Interaction of antithrombin III with preadsorbed albumin–heparin conjugates

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The adsorption of antithrombin III (AT III) onto polystyrene surfaces preadsorbed with albumin or albumin–heparin conjugates was studied using a two step enzyme immuno assay. When AT III-buffer solutions were used, the highest adsorption values were measured on high affinity albumin–heparin conjugate pretreated surfaces. Less AT III adsorption was found on nonfractionated albumin–heparin conjugate preadsorbed surfaces. AT III adsorption could also be detected on low affinity conjugate and albumin coated surfaces. When AT III was adsorbed from plasma or plasma dilutions with buffer, only AT III on surfaces preadsorbed with high affinity or nonfractionated albumin–heparin conjugate was found. These results demonstrate that the heparin moiety of the conjugate is directed to the solution phase whereas the albumin moiety contacts the polystyrene surface.

Keywords: Polymers, heparin, albumin–heparin conjugates, antithrombin III, *protein adsorption*

The mechanism by which heparin acts as an anticoagulant has been the subject of many papers. It is now generally accepted that heparin requires the participation of the natural coagulation inhibitor antithrombin III (AT III) to express its activity. As shown by spectroscopic techniques[1–3], heparin binds strongly to AT III and induces a conformational change of this protein. The heparin–AT III complex rapidly neutralizes the activity of activated clotting factors (thrombin, factors XIIa, XIa, Xa, IXa) yielding an inactive heparin–AT III-clotting factor complex. Subsequently, heparin is released from this ternary complex and is then available for further reactions. A review concerning the mechanism of the anticoagulant action of heparin was recently published by Björk and Lindahl[4]. The binding of AT III to heparin is not only important for the anticoagulant activity, but also plays a role in heparin–platelet interactions as was observed for heparin in solution[5, 6] as well as for immobilized heparin[7]. Although the mechanism of the anticoagulant action of heparin in solution is well known, the reasons for the observed enhanced antithrombogenicity of materials after heparinization are still not fully understood. Both the release of heparin from the surface into the blood phase as well as the action of surface bound heparin have been mentioned as causes for the observed enhanced antithrombogenicity[8–10]. Although the role of AT III binding onto heparin in solution has been extensively investigated, the adsorption of AT III onto heparinized materials has received less attention[11, 12].

Recently we reported on the use of albumin–heparin conjugates for the improvement of the blood compatibility of polymeric surfaces either by preadsorption or by covalent coupling of the conjugates[13]. The *in vitro* blood compatibility of materials preadsorbed with albumin–heparin conjugates was strongly improved. Compared with control materials, platelet adhesion onto materials preadsorbed with albumin–heparin was reduced[14] and it was shown that these surfaces also inhibited the surface induced coagulation[15, 16].

As pointed out above, binding of AT III onto heparinized materials is possibly important for both the neutralization of formed activated clotting factors at the blood–polymer interface and for reduction of immobilized heparin–platelet interactions. In this paper we describe the results of the adsorption of AT III from plasma and from buffer containing purified AT III onto polystyrene preadsorbed with different albumin–heparin conjugates and albumin. A two step enzyme immuno assay (EIA) as described by Breemhaar *et al.*[17, 18] was used to detect adsorbed AT III.

MATERIALS AND METHODS

Materials

All experiments described here were carried out using multiwell polystyrene microtitre plates (110 wells) obtained from Organon Technika, Turnhout, Belgium.

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Reagents

Human serum albumin (A 9511) was obtained from Sigma, St. Louis, USA. Heparin (170 U/mg) from porcine mucosa was obtained from Diosynth, Oss, The Netherlands. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was a product of Aldrich, Beerse, Belgium. Purified human antithrombin III (AT III) was a generous gift of the American Red Cross. Human CPD plasma was obtained from the bloodbank Twente and Achterhoek, Enschede, The Netherlands. Rabbit sera directed against human albumin, human AT III and sheep serum directed against rabbit immunoglobulin (ShaIgG) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Horse radish peroxidase was purchased from Boehringer, Mannheim, FRG. Urea peroxide was a product of Organon Technika, Turnhout, Belgium. 3, 3', 5, 5'-Tetramethylbenzidine was purchased from Fluka, Switzerland. *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Synthesis of the enzyme–anti-immunoglobulin conjugate

Sheep anti-rabbit immunoglobulin G was covalently coupled to horse radish peroxidase using SPDP as a coupling agent. The procedure was described in detail by Carlsson *et al.*[19].

