

Purification of a Modified Form of Bovine Antithrombin III as an HIV-1 CD8⁺ T-cell Antiviral Factor*

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CD8⁺ T-cells secrete soluble factor(s) capable of inhibiting both R5- and X4-tropic strains of human immunodeficiency virus type 1 (HIV-1). CCR5 chemokine ligands, released from activated CD8⁺ T-cells, contribute to the antiviral activity of these cells. These CC-chemokines, however, do not account for all CD8⁺ T-cell antiviral factor(s) (CAF) released from these cells, particularly because the elusive CAF can inhibit the replication of X4 HIV-1 strains that use CXCR4 and not CCR5 as a coreceptor. Here we demonstrate that activated CD8⁺ T-cells of HIV-1-seropositive individuals modify serum bovine antithrombin III into an HIV-1 inhibitory factor capable of suppressing the replication of X4 HIV-1. These data indicate that antithrombin III may play a role in the progression of HIV-1 disease.

Soluble inhibitory factors produced by CD8⁺ T-cells have been shown to inhibit HIV-1¹ replication and may play a critical role *in vivo* in antiviral host defense (1). These inhibitory factors include CC-chemokines (2–4), which bind to the CCR5 coreceptor and inhibit R5 viral entry into cells (1) (5–7), as well as less well characterized soluble factor(s) produced by CD8⁺ T-cells and termed CD8⁺ T-cell antiviral factor(s) (CAF), which are capable of inhibiting both R5 and X4 HIV-1 (8–15).

Recently, we demonstrated that there are two factors produced by activated CD8⁺ T-cells capable of inhibiting the X4 strain HIV-1_{IIIB} (16). These factors are distinctive in size and the ability to bind heparin. One of these factors bound heparin at physiological salt concentration, eluted at 350 mM NaCl, and was retained by a 50-kDa cut-off Centricon filter. The other factor did not bind heparin at physiological salt concentration and was filtered through a 50-kDa cut-off Centricon filter. The HIV-1 inhibitory activity of these factors was higher with bulk CD8⁺ T-cells of seropositive individuals and HIV-1-specific

cytotoxic T-lymphocytes (CTL) compared with bulk CD8⁺ T-cells of HIV-1-seronegative individuals (16). In the present study we identified the heparin binding inhibitory activity as a CD8⁺ T-cell modified form of antithrombin, which is produced in higher amounts by HIV-1-specific CTL and bulk CD8⁺ T-cells of seropositive individuals than by bulk CD8⁺ T-cells of seronegative individuals. In this study for the first time we demonstrate that CD8⁺ T-cells can activate a serum protein to become inhibitory for HIV, a possibility that has not been addressed previously.

EXPERIMENTAL PROCEDURES

HIV-specific CTL Clones and Bulk CD8⁺ T-Cells—Polyclonal CD8⁺ cells that were 90–99% CD3⁺ and CD8⁺-positive were generated by fluorescence-activated cell sorting from the six seronegative and six HIV-1-seropositive long-term nonprogressors by positive selection with anti-CD8 antibody-coated immunomagnetic beads (PerSeptive Biosystems, Framingham, MA) as described (16). HIV-1-specific CTL clones were used as described (16). Bulk CD8⁺ cell lines from seropositive and seronegative persons were established by incubating purified CD8⁺ cells (2×10^6) with 2×10^7 irradiated allogeneic feeder cells (peripheral blood mononuclear cells) and 0.25 μ g of phytohemagglutinin (Murex, Dartford, UK)/ml for 3 days. Cells were maintained in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 10 mM HEPES, 2 mM glutamine, 100 units of penicillin/ml, 10 μ g of streptomycin/ml, and 50 units of interleukin-2/ml (R10-50). After 2 weeks, 0.5×10^6 cells/ml were stimulated by using CD3 cross-linking in a 1:4 ratio of cells to goat anti-mouse antibody-coated beads (PerSeptive Biosystems) saturated with a mouse anti-human 12F6 CD3 antibody (17) (2 μ g of antibody/ 10^6 cells). The supernatant fluid was harvested after 4 h by centrifugation at $3000 \times g$ for 10 min. The serum-free medium contained 2% (w/v) bovine serum albumin (BSA, Sigma).

