79
15	Aco_{61}-HSA	76.2	0.30	0.5	81.4	2.1	0.83
16	$DTPA_{16}$-HSA	72.6	0.13	Toxic	Toxic	nd	nd
17	$DTPA_{28}$-HSA	76.8	0.23	Toxic	Toxic	nd	nd
18	$DTPA_{46}$-HSA	83.1	0.38	Toxic	Toxic	nd	nd
19	$DTPA_{54}$-HSA	85.9	0.45	Toxic	Toxic	nd	nd
20	Suc-RSA	73.1	0.20	2.5	>3420	nd	nd
21	Suc-CSA	73.1	0.20	8.6	>3420	nd	nd
22	Suc-BSA	73.1	0.20	1.9	>3420	1874	0.25
23	Suc-SSA	73.1	0.20	1.9	75.2	205	nd
24	Suc-orosomucoid	22.6	0.13	5700	>11 000	nd	nd
25	Suc-transferrin	80.8	0.17	45.8	198	>3100	0.31
26	Suc-γ-globulin	158.0	0.14	13.3	46.8	nd	nd
27	Suc-immunoglobulin	158.0	0.14	5.7	34.8	nd	nd
28	Suc-lysozyme	14.8	0.09	858	>16900	>16 900	0.06
29	Suc-β-casein	25.2	0.12	2218	>9920	nd	nd
30	Aco-β-casein	25.8	0.17	508	>9690	nd	nd
31	Suc-conalbumin	83.7	0.18	3.2	>2990	nd	nd
32	Aco-conalbumin	86.2	0.26	1.0	894	nd	nd
33	Suc-bLf	81.4	0.16	12.3	>3070	388	nd
34	Aco-bLf	84.1	0.24	2.0	>2970	nd	nd
35	DTT-Suc-HSA	73.1	0.17	25.9	>3420	nd	nd
36	DTT-Aco-HSA	76.2	0.20	1072	>3280	nd	nd

Mean values of at least six experiments.
nd, not determined. All compounds showed no toxic effects up to a concentration of 3000 nM (corresponding to approx 250 $\mu g \cdot ml^{-1}$), except for the DTPA and the DTPA derivatized albumins, which showed toxicity at a comparable concentration to the IC_{50}, resulting in selectivity indices of 1.
* The inhibitory index for anti-gp120 mAb binding inhibition (see (Materials and Methods) (gp120–CD4 interaction)).

with phosphate buffered saline pH 7.4 at a flow rate of 0.5 $ml \cdot min^{-1}$.

The relative net charge of the modified proteins was determined on the FPLC system equipped with a Mono-Q 5/5 anion exchange column (Pharmacia). Buffer A was a 0.02 M Tris–HCl buffer pH 7.4 and buffer B consisted of buffer A and 1.0 M NaCl at a pH of 7.4. Elution was performed at 0.25 $ml \cdot min^{-1}$ with a gradient from 100% A to 100% B in 30 min. The gradient started at 4 min after injection.

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on an AVIV circular dichroism spectrometer model 62A DS equipped with an Apple Power Macintosh 7100/66 computer. The instrument was calibrated with benzene vapor (wavelength) and *d*-camphorsulphonic acid (magnitude). Spectra are the average of five scans recorded using a bandwidth of 1 nm, a step width of 1 nm and a 1-s averaging time per point. Measurements were performed at 20°C and the spectra were recorded using a 1 mm path length; protein concentration in all cases was 0.1 $mg \cdot ml^{-1}$ in water. CD spectra were analysed for secondary structure using the program CONTIN, described by Provencher and Glöckner [33].

Anti-HIV Screening

Cells, virus and antiviral assays. MT-4 cells were used for the anti-HIV-1, anti-HIV-2 and anti-SIV assay as was earlier described by Pauwels *et al.* [34]. Briefly, serial fivefold dilutions of the test compounds were made directly in a 96-well microtitre tray. Untreated control HIV/SIV- and mock-infected cell samples were included for each compound. Fifty microlitres of HIV/SIV (100 $CCID_{50}$) or control medium were added to either infected or mock-infected cells. Exponentially growing MT-4 cells were centrifuged for 5 min at $140 \times g$ and resuspended at 6×10^5 cells/ml, and 50 µl volumes were then transferred to the microtitre tray wells. The cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Five days after infection the viability of mock- and immunodeficiency virus-infected cells was examined by a colorimetric assay (the MTT method). The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the compound that protected HIV infected cells by 50%, whereas the 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the compound that reduced the viability of mock-infected cells by 50%.

