Possible Mechanism for in Vitro Complement Activation in Blood and Plasma Samples: Futhan/EDTA Controls in Vitro Complement Activation

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Background: Ongoing in vitro complement (C) activation in citrate or EDTA plasma has prevented an accurate analysis of C-activation products generated in vivo. The aim of this study was to characterize handling and storage conditions required to prevent in vitro C activation in blood and plasma samples collected with Futhan/EDTA.

Methods: Biotrak™ RIAs were used to quantitatively measure C3a and C4a in blood and/or plasma samples from healthy individuals (controls) and from liver transplant patients. Blood samples were routinely drawn into either EDTA (1 g/L) tubes or into tubes containing both EDTA (1 g/L) and Futhan (0.1 g/L) and immediately centrifuged at 2000 g for 15 min at 4 °C.

Results: In controls, C4a, but not C3a, in fresh samples (time 0) was higher in EDTA plasma than in Futhan/EDTA plasma (n = 20; P = 0.002). Futhan/EDTA prevented C3a and C4a generation in blood and plasma samples held at room temperature (22–23 °C) for 1 h and in plasma held for 24 h at 4 °C or −70 °C. The mean C3a concentration (1.76 mg/L; n = 19) at time 0 in EDTA plasma samples from liver transplant patients was significantly higher than for controls (0.34 mg/L; n = 11). In these patients, the mean C3a in EDTA samples increased to 13.8 mg/L after 60 min at room temperature, but there was no change in the C3a concentration of an EDTA plasma from a control. In the patients, C3a concentrations were lower in Futhan/EDTA plasma than in EDTA at time 0 and after 60 min at room temperature (1.40 and 2.02 mg/L, respectively). The mean patient C4a was 4.02 mg/L in EDTA plasma at time 0 vs 0.24 mg/L for controls; it increased to 16.9 mg/L after 60 min at room temperature compared with 0.76 mg/L for controls. The mean patient C4a was 0.83 mg/L in Futhan/EDTA plasma at time 0 vs 0.1 mg/L for controls. Neither patient nor control C4a concentrations increased vs time in Futhan/EDTA.

Conclusion: The combination of Futhan (0.1 g/L) and EDTA (1 g/L) eliminates in vitro C activation.

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The blood complement (C)1 system has a wide array of functions associated with a broad spectrum of host defense mechanisms, including both antimicrobial and antiviral actions (1, 2). Products derived from the activation of complement components include important non-self-recognition molecules C3b, C4b, and C5b as well as the anaphylatoxins C3a, C4a, and C5a, which induce a variety of cellular immune responses (3–8). These anaphylatoxins can also act as potent proinflammatory agents (9, 10). The role of complement in immunopathogenesis is well documented for several autoimmune diseases, such as rheumatoid arthritis (11, 12), lupus erythematosus (SLE) (13, 14), and acute glomerulonephritis (15, 16). Other pathologies that involve activation of the complement system include sepsis (17, 18), respiratory distress syndrome (19, 20), and multiorgan failure (21, 22). Recent interest in C activation associated with transplanted organ rejection (23–25) points out the need for a reliable and accurate assay system for monitoring in vivo C activation in these and various other patient populations. Unfortunately, a

1 Nonstandard abbreviations: C, complement; C3a and C4a, activation fragments from complement components C3 and C4; SLE, systemic lupus erythematosus; Futhan, 6-amidino-2-naphthyl-p-guanidinobenzoate dimethanesulfonate (FUT-175 or nafamostat mesilate); MBL, mannose-binding lectin; and MASP, MBL-associated serine protease.
major complication exists in the form of extensive in vitro C activation that occurs in drawn EDTA blood samples. This in vitro activation has done much to impede development of reliable complement assays both for research and clinical applications.

In designing a routine diagnostic complement assay for the clinical laboratory, sample stabilization becomes a key issue. It is well established that neither of the major C-activation pathways (i.e., the classical and alternative pathways) can function in the presence of the metal chelator EDTA (26). Consequently, understanding the mechanism of in vitro C activation is as important as developing a method to control it.