Assay for Inhibition of Viral Replication—Human T cells and H9 cell line were acutely infected with X4 HIV-1_{IIIB}, human macrophage-like PMI cells were infected with R5 HIV-1_{JR-CSF}, and macaque T-cell line CEM-174 was infected with SIV-239 or SHIV_{KU-1} at a multiplicity of infection of 10^{-2} TCID₅₀/ml and resuspended in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (R20). The cells were then plated in 2 ml of R20 at 5×10^5 cells/ml in a 24-well plate. Cell supernatant (1 ml) was removed every 3 days and replaced with medium supplemented with fractions (10–100 μ l) of the eluates of the purification process or antithrombin. After 9 days the concentration of p24 for the HIV-1 or p27 antigen for the SIV and SHIV strains were measured with an HIV-1 p24 enzyme-linked immunosorbent assay kit (ELISA; PerkinElmer Life Sciences) or SIV core antigen ELISA kit (Beckman-Coulter, Miami, FL). The percentage inhibition was calculated against the medium control, which had p24 or p27 levels of >100 ng/ml after 9 days of testing.

Purification of Viral Inhibitory Activity—After 4 h, CD3 antibody stimulation at 37 °C supernatant was collected, centrifuged, and applied to a heparin-Sepharose column (5-ml HiTrap heparin-Sepharose column, Amersham Biosciences). The column was eluted with a continuous gradient to 1 M NaCl in phosphate-buffered saline (pH 7.4) as described earlier (16). Inhibitory fractions were pooled and concentrated with a Centricon 50-kDa cut-off centrifugal concentrator. The sample was applied to a Superdex 200 column (3.2 \times 300 mm, Amersham Biosciences), and active 40-kDa fractions were collected as described earlier (16). After the heparin-Sepharose column, the inhibitory

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¹ The abbreviations used are: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SHIV, simian-human immunodeficiency virus; ATIII, antithrombin III; CAF, CD8⁺ T-cell antiviral factor(s); TCID₅₀, 50% tissue culture infective dose; ID₅₀, protein concentration causing a 50% decrease in virus antigen production; CTL, cytotoxic T-lymphocytes; BSA, bovine serum albumin; R20, 20% heat-inactivated fetal calf serum; HPLC, high-pressure liquid chromatography; NF- κ B, nuclear factor κ B; ELISA, enzyme-linked immunosorbent assay; S, stressed; R, relaxed; L, latent.

