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CLINICAL INVESTIGATION

Compartmental distribution of complement activation products in artificial kidneys

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Compartmental distribution of complement activation products in artificial kidneys. The compartmental distribution of the human anaphylatoxins C3a and C5a has been defined during simulated hemodialysis performed with various types of hemodialyzers. New cuprophan hollow fiber dialyzers were found to activate human complement very readily in vitro, while re-used cuprophan dialyzers displayed only modest complement activating potential. The C3a and C5a antigens, formed as a result of complement activation in these dialyzers, accumulated predominantly in the blood path and were not adsorbed extensively on the membrane surface or transported into the dialysate compartment. Cellulose acetate membranes also produced complement activation in vitro, but to a lesser degree than new cuprophan hollow fibers. However, these membranes exhibited a significant capacity to bind the anaphylatoxins to their surface. Polyacrylonitrile membranes appeared to be unique in that they not only failed to activate complement significantly, but they rapidly adsorbed large quantities of C3a and C5a. These findings demonstrate that hemodialysis membranes may differ with regard to their complement activating potential as well as their ability to remove circulating anaphylatoxins from the blood path. Clinical measurements of anaphylatoxin production during hemodialysis reflect these dynamic events.

Complement activation, which results in the formation of the human anaphylatoxins C3a and C5a, is known to occur during hemodialysis performed with certain types of artificial kidney membranes [1–4]. Available data strongly suggest the anaphylatoxins to be the etiologic factors in dialysis leukopenia [1–3, 5–7]. They have also been proposed to cause some of the dialysis–related complications such as hypoxemia and the first–use syndrome [6–9]. Prior clinical investigations have further suggested that dialyzers composed of new cuprophan hollow fibers tend to activate complement much more readily than either re-used cuprophan dialyzers [2] or plate dialyzers composed of polyacrylonitrile [1]. Cellulose acetate hollow fiber dialyzers appear to display an intermediate ability to activate complement [3].

These conclusions were based on studies performed by quantitatively measuring the plasma levels of C3a and C5a antigens found in the blood of patients during maintenance hemodialysis performed with each type of dialyzer. Hypothetically, under these conditions the measured plasma level of either C3a or C5a might be governed by several different factors including: 1) their rate of production at the membrane surface; 2) their transport into the dialysate compartment; 3) their adsorption on the membrane itself; 4) the rate of catabolism in the patient's body; and 5) in the case of C5a, the rate and extent of binding of this bioactive molecule to specific receptors found on the granulocytes of peripheral blood [10]. To test the first three of these hypotheses and further define the properties of hemodialyzers, we have conducted a series of investigations that were aimed at defining the rate of transport and sorption of the anaphylatoxins during simulated hemodialysis performed with several different commonly–employed hemodialyzers.

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Methods

Artificial kidney membranes

Three different commercially available artificial kidney membranes were used in this study. They were cuprophan hollow fiber (CF-1511, Travenol Laboratories, Inc., Deerfield, Illinois, USA, surface area = 0.8 M^2), cellulose acetate sheet (Hemofresh, Daicel Chemical Industry, Ltd, Himeji, Japan, surface area = 0.7 M^2), and polyacrylonitrile sheet (1210H, Hospal, East Brunswick, New Jersey, USA, surface area = 1.0 M^2). The cellulose acetate membrane (Hemofresh) is normally used for hemofiltration purposes. However, there are two ports in the ultrafiltrate compartment such that dialysate can enter and exit as in a hemodialyzer. The polyacrylonitrile membrane (1210H) can be used for either hemodialysis or hemofiltration purpose.

The re-used cuprophan membrane hemodialyzers utilized in this study (CF-1511) were obtained after routine patient use. All patients had been on chronic hemodialysis for at least six months and were free of intercurrent acute illness. None was taking either corticosteroid or nonsteroidal antiinflammatory agents. After a single use by the patient, the cuprophan dialyzers were rinsed with water and stored in 2% formalin.

Prior to use in these experiments, the blood path of the dialyzers was rinsed with one liter of sterile normal saline and then rinsed with air to remove as much saline as possible. Effluents from the re-used cuprophan dialyzers were tested with Schiff's reagent to insure that residual formalin was less than 10 ppm as accepted in clinical practice.

Preparation of pooled human serum

Whole blood was collected from normal human volunteers in plastic collection bags containing citrate (CDPA-1). The plasma obtained by centrifugation was pooled, recalcified with 2 M calcium chloride, and allowed to clot at 37°C. Serum was then expressed from the fibrin clots and stored in 250 ml aliquots at −70°C.