Albumin–heparin conjugates

Albumin and heparin were coupled using a water soluble carbodiimide (EDC). Unreacted albumin and heparin were separated from albumin–heparin conjugates using DEAE cellulose and Cibacron Blue Sepharose chromatography respectively, as described in detail previously[13]. High and low AT III affinity albumin–heparin conjugates were obtained by fractionation using immobilized AT III[13]. The albumin–heparin conjugates obtained were characterized with respect to the weight percentage of heparin in the conjugates using a metachromic assay[13,20] and the anticoagulant activity using an APTT (activated partial thromboplastin time) assay[13].

Adsorption of albumin, albumin–heparin conjugate and AT III onto polystyrene

Solutions of albumin, albumin–heparin conjugate and AT III were made in PBS (phosphate buffered saline, 0.9% NaCl, 10 mM Na_2HPO_4 adjusted with HCl to pH 7.4). AT III concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient $E^{1\%}_{280\ nm} = 6.10^{21}$.

The wells of polystyrene microtitre plates were contacted for 1 h at 20°C with 75 μl of serial dilutions of albumin (concentrations varied from 10^{-5}–10 mg/ml), albumin–heparin conjugate (concentrations varied from 10^{-5}–10 mg/ml) or AT III (concentrations varied from 10^{-5}–2×10^{-1} mg/ml) solutions in PBS.

After the addition of the protein solutions, the plates were covered in order to prevent evaporation. The protein solutions were then discarded and each well was sequentially washed 4 times with 150 μl aliquots of PBS. After washing, 50 μl of a solution of anti-albumin (first antibody) was added to the wells which were previously exposed to the albumin and albumin–heparin conjugate solutions and 50 μl of a solution of anti-AT III was added to the wells which were previously exposed to the different AT III solutions. The solutions of anti-albumin and anti-AT III were prepared by dilution of rabbit serum directed against human albumin and human AT III respectively a 100 times in PBS–BSA [PBS containing 1% (w/v) of bovine serum albumin]. The adsorbed proteins were incubated with the corresponding antibody solution for 1 h. Subsequently the antibody solution was discarded and each well was sequentially washed 4 times with 150 μl aliquots of PBS. Then 50 μl of an appropriate dilution of enzyme coupled anti-immunoglobulin (second antibody) in 0.02 M Tris/HCl buffer containing 1% (w/v) polyethylene glycol 6000 (obtained from Fluka, Switzerland) was added to each of the wells. After an incubation time of 1 h, the solution of the second antibody was discarded and each well was washed 4 times with 150 μl of PBS after which 100 μl of substrate solution was added to each well. The substrate solution was prepared by diluting a 3, 3', 5, 5'-tetramethylbenzidine (10 mg/ml DMSO) solution 100 times in buffer (0.6 M Na acetate, pH 6.0) which contained 1 ml of a stock ureum peroxide solution (1 tablet per 10 ml H_2O) per 100 ml. The polystyrene plate was placed in the dark for 30 min. followed by the addition of 2 M H_2SO_4 to stop the enzymatic reaction. Finally the intensity of the yellow colour formed was measured at 450 nm using a colorimeter (Vitatron) equipped with a 80 μl flow through cell.

Adsorption of AT III onto polystyrene preadsorbed with albumin or albumin–heparin conjugate

To evaluate AT III interactions with adsorbed albumin or albumin–heparin conjugates, 75 μl of a solution of albumin or albumin–heparin conjugate (5 mg/ml in PBS) was first pipetted into the wells of a polystyrene plate. After 1 h incubation the albumin and albumin–heparin conjugate solutions were discarded and the wells were washed with PBS as described above. Then 60 μl of AT III solutions (10^{-5}–2×10^{-1} mg/ml in PBS) or plasma (undiluted–10^5 times diluted in PBS) were added to the preadsorbed surfaces and incubated for 1 h. The plasma and AT III solutions were then discarded and the wells were washed with PBS using the procedure described above. Subsequently 50 μl of anti-AT III solution was added and the remainder of the test was conducted as described before.