HIV-1 (strain IIIb), HIV-2 (strain ROD) or SIV (strain mac251) were obtained from the culture supernatant of persistently virus infected HUT-78 cells. The virus titre of the supernatant was determined in MT-4 cells. The virus stock was stored at −70°C until used.

Correlation patterns between antiviral activity and charge densities of the proteins were calculated using the non-linear curve fitting program Multifit (developed by Dr J.H. Proost, Department of Pharmacokinetics and Drug Delivery, Groningen, Netherlands).

gp120–CD4 interaction. HIV-1 infected MT-4 cells (2×10^5/100 µl), grown and cultivated as described above, were washed twice using RPMI containing 10% FCS. The cells were incubated with 25 $\mu g \cdot ml^{-1}$ negatively charged proteins at 20°C for 15–20 min in RPMI/10% FCS and washed again using RPMI to remove residual compound. Staining with anti-gp120 mAb (9284, DuPont de Nemours, Brussels, Belgium) was performed for 45 min at 37°C. The cells were washed again two times using PBS followed by an incubation with FiTC-conjugated anti-mouse immunoglobulin antibody (Prosan, Ghent, Belgium) for 45–50 min at 37°C. After this the cells were washed twice with PBS, resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS, and analysed by flow cytometry, as described previously by Schols *et al.* [35].

The inhibitory index for anti-gp120 mAb binding inhibition (IIvb) was calculated according to the formula $1 - (MFLxx - MFInx)/(MFIpc - MFInc)$, in which MFLxx is the mean fluorescence intensity (MFI) of an infected sample in the presence of compound (25 $\mu g \cdot ml^{-1}$). MFInx represents the uninfected sample in the presence of compound, MFIpc and MFInc represents the positive and negative control, respectively.

Correlation patterns between the gp120–CD4 interaction and charge densities of the proteins were calculated using the non-linear curve fitting program Multifit (developed by Dr J.H. Proost, Department of Pharmacokinetics and Drug Delivery, Groningen, Netherlands).

Anti-HCMV Screening

Cells and virus. Human foetal lung fibroblasts (FLF), between passage seven and 17, were used as target cells for infection with HCMV strain AD169. Fibroblasts were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% foetal calf serum

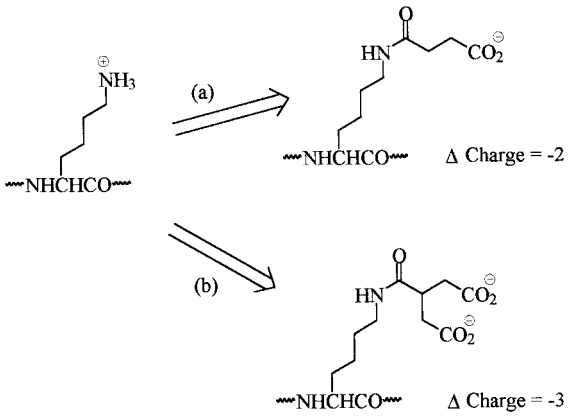

Figure 1 Derivatization reaction of the terminal amino functions in the protein backbone and anhydrides of succinic and *cis*-aconitic acid.

(Gibco) and 60 $\mu g \cdot ml^{-1}$ gentamicin sulphate (Gibco) at 37°C, 100% humidity and 5% CO_2. Infective virus was prepared by infecting subconfluently growing FLF in large (150 cm^2) tissue culture flasks (Costar, Cambridge, UK) at MOI (multiplicity of infection) = 0.1. Infection was allowed to proceed until the cells showed a maximal cytopathic effect (c.p.e.) and started lysing; this was usually approximately 10 days post-infection. These cells were detached from the flasks and centrifuged together with the medium. The resulting supernatant was frozen in liquid nitrogen and stored at −80°C and used for the infections in the experiments described below.