Preparation of radioiodinated polypeptides

Human $C3a_{desArg}$ and $C5a_{desArg}$ were purified to homogeneity from zymosan-activated human serum as previously described [11]. Equine cytochrome C was purchased from Sigma Chemicals (St. Louis, Missouri, USA). These polypeptides (10 μg) were radioiodinated with 1 mCi of either carrier-free $Na^{125}I$ or $Na^{131}I$ (Amersham, Arlington Heights, Illinois, USA) by a solid-phase lactoperoxidase-glucose oxidase (Enzymobeads) method as described by the manufacturer (BioRad, Richmond, California, USA). Free radioisotope was separated from protein-bound by gel filtration chromatography. The specific activity of each preparation was determined to be: ^{125}I-$C3a_{desArg}$, 80 μCi/μg; ^{125}I-$C5a_{desArg}$, 30 μCi/μg; and ^{131}I-cytochrome C, 20 μCi/μg. In each case, the radiolabelled polypeptide contained < 5% free iodine as defined by the precipitability of the protein-bound radioisotope in the presence of 10% trichloroacetic acid.

Simulated hemodialysis circuits

Simulated hemodialysis was performed in the following manner. A 250 ml aliquot of pooled human serum was placed in a sterile beaker, warmed to 37°C and pumped through a test dialyzer at a flow rate of 100 ml/min. Venous outflow from the dialyzer was returned to the stirred serum pool in the beaker, thus permitting measurement of the anaphylatoxin antigens as they accumulated during the period of experimentation. Simultaneously, 250 ml of dialysate (Eri-lyte, Erika Inc., Kockleigh, New Jersey, USA) was warmed to 37°C and recirculated through the dialysate compartment of the dialyzer in a counter current direction at a flow rate of 100 ml/min. Again, the dialysate compartment loop was a closed system, thus permitting measurement of transported molecules which accumulated outside the blood path of the dialyzer. Ultrafiltration through the membrane was minimized by minute-to-minute monitoring of the compartment volumes and adjustment of the ultrafiltration pressure throughout the experiment.

Complement activation and anaphylatoxin formation taking place within the dialyzer was evaluated by removing serum from the recirculated pool and mixing it with one-twentieth volume of 0.2 M disodium ethylenediamine tetraacetic acid (EDTA) to terminate complement activation. These samples were then assayed by radioimmunoassay (Upjohn, Kalamazoo, Michigan, USA) techniques to quantitate the serum levels of C3a antigen. Control experiments were performed by recirculating serum through an identical circuit consisting of polyvinylchloride tubings, but without the presence of a dialyzer. All serum samples were obtained in duplicate.

Experiments designed to define the fate of radiolabelled polypeptides were conducted in a similar manner. However, EDTA (final concentration, 10mM) was added to the pooled serum prior to recirculation to prevent complement activation during the course of these investigations. Immediately prior to recirculation of the serum, 1 to 2 μCi of radiolabelled peptide ^{125}I-$C3a_{desArg}$, ^{125}I-$C5a_{desArg}$ or ^{131}I-cytochrome was added to the 250 ml of EDTA-serum and then the study was initiated as described above. After varied intervals of time, 1 ml aliquots were removed from both the serum pool (blood path) and dialysate compartments. These samples were then counted in a gamma counter.

Calculations

The mass of the tracer substance in either the blood or dialysate compartment at any given time point was given by the product of the concentration and measured volume of the fluid in that compartment. This mass was corrected for the cumulative amount of tracer removed by sampling from that compartment at that time point. The corrected mass was then expressed as a percentage of total mass added to the serum at the beginning of the experiment. Mass not detected in either the serum or dialysate was assumed to be adsorbed onto the artificial kidney membrane surface. At any given time point, these relationships are expressed as follows:

$$\% M_m = [1 - (M_s + M_d)/C_T] \times 100$$

where $\% M_m$ = Mass adsorbed onto the membrane expressed as a percentage of the total mass in the system (C_T).

M_s = Mass corrected for sampling loss in the serum.

M_d = Mass corrected for sampling loss in the dialysate compartment.

Differences in concentration at different time points were tested by Student's paired t statistics. A P value ≤ 0.05 was considered as statistically significant.

