Heparin, a polyanion, exerts its main activity to inhibit coagulation through a serine protease inhibitor, antithrombin III. Previous studies have clearly shown that heparin in the absence of antithrombin III also has the capacity to regulate C activity. The present studies examined the ability of purified human antithrombin III to regulate classical and alternative pathways of C alone and in the presence of heparin. Antithrombin III alone inhibited generation of both pathways in a dose-related manner; antithrombin III at 8 μg/10⁶ cellular intermediates inhibited generation of the classical and alternative pathway convertases by 60 and 42%, respectively. Antithrombin III and heparin augmented each other's capacity to inhibit generation of both convertases in a dose-related manner. Antithrombin III did not appear to inhibit on the basis of charge because it is only slightly anionic (isoelectric pH value, 5.0); instead, antithrombin III acted as a protease inhibitor of the proteolytic enzymes of the C cascades. Antithrombin III acted only to inhibit formation of the alternative pathway convertase but had no activity on terminal lysis by this pathway; similarly, antithrombin III inhibited preformed EAC1.4b,2a,3b but had no activity on classical pathway cellular intermediates containing additional components. Finally, antithrombin III inhibited consumption of factor B hemolytic activity in a reaction mixture that also contained factor D and C3b, suggesting that factor D activity was also inhibited. These studies demonstrate the capacity of antithrombin III to regulate C activity and suggest that, in concert with heparin, antithrombin III may play an important role in the regulation of C in vivo.

ATIII, a SERPIN, acts with heparin, a polyanion, acidic polysaccharide, to inhibit coagulation (1). In the absence of the polyanion heparin, synthetic ATIII analogues have been shown to regulate activity of serine proteases of the C system (2, 3). Investigators have suggested that ATIII may also bind to C5b-9, may inhibit Cts in the presence of heparin, and may form a trimolecular complex with thrombin and S-protein (4-6). Previous studies have clearly demonstrated the ability of heparin and heparin oligosaccharides to act on both the classical and the alternative pathways of C to regulate C activity (7-11). This activity is similar to other activities of heparin that are unrelated to its anticoagulant activity and do not require the presence of ATIII as a cofactor (12, 13).

Heparin and ATIII have not been studied for their ability to regulate each other's activity on C. Furthermore, native ATIII has not been examined extensively for ability to regulate formation and function of the classical and the alternative pathways of C. The present study was designed to examine the ability of native human ATIII, in the presence or absence of heparin, to act on the C system.

MATERIALS AND METHODS

Purification of human ATIII: The ATIII used in these studies was prepared for chemical homogeneity (from human plasma) by (i) affinity chromatography on Sepharose-agarose (Bio-Rad, Richmond, Calif.) and (ii) gel filtration on DEAE-cellulose (Bio-Rad). The ATIII was eluted with Tris, pH 7.0, and concentration were assessed as described previously (15). The ATIII showed a single band on anodization of Coomassie blue-stained gels that had been subjected to SDS-PAGE; gels scanning showed the ATIII to be greater than 95% pure. A cluster of five bands between pH 4.9 and 5.3 was seen on IEF; this cluster could be reduced to a single band at pH 5.0 when the preparation was treated with neuraminidase. Less than 1% heparin was present by weight when this ATIII preparation was examined for heparin contamination by carbazole assay for uronic acid content (16).