Anti-HCMV testing of antivirals. The anti-HCMV screening was done as described [36]. In brief: antivirals were dissolved in PBS at 2 $mg \cdot ml^{-1}$ immediately prior to the experiments. One day prior to infection with HCMV, subconfluent growing FLF were detached using trypsin, suspended in culture medium at 100000 FLF/ml and seeded into a flat bottom 96-well tissue culture plate (Costar) at approximately 10000 cells/well. The next day the medium was refreshed and the antiviral protein to be tested was added to 50 µl of medium. Initially, antivirals were tested at eight concentrations in

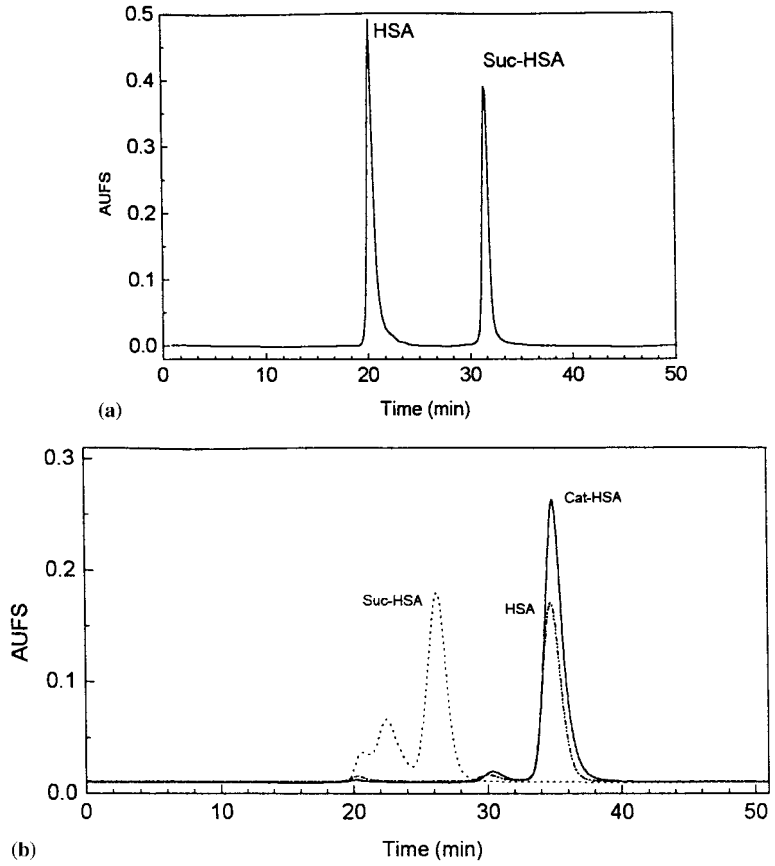

Figure 2 (a) FPLC characteristics of HSA and Suc-HSA using a Mono-Q 5/5 anion exchange column. The retention time is related to the net negative charge. (b) FPLC characteristics of HSA, Suc-HSA and Cat-HSA on a Superose 12 column.

twofold concentration dilution series starting at 125 or 250 $\mu g \cdot ml^{-1}$ (final concentration after addition of virus). After 30 min incubation, 50 µl of HCMV diluted in medium was added at MOI = 1 and infection was allowed to proceed for 72 h. The experiments were done at least in fivefold to compensate for the biological variations of this system.

RESULTS

Plasma and milk proteins of several species and sources were derivatized using anhydrides of succinic- and *cis*-aconitic acid. These derivatizations resulted in the introduction of one COOH or two COOH groups per reacted NH_2 function in the proteins, respectively. The reaction schemes for these derivatizations are given in Figure 1.

FPLC analysis of Suc-HSA (Figure 2a) using a Mono-Q anion exchange column showed a 10 min increase of retention time compared with the parent protein HSA, indicating that the net negative charge of the protein has considerably increased. Comparable results were obtained after anionization of the other proteins used in this study (results not shown).

Figure 2b shows that cationized-HSA (Cat-HSA) had a comparable molecular mass with HSA and that the dervatization did not affect the degree of polymerization. In fact Cat-HSA was for at least 95% a monomeric protein. However, acylation of albumin resulted in a decrease of the monomeric protein fraction. In all cases the polymerization was less than 15%. Interestingly, monomeric and polymeric fractions showed the same chromatographic behaviour on the Mono-Q anion exchange column, indicating that their negative charge density does not differ significantly. Figure 2b also showed a retention time shift of Suc-HSA, suggesting a larger molecular weight of this modified protein. This observation could not be supported by various electophoresis techniques, indicating that the size, conformation and charge of the proteins affect the retention of the acylated proteins on the Superose column.

Anti-HIV Effects

Complete succinylation or aconitylation of the various proteins showed that nearly all compounds became potent inhibitors of HIV-1 and to a lesser extend of HIV-2 and SIV replication (Table 1).