Results

Complement activation during simulated hemodialysis

The concentration of C3a antigen found in the recalcified pooled human serum employed in these studies was 1773 ± 94 (SEM) ng/ml. This value is significantly greater than that of normal human plasma (76 ± 15 ng/ml) [1], and is thought to result from complement activation that occurs during the recalcification and clotting processes [12]. However, the functional integrity of the serum complement system was demonstrated by incubation of this recalcified serum with zymosan for 30 min at 37°C, which resulted in significant generation of C3a (final concentration, 35.8 ± 0.9 μg/ml). Therefore, pooled human serum prepared in this manner may be utilized to evaluate the complement-activating potential of hemodialyzers or other artificial medical devices.

Recirculation of the serum through the polyvinylchloride tubing alone without a dialyzer in the in vitro circuit did not result in changes of C3a antigen concentration.

The changes in the serum concentration of C3a antigen that were produced during recirculation of this pooled serum through different dialyzers is shown in Figure 1. New cuprophan hollow fiber dialyzers promoted a steady generation of C3a in the blood compartment throughout the duration of the experiment, reaching a value of 6541 ± 259 (SEM) ng/ml above baseline at 90 min of recirculation. However, even after this period, steady-state levels of C3a antigen were not achieved,

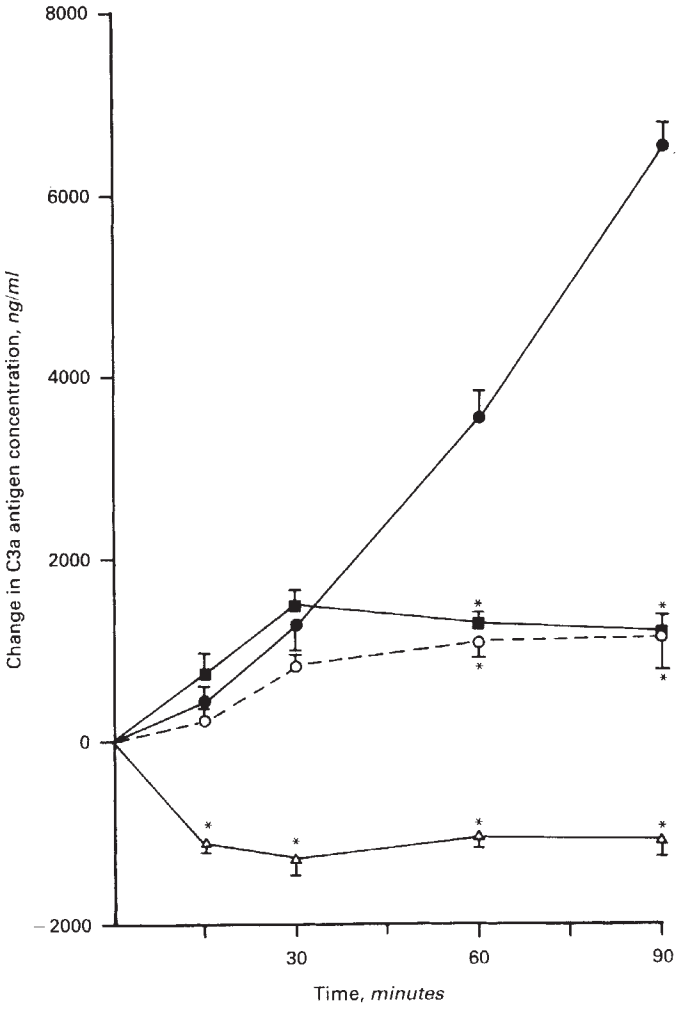

Fig. 1. *Changes in serum C3a antigen concentration produced by in vitro recirculation of serum through different hemodialyzers.* Hollow fiber dialyzers composed of new cuprophan (●), cellulose acetate (■) and re-used cuprophan (○) produce increased concentrations of $C3a_{desArg}$. By contrast, use of polyacrylonitrile plate dialyzers (△) is associated with a decrease in C3a antigen concentration. Data points represent the mean ± SEM of duplicate determinations in three separate experiments. *Denotes values different ($P < 0.05$) from the corresponding values generated by new cuprophan.

suggesting that neither the membrane's activating capacity nor the serum pool's complement had been exhausted.

The generation of C3a antigen produced by either re-used cuprophan or cellulose acetate membranes was measurable, but relatively modest when compared to that produced by new cuprophan hollow fibers. Additionally, after 30 min of recirculation, steady-state concentrations of C3a were achieved with both of these types of dialyzers.