Buffers and components. GVB (17), GVB containing 0.5% dextran, DGBV®, or GVB containing 40 mM EDTA were used as buffers in the hemolytic assays. Properdin was isolated (18) from 2.5 liters of outdated human plasma, which had been collected at the University of Iowa Blood Donor Center in sodium citrate and which was oxidized by the addition of 1.0 M CaCl₂. The clot was removed by centrifugation and filtered through muslin. The resulting material was then dialyzed against 2 mM Tris, 2 mM EDTA, pH 7.5 buffer to produce a cagobulin precipitate, which was centrifuged. The precipitate was redissolved in veronal buffered saline containing an additional 0.15 M NaCl, dialyzed against 0.1 M Tris, 0.1 M EDTA, pH 9.0 buffer and loaded onto a 5 X 100-cm column containing QAE-A50 Sephadex ion-exchange medium (Pharmacia Fine Chemicals, Piscataway, N.J.), which had been equilibrated in 0.1 M Tris, 10 mM EDTA, pH 9.0 buffer. Properdin was found in the wash from this column, which was adjusted to pH 7.0 and ionic strength 3.0 mM and applied to a 2.5 X 100-cm column containing SP-C25 Sephadex ion-exchange medium (Pharmacia) eluted in 0.1 M sodium acetate, 0.4 M NaCl, 3.0 mM buffer. Properdin was eluted using a linear gradient to 0.65 M NaCl. The resulting material was concentrated by Amicon ultrafiltration (Banters, MA) and passed over a 2.5 X 100-cm column containing C-200 Sephadex Superfine (Pharmacia). The resulting material was pure by immunoelectrophoretic and functional criteria. The properdin was divided into small aliquots and frozen at -70°C for later use.
Factor III for these studies was obtained (19) from 1 liter of outdated human plasma that had been collected in sodium citrate. The plasma was adjusted to pH 7.5, ammonium sulfate (320 g/l) was added and stirred for 1 h at 4°C, and the mixture was centrifuged. The precipitation was then removed, and the supernatant was adjusted to pH 7.0 and dialyzed against 0.05 M sodium acetate, 2 mM EDTA, pH 6.0 buffer. The supernatant was then applied to a 5 x 100 cm column containing CMS-2 cellulose ion-exchange medium (Whatman, Clifton, N.J.) equilibrated in 0.05 M sodium acetate, 2 mM EDTA, pH 6.0 buffer, and the column was washed with two bed volumes of starting buffer and eluted with a 10-liter linear gradient to a limiting buffer, which contained 0.5 M sodium acetate, 2 mM EDTA, 0.25 M NaCl pH 7.0. Fractions containing factor B were pooled and concentrated by Amicon ultrafiltration, adjusted to pH 7.0, and dialyzed against 0.01 M Tris, pH 7.0 buffer, and loaded onto a 2.5 x 100 cm column containing DEAE-cellulose ion-exchange medium (Whatman) equilibrated in 0.01 M Tris, pH 7.0 buffer. The column was washed with two bed volumes of 0.01 M Tris, pH 7.0 buffer, and factor B was eluted using a 1-Liter linear gradient to 0.15 M NaCl, pH 7.0. The resulting material was pure by immunoelectrophoretic and functional criteria. The factor B was divided into small aliquots and frozen at -70°C for later use.

Factor D was obtained (20) from 250 ml fresh human serum. The serum was adjusted to pH 7.5 and to 3.5 M NaCl by the addition of 0.15 M NaCl. Two liters of ice-cold distilled water were added to the column containing QAE-Sephadex equilibrated in 10 mM Tris, 50 mM NaCl, 2 mM EDTA, pH 7.5 buffer. The column was washed with two bed volumes of starting buffer, and factor D was eluted using an 8-liter linear gradient to 10 mM NaCl, 2 mM EDTA, pH 7.5. Fractions containing factor D were pooled and concentrated using Amicon ultrafiltration and loaded onto a 2.5 x 100 cm column containing G-75 Sephadex Superfine (Pharmacia), which had been equilibrated with Tris-NaCl buffer solution (10 mM Tris buffer, pH 7.5, 100 mM NaCl, 2 mM EDTA, and 0.025% gelatin). Fractions containing factor D were then pooled and concentrated and rechromatographed using the G-75 column. Finally, the fractions containing factor D were pooled, concentrated, and made into small aliquots, and frozen at -70°C for later use. The resulting material was pure by immunoelectrophoretic and functional criteria.

C3 was purified to homogeneity and quantitated as described previously (5). C1, C2, C5, C6, C7, and C9 were similarly purified (22) or were purchased from Diametex, Miami, FL. C3b was generated from purified C3 using cobra-factor convertase as described (7). Rat and guinea pig serum as sources of terminal components for the alternative and classical pathway assays, respectively, were purchased from Rockland, Inc. (Gilbertville, PA) and diluted in 40 mM EDTA (C-EDTA).

