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Ex vivo complement protein adsorption on positively and negatively charged cellulose dialyser membranes

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An *ex vivo* test system was used to measure complement protein C3 and factor B adsorption onto small dialyser modules made from regenerated and modified cellulosic hollow fibre membranes in which positive diethylaminoethyl (DEAE) or negative carboxymethyl (CM) groups were introduced into the cellulose matrix. The extracorporeal system, which included test dialysers and the dialysis environment, allowed the use of labelled proteins without contaminating the blood donors which were connected in an open-loop fashion to the extracorporeal test system. The modules were removed at selected time points from the extracorporeal system for radioactivity counting. The results were used to evaluate the mechanisms involved in complement reactions to foreign surfaces. The system therefore allowed the analysis of complement protein adsorption occurring in the dialyser modules and its relationship to the complement generation rate in the extracorporeal system to be evaluated. It was possible to demonstrate that significant complement C3 and factor B adsorption occurred in the test modules made of cellulosic membranes. Complement adsorption as a function of the pH and the release reaction of the adsorbed C3 and factor B after membrane blood perfusion were therefore found to be variable according to the cellulosic membrane type and the presence of positive or negative charged groups within the cellulose matrix. The data obtained from the *ex vivo* model therefore provided additional evidence on the discussion of the mechanisms involved in the increased complement activation by regenerated cellulose and in its attenuation by DEAE- or CM-modified cellulose.

1. Introduction

An *ex vivo* model designed to investigate the enhancement of blood compatibility properties of dialysis membranes produced by modification of cellulose membranes was used in the analysis of membrane-induced complement activation by measurement of complement generation and complement protein adsorption using labelled proteins. The focus was on the elucidation of mechanisms involved in the attenuation of complement activation reactions by modified cellulosic membranes. As previously reported [1–6], the chemical modification of regenerated cellulose membranes based on the substitution of positively charged groups such as diethylaminoethyl minimizes complement activation by more than 50%. However, the substitution with negatively charged groups such as carboxymethyl or acetate has also been shown to attenuate complement activation to a similar extent [7, 3].

Complement activation has been shown to occur via the alternative pathway by biologically active surfaces such as zymosan or bacterial polysaccharides and that covalent binding of the complement protein C3 on foreign surfaces was the prerequisite [8–11]. When the magnitude of the complement activation was considered, dialysers made with modified cellulose such as cellulose acetate and Hemophan (DEAE-substituted cellulose) greatly reduced complement activation compared to unmodified regenerated cellulose. It was therefore suggested that the inhibition of complement activation by modified cellulosic membranes as compared to unsubstituted regenerated cellulose may be related to the competition between the complement components Factor B and β1H binding reaction on the membrane surface [12–14]. These observations were similar to those previously reported on complement binding reactions of membrane-associated sialic acid and heparin [15–17]. In these investigations, it was demonstrated that the relative affinity of B and β1H for particle-bound C3b was influenced by the chemical structure of the surface and this determined whether or not particles have the capacity to activate complement. Following the key role of the C3 reaction with complement activating surfaces described

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by Sim [8], the C3-binding with complement activating foreign surfaces was shown to occur by hydroxylamine ester binding. However, in the protein–membrane interactions during the first contact of the blood with dialyser membranes, interactions by ionic exchanges or hydrophobic interactions are not excluded, since many dialyser membranes have hydrophobic characteristics or/and charged groups introduced into the matrix. Therefore, an effective formation of C3-convertase as demonstrated by zymosan may occur only by proteolytic activation or by conformational change of the native C3 forming a reactive carboxyl group, which may be covalently bound on to the surface [9, 10, 18]. Quantitative and qualitative determination of complement adsorption occurring during haemodialysis and the relationship to the complement generation rate in the extracorporeal system may elucidate the phenomenon involved in complement activation. The objective of this study was the application of an *ex vivo* model to investigate the following:

1. Measurements of the adsorbed complement proteins C3 and/or C3 fragments, and factor B on cellulosic hollow fibre membranes, that is regenerated cellulose, and two different modified cellulose (positively charged DEAE-cellulose and a negatively charged carboxymethyl-cellulose).
2. Determination of the binding forces involved in C3 and factor B adsorption. The characterization of the membrane-associated C3 and factor B as well as their binding reactions in a simplified system involving the binding of labelled ^{125}I-C3 and ^{131}I-factor B to dialyser modules. Radioactivity counting was performed directly on the hollow fibres.

2. Materials and methods

2.1. Dialyser membranes

For the purpose of the study, DEAE-modified cellulose (three subtypes), CM-modified and regenerated cellulose membranes were prepared. The DEAE-modified cellulose subtypes were mixtures containing 5% (Hemophan type: DEAE-C$^+$), 2.5% (type: DEAE-C-) and ~1% (low DEAE-cellulose) of 20% substituted DEAE-cellulose and regenerated cellulose (RC). The substitution degree of CM-cellulose subtype was not specified by the manufacturer (Akzo). The modules used were made with a total membrane surface area of 0.035 m^2, consisting of 174 capillaries, each of 23 cm length.

2.2. *Ex vivo* and *in vitro* experiments

Sterile preparation of the extracorporeal systems and the *ex vivo* procedure were performed as previously described [4]. Venous blood from normal volunteers was pumped through the mini dialysers at a rate of 6 ml/min. A reduction factor for the extracorporeal system was approximately 28. In this study, blood was heparinized at 0.40 U/ml before being perfused through the module. In addition, blood was spiked with different amounts (counts/min) of ^{125}I-C3 (~20 000 counts/min/10 μl) and ^{131}I-factor B (~5 000 counts/min/10 μl) giving an overall count of 100 000 counts/min/ml of blood before perfusion starts. The dialysate compartment was filled with a standard acetate buffered dialysate and closed by clamps.