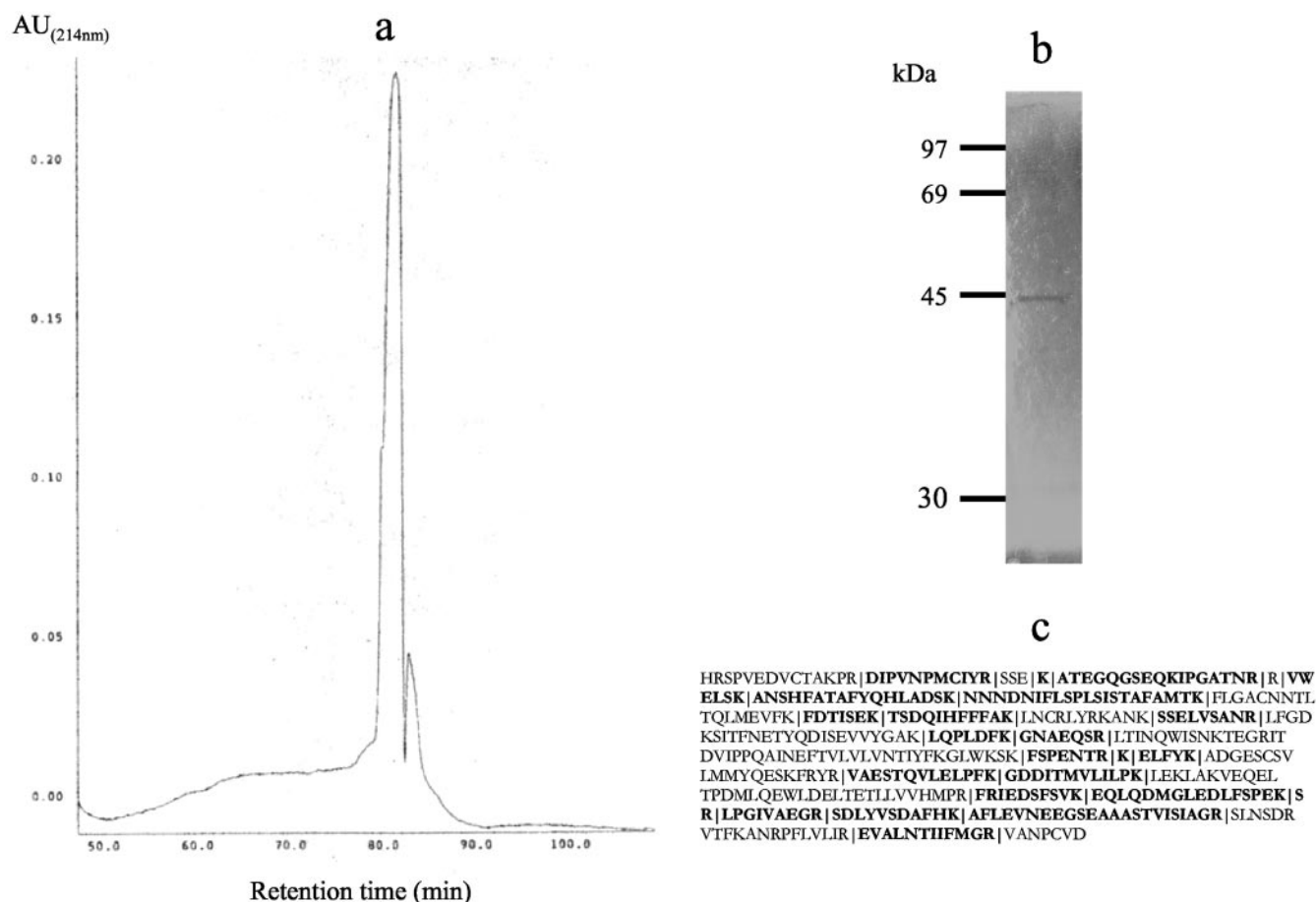


FIG. 1. **Identification of an HIV-1 inhibitor.** *a*, C4 HPLC profile of 40-kDa Superdex 200 HIV-1 inhibitory eluate (AU, arbitrary units at 214 nm). *b*, SDS-PAGE (reduced silver-stained protein) of purified activity (eluate with $ID_{50} = 5.5 \mu\text{g/ml}$) after heparin-Sepharose and Superdex 200 purification. *c*, sequence data of in-gel trypsin-digested protein band. 51% of bovine antithrombin (GenBankTM accession no. GI1168462) identified with masses of 24 different peptides (in **bold type**) after microsequence analysis by reverse-phase HPLC nanoelectrospray tandem mass spectrometry of the tryptic digest. The trypsin cleavage sites found for the identified peptides are indicated with a vertical line (1).

activity was purified 215 times, and after the Superdex 200 column 909 times (16). Fractions that inhibited HIV-1 were applied to a Vydac RP-4 HPLC column equilibrated with H_2O and 0.1% trifluoroacetic acid and tested for purity. Bound protein was eluted with a gradient of acetonitrile in trifluoroacetic acid as described earlier (18). Additionally, the purity of the 40-kDa Superdex 200 eluates was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) with silver staining (16). The protein concentration of the eluates were determined by the bicinchoninic acid method according to the manufacturer's procedure (Pierce). Fractions with >95% purity as tested by C4-HPLC and silver staining were used for the inhibition tests to determine the ID_{50} (concentration ($\mu\text{g/ml}$) of protein necessary for 50% decrease of virus antigen measured by ELISA).

Amino Acid Sequencing of Inhibitory Activity—For amino acid sequencing following separation of the 40-kDa Superdex eluates by SDS-PAGE, the gel was treated with transfer buffer and blotted onto nitrocellulose paper (19). After blotting the nitrocellulose paper was stained with Ponceau Red (Bio-Rad) according to the manufacturer's procedure. The 43-kDa protein stain was cut out and digested with trypsin (Sigma). The tryptic digest microsequence analysis was done by reverse-phase HPLC nanoelectrospray tandem mass spectrometry ($\mu\text{LC/MS/MS}$) on a Finnigan LCQ quadrupole ion trap mass spectrometer.