It has been known for some time that amidine and guanidine derivatives will inhibit C-mediated hemolysis (i.e., C activation) (27). Fujii and Hitomi (28) designed, synthesized, and characterized a potent synthetic inhibitor of complement and coagulation proteinases, based in part on this information. This inhibitor is 6-amidino-2-naphthyl-4-guanidinobenzoate dimethanesulfonate, commonly referred to in the literature by the trade name Futhan or FUT-175, or by the generic name of nafamostat mesilate. The advantage of this particular proteinase inhibitor as a blood additive is that it has an unusually broad specificity for serine proteinases, being a potent inhibitor of both coagulation and complement proteinases. Futhan inhibits thrombin, plasmin, and kallikrein (both plasma and pancreatic), all of which can degrade C components C3, C4, and/or C5 (29, 30). Previous studies have focused on identifying the complement proteinases that are targeted by Futhan, which include C1r, C1s, and factors B and D (31). This inhibitor was also effective toward Hageman factor and factor Xa at submicromolar concentrations (32). Several studies have examined the in vivo protective effects of Futhan in various models of immunological reactions including Forssman shock in guinea pigs, passive cutaneous anaphylaxis in rats, and delayed hypersensitivity reactions and endotoxin shock in mice (33, 34). Because Futhan inhibits C-mediated hemolysis by both the alternative and classical pathways (35), it was concluded that the major effect of this compound on in vivo immunological reactions resulted from direct inhibition of enzymes in the C-activation pathways.

A new pathway of C activation, called the lectin pathway, was discovered recently (36). This pathway involves a mannan-binding protein or lectin (MBL) that is identical to the bactericidal Ra-reactive factor that binds to the Ra polysaccharides on various strains of bacteria (37). MBL is a multichain, multisubunit protein that functions similarly to the C1q component of the classical pathway. There are two proteinases associated with MBL: MBL-associated serine proteinase-1(MASP-1) and MASP-2 (38). The MBL:MASP-1-MASP-2 complex is activated via MBL binding to neutral sugars, activating the MASP enzymes, which then cleave components C2, C3, and C4 to initiate the classical C pathway (36, 37). These MASP enzymes are likely candidates for the proteases that promote C activation in EDTA plasma.

Although extensive studies have been undertaken to characterize Futhan activity in vivo and to identify the various proteinases that it inhibits, its effectiveness as an inhibitor of C activation in drawn blood/plasma samples, particularly for patient samples, is less well characterized. Futhan effectively stabilizes freshly drawn blood samples from healthy individuals, permitting direct measurement of the anaphylatoxins C3a and C4a (39). The effectiveness of Futhan for controlling in vitro C activation in patient blood in which the activating enzyme concentration can be relatively high has not been established. In the present study, we describe parameters for using Futhan, along with EDTA, as effective additives for preventing C activation during routine handling and processing of blood and plasma samples. It was important to show that the combination of Futhan and EDTA is effective when in vitro C activation is relatively high to establish clinical utility of the procedure. Reliable estimates of the circulating concentrations of C3a, C4a, and C5a can be used as an index of in vivo C activation only if in vitro activation is totally eliminated. Because improper handling of blood samples and variable intrinsic proteinase activity can lead to substantial in vitro C activation, even in EDTA plasma, an additional inhibitor must be used. Consequently, we developed a blood-drawing and handling protocol that stabilizes blood or plasma from activation of either component C3 or C4. The ability of Futhan/EDTA to stabilize complement in frozen (−70°C) plasma samples for months or at 4°C for >24 h permits research and clinical samples to be stored for later measurement of total circulating C-component concentrations or for determining the concentrations of the C-activation fragments C3a, C4a, and/or C5a.

When applied to patient blood samples, the Futhan/EDTA blood-drawing protocol provided evidence that circulating C3a and C4a in liver (allograft) transplant patients were significantly increased compared with control (i.e., “normal”) individuals. None of these patients were experiencing rejection episodes but were considered clinically stable on their respective treatment regimens. The more striking result was that in vitro conversion of C3 and C4 vs time was markedly higher in the EDTA blood or plasma from these patients than observed in EDTA plasma from control individuals.