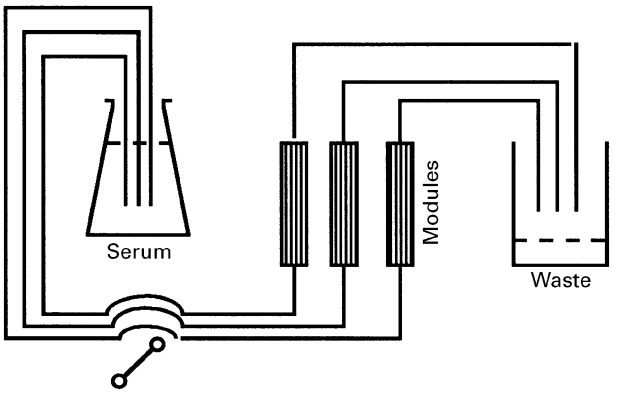

Figure 1 *In vitro* model for testing complement protein adsorption. ^{125}I-C3 (~20 000 cpm/10 μl) and ^{131}I-factor B (5000 cpm/10 μl) were added into pooled serum from healthy subjects. The test modules containing 174 hollow fibre membranes with a length of 2 cm were perfused at a flow rate of 0.5 ml/min. The experiments were performed at 37°C while the dialysate compartment was filled with a standard acetate buffered dialysate and closed by clamps.

An *in vitro* incubation model utilizing plasma instead of whole blood was designed as a control system for comparison with *ex vivo* system experiments. As shown in Fig. 1, the mini dialysers were perfused with pooled human serum at 0.5 ml/min. The dialysate compartment was filled with acetate dialysate and remained closed during experiments. ^{125}I-C3 and ^{131}I-factor B were added to the pooled serum before experiments. The general assumption was that the *in vitro* model offered an essential control with respect to membrane protein interaction in the absence of blood cells.

2.3. Purification of C3 and factor B

The purification procedure was as described previously [19]. 1 M $BaCl_2$ was added to 5 l human plasma (40 ml $BaCl_2$ per 1000 ml plasma) in order to precipitate vitamin K-protein complexes. The supernatant was dialysed against buffer A (25 mM potassium phosphate (pH7); 5 mM EDTA; 25 mM 6-aminohexonate). A preparative chromatography of C3 was performed by ionic exchange using a DEAE-column (DEAE-Sephadex A20, 20 cm × 5 cm). The C3 protein fraction as determined by the heamolytic activity and by a purified C3 probe (Sigma), was eluted by a linear gradient of NaCl solvent (gradient: 0–450 mM) in buffer A. The partially purified C3 was dialysed against a buffer B (20 mM Tris acetate (pH6.8); 5 mM EDTA; 10 mM NaCl; 25 mM 6-aminohexonate). Further purification of C3 was performed by ionic exchange chromatography using a carboxymethyl-Sephadex column (CM-Sephadex C50, 20 cm × 5 cm) by a gradient elution with NaCl solvent (0–300 mM) with the buffer solution B. The C3 fraction was dialysed against water prior to lyophylized. Final purification of the lyophylized

C3 fraction was achieved by size exclusion chromatography using Sephadex column (Sephadex G-50, 12 cm × 0.8 cm).

Plasma preparation and DEAE-chromatography as used for C3 was performed to separate factor B. The factor B fraction was diluted with water (1 : 1) prior to chromatography separation with CM-column (CM-Sephadex 30 cm × 3.1 cm) and eluted with a NaCl solvent by a linear gradient (0–800 mM) against 1 : 1 diluted buffer solution A. Factor B was dialysed against 15 mM imodazole; HCl (pH 7.3); 0.03 M NaCl; 2 mM benzamidine buffer. Final purification was achieved by a benzamidine Sepharose column (20 cm × 1.7 cm) prior to dialysis and lyophylized.

2.4. Radio-iodination of C3a, C3 and factor B by modified chloramine-T labelling method [20]

500 μg lyophylized C3a (a gift from Behring), C3 or factor B were added to 1 ml buffer solution (potassium phosphate; 100 mM EDTA; pH 7.3) containing 1 mCi Na ^{125}I (for C3) and 1 mCi ^{131}I (for factor B), 100 μml dimethyl sulphoxide, and 50 μml chloramine-T (2 md/ml). The reaction occurred for 4 min at 0°C and was stopped by the addition of 200 μl sodium metabisulphite (10 mg/ml). Free and labelled ^{125}I-C3 and ^{131}I-factor B were separated by gel filtration chromatography (Sephadex G-50 12 cm × 0.8 cm). The labelling was estimated as approximately 6–8 ^{125}I atoms incorporated to 100 molecules of C3 and 4–6 ^{131}I incorporated to 100 molecules of factor B.

2.5. Quantification of C3a, C3 and factor B in plasma

Complement C3a was measured by radioimmunoassay (Amersham); C3 was measured by RID (Behring) and factor B by radioimmunoassay (developed in-house).

2.6. Gamma-counting by window technique

After *ex vivo* perfusion with blood or *in vitro* perfusion with plasma of the mini dialysers, modules were rinsed for 2 h with different detergent solutions. The modules were then rinsed with two litres of saline in order to remove the free labelled proteins. Then, the hollow fibres were extracted from the modules, cut and placed into the counting chamber of a gamma counter. A double radio detection of ^{125}I-C3 and ^{131}I-factor B were measured in a gamma-counter for which two windows were adjusted to detect both energy levels. A correction for interference in both energy scales was performed for each window with the ^{125}-I and ^{131}-I separately.

2.7. Determination of protein adsorption

Protein adsorption in the module was estimated using the following formula

$$P_{ad}[\text{mol}] = (P_{ad}[\text{cpm}]/P_0[\text{cpm}]) \cdot P_{total}[\text{mol}] \quad (1)$$

P_{ad}[mol] was the total amount of protein adsorbed onto the module; P_{ad}[cpm] was the measured membrane-related protein in counts/min/module; P_0[cpm] was the amount of labelled protein in plasma in counts/min/10 μl; P_{total} was the amount of unlabelled protein contained in the plasma volume in the blood compartment of the module.

P_{total}[mol] was determined as follows:

$$P_{total}[\text{mol}] = P_{con} \cdot V_p \quad (2)$$

where P_{con} was the protein concentration, and V_p the total plasma volume in the blood compartment of the module.

The plasma volume of modules was determined as follows:

$$V = r^2 \cdot L \cdot n(100 - H_k)/100 \quad (3)$$

where $2r$ is the internal diameter of the hollow fibres, L is the length of the hollow fibre, n is the number of hollow fibres contained in the module, H_k is the blood haematocrit.