Succinylation of the albumins from chicken, rat, bovine, simian and human origin (compounds **10**, **20**–**23**, Table 1) resulted in activities with comparable 50% effective concentrations on the inhibition of HIV-1 induced cytopathicity (IC_{50} values) of about 2 nM. Partial succinylation of human serum albumin, showed that at least 58 of the available 61 NH_2 groups in the albumin molecule need to be derivatized in order to get the maximum activity. Succinylation of less than 45 amino groups yielded basically inactive compounds.

A more pronounced anti-HIV effect was found if albumin was derivatized with *cis*-aconitic anhydride. A significant activity was observed if only 37 of the available NH_2 groups in albumin were modified (compounds **11**–**15**). The antiviral activity of Aco_{37}-HSA could be increased about 20 times, if all NH_2 were derivatized. Aconitylation of albumin resulted in one of the most active compounds currently available for anti-HIV purposes.

Further increasing the negative charge of albumin by using diethylenetriaminepentaacetic anhydride (DTPA), in which four COOH groups are introduced per NH_2, function resulted in rather toxic HSA derivatives.

Irreversible cleavage of the di-sulphide bridges in the acylated albumins was performed using dithiothreitol. The destruction of the secondary structure was confirmed by CD analysis and resulted in a four and 2000-fold decrease in anti-HIV-1 activity for DTT-Suc-HSA and DTT-Aco-HSA, respectively.

We calculated the relative charge density of the acylated proteins by dividing the added net charge through the number of amino acids in the protein (δ charge/AA). In Figure 3 the antiviral activity versus the δ charge/AA for HIV-1 is plotted. The observed pattern could be fitted with a sigmoidal function ($r^2 = 0.694$).

Potent inhibitory effects were also found for the succinylated immunoglobulins, compounds **26** and **27** (Table 1). They were particularly active against HIV-1 and HIV-2. This is in line with the hypothesis that the negative charge density is an essential feature. On the other hand Aco-conalbumin (compound **32**) was less active than anticipated on the basis of the above mentioned sigmoidal patterns. We also examined the antiviral activities of several compounds against HIV-2 and SIV. As in the case of the anti-HIV-1 activity, the anti-HIV-2 activity correlated with the charge density on the particular proteins (Figure 4, $r^2 = 0.560$). In general a higher net negative charge resulted in an increased anti-HIV-2 effect. Invariably, the negatively charged

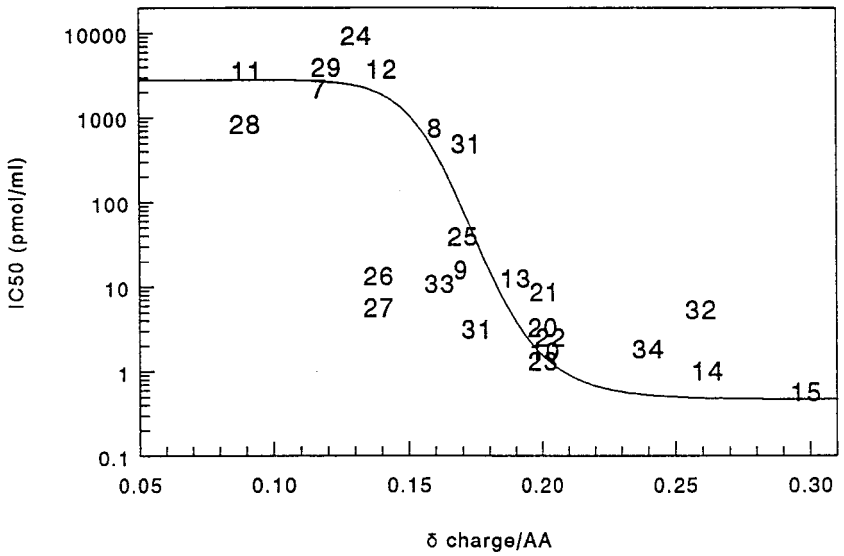

Figure 3 Correlative pattern of the antiviral activity expressed as IC_{50} (pmol·ml^{-1}) against HIV-1 IIIb replication of a series of acylated plasma proteins and their charges, charge density (number of extra negative charges introduced per number of amino acids in the respective proteins). The numbers indicate the derivatized and native proteins as mentioned in Table 1.