C reactions. EAC 4b and EAC 3b cellular intermediates were prepared as described (23, 24) and EAC 3b were produced by incubating 1 x 10⁶ EAC 4b with an excess of guinea pig C2 and 100 µg C3. In each experiment using these cellular intermediates, inhibition was based upon a uninhibited control tube, which contained about 10⁶ C56789-c5b-9. Effect of ATIII and/or heparin on alternative pathway activity. Briefly, the regulatory activity of ATIII and/or heparin on the alternative pathway was assessed by incubating 1 x 10⁶ EAC 4b, 3b with 0.031 ng factor B, 80 ng factor P, and excess factor D with and without ATIII and/or heparin (1 µg EAC 4b, 3b) in 0.3 ml DDBV for 30 min at 30°C in a shaking water bath. Then 0.7 ml C-EDTA was added and incubation was continued for an additional 50 min at 37°C. Next, 1.5 ml saline was added to each tube except the 100% lysed tube, which received water. The tubes were shaken and centrifuged. Lytic activity of the cellular intermediates was determined by measuring the optical density of the supernatant at 414 nm to determine the average number of lytic sites per cell. The reagent blank tube contained cellular intermediates and C except that factor B was omitted. Noninhibited tubes contained cellular intermediates and C. Effect of ATIII and/or heparin on classical pathway activity. Briefly, the regulatory activity of ATIII and/or heparin was examined by incubating a reaction mixture consisting of 1 x 10⁶ EAC 1b, 2b, 0.8 units factor B, 600 µg factor D, and 100 µg C7 with or without ATIII and/or heparin in 0.4 ml DDBV for 30 min at 30°C. Then, 10 units each of C8 and C9 were added in 0.1 ml DDBV. Incubation was continued for 60 min in a shaking water bath at 37°C. 1.5 ml saline was added to each tube except the 100% lysed tube, which received water. The tubes were then shaken and centrifuged, and lysis was determined. The reagent blank tube contained cellular intermediates and C except that C2 was omitted. Noninhibited tubes contained cellular intermediates and C but no ATIII and/or heparin. Effect of ATIII on terminal lysis of cellular intermediate containing alternative pathway convertase. Activity of ATIII on terminal was assessed (1) 1 x 10⁶ EAC 4b, 3b, 0.4 ng factor B, 220 ng factor P, and excess factor D with and without ATIII were incubated in 0.4 ml DDBV for 30 min at 30°C. Next, 1 ml of iced cold DDBV was added and the cells were shaken and centrifuged. The cells were washed twice, resuspended in 0.1 ml 40 mM EDTA and 0.3 ml of C-EDTA, and lysozyme was assayed. Next, 1 ml of iced cold DDBV was added and the cells were shaken and centrifuged. The cells were washed twice, resuspended in 0.1 mM 40 mM EDTA with and without ATIII, and 0.3 ml C-EDTA was added. Incubation was continued for 60 min at 30°C and lysis was assessed.

Effect of ATIII and/or heparin on classical pathway activity. The fluid phase model for the formation of C3b Bb. We examined the ability of ATIII to interfere with fluid phase consumption of factor B in a reaction mixture that also contained C3b and factor D. For these experiments, 1.6 µg C2b and about 26 ng factor D alone with and without various concentrations of ATIII were warmed to 30°C in 150 µl DDBV. At time zero, 2 µg factor B in 50 µl DDBV was added and at timed intervals, 10 µl aliquots were removed, added to 1 ml ice-cold DDBV, and subsequently assayed for factor B hemolytic activity as described previously (5).
30°C, following which lysis was assessed.

Comparison of effects of ATIII on early events in the classical pathway. 1) 1 x 10^5 EAC1.4b were incubated with various concentrations of ATIII or buffer alone in 0.2 ml DGBB^-m for 30 min at 30°C. Next, 1 ml of ice-cold DGBB^-m was added and the cells were shaken and centrifuged; the cells were then washed twice, resuspended in 0.3 ml DGBB^-m containing 0.125 unit C2, and incubated for 3 min at 30°C. Then 0.3 ml C-EDTA was added and incubation was continued for 60 min at 37°C, after which lysis was determined.

2) 1 x 10^5 EAC1.4b and 0.25 unit C2 were incubated with various concentrations of ATIII or buffer alone in 0.3 ml DGBB^-m for 30 min at 30°C. Next, 1 ml of ice-cold DGBB^-m was added, the cells were shaken and centrifuged; the cells were then washed twice, resuspended in 0.3 ml DGBB^-m, 0.3 ml C-EDTA was added, incubation was continued for 60 min at 37°C, and then lysis was determined.

RESULTS

Regulation of alternative pathway activity. First, we tested the ability of ATIII with or without heparin to regulate alternative pathway convertase activity (Fig. 1). Heparin alone at 1 μg/10^6 EAC4b,3b inhibited lysis by 57%. ATIII alone inhibited lysis is a dose-related manner. Heparin and ATIII augmented each other's capacity to inhibit lysis. The data are presented in μg/10^6 cellular intermediates because previous studies have shown that the volume of the reaction mixture has little effect on the activity of a given amount of inhibitor (11).