3. Statistics

All the results were expressed in terms of the differences between mean values as evaluated using Student's paired t-test, a two-way analysis of variance and Mann–Whitney test on the median values. The analysis was performed using a Minitab V 8.0 statistical package (Minitab Inc., State College, Pennsylvania). The results were reported significantly different when $p < 0.05$ at 95% confidence interval.

4. Results

4.1. C3-adsorption and the kinetics of the C3a formation

Fig. 2 shows the plasma values of C3a obtained during 40 min of *ex vivo* perfusion through regenerated cellulose, a subtype of DEAE-modified cellulose (DEAE-C +) and CM-modified cellulose. The value at 0 min was obtained at the module inlet, whereas the values during dialyser perfusion were measured at the module outlet. As indicated in Fig. 2, 15–20 min into the perfusion using the regenerated cellulose module, the outlet plasma C3a level reached a steady state after levels approximated 20 times the initial value. The observed steady state occurred only with the *ex vivo* conditions in view of the inevitable exclusion of feedback mechanisms involved during *in vivo* blood recirculation. During perfusion with the DEAE-modified cellulose subtypes, outlet plasma C3a concentrations reached a steady state after ~5 min but the magnitude of the increase was comparatively lower than the regenerated cellulose modules (~10–15 times). During perfusion with CM-cellulose, generation of C3a was observed to be minimal during the first 5 min, an observation that suggests that there is an apparent masking of the true kinetics of C3a generation. Probably the generated C3a was adsorbed onto the surface during the first 5 min. Steady state C3a generation was also observed after 5 min of *ex vivo* for all membrane types. There was an overall reduction in complement

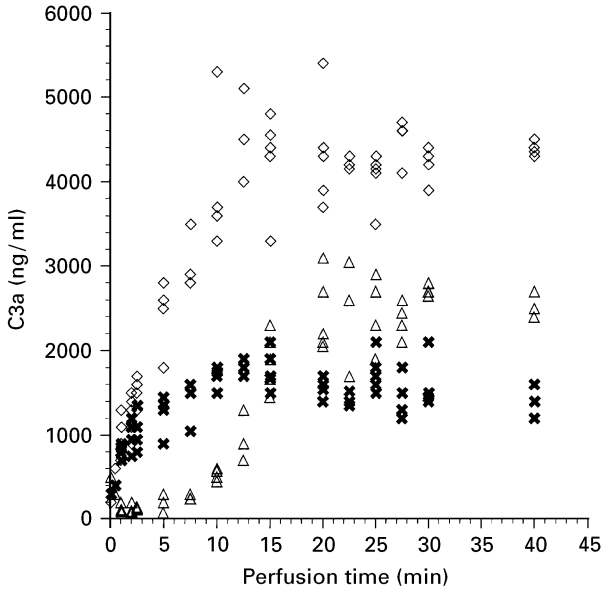

Figure 2 Kinetics of C3a plasma concentration at the outlet of the dialyser during *ex vivo* perfusion (factor of reduction 1: 28; blood flow 6 ml/min, membrane surface area 0.035 m^2). ◇ RC; ✖ DEAE-C; △ CM-C.

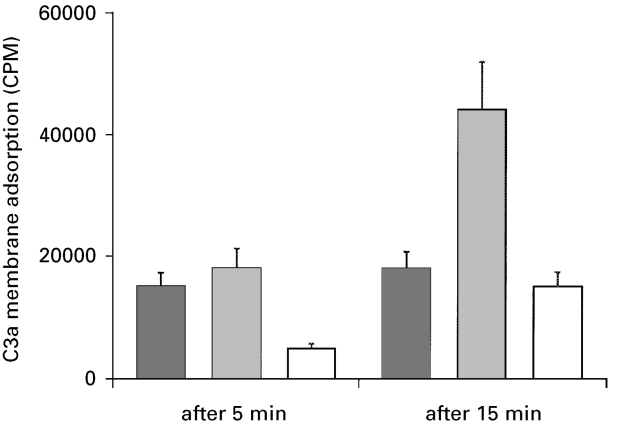

Figure 3 C3a-membrane adsorption after dialyser module incubation with human serum (at 37°C, pH = 7). At the initial time ($t = 0$) a total amount of 1.34×10^5 cpm of ^{125}I-C3a was counted in the blood compartment of the dialyser module. The ratio between labelled and unlabelled C3a before incubation was ~1/1000. ■ RC; ▒ CM-C; □ DEAE-C.