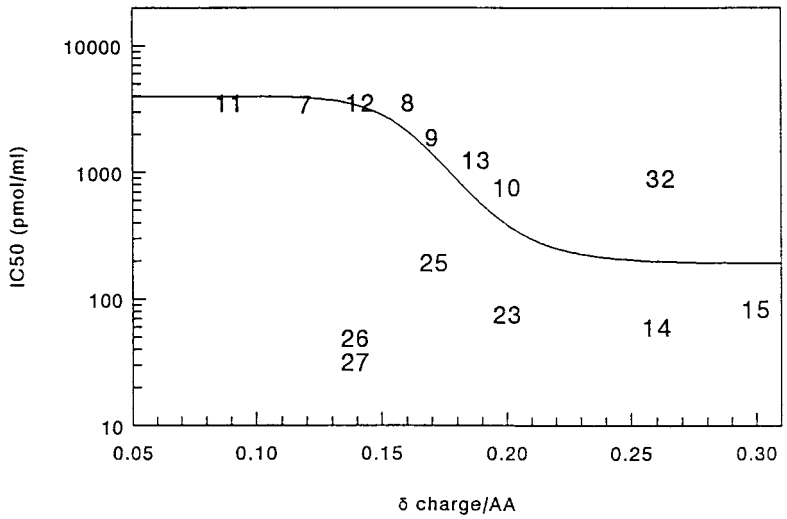

Figure 4 Correlative pattern of the antiviral activity expressed as IC_{50} (pmol·ml^{-1}) against HIV-2 ROD replication of a series of acylated plasma proteins and their charges, charge density (number of extra negative charges introduced per number of amino acids in the respective proteins). The numbers indicate the derivatized and native proteins as mentioned in Table 1.

plasma and milk proteins showed the highest potency against HIV-1 IIIb, followed by SIVmac251 variant, whereas the activity against HIV-2, was only modest. Differences in the exposure of the viral glycoproteins and interaction of the acylated proteins with these glycoproteins in HIV-1, SIV and HIV-2 may explain the observed differences in antiviral activity.

Apart from Lf none of the native proteins produced a significant inhibition of HIV-1 replication (Table 2). Taking into account the high homology in amino acid configuration with Lf, conalbumin itself had no intrinsic activity against HIV-1. Its modest antiviral effect may be explained by the absence of a negatively charged domain linking the N and C lobe as present in hLf and bLf [37]. This essential

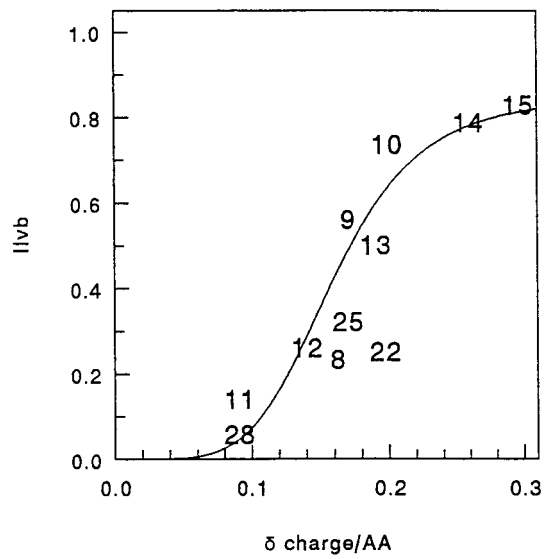

Figure 5 Correlation between potency of inhibition of the gp120–CD4 interaction and the charge density of a series of proteins. The numbers represent the native and reacted proteins as mentioned in Table 1.

domain in the Lf molecule is thought to be responsible for the anti-HIV activity of the two Lfs [21].

Mechanisms of Anti-HIV Effect

Eleven native and charge modified proteins that differ both in charge density and in anti-HIV-1 activity were selected for a study of *in vitro* inhibition of gp120–CD4 binding. From the binding studies we calculated the inhibitory index for anti-gp120 mAb binding (IIvb; Table 1) as described in the 'Materials and Methods' section. As can be seen from Table 1, all negatively charged proteins inhibited gp120–CD4 binding, whereas the native proteins did not (results not shown). Clearly, the inhibitory potency seemed to be dependent on the negative charge density on the proteins (Figure 5) and correlated positively with the *in vitro* IC_{50} values.

Anti-HCMV Effects

All native and acylated proteins were tested for their anti-HCMV activity. Except for Lf, all native and acylated proteins tested were found to be inactive. After cationization, most of the other plasma and milk proteins also showed activity against HCMV, with IC_{50} values ranging from 0.03 to 0.5 μM (Table 2). Cationized-HSA was found to be inactive against HCMV. Compared to the other cationized proteins, its overall charge was slightly negative under the experimental pH conditions (pH 7.4). However, most cationized compounds showed at the same time increased cell toxicity, leading to modest selectivity indices (IC_{50}/CC_{50}) varying from 6 to 70.