By contrast, the results obtained with the polyacrylonitrile membrane were significantly different from the others studied. When plate dialyzers equipped with this membrane were employed, serum levels of C3a antigen showed an actual decline from baseline levels rather than an increase. A significant diminution in C3a levels was observed as early as 15 min and persisted until the end of the recirculation experiment. A polyacrylonitrile membrane in hollow-fiber format supplied by another manufacturer (PAN15, Asahi, Tokyo, Japan), as well as polysulfone (D30, Amicon, Danvers, Massachusetts, USA) and polycarbonated (20218, Gambro, Lund, Sweden) membranes also promoted a decrement in serum $C3a_{desArg}$ concentration (data not shown).

Distribution of radiolabelled polypeptides during simulated hemodialysis

The compartmental distribution of the complement fragments in different types of dialyzers was defined with ^{125}I-$C3a_{desArg}$ and ^{125}I-$C5a_{desArg}$ employed as tracers. Additionally, ^{131}I-cytochrome C, which is similar in charge and molecular wt to both C3a and C5a, was employed as a control tracer molecule to determine if the observed phenomena were of a general or specific nature.

The time-dependent distribution of these isotopically-labeled polypeptides in new cuprophan dialyzers is shown in Figure 2. These studies demonstrate that distribution and equilibration of all three polypeptides took place during the first 30 min of the experiment. At equilibrium, approximately 80% of C3a, C5a, or cytochrome C remained in the blood compartment. Transport of these low molecular wt polypeptides into the dialysate compartment was negligible (approximately 5% of total) and about 10 to 20% of the tracers appeared to be bound to the membrane surface after 90 min of recirculation. The time-dependent distribution of each of these polypeptides in re-used cuprophan dialyzers (Fig. 3) was similar to that observed with new dialyzers.

Both cellulose acetate (Fig. 4) and polyacrylonitrile (Fig. 5) membranes behaved very differently from cuprophan hollow fibers. With both of these types of membranes, there was a greater time-dependent disappearance of ^{125}I-C3a, ^{125}I-C5a, and ^{131}I-cytochrome C from the blood compartment and corresponding accumulation of the tracers on the membrane itself. Polyacrylonitrile membranes in particular exhibited a pronounced tendency to bind these molecules very rapidly, with equilibrium being reached within 15 min after the onset of recirculation. At equilibrium, polyacrylonitrile membranes bound > 90% of the available C3a and > 80% of the C5a. By contrast, only about 40% of the cytochrome C tracer was adsorbed on these membranes. Transport of these marker polypeptides into the dialysate compartment by either cellulose acetate or polyacrylonitrile membranes was low and comparable to that observed with cuprophan membranes.

Discussion

Activation of the complement cascade occurs during clinical hemodialysis with cuprophan membranes, probably via the alternative pathway [1, 5]. We have suggested that this process is initiated with the attachment of metastable C3b onto the cuprophan membrane surface and subsequent formation of a surface-bound C3 convertase [1]. The C3 convertase then converts C3 to C3b, releasing the cationic fragment C3a into the circulation. Addition of another C3b onto the C3 convertase complex forms a C5 convertase which is capable of converting C5 to C5b and the cationic glycopolypeptide C5a [13]. Once C3a and C5a are released into the circulation, they are rapidly converted by serum carboxypeptidase into their respective