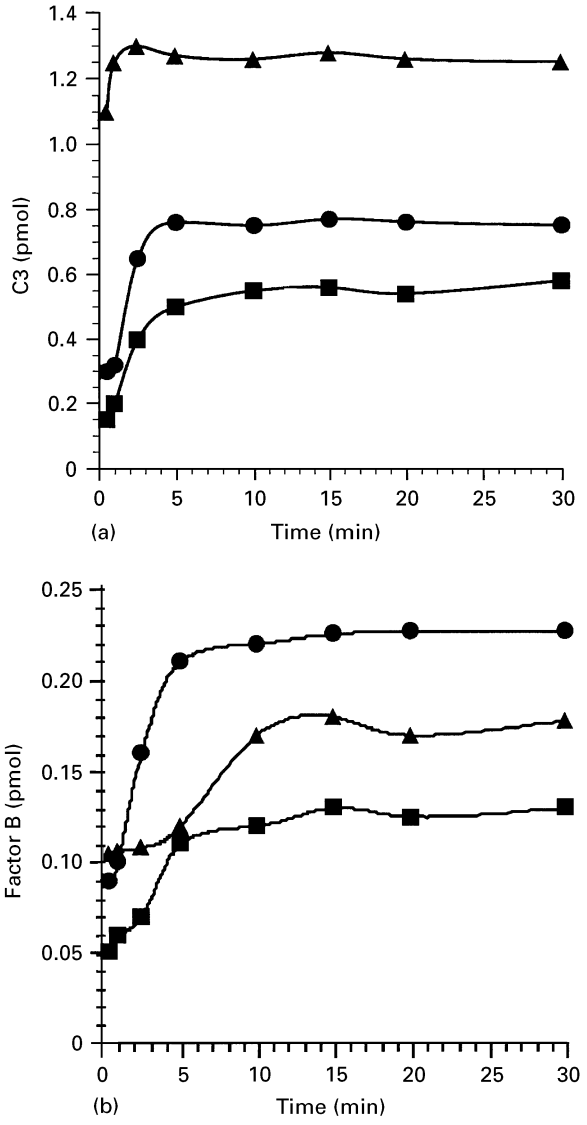

Figure 4 Complement C3 (a) and factor B (b) adsorption (in pmol per hollow fibre unit) onto different dialyser membranes during *ex vivo* (at 37°C, pH 7.4). The dialyser modules tested were: RC (■), CM-C (▲) and DEAE-C (●). For each perfusion the dialyser was removed and prepared for counting. Each volunteer ($n = 8$) was investigated three times with each module. Complement plasma concentrations were C3 = 1.2–1.5 mg/ml; factor B = 0.23–0.26 mg/ml, ^{125}I-C3 was ~10 000 cpm/10 μl plasma; ^{131}I-factor B was ~7500 cpm/10 μl plasma.

activation with CM-cellulose membranes as compared to regenerated cellulose by about 50%.

Fig. 3 shows the membrane-associated ^{125}I-C3a expressed in counts per min (cpm) during *in vitro* module incubation with serum in order to investigate the magnitude of C3a adsorption after complement activation. Data could not be calculated in moles since the proportion between labelled and unlabelled C3a changed with the incubation time because of complement activation. Therefore, results should qualitatively illustrate the differences between regenerated cellulose, DEAE-modified cellulose and CM-modified cellulose with respect to their ability to adsorb C3a. As illustrated in Figs. 3–5, CM-modified cellulose adsorbed more of the component C3a than DEAE-cellulose. In view of the fact that the isoelectric point of C3a is > 8, it follows that under physiological pH C3a adsorption in the negatively charged CM-C matrix may have occurred by cationic exchange. This may explain why the generation rate by CM-modified cellulose indicated a decrease during the 5 min of perfusion. The results using the synthetic sodium methallyl-acrylonitrile copolymer (AN69), which is negatively charged, also demonstrated that significant amounts of C3a are adsorbed [21].

4.2. Kinetics of C3 and factor B adsorption

The kinetics of C3 and factor B membrane adsorption expressed in mol/hollow fibre during *ex vivo* perfusion were as shown in Fig. 4: protein C3 and factor B adsorption were saturated after 5 min blood perfusion. Therefore, the capacity to adsorb C3 or factor B was different in the tested modules. The DEAE-C- and

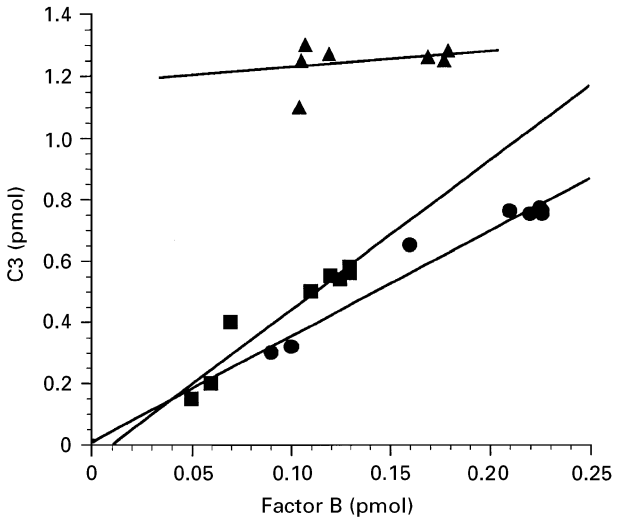

Figure 5 Relationship between C3 (or C3b) and factor B by different cellulosic membranes (■ RC; ▲ CM-C, ● DEAE-C). Specifications for the experiments as Fig. 4.

CM-C membranes indicated a higher complement C3 or C3b adsorbing potential than regenerated cellulose. On the other hand, the membrane–associated factor B by DEAE-C was remarkably elevated compared to those measured on RC and CM-C.

Fig. 5 shows the relationship between the adsorbed C3 or C3b and factor B by the three membranes. The relationship between the adsorbed complement proteins on RC, CM-C and DEAE-C ($r^2 > 0.9$), it was observed that the respective patterns of the ratio of factor B/C3 for each membrane were in the following order: DEAE-C > RC > CM-C. The results therefore suggested that the factor B/C3 ratio of the adsorbed complement proteins could not be utilized as a scale for membrane associated C3bB complex, a known indicator of the complement activation via the alternative pathway.

4.3. Effect of heparin on complement protein adsorption

Fig. 6 shows the effect of heparin adsorption on the membrane–associated C3 and factor B using the *in vitro* incubation model. In the experiments three categories of membrane preparation were performed;

Category A: the membranes were washed with saline alone for 1 h;
Category B: membranes were rinsed with 2.5 IU/ml heparin in saline for 1 h; and
Category C: membranes were incubated with 800 IU/ml heparin in glycine buffer at pH 2.7 for 2 h in an attempt to obtain optimum membrane-associated heparin on DEAE-C.

The method used for category C has been previously reported [1]. As indicated in Fig. 6, membrane heparin-binding increased the capacity of the membrane associated factor B for both CM-C and DEAE-C ($p < 0.001$). In contrast, no effect can be seen on an increase of membrane–associated C3 or C3b for all