Production of the Different Antithrombin Configurations—Commercially available ATIII (human or bovine, 0.2–0.4 units/ μg , Sigma), which is purified by heparin-sulfate binding (1 M NaCl eluates), contained the “stressed” (S) configuration. To determine which form of ATIII is capable of inhibiting HIV-1, we further produced a relaxed (R), pre-latent and two latent (L) forms of ATIII from the S form. The R form (Fig. 2A) was produced by incubating S bovine or human antithrombin with porcine pancreatic elastase (Calbiochem) as described (20), and the pre-latent form was produced as described earlier (21). The two different L forms of ATIII were produced by incubating S antithrombin with

0.9 M guanidine (22) or in 0.25 M trisodium citrate at 60 °C for 18 h (23). After incubation, each L form was dialyzed three times against a 1000 \times volume of phosphate-buffered saline. The NH_2 -terminal heparin binding site of S ATIII was cleaved through partial digestion as described (24) using an immobilized V-8 protease kit (Pierce) for 1 h at 4 °C.

Statistical Analysis—The standard error is shown by error bars.

RESULTS

A Modified Form of ATII Is Purified from CTL Cultures as an Inhibitor of HIV-1 Replication—We purified to homogeneity with a purification factor of 909 \times (16) the earlier described 40-kDa (gel filtration) heparin binding HIV-1 inhibitory activity (16) found in activated CD8^+ T-cell supernatants from HIV-specific CTL clones or bulk CD8^+ T-cells. This HIV-1 inhibitory activity, purified using heparin-Sepharose and Superdex 200 size exclusion chromatography, showed one protein peak by C4-HPLC (Fig. 1a). The fractions from the Superdex 200 column that contained anti-HIV-1 activity were pooled, analyzed by SDS-PAGE, and silver-stained under reducing and nonreducing conditions, which revealed a single molecular species migrating at 43 kDa (Fig. 1b). The ID_{50} of these eluates was 5.5 $\mu\text{g/ml}$ (16). Microsequence analysis by reverse-phase HPLC nanoelectrospray tandem mass spectrometry of the tryptic digest revealed the identity of bovine antithrombin III (ATIII), which suggests that activated CD8^+ T-cell of seropositive individuals or HIV-specific CTL clones can modify this serum protein. The 24 different pep-