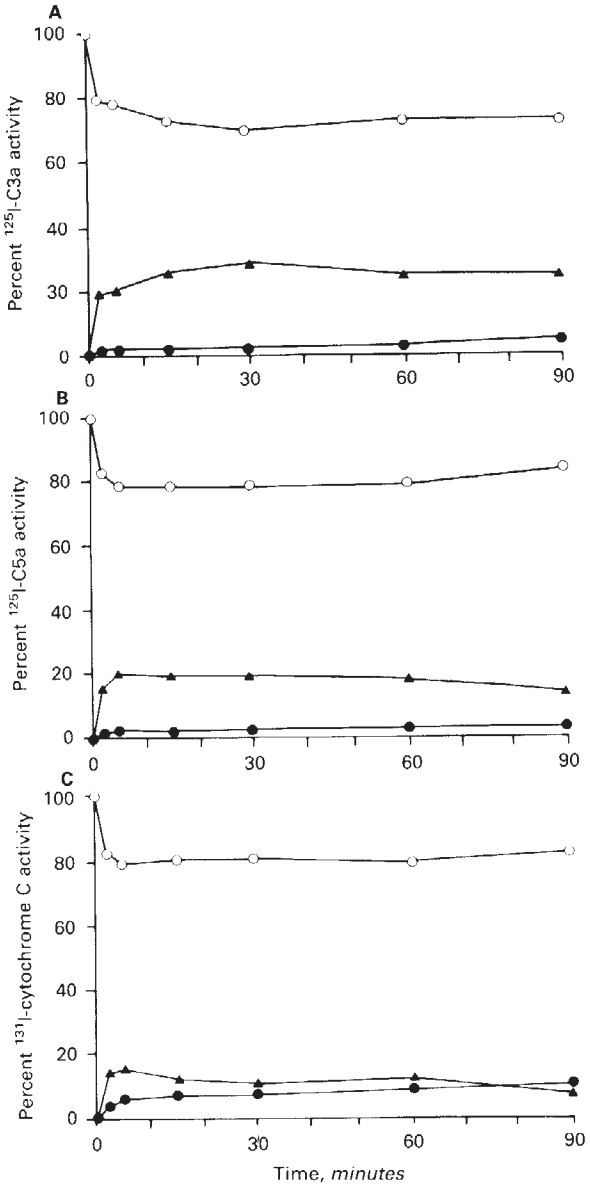

Fig. 2. *Time–dependent distribution of ^{125}I-C3a$_{desArg}$ (A), ^{125}I-C5a$_{desArg}$ (B), and ^{131}I-cytochrome C (C) in the blood path (○), dialysate compartment (●), or adsorbed onto the membrane surface (▲) when EDTA-serum containing tracers is recirculated through new cuprophan hollow fiber dialyzers.*

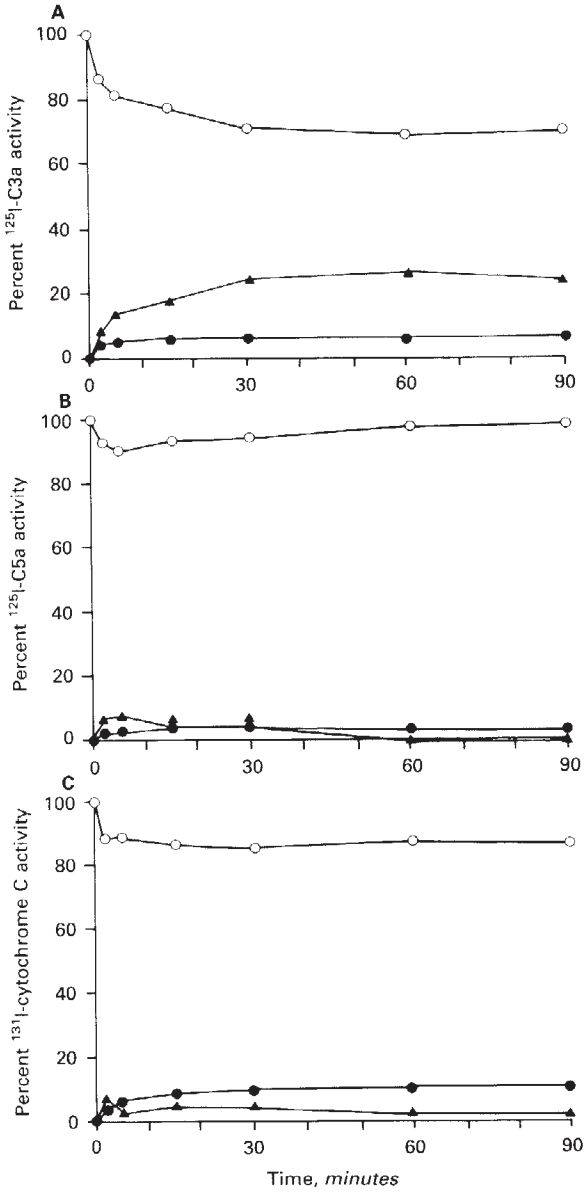

Fig. 3. *Time–dependent distribution of ^{125}I-C3a$_{desArg}$ (A), ^{125}I-C5a$_{desArg}$ (B), and ^{131}I-cytochrome C (C), in the blood path (○), dialysate compartment (●), or adsorbed onto the membrane surface (▲) when EDTA-serum containing tracers is recirculated through re-used cuprophan hollow fiber dialyzers.*

stable desArg derivatives. The antisera in the currently available radioimmunoassay (RIA) recognize both the anaphylatoxins and their desArg derivatives [12].