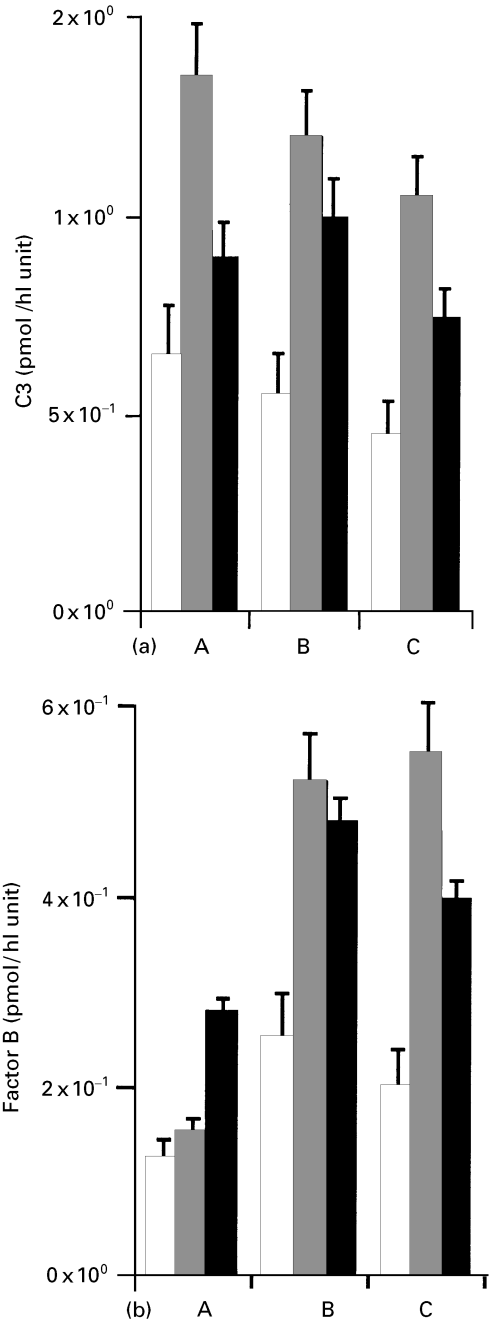

Figure 6 Effect of heparin on (a) membrane-associated C3/C3b and (b) factor B after *in vitro* incubation of dialyser membrane ($n = 7$) with human serum (incubation time 15 min, at 37°C, pH7). Complement plasma concentrations were C3 = 1.2–1.5 mg/ml; factor B = 0.23–0.26 mg/ml, ^{125}I-C3 was ~12 000 cpm/10 μl plasma; ^{131}I-factor B was ~9000 cpm/10 μl plasma. □ RC; ▒ CM-C; ■ DEAE-C.

membranes. A slight reduction in C3 binding efficiency can be seen by CM-C and RC.

4.4. Binding properties of the adsorbed C3 and factor B

The analysis of the binding properties involved with the membrane-associated C3 or C3 fragment and factor B required experiments to be conducted on the release of the adsorbed proteins by specific detergents. In accordance with previously reported studies by Twose *et al.* in 1980 [23] on complement binding to

TABLE I Remaining membrane-associated complement protein C3 and factor B in the dialyser module after *ex vivo* perfusion and incubation with detergent. After 15 min *ex vivo* perfusion (6 ml blood flow, dialyser membrane surface area 0.035 m^2, a reduction factor of 1:28), modules were rinsed with saline and incubated for 120 min using different detergents. Complement plasma concentrations were C3 = 1.2–1.5 mg/ml; factor B = 0.23–0.26 mg/ml, ^{125}I-C3 was ~60 000 cpm/10 μl plasma; ^{131}I-factor B ~17 000 cpm/10 μl plasma. The detergents were: Detergent A: 6 M urea/0.5% SDS solution; Detergent B: 50 mM diethanolamine/ HCl (pH = 11.5) /100 mM NaCl/1% triton X; Detergent C: 1% triton X by pH = 7 for 2 h incubation followed by an incubation with detergent B.

	Membranes	C3 adsorption (pmol)	Factor B adsorption (pmol)
Without detergent	RC	113	21
	DEAE-C	157	49
	CM-C	235	26
		% C3 removal	% factor B removal
Detergent A (6 M urea/0.5% SDS)	RC	54	< 0.1
	DEAE-C	< 0.1	< 0.1
	CM-C	63	< 0.1
Detergent B (50 mM diethanolamine/HCl (pH 11.5)/100 mM NaCl/ 1% Triton X)	RC	88	67
	DEAE-C	82	64
	CM-C	89	34
Detergent C (1% Triton X at pH7, wash followed by incubation with detergent B)	RC	89	99
	DEAE-C	84	75
	CM-C	91	47

surfaces such as Sepharose-trypsin, the more efficient conditions for the release of covalently bound C3 were the incubation of the materials at 37°C in 50 mM-diethanolamine; HCl; 100 mM-NaCl, pH 11.5 buffer. The addition of the detergent Triton-X (1%) enhances the release. Proteins adsorbed by ionic forces may also be released by this detergent. The membrane–associated proteins involved in hydrophobic interactions were released after incubation at 37°C with 6 mol urea/0.5% sodium dodecyl sulphate (SDS). In the following experiment, we investigated the release of the membrane–associated ^{125}I-C3 and ^{131}I-factor B after incubation with these detergents as shown in Table I. The release of C3 bound to cellulosic membranes was found to be in the following order: RC (~50%) and CM-C (~60%) when using detergent A, specific for removing adsorbed protein by hydrophobic interaction. In contrast, no release of C3 was detected from the DEAE-C membrane, whereas in the different cellulosic membranes no release of factor B was detected with detergent A. The efficiency of releasing the membrane-adsorbed C3 was demonstrated by all dialyser membranes using detergents B or C: 88–89% by RC; 89–91% by CM-C and 82–84% by DEAE-C. Therefore, the efficiency of C3 elimination was correlated only with that of factor B by RC (~100%). At the molar basis the efficiency of the release of factor B by CM-C (~47%) and DEAE-C (~75%) was relatively decreased as compared to C3 protein.

4.5. Serum pH effect on the membrane-associated C3 and factor B

The effect of the serum pH was also investigated in an effort to determine whether the modification of cellulose based on the substitution of charged groups, significantly adsorbs complement proteins by ionic forces. In this respect, DEAE-C and CM-C matrix with the charged groups positive DEAE and negative CM may be significantly involved as ionic or cationic exchangers during blood-membrane interaction. Since proteins are amphoteric polyelectrolytes, variation of the pH may increase or decrease protein adsorption on DEAE-C and CM-C by exchanging anions or cations bound to the matrix. Fig. 7 shows the corresponding membrane-bound complement proteins after 5 min incubation of human serum with dialysers in vitro. Results demonstrated the significance of the charged groups of both modified cellulose membranes: C3 and factor B adsorption are increased with the pH (4–7.5) on DEAE-C, but in contrast C3 and factor B adsorption are decreased on CM-C by varying the pH (7–12), suggesting that cations are exchanged. As shown, at physiological conditions significant C3 adsorption by ionic forces is involved in DEAE-C.