Regulation of classical pathway activity. Next, we examined the potential for ATIII with or without heparin to regulate classical pathway convertase activity (Fig. 2). Heparin alone at 10 μg/10^6 EAC4b,3b inhibited lysis by 38%. ATIII alone inhibited lysis is a dose-related manner. Again heparin and ATIII augmented each other's capacity to inhibit lysis. In a parallel experiment, we determined that extensive dialysis of ATIII against saline did not significantly diminish the ability of the ATIII to regulate C-activity, demonstrating that the ATIII buffer was not responsible for the inhibitory activity seen in the ATIII preparations. However, we did observe that as the ATIII was frozen and thawed, its activity on C was decreased; this problem was overcome by separating and freezing the ATIII in small single use aliquots for these studies.

Effect of ATIII on terminal lysis of cellular intermediates containing alternative pathway convertase. We examined whether the effect on the alternative pathway was on the formation of the convertase or whether it was on terminal lysis (Fig. 3). In this experiment, ATIII was added either: 1) during the formation of the cell-bound convertase and the cells were washed before terminal components were added; or 2) with the terminal components. Again, ATIII inhibited the formation of the alternative pathway convertase in a dose-related manner but had no activity when added with the terminal components.

Ability of ATIII to regulate the fluid phase model for the formation of alternative pathway convertase (C3b,Bb). ATIII was examined for capacity to interfere with the fluid phase consumption of factor B in a reaction mixture that also contained C3b and D, a model for fluid phase C3b,Bb formation and decay (Fig. 4). ATIII interfered with factor B consumption in a dose-related manner from 3 to 0.3 μg/reaction mixture, suggesting an activity on factor D.

Effect of ATIII on various classical pathway convertases. Next, we formed various classical pathway convertases on sheep E in the presence of ATIII to determine the magnitude of an effect that ATIII would have on lysis of these cellular intermediates (Fig. 5). ATIII had comparable activity on the formation of EAC1.4b,2a, EAC1.4b,2a,3b, and EAC1.4b,2a,3b,5b.

Effect of ATIII on various preformed convertases of
Figure 4. Ability of various concentrations of ATIII to inhibit fluid phase consumption of hemolytic factor B activity in a reaction mixture that also contained factors D and C3b. Factor B activity remaining was plotted over time. ATIII interfered with factor B consumption in a dose-related manner.

Figure 5. Effects of ATIII on the formation of EAC1.4b,2a (●), EAC1.4b,2a,3b (●), and EAC1.4b,2a,3b,5b (●). ATIII had comparable activity on the formation of EAC1.4b,2a, EAC1.4b,2a,3b, and EAC1.4b,2a,3b,5b.

Figure 6. Effects of ATIII on preformed EAC1.4b,2a,3b (●), EAC1.4b,2a,3b,5b (●), EAC1.4b,2a,3b,5b,6 (●), and EAC1.4b,2a,3b,5b,6,7 (●). Preformed EAC1.4b,2a,3b were inhibited from subsequent lysis, whereas cellular intermediates that contained additional C proteins were not inhibited by ATIII.

the classical pathway. We examined the ability of ATIII to act on preformed convertases of the classical pathway to determine the steps at which this SERPIN had activity (Fig. 6). As expected, preformed EAC1.4b,2a,3b were inhibited from subsequent lysis, whereas cellular intermediates that contained additional C proteins were not inhibited at all by ATIII. In a companion experiment, we compared the effects of ATIII on preformed EAC1.4b,2a,3b with an effect on the formation of the EAC1.4b,2a convertase (Table 1). ATIII had comparable activity, suggesting that in both cases it acted on the C1 to render it incapable of acting on C2.

**DISCUSSION**

Heparin and other polyanions have been studied extensively for capacity to regulate C (7–11). Heparin clearly has the ability to regulate multiple steps in both the classical [25–28] and the alternative pathways (7, 9–11) of C and has been shown to; potentiate the activity of C1 esterase inhibitor (8); interfere with C1q binding to immune complexes (25); inhibit C1r interaction with C4 and C2, and C2 binding to C4 (27, 28); suppress reactive lysis (29); inhibit generation of cell bound and fluid phase alternative pathway convertase (7); inhibit preformed alternative pathway convertase (7); and impair factor H-mediated decay of the alternative pathway convertase (7). The ability of heparin to inhibit the generation of alternative pathway convertase is independent of its anticoagulant activity and of its ATIII binding (9, 10) activity. Nevertheless, it remained unproven whether heparin and ATIII would block each other's activity on C; whether would have additive activity on C, or whether would be synergistic in regulating C activity.