Such antigens detectable by RIA are readily found in the venous plasma of patients undergoing cuprophan hemodialysis [1–4]. Peak venous plasma concentration of C3a antigen usually occurs at 15 min after the onset of treatment achieving values over 15–fold from baseline, and begin to decline thereafter. It has further been shown that different types of dialysis membranes are associated with drastically different plasma concentrations of the anaphylatoxin antigens [1–3]. However, the dynamic events occurring in the dialyzer as they might affect the final concentration in the plasma have not been studied.

The in vitro system described herein was designed to assess, in a controlled manner, the biocompatibility of artificial kidney membranes from the standpoint of anaphylatoxins. It has certain clear advantages over measurements taken from patient's blood during hemodialysis. First, patient variability in generation and catabolism of the complement fragments can be eliminated using test serum from a single batch. This makes for improved ability to discriminate between different membranes. Secondly, using serum as the test solution avoids largely the confounding effect of anaphylatoxin adsorption by the cellular elements in the blood. Thirdly, this system allows assessment of the compartmental distribution, that is, loss into the dialysate and onto the membrane, of complement activation products in

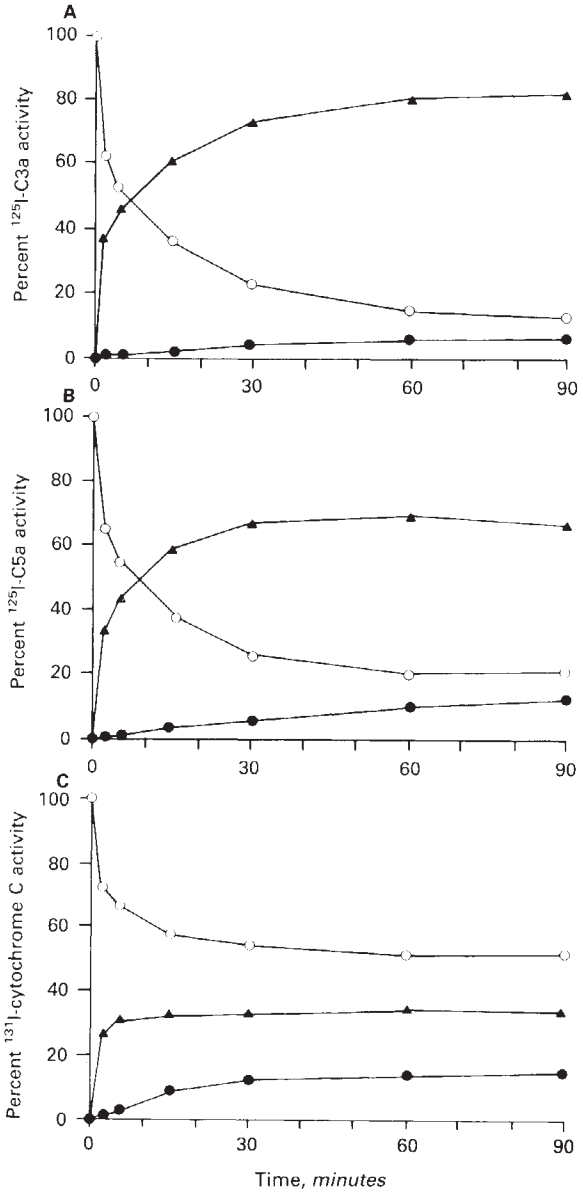

Fig. 4. *Time–dependent distribution of ^{125}I-C3a$_{desArg}$ (A), ^{125}I-C5a$_{desArg}$ (B), and ^{131}I-cytochrome C (C) in the blood path (○), dialysate compartment (●), or adsorbed onto the membrane surface (▲) when EDTA-serum containing tracers is recirculated through cellulose acetate hollow fiber dialyzers.*