RESULTS

Table 1 presents the results of the fractionation of albumin–heparin conjugate with immobilized AT III. Since the compositions of the different conjugates do not differ substantially, only the adsorption isotherm of nonfractionated albumin–heparin conjugate on polystyrene was determined and compared with the one obtained for albumin.

Table 1 Characterization of albumin–heparin conjugates

Conjugate	Weight % of heparin	Anticoagulant activity* in U/mg heparin
nonfractionated	10.9 ± 0.5	76 ± 6
high affinity	10.8 ± 0.6	150 ± 12
low affinity	9.3 ± 0.4	13 ± 4

*Using an APTT assay with heparin (170 U/mg) as a standard.

Figure 1 shows the adsorption isotherms observed for albumin and albumin–heparin conjugate on polystyrene as studied with the two step immuno assay. At higher concentrations ($> 10^{-1}$ mg/ml), extinctions are statistically indistinguishable for albumin and the albumin–heparin conjugate, whereas at lower concentrations (10^{-5}–10^{-2} mg/ml) slightly lower extinctions for the albumin–heparin conjugate were usually observed (5 independent experiments).

Figure 2 shows the adsorption isotherm of AT III onto a polystyrene surface. When plasma or plasma dilutions (up to 10^6 times) were used as a source of AT III, no adsorption of AT III onto polystyrene could be detected.

Figure 3 shows the results of the adsorption of AT III onto polystyrene preadsorbed with albumin, nonfractionated albumin–heparin conjugate and low and high affinity albumin–heparin conjugate. Besides a significant adsorption of AT III onto nonfractionated and high affinity albumin–heparin conjugate, surprisingly, also on albumin and low affinity albumin–heparin conjugate preadsorbed polystyrene some adsorption of AT III was observed. As an internal control, AT III adsorption from PBS containing a

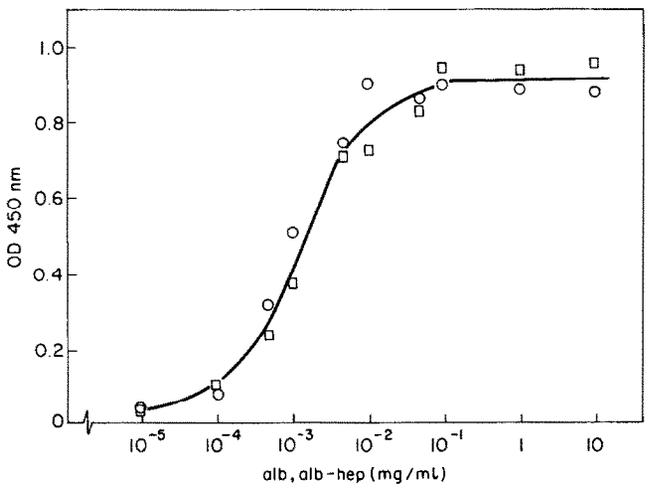

Figure 1 Adsorption isotherms of albumin (○) and albumin–heparin conjugate (□) on polystyrene. All plotted points are the mean of 5 measurements.

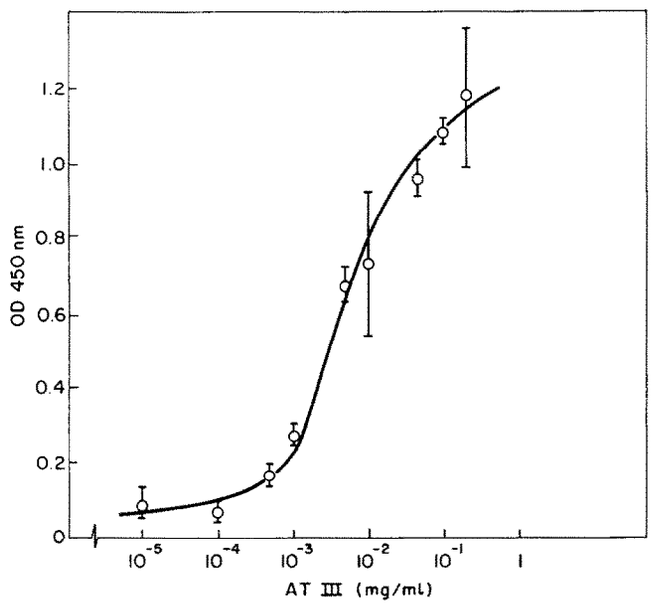

Figure 2 Adsorption isotherm of AT III on polystyrene. All plotted points are the mean (± S.D.) of 3–4 measurements.