**Materials and Methods**

**REAGENTS**

Futhan was obtained from Banyu Pharmaceutical. The complement C3a (code RPA 518) and C4a (code RPA 519) Biotrak RIA assay kits were obtained from Amersham Life Sciences. EDTA Na₄ (Sigma grade) was purchased from Sigma. All other chemicals, buffer salts and reagents were analytical reagent grade.
BLOOD SAMPLES
Blood samples of 5–10 mL were drawn from healthy donors, liver transplant and SLE patients under an approved Human Subjects protocol no. 96-293, and after the individuals had signed an informed consent form agreeing to be voluntary donors. The healthy donors were a random group of male and female individuals between the ages of 27 and 48 years. Liver transplant patients were selected from a group of patients being routinely followed at The Scripps Clinic Foundation by Dr. John Brems, currently at Loyola University Medical Center, Maywood, IL. None of these organ transplant patients were experiencing acute rejection episodes or other detectable liver malfunctions at the time that the blood was drawn.

BLOOD PROCESSING PROTOCOLS
Blood samples were either drawn into EDTA tubes2 (Venoject; Terumo Corp.) or Futhan/EDTA tubes (Venoject; Terumo Europe). The plasma was collected immediately by centrifugation at 2000g for 15 min at 4 °C unless otherwise indicated. The plasma samples were either processed immediately for analysis or snap frozen in liquid nitrogen and stored at −70 °C. Frozen samples were thawed at 4 °C and processed as described in the text.

ASSAY PROCEDURE AND DATA ANALYSIS
Equal volumes of the plasma sample and the precipitating reagent supplied in the Biotrak RIA kits were mixed by thorough agitation and incubated at room temperature for 5 min. The mixture was then centrifuged at 2500g for 15 min at 4 °C. The supernatant from each sample tube was collected for analysis in the RIA assay. A 50-μL aliquot of the original or diluted supernatant was mixed with 50 μL of the Biotrak assay buffer in a 12 × 75 mm polypropylene tube. The 125I-labeled C3a or C4a and the specific antibody solutions (50 μL of each) were added and incubated at room temperature for 30 min. A 50-μL aliquot of the second antibody (goat anti-rabbit) was added to the tube, and the mixture was incubated for an additional 30 min at room temperature. Isotonic saline (2 mL) was added, and the tube was centrifuged at 2000g for 10 min at 4 °C to pellet the antigen-antibody complexes. The supernatants in the tubes were decanted, and the tubes were counted for 1 min in a Cobra Autogamma Model 5002 (Packard Instruments) scintillation counter. Analysis of each sample was performed in duplicate. Data were analyzed using RiaSmart software supplied by Packard Instruments and installed in the Cobra Autogamma counter.

Results
We examined the concentrations of Futhan that were required to protect EDTA plasma samples from in vitro C activation vs time. When no Futhan was present, the concentrations of C3a in EDTA plasma were only slightly increased compared with samples containing Futhan, even after standing for 24 h at 4 °C. Fresh EDTA-plasma samples were collected just before analysis (EDTA/p0). The whole blood (EDTA/b1) and plasma (EDTA/p1) samples were stored at room temperature for 1 h, and a separate set of plasma samples were stored for 24 h at 4 °C (EDTA/p4) or frozen (EDTA/pF) at −70 °C and thawed before analysis. The C3a values for these samples were not significantly different for any of the various handling conditions, and adding Futhan to the EDTA samples had only a minimal effect because there appeared to be little C3 activation in nondiseased plasma under these conditions (see Fig. 1). Although these data were obtained from the plasma of a single individual, similar data have been obtained from blood and plasma of other normal donors (see Figs. 2 and 3). When Futhan concentrations were 0.2 g/L, the C3a concentrations in all of the samples were actually somewhat higher than at the lower Futhan concentrations. This activation may be a result of nonspecific effects related to exposing the plasma to increased salt concentrations.