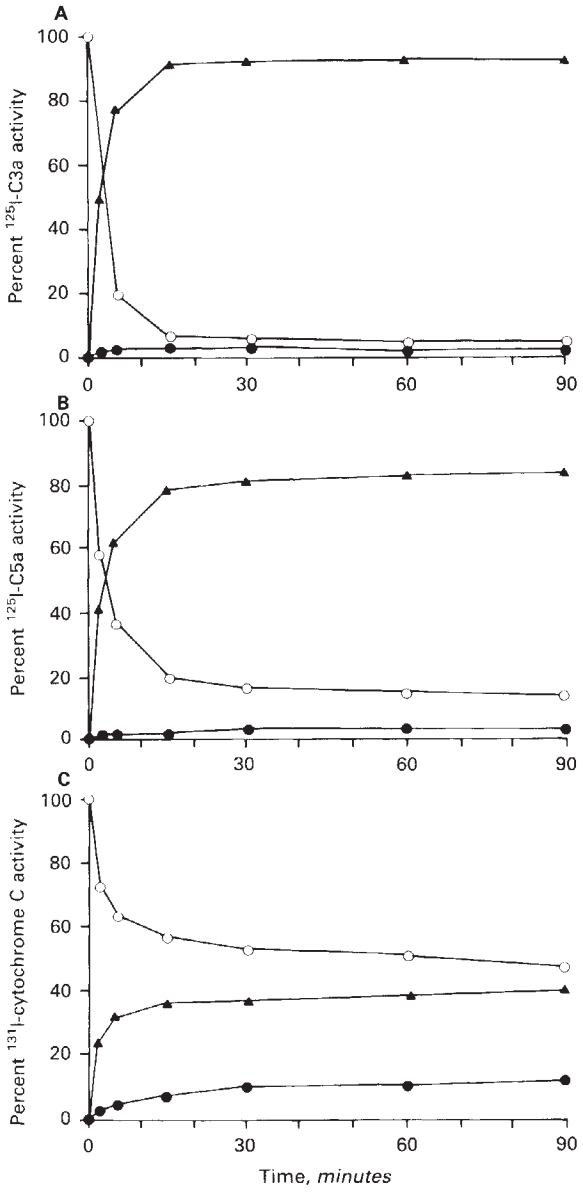

Fig. 5. *Time–dependent distribution of ^{125}I-C3a$_{desArg}$ (A), ^{125}I-C5a$_{desArg}$ (B), and ^{131}I-cytochrome C (C) in the blood path (○), dialysate compartment (●), or adsorbed onto the membrane surface (▲) when EDTA-serum containing tracers is recirculated through polyacrylonitrile plate dialyzers.*

the artificial kidneys, as it might affect the final serum concentration.

We found that new cuprophan membranes indeed generate C3a$_{desArg}$ vigorously in this in vitro system in a manner analogous to the apparent in vivo circumstance, with serum concentration exceeding 6000 ng/ml above baseline after 90 min of recirculation. Under the identical circumstances, re-used cuprophan and cellulose acetate membranes produced significantly lower concentration of C3a$_{desArg}$ than new cuprophan membranes at the end of recirculation. However, during the initial 30 min the rate of rise of all three types of membranes was not significantly different. Presumably, the reactivities of available C3b binding sites on these membranes are rather similar, but the difference in the number of sites limits the total amount of C3a$_{desArg}$ which can be eventually generated. This similarity between new cuprophan and cellulose acetate has been observed in vivo. Clinical data on the cellulose acetate hemofiltration membrane used in the present study have not been reported in the literature. Measurement of plasma C3a$_{desArg}$ concentration in two of our patients showed values of 1170 ng/ml and 769 ng/ml at 15 min, which were again significantly lower than those seen with new cuprophan membranes.

Attenuation of complement activating potentials by the reuse of cuprophan membrane in the present study agrees with that seen in the patients [2]. It is thought to be not due to the formalin per se, since new cuprophan membranes treated with

formalin alone still activated complement vigorously. The lower level of activation by re-used cuprophan membrane is thought to be due to a coating of the membrane surface with C3b-like molecules, preventing further deposition of active C3b and formation of C3 convertase.

In contrast to these membranes, recirculation of serum through polyacrylonitrile membrane in vitro produced a decrement in serum $C3a_{desArg}$ concentration which was evident in the first 15 min. This was different from that observed in dialysis patients where plasma $C3a_{desArg}$ concentration increased rather than decreased with the same membrane, although the magnitude of increment was only about 10% of that for new cuprophan membranes [1]. This discrepancy warrants explanation. The decrease seen with polyacrylonitrile in vitro suggested that rate of removal from the serum was faster than the rate of formation. There is no reason to suspect that the rate of removal was faster in vitro than during clinical hemodialysis. The difference was more likely to be due to the slower rate of formation in the present study because of the slower serum flow. Differences in the concentration of substances regulatory for complement activation such as factor H, I and properdin were not assessed in the present study. Our preliminary data, however, suggest that complement activation occurs somewhat slower in recalcified serum than fresh serum.