Lf obtained from human and bovine origin was the only native protein that was active against cytomegalo virus replication. Interestingly murine Lf was found to be inactive. The *N*-terminal deletion variant hLf^{-2N}, lacking two *N*-terminal amino acids was moderately active whereas the variant omitting three *N*-terminal amino acids (hLf^{-3N}) was completely inactive against CMV. The heterologous protein isolated from egg white, conalbumin was found to be inactive as well.

The anti-CMV activity of Lf was enhanced about ten times upon cationization (see Table 2). Amination to a higher extend, of 140 amino groups marginally improved the antiviral selectivity index of this protein: this drastic modification at the same time increased the toxicity to the cells.

CD Analysis of HSA, Lf and their Derivatives

Analysis of the CD spectra as described by Provencher and Glöckner [33] gives estimates of the amount of α-helix, β-sheet and remainder (random coil and beta-turn) in the protein. Figure 6 shows the CD-spectra of Aco_{28}-HSA and Aco_{61}-HSA. The spectra for HSA and Aco_{28}-HSA were very similar, indicating little change in secondary structure following partial aconitylation. This was confirmed by quantitative analysis of the spectra (Table 3) that showed changes in the order of only 3% or less. The spectrum of Aco_{61}-HSA, however, was clearly different from the other two (Figure 6) and the analysis showed a strong reduction of α-helix content from 61 to 26% with increasing numbers of the other structural elements (Table 3). We concluded that a high degree of aconitylation to some extent seems to unfold the HSA molecule. As expected this effect is even stronger if the Aco_{61}-HSA is treated with dithiotreitol after aconitylation (Table 3). Interestingly, the effect of an extensive succinylation of HSA in Suc_{47}-HSA and Suc_{61}-HSA on α-helix, β-sheet and remainder was much less pronounced as compared to Aco_{61}-HSA. The α-helix content was reduced from 61% for HSA to 48 and 40% for Suc_{47}-HSA and Suc_{61}-HSA, respectively. The introduction of two COOH groups per reacted $-NH_2$ group in Aco-HSA compared to the single COOH group in the case of Suc-HSA may explain these differences. The more strongly polyanionic

Table 2 Anti-HIV and Anti-HCMV Activity and IEP Values of the Charge Modified Proteins

Compound	p*I* (range)	HIV-1		HCMV	
		IC_{50} (nM)	CC_{50} (nM)	IC_{50} (nM)	CC_{50} (nM)
Controls					
Poly-l-lysine	>9.5	>25 000	>25 000	0.6	2.3
Dextran-sulphate 5 kDa	<4.5	4	>50 000	1100	>50 000
Suc-HSA	<4.5	5.4	>3420	>3420	>3420
Aco-HSA	<4.5	0.5	>3280	>3280	>3280
Neo(glyco)proteins					
Cat-HSA	6.3–7.3	>3700	>3700	>3700	>3700
Cat-asialofetuin	7.0–8.5	>1960	1960	132	2650
Cat-transferrin	8.8	>262	262	141	1600
Cat-γ-globulins	9.0	>1670	>1670	34	1490
Cat-orosomucoid	>9.5	nd	nd	278	>17 800
Cat-lysozyme	>9.5	>22 700	>22 700	509	3000
Cat-β-lactoglobulin A	8.8–9.3	1380	6722	283	13 300
Cat-β-lactoglobulin B	8.8–9.3	1500	6050	383	>13 900
Cat-α-lactalbumin	9.0	>1714	1710	435	8790
Cat-β-casein	>9.5	>10 900	>10900	>10 900	>10 900
Cat-conalbumin	>9.5	>3250	>3250	51.9	>3250
bLf	6.5–7.5	500	>3125	450	>3125
hLf	6.5–7.6	930	>3125	1125	>3125
mLf	nd	nd	nd	>3125	>3125
hLf^{-2N}	6.5–7.6	nd	nd	630	>3125
hLf^{-3N}	6.5–7.6	nd	nd	>3125	>3125
Cat_{129}-bLf	7.2–8.2	>1525	>1525	150	>3125
Cat_{140}-bLf	7.5–8.5	nd	nd	88.8	2075

Mean values of at least six experiments.
nd, not determined.

character of Aco-HSA may lead to stronger internal repulsive forces within the protein backbone, leading to changes in the secondary structure without complete unfolding of the protein. Irreversible cleavage of the di-sulphate bridges in the modified albumins (DTT-Aco_{61}-HSA) resulted in a complete destruction of the secondary structure.