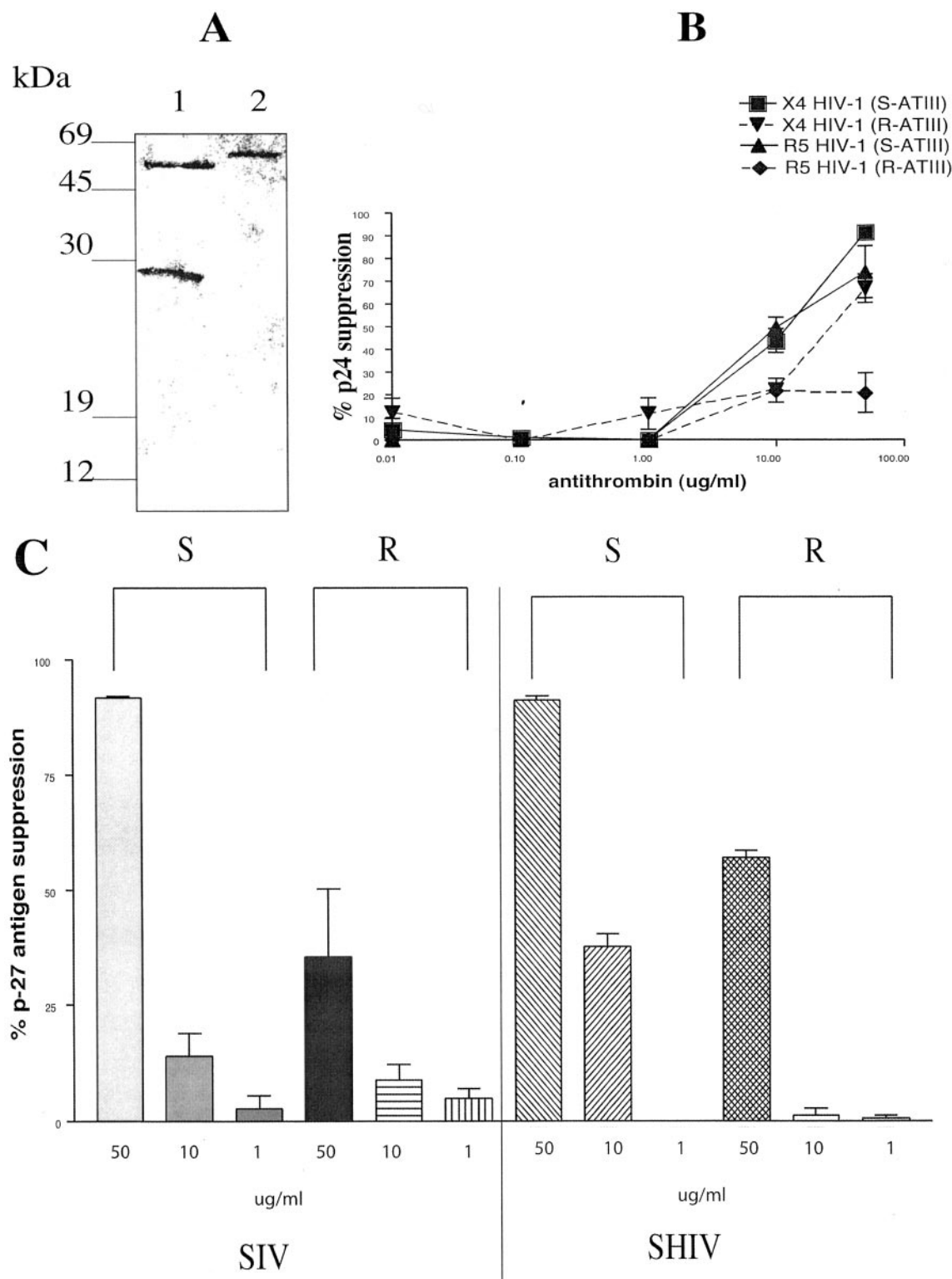


FIG. 2. **SDS-PAGE of S and R form of ATIII (A), inhibition of X4 HIV-1 (B), and inhibition of SIV and SHIV with the S and R form of ATIII (C).** A, SDS-PAGE of reduced Coomassie Blue-stained porcine elastase-digested (lane 1) and undigested ATIII (lane 2). Porcine elastase cleaves ATIII in an ~3-kDa peptide (not shown) and an ~50-kDa protein. Porcine elastase runs at ~28 kDa (second protein seen in lane 1). B, X4 HIV-1, R5 HIV-1. C, SIV (SIV₂₃₉) and SHIV (SHIV_{KU-1}) inhibition with S and R form (S-ATIII, R-ATIII). X4 HIV-1, SIV, and SHIV were inhibited by both forms. No inhibition was seen for the relaxed form of the R5 virus. Virus inhibition was calculated using the buffer controls or the enzyme controls. Virus antigen in the controls at this time were above 100 ng/ml. The standard error is shown for 2–10 independent experiments.

tides sequenced (shown in *bold type* in Fig. 1c) were identical to bovine antithrombin III.

HIV-1 Inhibitory Activity of Known Forms of ATIII—Having identified a form of bovine ATIII as an inhibitor of HIV-1 replication, we determined which of the described forms of

ATIII is able to inhibit HIV-1. We therefore investigated whether native or enzymatically modified forms of ATIII can inhibit HIV-1 replication. Under physiological conditions, ATIII exists in different forms. ATIII circulates in a quiescent L form, in which its reactive COOH-terminal loop is not fully

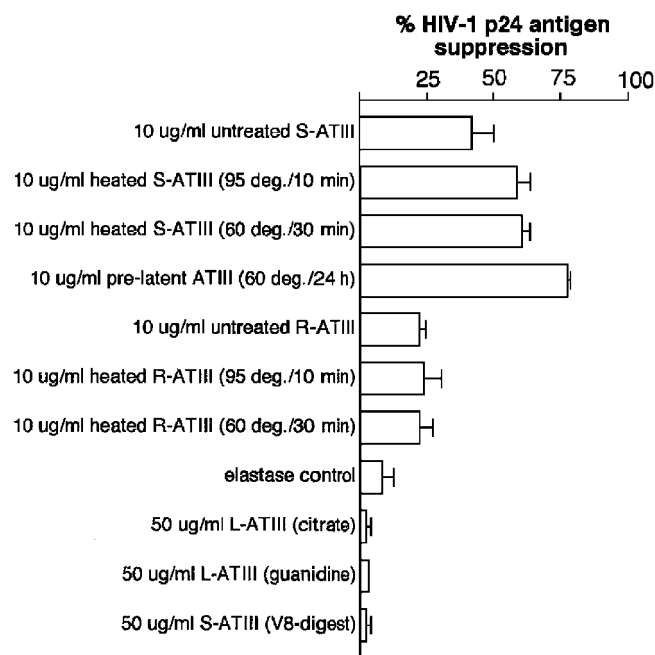


FIG. 3. **X4 HIV-1 inhibition with various forms of ATIII.** X4 HIV-1 inhibition with: heat-treated S and R form (95 °C, 10 min and 60 °C, 30 min treatment), a pre-latent form (60 °C, 24 h), with porcine elastase alone as a control, the L forms produced either through citrate or guanidine treatment, and a V8 protease-digested S form. Whereas the L forms could not inhibit HIV-1, the antiviral activity of the S form and the R form was heat-stable. V8 protease treatment abolished antiviral activity. HIV-1 p24 antigen levels in the controls were above 100 ng/ml. Standard error is shown for two independent experiments.