The distribution of the $C3a_{desArg}$ and $C5a_{desArg}$ molecules in the artificial kidney membrane system were shown with the tracer experiments in which continuous complement activation was prevented by the divalent ion chelator, EDTA. Transfer of $C3a_{desArg}$ and $C5a_{desArg}$ into the dialysate compartment, as seen in the tracer experiments, was not prominent with any of the membranes, since these polypeptides carry a cationic charge and molecular wt around 10,000 daltons. The modest membrane adsorption together with the large capacity to activate complement probably account for the high concentration of $C3a_{desArg}$ seen with new cuprophan in clinical dialysis and the present in vitro study. In contrast, polyacrylonitrile membranes adsorbed significantly more $C3a_{desArg}$ molecules which contribute to the fall of $C3a_{desArg}$ concentration in Figure 1. The adsorption of $C3a_{desArg}$ by polyacrylonitrile membrane, however, seemed to be saturable and was estimated to be 450 μg, approximating only 20% and 2% of the estimated total amount present in the plasma at the time of peak concentration in patients dialyzed with polyacrylonitrile and new cuprophan membrane, respectively [1]. Therefore, the activation potential, rather than the adsorption capacity, seems to be the major determining factor of the net plasma concentration of $C3a_{desArg}$ in the dialyzed patients.

Re-used cuprophan membranes were associated with serum $C3a_{desArg}$ concentration similar to that of cellulose acetate, but the amount adsorbed was far less, being similar to that of new cuprophan. In other words, re-use attenuated the complement activating potential of cuprophan membranes but did not change the adsorption capacity. The two phenomena are not associated.

The specificity of $C5a_{desArg}$ and $C3a_{desArg}$ binding to these membranes were assessed by comparison with cytochrome C, a molecule with similar molecular wt and isoelectric point. Although not closely correlated, cellulose acetate and polyacrylonitrile membranes adsorbed $C3a_{desArg}$, $C5a_{desArg}$ and cytochrome C more than new or re-used cuprophan. This suggested that the nature of binding between the complement fragments and the artificial kidney membranes was rather nonspecific. It is tempting to postulate that the large propensity of polyacrylonitrile membranes to adsorb cationic proteins is related to the anionic nature of its surface. However, there are two lines of evidence against this hypothesis. First, the polyacrylonitrile membrane with a neutral surface charge (PAN15, Asahi) also promoted a decrement in serum $C3a_{desArg}$ concentration when tested in vitro (unpublished observation). Secondly, it has been shown recently that even immunoglobulin G (isoelectric point = 7.3) and fibrinogen (isoelectric point = 5.4) are adsorbed significantly by the anionic polyacrylonitrile membrane (Hospal) [14]. It seems, therefore, that polyacrylonitrile membrane has a propensity to adsorb a large variety of plasma proteins nonspecifically.

Measurements on the serum C3a antigen concentrations in the present study are in general agreement with those obtained during clinical hemodialysis [1–4]. The highest concentration is associated with new cuprophan and the lowest with polyacrylonitrile, with re-used cuprophan and cellulose acetate in between. However, generation rate by an artificial membrane cannot be determined by plasma concentration alone. Removal by the patient, membrane and dialysate are all factors which need to be considered, as indicated above. For example, although loss of anaphylatoxins into the dialysate compartment through hemodialysis membranes is small in general, the loss through a plasmapheresis membrane could be substantial.

Hemodialysis leukopenia is most likely caused by the formation of the anaphylatoxins. Although not universally agreed upon, some clinical and animal studies also suggest that complement activation is important in the pathogenesis of certain dialysis–related complications, such as hypoxemia, pulmonary hypertension and the first–use syndrome [3, 4, 6–9]. How these variabilities of artificial membranes in adsorbing and removing RIA-detectable anaphylatoxin antigens affect the welfare of the dialysis patients is unknown at present.

In conclusion, artificial kidney membranes possess different potentials to activate the complement system. Such activation potentials can be tested conveniently in vitro as described. Besides generation of the complement activation products, artificial kidney membranes also adsorb these molecules onto their surfaces. The capacities of adsorption also differ among membranes. These two phenomena are not necessarily related. Membranes which produce the least amount of anaphylatoxins and simultaneously adsorb the most may be considered as most biocompatible from the standpoint of complement activation.

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