Using the *in vitro* model, the effect of ionic strength was investigated by varying the relative serum salt concentration from 150 to 600 mM and the findings for C3 binding and C3a generation were as shown in Fig. 8. The ^{125}I-C3 binding efficiency shows a gradual decline with increasing ionic strength for all membrane types. Nevertheless, the overall effect is minimal for RC and CM-C with average reductions of 20% on RC, 40% on CM. By contrast, there was relatively higher reduction of C3 binding on DEAE-C by about 80% with increasing ionic strength. This suggested that the effect of charged moieties on C3 adsorption onto membranes was more important with the DEAE-modified cellulose.

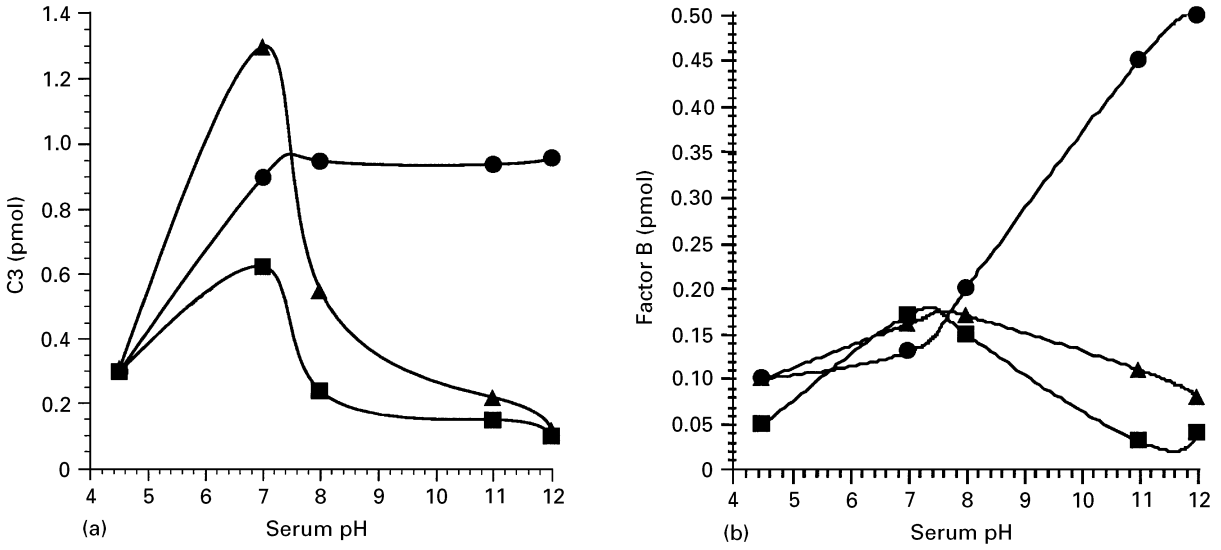

Figure 7 Effect of serum pH on (a) membrane-associated C3/C3b and (b) factor B after *in vitro* incubation of dialyser membrane with human serum (incubation time 5 min, at 37 °C). Complement serum concentrations were C3 = 1.2–1.5 mg/ml; factor B = 0.23–0.26 mg/ml, ^{125}I-C3 was ~10 000 cpm/10 μl plasma; ^{131}I-factor B ~8000 cpm/10 μl plasma. ■ RC; ▲ CM-C; ● DEAE-C.

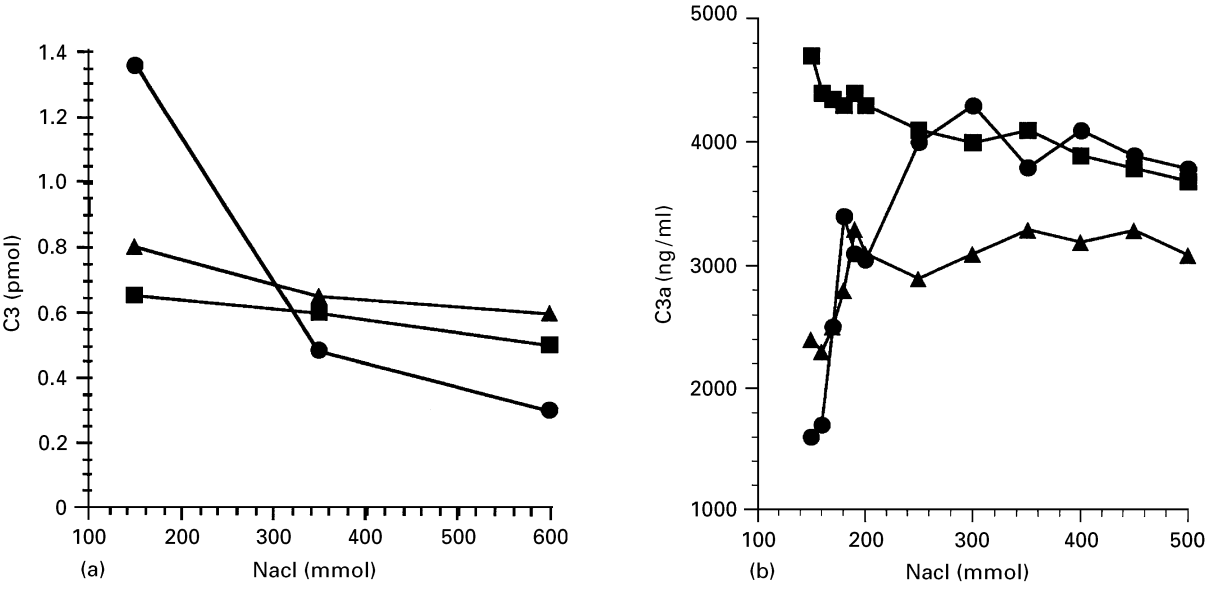

Figure 8 Effect of NaCl on (a) membrane-associated C3 and (b) C3a generation after 15 min dialyser modules perfusion. ■ RC; ▲ CM-C; ● DEAE-C.