Covalent attachment of, for example, azidothymidine-monophosphate to HSA and also cationization of HSA had little effect on the secondary structure of the protein (Figure 6, Table 3), indicating that these derivatizations do not result in gross conformational changes in the HSA molecule.

Succinylation of Lf had little effect on the secondary structure (Table 3) indicating that this protein remains in a virtually native conformation after acylation. The effect on its secondary structure became more pronounced when succinylated Lf was treated with dithiotreitol with a reduction in the α-helix from 27 to 16% and of the β-sheet from 30 to 24%. As was the case for HSA, the effect of aconitylation of Lf is stronger than that of succinylation. In Aco-Lf the α-helical content was reduced from 27 to 16% and the amount of β-sheet increased from 30 to 37% while these values were 21 and 31% after succinylation (Table 3).

DISCUSSION

From the antiviral data of the various succinylated albumins derived from five different albumin sources, we conclude that the moderate difference in amino acid composition of these albumins does not significantly influence the anti-HIV-1 activity. In contrast, irreversible cleavage of the di-sulphide

bridges in the acylated proteins and destruction of the secondary structure of the proteins, led to a marked decrease of the antiviral potency. It is relevant to note that acylation of poly-l-lysine, produced a polypeptide with the highest net charge modification per molecule. Yet, this compound did not show any anti-HIV activity *in vitro* [18].

Interestingly, the acylated proteins were in general ten times more active on a molar basis than the dextran sulfate tested in our series. Therefore, in addition to a polyanionic character, hydrophobic amino acids, and/or partially intact globular structures of the derivatized proteins seem to be requirements for a proper anti-HIV effect. Anti-HIV activities were also recently described for synthetic polypeptides containing high amounts of the acidic amino acids aspartate and glutamate [38]. However, in contrast to the negatively charged albumins, these synthetic polypeptides exhibited no activity against clinical isolates of HIV [19].

Aconitylation of the proteins and especially of HSA resulted in the highest activity against the immunodeficiency viruses tested. The IC_{50} value for Aco_{61}-HSA was about 0.5 nM, corresponding to a concentration of about 0.04 $\mu g \cdot ml^{-1}$. The 50% cytotoxic concentrations for the succinylated and aconitylated proteins were all above 3500 nM, indicating to a low cytotoxicity of the compounds.

The antiviral activity could not be further increased upon derivatization using DTPA. In addition DTPA derivatized albumins turned out to be quite toxic compounds. The general toxic effect of the DTPA derivatives in the *in vitro* antiviral test system can be explained by the strong chelating activity of DTPA towards essential inorganic cations, an effect that may occur in spite of the loss of a single carboxylic group through the coupling reaction [39]. In general the antiviral activities of the negatively charged proteins against HIV-2 and SIV were about 10–100 times lower than against HIV-1. We recently reported that binding of negatively charged albumins to the V3 domain of the viral envelope protein gp120 as well as to receptors on the T-lymphocyte and macrophage cell surfaces may contribute to their potent anti-HIV activity [21,25]. We also showed that Suc-HSA, preferentially interferes with virus/cell fusion whereas Aco-HSA, as an even more negatively charged protein, exhibited a more pronounced effect on virus/cell binding [18].

In contrast to acylation, cationization produced an enhanced effect against HCMV replication. Unfortunately, cationization also affected the toxicity of the compounds.