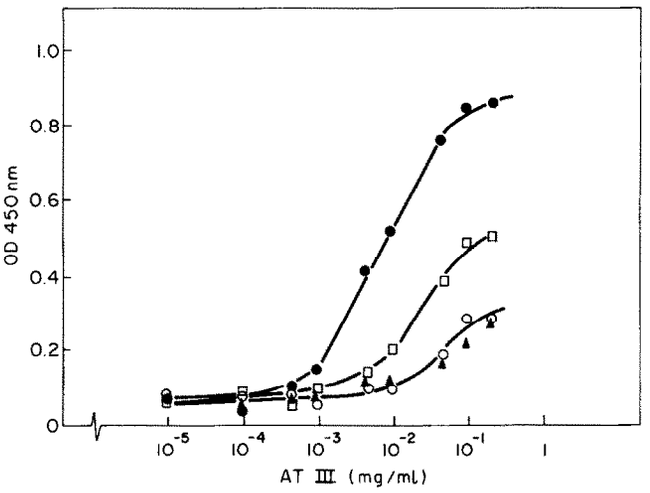

Figure 3 Adsorption of AT III from buffer containing different concentrations of purified AT III onto polystyrene preadsorbed with albumin (○), nonfractionated albumin–heparin conjugate (□), high affinity albumin–heparin conjugate (●) and low affinity albumin–heparin conjugate (▲). All plotted points are the mean of 3–4 measurements.

physiological concentration of AT III (0.2 mg/ml[22]) was studied for each polystyrene plate used. The extinctions observed for adsorption of AT III onto preadsorbed albumin and albumin–heparin conjugate surfaces can then be related to the extinctions obtained for AT III adsorption from a 0.2 mg/ml solution onto bare polystyrene. From *Figure 3* it can be calculated that the percentage AT III adsorption of surfaces preadsorbed with albumin, low affinity albumin–heparin conjugate, nonfractionated albumin–heparin conjugate and high affinity albumin–heparin conjugate was 21%, 21%, 38% and 65% respectively. Dependent on the batch of polystyrene plates and AT III used, the AT III adsorption ratios differed widely. For preadsorbed albumin, values between 15 and 70% were observed and for preadsorbed albumin–heparin conjugate (nonfractionated) these values varied from 35–100%. In spite of the relatively large differences in AT III adsorption percentages observed on the same protein coating, in every experiment the albumin–heparin conjugate preadsorbed surfaces showed a significant higher adsorption of AT III than the albumin preadsorbed surfaces. The adsorption of AT III onto albumin preadsorbed surfaces could not be reduced by increasing the concentration of the albumin solution (up to 10 mg/ml). In addition, no reduction in adsorption of AT III onto albumin preadsorbed surfaces was observed when after the incubation of the albumin preadsorbed surfaces with AT III the wells were washed with PBS containing 0.75 M NaCl.

Figure 4 shows the adsorption behaviour of AT III on surfaces preadsorbed with albumin and different albumin–heparin conjugates using plasma or plasma dilutions as a source of AT III. From this figure it appears that high affinity and nonfractionated albumin–heparin conjugate preadsorbed surfaces bind substantial amounts of AT III. On the other hand on surfaces preadsorbed with albumin and low affinity albumin–heparin conjugate no AT III adsorption from plasma could be detected with the EIA technique used.