5. Discussion

This study evaluated quantitatively and qualitatively complement adsorption on hollow fibre haemodialysis membranes using an *ex vivo* system, and a relationship between complement proteins adsorption and the generation of active complement fragments in the extracorporeal system was determined. The open-loop *ex vivo* system allowed the use of labelled proteins without contaminating the donors. The modules were removed at selected time intervals from the extracorporeal system for radioactivity measurements. It was therefore possible to demonstrate that significant complement C3 and factor B adsorption occurred in the tested modules and that the magnitudes of complement adsorption was a function of the pH. The release reaction of the adsorbed C3 and factor B after membrane blood perfusion, was shown to be variable in accordance with the cellulosic membrane type. The set of data obtained in the *ex vivo* model was useful and has provided evidence to further clarify the mechanisms involved in the increased complement activation by regenerated cellulose and in its minimization by modified cellulose.

Radioactivity counting of ^{125}I-labelled C3 and ^{131}I-labelled factor B bindings in the hollow fibres demonstrated that the extent of complement protein adsorption may be time dependent and that the generation of C3a was limited to the first 15 min of module perfusion. As demonstrated in this study, the process of adsorption appears to increase with time up to a saturable level or steady state at 5 min. The findings also suggest that the complement activating potential of cellulosic dialyser membranes was not primarily dependent on the total amount of adsorbed C3 and

factor B. Total membrane-adsorbed C3 was found to be dependent on the charge of the cellulose matrix magnitudes and were of the following order: CM-C > DEAC-C > RC. The corresponding membrane-adsorbed factor B could be classified as follows: DEAE-C > RC > CM-C. In contrast, the respective complement activating potential of the tested cellulosic membranes were as follows: RC > CM-C > DEAE-C. The results also suggests that the greater proportion of the C3 adsorbed by all cellulosic membrane types was not bound covalently.

These findings are in agreement with those previously reported on the analysis of C3 fragments in dialysis membranes during clinical haemodialysis [22]. Likewise, the quantification of the total membrane-associated C3 found by other studies [22] using SDS-PAGE techniques was in the same range as the presented results from the *ex vivo* findings as shown below: Cheung *et al.* [22] ~ 1.5 mg/1.1 m^2 RC membrane surface area after 4 h haemodialysis; in this study ~ 1 mg/m^{2}RC membrane surface area after 15 min *ex vivo*. The presented results underline that complement protein adsorption was optimal at the early phase of blood–membrane contact. Following our results based on the analysis of complement activation during the first 15 min of blood–membrane interaction, it is conceivable that complement activating potential of foreign surfaces was possible even when there was a small proportion of covalently bound C3. Based on the C3 protein adsorption kinetics, the calculation of the percentage of C3 bound can be estimated as a simple percentage of the radioactivity. The binding of C3 to regenerated cellulose approached saturation when about 1% of the supplied bulk C3 (amount of C3 contained in the hollow fibres) was bound. In this respect, a better approach in the calculation of the covalently bound C3 to RC could be the difference between the release of C3 by detergents C and A (Table I). Therefore, a saturation point of the covalently bound C3 was reached at an approximate optima of 0.5% of the bulk C3, which is measured from the average of the measured membrane-associated C3 protein (~ 1.1 nmol/m^2). This is the amount of membrane-adsorbed C3 at pH4 which may represent the optimal efficiency of covalently-bound C3. In accordance with some reported findings [8] using trypsin Sepharose as a complement activating surface, C3 binding efficiency increased as pH was reduced, suggesting that this may be related to the effect of differential reductions due to protonation or nucleophilic properties of hydroxyl groups to which C3 binds and/or of the water molecules that compete with surface hydroxyl groups. The average amount of C3 bound (~ 1.1 nmol/1 m^2) as estimated on regenerated cellulose represents a 1/20 factor of the C3 covalently bound to the Sepharose trypsin surface [8]. In another study, the amounts of covalently bound C3/m^2 of membrane were shown to represent only a factor of approximately $0.2–0.3 \times 10^{-3}$ of the total activated C3 during clinical haemodialysis [23] which was equivalent to approximately 5 μM C3a generated during 4 h of haemodialysis. The logical conclusion would be that the largest part of the activated C3a rises in the fluid phase. The presented hypothesis based on the estimation of the covalently bound C3 on RC by the *ex vivo* was supported by the following findings:

(1) Adsorbed C3 by RC was released by detergents A and B at different magnitudes.
(2) RC membrane-adsorbed C3 was reduced by increasing the pH, a finding similar to the covalent-binding reaction of C3 to Sepharose-trypsin [8].
(3) Factor B release reaction from RC occurred only with detergent B and correlated well at the molar basis (~ 0.6 nmol/m^2) with the estimated covalently-bound C3. Therefore, it could be speculated that factor B may be associated with the covalently bound C3b as a complex C3b, B.

According to the findings presented, it is possible to draw the conclusion that the covalent binding of C3 to the hydroxyl groups of the cellulosic membranes was altered by the modification of cellulose membranes (DEAE-C and CMC), and that the alteration contributes significantly ($p < 0.05$) to the reduction of the covalent binding of C3. This alteration was possibly due to an increased adsorption of complement and other proteins by hydrophobic interaction as demonstrated in the case of CMC or by ionic forces (DEAE and CM-C). Therefore, hydrophobic or hydrophilic complement interaction also occur in regenerated cellulose, since as demonstrated, only an optimum value of 50% of the adsorbed C3 was estimated as being covalently bound.