We found that native human ATIII inhibited activity of both the classical and the alternative pathways of C in a dose-related manner (Figs. 1 and 2). The presence of a fixed amount of heparin augmented the ability of ATIII to inhibit lysis of both pathways in a dose-related manner.

We examined the mechanisms by which ATIII acted to exert this activity and found that ATIII inhibited the fluid phase consumption of factor B in a reaction mixture that also contained C3b and factor D. In this system, which is a model for the fluid phase assembly of the C3b,Bb complex and which uses only purified components, C3b binds to factor B (to produce C3b,B) which is then cleaved by factor D (to produce C3b,Bb); it is likely that the ATIII inhibited factor D serine protease activity (Fig. 4).

When ATIII was incubated with preformed convertases, it was able to inhibit EAC1.4b, EAC1.4b,2a, and EAC1.4b,2a,3b from being lysed but had no activity on cellular intermediates that contained additional C proteins [Fig. 6]. As expected, ATIII inhibited formation of

**TABLE 1**

<table>
<thead>
<tr>
<th>ATIII Concentration (μg/10^7 cellular intermediates)</th>
<th>% Inhibition of Lysis</th>
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</thead>
<tbody>
<tr>
<td>ATIII preincubation with EAC1.4b</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>4 x 10^{-7}</td>
</tr>
<tr>
<td>1.25</td>
<td>2 x 10^{-7}</td>
</tr>
<tr>
<td>0.6</td>
<td>1 x 10^{-7}</td>
</tr>
<tr>
<td>0.3</td>
<td>5 x 10^{-8}</td>
</tr>
<tr>
<td>ATIII present during formation of EAC1.4b,2a</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>4 x 10^{-7}</td>
</tr>
<tr>
<td>1.25</td>
<td>2 x 10^{-7}</td>
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<tr>
<td>0.6</td>
<td>1 x 10^{-7}</td>
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<tr>
<td>0.3</td>
<td>5 x 10^{-8}</td>
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*Assumes a m.w. of 60,000 and that 10^7 cellular intermediates are in 0.1 ml buffer.*
EAC1,4b,2a, EAC1,4b,2a,3b, and EAC1,4b,2a,3b,5b, if present throughout the processes of convertase formation (Fig. 5). These experiments suggest that ATIII acted on the enzymes of the C system (e.g., C1, C2, B, and D), whereas additional steps were not affected by incubation with ATIII.

These studies support previous observations by Glover et al. (2) and Schasteen et al. (3), which suggest that portions of the ATIII molecule might have the capacity to regulate C activity. In those earlier studies, synthetic fragments that represented the C-terminal sequence of ATIII (and of other SERPIN) had activity at micromolar concentrations on the enzymatic activity of factors D and C1s (2, 3). In contrast, synthetic peptides, based upon the active site in C9 that is cleaved by convertase, were poor inhibitors of C activity except at millimolar concentrations (2, 3). However, Schasteen et al. (3) found that native ATIII did not inhibit C activity in either pathway at 5 μM concentrations. Unfortunately, the conditions under which their ATIII was tested were not presented in their manuscripts (2, 3). Perhaps their ATIII had been frozen and thawed multiple times, which would have caused the ATIII to have decreased activity. Although the data are not presented here, we did examine commercially available ATIII (Sigma Chemical Co., St. Louis, MO) early in this study and found that it, too, had activity in the micromolar range on C activity.

Studies by Ogston et al. (5) showed that ATIII in the presence of heparin was able to inhibit C5a esterase activity; this activity required the presence of both ATIII and heparin (5). ATIII has also been shown to be decreased in disseminated intravascular coagulation and shock, conditions in which there may be brisk C activation (30). ATIII may also act on the terminal pathway of C; ATIII has been found attached to the membrane attack complex (C5b-9) (4). Furthermore, S-protein, which inhibits the membrane attack complex in plasma, may bind to both thrombin and ATIII, although S-protein did not appear to bind to ATIII when thrombin was not also present (6).

The present studies provide a link between the coagulation system and C. ATIII is the most important SERPIN that regulates blood coagulation and has been studied extensively for a heparin binding domain. Amino acids 114 to 156 of ATIII are thought to contain the binding site for heparin, based upon ATIII fragment studies and upon predictions made from molecular modeling of the ATIII molecule (31, 32); residues 126 to 140 are predicted to be on the external portion of the molecule. The SERPIN site is found at residues 384 to 385 and consists of the sequence... tremendously important that a deeper understanding of these interactions is needed to further advance our understanding of the regulation of C activity.

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