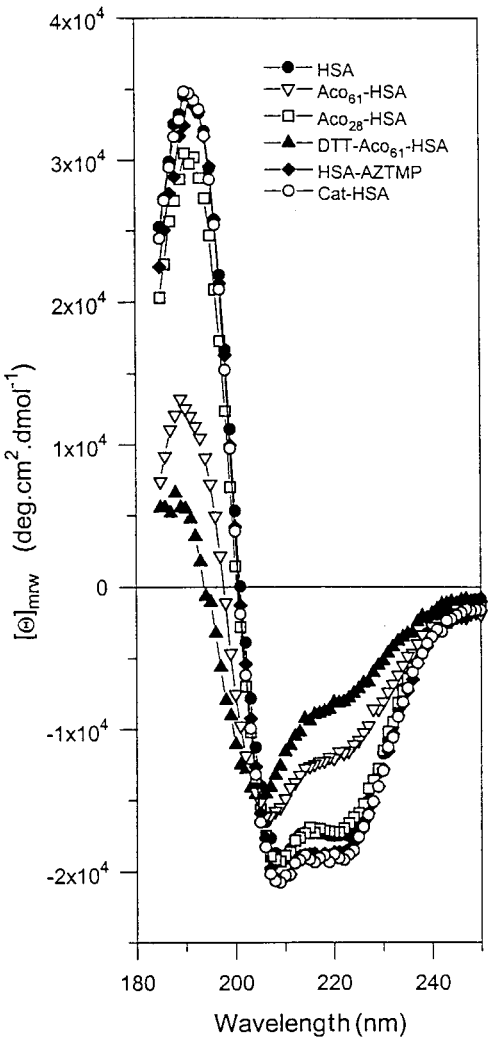

Figure 6 CD spectra of HSA and two charge-modified derivatives.

The difference in anti-HCMV activity between bLf, mLf and hLf could at least partially be explained by differences in the *N*-terminal amino acid sequence in the molecules. Human Lf contains an unique *N*-terminal 'first' basic cluster of four arginine residues (Arg^2-Arg^5), whereas bLf and mLf contain only two basic amino acids or even a single basic residue, respectively. The so-called second basic cluster in hLf (Arg^{25}-Arg^{31}) contains four basic residues, whereas bLf and mLf contain three and two residues in the aligned sequence. Murine Lf, like bovine transferrin, was inactive against HCMV. Moreover, deletion of the three *N*-terminal residues of hLf (hLf^{-3N}) resulted in a complete loss of hLf anti-HCMV activity. These results suggest that the first *N*-terminal basic cluster in hLf and bLf plays a

Table 3 Percentage Protein Folding in Native and Modified HSAs and Lfs

Compound	α-helix	β-sheet	Remainder
HSA	61	6	33
Aco_{28}-HSA	58	8	34
Aco_{61}-HSA	26	29	45
Suc_{47}-HSA	48	17	35
Suc_{61}-HSA	40	21	39
DTT-Aco_{61}-HSA	17	35	48
Cat-HSA	63	1	36
AZTMP-HSA	69	3	28
Lactoferrin	27	30	43
Aco-lactoferrin	16	37	47
Suc-lactoferrin	21	31	48
Cat-lactoferrin	32	29	29
FiTC-lactoferrin	32	29	29
AZTMP-lactoferrin	27	34	39

major role in their anti-HCMV activity and that an intact hLf terminus is required for the anti-HCMV activity. In other studies marked differences in potency of various Lfs against distinct viruses were reported [40]. In addition *N*-terminal proteolysis of commercially available Lfs and/or the presence of contaminants [41,42] may largely influence the outcome of anti-HCMV screening *in vitro*.

The anti-HCMV activity of native hLf as found in the present study was enhanced about ten times (Table 2) upon cationization, suggesting that besides the cationic groups of the *N*-terminus, positive charges in the rest of the molecule may add to the antiviral profile. The effect of the cationized proteins is likely to be due to shielding of the heparan-sulphate-binding site for HCMV on the target cells resulting in a block of viral entry [43]. More drastic cationization of hLf marginally affected the antiviral selectivity index ($SI = IC_{50}/CC_{50}$) of this protein since this modification at the same time increased the toxicity to the cells. Conalbumin, like mLf and bovine transferrin, did not inhibit HCMV as was anticipated on the basis of the absence of an *N*-terminal first basic cluster as present in hLf.

Of the cationized proteins, only Cat-β-lactoglobulins A and B showed some anti-HIV-1 activity (IC_{50}s were about 0.3 μM). These cationic lactoglobulins may interfere with the viral adherence to the cells by an electrostatic interaction with, for example, the negatively charged V2 domain and/or parts of the CD4 receptor.

The relatively small changes in secondary structure of HSA and bLf after succinylation or even following covalent attachment of the dye fluorescein or drugs like AZT, also shown by CD, may explain the low immunogenicity of these protein–drug conjugates as recently described by us [44].

The derivatized plasma and milk proteins investigated in the present study provide interesting lead compounds for the development of antiviral polypeptides. Taking into account their abundant availability and large-scale production, these industrial proteins can, through simple chemical derivatization, provide relatively cheap antivirals for systemic or local administration. In addition they can in principle also be employed as intrinsically active carriers for other antiviral drugs (i.e. nucleoside analogues, protease inhibitors, glycosidase inhibitors and others). Such dual targeting preparations may interfere with HIV/HCMV replication at multiple steps of the replication cycle: virus/target cell binding, virus/cell fusion as well as in the production of new viral particles.

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