exposed and cannot bind target proteins. When ATIII binds to heparin, however, a stressed conformation, the S form, is induced exposing the reactive COOH-terminal loop and increasing thrombin binding affinity by 100-fold. The thrombin-ATIII complex then slowly dissociates, and the reactive loop of ATIII is cleaved by the released thrombin. The cleaved ATIII consists of disulfide-bonded A and B chains and does not bind target proteases. Additionally, this cleavage induces a conformational change to a relaxed, R form, in which the reactive loop is irreversibly inserted into an A- β sheet (25). An R form of ATIII was purified based on its antiangiogenic activity capable of inhibiting tumor growth. This form of ATIII is cleaved between Ser³⁸⁶ and Thr³⁸⁷ and can be generated by digesting with porcine elastase (20). Other enzymes can cleave ATIII and produce additional R forms such as thrombin (Arg³⁹⁴-Ser³⁹⁵), pancreatic elastase (Val³⁸⁸-Ile³⁸⁹) and human neutrophil elastase (Ile³⁹¹-Ala³⁹²) (26, 27). A "pre-latent" antithrombin with anti-angiogenic activity has also been described in which the antithrombin activity and a high heparin binding affinity are preserved (21). To determine which form of ATIII is capable of inhibiting HIV-1, we produced the R (Fig. 2A, lane 1), pre-latent, and L forms of ATIII from a commercially available S form (Fig. 2A, lane 2) and tested their ability to inhibit HIV and SIV/SHIV. We found that the R form of antithrombin inhibited X4 virus with half-maximal inhibition (ID₅₀) at ~25 μ g/ml. The S form was even more potent and had an ID₅₀ of 10 μ g/ml (~3 units/ml) (Fig. 2B). This is similar to the ID₅₀ (5.5 μ g/ml, 130 nM) we found for the CD8⁺ T-cell-modified antithrombin purified from CTL-conditioned medium and also to the ID₅₀ (89 nM) found for SDF-1 using the same *in vitro* system (16). No substantial inhibition of the R form (suppression <25%) was found for the R5 virus tested (Fig. 2B). The S form inhibited SIV and SHIV replication 92 and 91%, respectively, at a concentration of 50 μ g/ml (~15 units/ml). The R form inhibited these strains