DISCUSSION

No substantial differences in adsorption behaviour of albumin and albumin–heparin conjugate on polystyrene

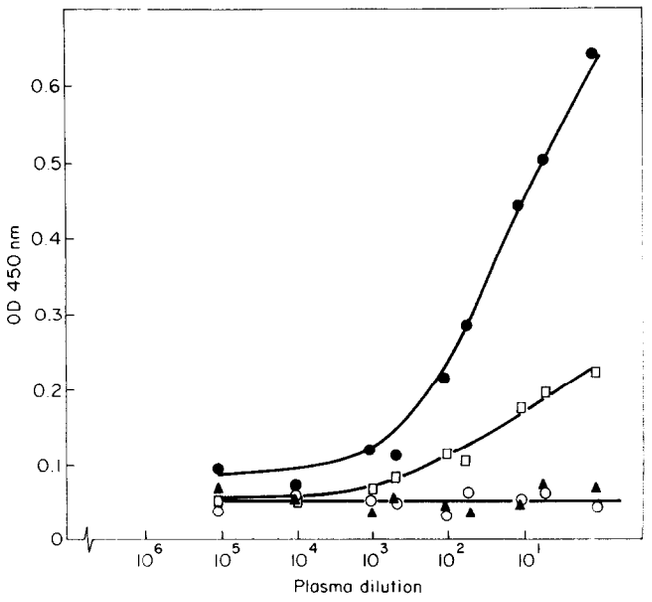

Figure 4 Adsorption of AT III from plasma and plasma dilutions in buffer onto polystyrene preadsorbed with albumin (○), non-fractionated albumin–heparin conjugate (□), high affinity albumin–heparin conjugate (●) and low affinity albumin–heparin conjugate (▲). All plotted points are the mean of 3–4 measurements.

were observed, with the EIA technique used. The observation that at lower concentrations of albumin and albumin–heparin conjugate the extinctions for albumin–heparin conjugate were slightly lower than for albumin indicates that either the surface concentration of albumin–heparin conjugate is slightly lower or the immunological affinity of anti-albumin for the albumin–heparin conjugate is lower than for albumin. Possibly the binding of heparin to albumin blocked some of the anti-albumin binding sites present on the albumin molecule.

As known from affinity chromatography data, immobilized heparin is able to bind AT III[21] as well as a number of other plasma proteins such as factor IX and XI[15, 23]. As expected, polystyrene preadsorbed with high affinity and nonfractionated albumin–heparin conjugate binds a significant amount of AT III when exposed to buffer containing AT III or to plasma. In all experiments, higher amounts of AT III were bound to preadsorbed high affinity albumin–heparin conjugate than to non-fractionated conjugate. This can be explained by the observation that only 30–40% of nonfractionated heparin is able to bind AT III[24, 25]. Surprisingly, albumin and low affinity albumin–heparin preadsorbed surfaces also bind some AT III when exposed to buffer containing purified AT III. Three possible explanations for this observation can be given:

(1) adsorbed albumin binds AT III;
(2) the polystyrene surface was not completely coated with albumin, providing free surface for binding with AT III;
(3) AT III replaces albumin from the polystyrene surface.

The following observations are of importance: AT III bound to albumin preadsorbed polystyrene surfaces could not be removed by washing with 0.75 M NaCl which indicates that electrostatic interactions play a minor role; when polystyrene was precoated with albumin using solutions with a concentration of 10 mg/ml instead of 5 mg/ml no decrease in the adsorption of AT III was observed. Exchange of preadsorbed albumin with proteins in solution has been reported in the literature[26, 27] and because the EIA technique is very sensitive, only a small amount of AT III which has displaced albumin can result in a substantial extinction. No adsorption of AT III from plasma onto albumin preadsorbed surfaces could be detected, which indicates that a possible binding of AT III from buffer solutions to adsorbed albumin does not have a physiological meaning.

The fact that the heparin moiety of adsorbed albumin–heparin conjugate can bind AT III indicates that the adsorption of albumin–heparin conjugate mainly occurs *via* the albumin portion with the heparin moiety residing in the aqueous solution phase.

From the study, it can be concluded that after contact between plasma and albumin–heparin conjugate preadsorbed polystyrene, adsorption of AT III takes place. It was demonstrated that this specific adsorption was due to interaction between AT III and the heparin moiety of the conjugate. The binding is dependent on the type of heparin (low or high affinity for AT III) used. From the fact that AT III is specifically adsorbed from plasma onto non-fractionated and high affinity albumin–heparin conjugate preadsorbed surfaces, it can be concluded that for these surfaces AT III also plays a role in the subsequent interaction with clotting factors and platelets.

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