The phenomenon of the alteration of the C3 covalent binding may be different in DEAE-C and CM-C. The dialyser modules made with DEAE-C membranes reacted as true anionic exchangers during blood perfusion. Although the amount of charged groups in the DEAE-C matrix was, by magnitude, a factor of approximately 100 lower than that in conventional ionic exchanger matrix used for chromatography, protein adsorption during blood perfusion was found to be abundant. The magnitude of protein adsorption by ionic forces was demonstrated. While the total amount of adsorbed C3 and factor B increased with the pH, the pH adsorption curves of C3 and factor B were markedly different. At physiological pH, the tertiary amino groups attached to the DEAE-C membrane surface adsorb proteins and thus may hinder the attachment of C3b to the remaining unsubstituted OH-groups. It has to be mentioned that protein adsorption in DEAE-C at pH 4 was minimal and that detergent A (SDS + urea) could not release membrane-associated C3 and factor B. The reason why a small number of substituted ligands might suffice to reduce C3 covalent binding may possibly be due to protein size and the distance between two charged groups of the same dimension. When C3 (radius ~ 3 nm) and albumin (radius ~ 6 nm) were bound next to charged groups within a distance of ~ 10 nm, the hydroxyl groups in the vicinity will not be available for C3b covalent binding (i.e "camouflage" effect of the hydroxyl groups). However, complement activation also occurs in DEAE-C and the generation rate of C3a is reduced to 50–75% (as compared to RC) and the generation rate of C5a

remained undetectable. Therefore, a certain level of covalently bound C3 may occur before the camouflage of hydroxyl groups intensifies on the membrane surface. However, the extent of covalently bound C3 appear to be significantly reduced as compared to RC (1.2 nmol/m^2). As a theoretical possibility, the estimation of the number of C3 molecules covalently bound to DEAE–C was calculated to follows:

$$A_{C3} \times K_{C3} = \sim 0.5 \text{ nmol/m}^2$$

where A_{C3} is the amount of activated C3 during 4 h haemodialysis using Hemophan (A_{C3} was estimated as being $\sim$1.25–3.5 μmol), K_{C3} is the ratio between membrane-associated C3 and activated C3, and it can be estimated, for instance from regenerated cellulose, that $C3_{ads} \ll C3_{acti}$ ($K_{C3} = \sim 0.2–0.3 \times 10^{-3}$).Therefore, the theoretical estimated amount of the possible covalently bound C3 on DEAE-C membrane was 0.5 nmol/m^2. In the reported study [22] applying analysis by SDS-PAGE and Western blot techniques, the predominant form of C3 associated with cellulose acetate or regenerated cellulose membranes was the fragment C3c. In addition, a modest amount of C3d that contains the thiolester group has been found to be bound to the surface of regenerated cellulose membrane. However, it was concluded by the authors that the potential complement activity was not determined by quantitative or qualitative differences in the binding of activated C3 fragment.

The mechanisms involved in the adsorption of complement proteins on CM-C membranes seem to be less well understood than those involved on DEAE-C membranes. The negatively charged groups increase the hydrophobicity of the membrane surface as well as the anion exchange behaviour of the membrane matrix for lipoproteins, complement C3a and C5a. However, membrane adsorption of complement proteins occurs more by hydrophobic interactions than by ionic forces. The magnitude of CM-C membrane-adsorbed C3 after blood perfusion was markedly greater compared to RC or DEAE-C and as a consequence the release reactions of C3 or factor B using detergents after blood perfusion were different to that observed for DEAE-C as follows:

(1) Markedly greater amounts of membrane-adsorbed C3 ($\sim$1.4 nmol/m^2) were released using urea/SDS detergent (in DEAE-C no C3 was released using the same detergent).
(2) The adsorption of complement C3 onto CM-C was relatively greater than on DEAE-C when the pH was varied from 5–7, whereas varying the pH from 7–12 reduced C3 adsorption on RC by an approximately equal magnitude.
(3) The amount of factor B adsorption on CM-C membrane was similar to that by RC. The reason(s) probably include the adsorption of lipoproteins onto the negative groups of the membrane matrix thereby enhancing further complement protein adsorption by hydrophobic interactions.

The complement inhibitors of the C3 convertase such as factor H and factor I have been suggested to be involved in the minimization of complement activation in modified cellulose. The concept that reducing complement activation by altering the chemical composition of cellulose may involve the competitive binding of factor B and H on the covalently bound C3 has previously been reported by Kazatchkine *et al.* [16]. Complement binding reactions were analysed on heparin and salicic acid-modified surfaces. Other studies by Cheung *et al.* [22] and Ritz *et al.* [14] demonstrated separately that the membrane-associated factor H was enhanced in the DEAE-C membrane suggesting that modification of the cellulose has an effect on the complement regulatory proteins. In accordance with the findings presented in this paper, the specifity of factor H adsorption may be minimal in a system in which protein adsorption was predominantly imposed by ionic forces. As has been demonstrated in this study, the anionic exchange character of the DEAE-C surface matrix enhances protein adsorption in accordance with the net protein charges. It has also been demonstrated that the heparinization of the DEAE-cellulose did not reduce factor B adsorption, suggesting that spontaneous complement adsorption remains prevalent.

In conclusion, the attenuation of complement activation in modified cellulosic surfaces occurred while protein adsorption was enhanced. Therefore, a quick "camouflage" of the hydroxyl groups as well as a translocation of covalently bound C3 may reduce the potential to generate complement C3 and C5 convertase on the cellulose matrix. Existence of greater amounts of non-covalently bound C3 at physiological pH is therefore not a clear indicator of complement activating potential on cellulosic or on synthetic polymer surfaces, but merely an indicator of a shift of the complement activating potential. Protein adsorption may be enhanced by:

(1) Introducing positively charged groups or introducing hydrophobic features onto the surface.
(2) The introduction of negatively charged groups such as carboxymethyl or acetate.
(3) Other possibilities include the introduction of phenyl groups, which in theory should reduce the complement activating potential of the cellulosic membrane more effectively, while magnifying protein adsorption. The unmodified regenerated cellulose matrix characterized as hydrophilic exhibits a slow adsorptive capacity for which a critical optimum amount of covalently bound C3 molecules is attained within 5 min and remains accessible for the complement system, while protein adsorption remains inadequately slow. The critical amount of covalently bound C3 occurring during blood perfusion through regenerated cellulose dialysers should therefore be $\sim$1.2 nmol/m^2 surface area. The critical amount of factor B complexed to C3 at the RC membrane surface should be $\sim$0.6 nmol/m^2 surface area. However, in order to establish if these findings are consistent, further analysis is necessary to clarify whether the critical covalently bound complement C3 is more related to the membrane-bound C3c or to the C3d.

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