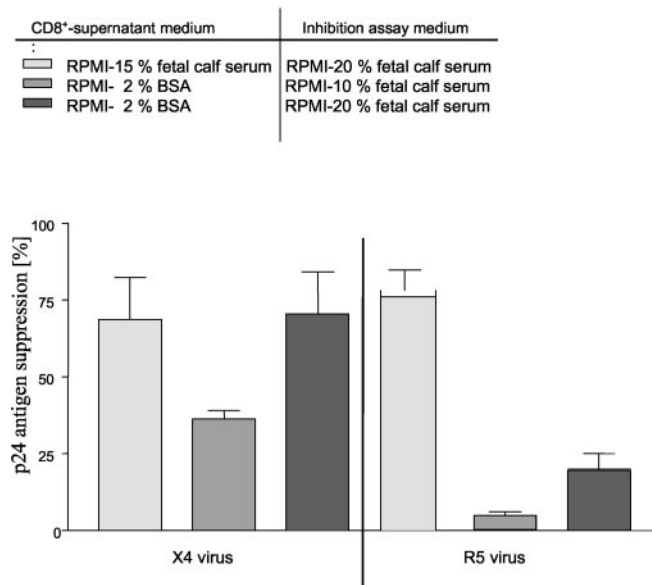


FIG. 4. **The CD8⁺ T-cell-produced heparin nonbinding activity activates a serum protein.** Inhibition of R5 and X4 viruses were measured with CD8⁺ T-cell supernatants containing serum proteins or containing only BSA. CD8⁺ T-cells were stimulated in 10% RPMI, 10% (v/v) fetal calf serum and then tested in an inhibition test with 20% (v/v) fetal calf serum (RPMI, 10% (v/v) fetal calf serum, 20% (v/v) fetal calf serum). Inhibition was detectable showing that there is a second factor in addition to the ATIII inhibiting HIV-1. We then stimulated CD8⁺ T-cells in 10% RPMI, 2% (w/v) BSA and tested the mixture in an inhibition test with 10% (v/v) fetal calf serum (RPMI, 2% (w/v) BSA, 10% (v/v) fetal calf serum), comparing the result with CD8⁺ T-cells that were stimulated in 10% RPMI, 2% (w/v) BSA; we then tested it in an inhibition test with 20% (v/v) fetal calf serum (RPMI, 2% (w/v) BSA, 20% (v/v) fetal calf serum). We found at least a doubling of activity in the serum of higher protein concentrations for both R5 and X4 viruses, which is consistent with our hypothesis. HIV-1 p24 antigen exceeded 2 ng/ml in the controls. The standard error is shown for four independent experiments.

to a lesser extent (36 and 57%, respectively) (Fig. 2C). Although the pre-latent form was effective in inhibiting X4 HIV replication, all L forms tested at up to 50 μ g/ml had no inhibitory activity (Fig. 3).

We also tested these various forms of ATIII for heat and enzyme sensitivity and found that both the S and R forms were heat-stable (95 °C, 10 min or 60 °C, 30 min) under physiological salt conditions (Fig. 3). We also found that partial V8 protease digestion, which specifically cleaves the heparin binding site (24), decreases the activity of the S form (Fig. 3), suggesting that this heparin binding domain is important for inhibition. We also found that bovine and human ATIII have a similar profile of inhibitory activity (data not shown). In summary, our data indicate that the purified CD8⁺ T-cell-modified form of ATIII is a unique form of ATIII with respect to size, heparin affinity, heat stability, and HIV inhibitory activity.

To exclude the toxic effects of ATIII in the HIV-1 inhibition assay, we analyzed the effects of the various ATIII preparations on cell growth and cell viability. These forms of ATIII used were not toxic to the H9 CD4⁺ T cells used for the inhibition tests and did not affect cell viability or growth as measured by trypan blue dye exclusion staining (data not shown), which is consistent with previous reports (20).

CD8⁺ T-cells of Seropositive Individuals Modify Serum ATIII into a Form Capable of Inhibiting HIV-1—In contrast to activated CTL supernatants or CD8⁺ T-cells supernatant of HIV-1 seropositive individuals, serum-containing medium alone and supernatants from unstimulated CD8⁺ T-cells grown in serum-containing medium did not substantially inhibit HIV-1_{IIB} replication (suppression <25%) even when applied to the

heparin-Sepharose column. The activity of CD8⁺ T-cells in HIV-1-seronegative individuals was below 10% inhibition after Superdex 200 gel filtration. Additionally, using untreated serum or supernatants from HIV-1-seronegative individuals, the 43-kDa form of ATIII was not detected following heparin-Sepharose chromatography and Superdex 200 chromatography by either SDS-PAGE silver staining or C4-HPLC (data not shown). These data strongly suggest that activated CD8⁺ T-cells of HIV-1-seropositive individuals modify ATIII into a form that is capable of inhibiting HIV-1. Because unprocessed ATIII is a glycoprotein with a molecular mass of 53–58 kDa, where 10% of the weight is glucosamine-based carbohydrate chains, and because the form of ATIII we purified with HIV-1 inhibitory activity migrates at 43 kDa by SDS-PAGE or 40 kDa by gel filtration, this led us to hypothesize that activated HIV-specific CTL or CD8⁺ T-cells of HIV-1-seropositive individuals modify ATIII into a form with HIV-1 inhibitory activity.

To test the hypothesis that the heparin binding anti-HIV-1 activity is produced through the modification of ATIII by a heparin nonbinding activity released by activated CD8⁺ T-cells, CD8⁺ T-cells were stimulated in serum-free medium and the supernatant was tested for suppressive activity. We found no HIV-1 inhibitory activity in the heparin-bound fractions (data not shown). However, there was still measurable inhibitory activity in the unfractionated supernatants that contained no serum (Fig. 4) when added to an HIV-1 inhibition assay that contained serum, suggesting that the non-heparin binding inhibitory factor was still released when CTL were cultured without serum proteins. To confirm that the non-heparin binding inhibitory activity modifies serum proteins, we tested the inhibitory activity of medium containing twice the amount of serum, reasoning that this would double the amount of substrate. Increasing the amount of fetal bovine serum proteins from 10 to 20% (v/v) doubled the inhibitory activity for both M- and T-tropic HIV (Fig. 4), suggesting that serum protein is responsible for the CTL antiviral activity and that CTL modify this serum protein.

DISCUSSION

We purified a form of bovine ATIII activated by HIV-1-specific CTL and bulk CD8⁺ T-cells of seropositive individuals that is ~10 kDa smaller than unprocessed ATIII as an inhibitor of HIV-1 replication, and as such, we identified a novel biological activity for ATIII. We demonstrated that the previously described S, R, and pre-latent forms of ATIII inhibited HIV-1, whereas the L form did not. The HIV-1 inhibitory activity of the S, R, and pre-latent ATIII was heat-stable. We also demonstrated that there is another factor produced by activated CTL that generates this modified form of ATIII and speculated that this might be the <50-kDa HIV-1 inhibitory factor that we had described previously (16). The failure of bulk CD8⁺ T-cells of seronegative individuals to produce this activity in equal amounts might be the result of a less activated or more naive population of CD8⁺ T cells from seronegative individuals (16), resulting in the production of less factor able to modify ATIII. To date, the search for the elusive CAF has focused on CD8⁺ T-cells secreted factors. For the first time we have demonstrated that CD8⁺ T cells can activate a serum protein to become inhibitory for HIV. Proteolytic processing with an increase of HIV-1 inhibitory activity has been demonstrated for the chemokine MDC (monocyte-derived chemokine), which is cleaved at its NH₂ terminus by CD26/dipeptidyl-peptidase IV (28–30). The modification of serum ATIII that we have described remains unresolved, and it seems plausible that part of CAF might in fact be a protease.

There is a growing body of evidence that ATIII has biological activity in addition to its functions in the coagulation cascade.

For example, ATIII has anti-inflammatory activity in sepsis (31), has anti-angiogenic activity and can inhibit tumor growth (20), and has chemotactic activity for neutrophils (32, 33). The mechanism of action of the diverse biological activity of ATIII, including its HIV-1 inhibitory activity, has not been elucidated. We do not believe that ATIII HIV-1 inhibits HIV-1 as a result of being a competitive agonist at CXCR4, because we did not observe, in our earlier experiments, that the purified protein down-regulates CXCR4 or induces a Ca²⁺ flux in primary CD4⁺ T-cells or H9 cells (16). However, ATIII has been shown to down-regulate NF-κB activity in monocytes (34), a feature that has also been described for CAF (8–15). ATIII has also been shown to bind to the syndecan family of proteoglycans, which may also mediate some of the biological activities of ATIII. In this regard, HIV-1, SIV, and SHIV have a requirement for syndecans for cell attachment, which facilitates HIV/SIV entry into cells (35, 36). In addition, another heparin-binding serine protease inhibitor, the secretory leukocyte protease inhibitor (SLP), is able to inhibit HIV-1. It has been suggested that SLP is an entry inhibitor but blocks a receptor distinct from chemokine receptors and CD4 (37–42). Furthermore, another serine protease inhibitor, α₁-antitrypsin, also has been described as an inhibitor of HIV-1. It was shown that α₁-antitrypsin HIV-1 inhibition occurs partly through blocking entry and partly through down-regulation of NF-κB activity. In our studies, we used α₁-antitrypsin for a comparison with ATIII but found no inhibitory activity for α₁-antitrypsin at up to 50 μg/ml (data not shown) in assays in which 10 μg/ml of ATIII was inhibitory. It is interesting to note that α₁-antitrypsin lacks the NH₂-terminal heparin binding site of ATIII and needs 100–500 times more protein (2–5 mg/ml) (43) than ATIII to achieve a similar HIV inhibition. Together with our findings regarding the sensitivity of the anti-HIV-1 activity of ATIII to partial V8 protease digest, which cleaves preferentially the NH₂-terminal heparin binding site (21), these data suggest that the heparin binding site may be important for the HIV-1 inhibitory effect.

Hypercoagulable states in HIV-1 patients have been associated with decreased ATIII levels and have been correlated with HIV-1 disease progression (44). Our data support the novel concept that ATIII may be protective in HIV-1 disease by inhibiting HIV-1 replication and also suggest that ATIII could be used therapeutically in conjunction with other antiviral agents to treat HIV-1 patients.

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