These same handling and storage conditions did have variable effects on C4 activation in the EDTA plasma (i.e., in the absence of Futhan). However, the characteristic activation of C4 in the control EDTA plasma was abolished when Futhan was added to these samples. Consequently, the major effect of adding Futhan to the control EDTA plasma was to reduce C4a, but not C3a, generation in vitro. Futhan concentrations as low as 0.01 g/L appeared to be effective in protecting the control plasma from C activation under the conditions described. These data indicated that activation of C in EDTA plasma resulted from either the classical or lectin pathway of activation because primarily C4a was generated. Furthermore, the plasma enzyme(s) clearly prefer cleaving C4 over C3.

Commercial blood-drawing tubes (EDTA/Futhan Venoject™ tubes; Terumo) containing 5 mg of disodium EDTA and 0.5 mg of Futhan per tube were designed for collecting 2.5–5.0 mL of blood. Although Futhan at 0.01 g/L was effective in protecting normal plasma from C4 conversion, it was anticipated that higher concentrations might be required to protect C4 in patient plasma in which the conversion of C4 (i.e., the converting enzyme concentrations) was significantly greater. The EDTA/Futhan vacuum tubes were developed to stabilize C in blood samples being drawn for either research or clinical applications.

Futhan is an inhibitor that exhibits a broad specificity for many of the blood serine proteinases. Therefore, it might be anticipated that Futhan would be superior to many other anticoagulants in preventing complement conversion in whole blood. General serine proteinase applications.

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2 The Futhan/EDTA tubes are produced by Terumo Europe and can be purchased from PerSeptive Biosystems, 500 Old Connecticut Path, Framingham, MA 01701. At present, the Futhan/EDTA tubes are approved only for research use in the United States.
inhibitors of the coagulation system such as benzamidine (an inhibitor of Factors VII and Xa, thrombin, plasmin, and C1s) or Trasylol (an inhibitor of thrombin, kallikrein, and plasmin) were compared to Futhan for their effectiveness in controlling C activation. It is well known that certain of the coagulation proteinases can convert C3 and

Fig. 1. Futhan inhibits in vitro C activation in EDTA (10 mmol/L) plasma.
Blood from a normal donor was drawn into EDTA, and 0–0.2 g/L Futhan was added as indicated. Plasma was recovered within 30 min of being drawn, and a precipitating agent was added to the time 0 plasma sample (EDTA/p0) to prevent further C activation. The EDTA plasma (EDTA/p1) and EDTA blood (EDTA/b1) samples were stored with or without Futhan for 1 h at 22 °C. A separate set of EDTA plasma samples was stored either at 4 °C for 24 h (EDTA/p4) or frozen at −70 °C for 24 h (EDTA/pF) and then thawed for analysis. C3a and C4a were determined in duplicate using the radioimmunoassay procedure; the mean value for each sample is reported.

Fig. 2. Effect of inhibitors on C3a and C4a in blood.
(A), C activation in blood was measured in the presence of several anticoagulants, both with (+EDTA) and without EDTA (0.5 g/L; −EDTA). Whole blood from a healthy donor was drawn into 0.15 mmol/L benzamidine, 20 μmol/L Trasylol, or 0.1 g/L Futhan and processed either immediately (0 min) or after 60 min (1 hr) at room temperature; samples were then analyzed for C3a and C4a. Benzamidine and Trasylol failed to prevent C3a generation in these blood samples, whereas Futhan appeared to be an effective inhibitor. When these same additives were combined with 10 mmol/L EDTA (+EDTA), there was no generation of C3a; however, extensive conversion of C4 occurred except when Futhan was present. Data reported as the means of duplicate analyses. B&T, benzamidine plus Trasylol. (B), EDTA (0.5 g/L) whole blood from a healthy donor was incubated at 4 °C with and without 0.1 g/L Futhan. C3a and C4a were determined in duplicate vs time over 23 h. C3a concentrations in EDTA blood (□) were only slightly higher than C3a concentrations in Futhan/EDTA blood (●) vs time. C4a concentrations in EDTA blood (□) continued to rise throughout the entire 23-h period, from 0.3 mg/L to >1.70 mg/L. When the blood was drawn into Futhan/EDTA (●), no C4a was generated over the entire 23h period.
C4 (39); therefore, it is important to inhibit both plasma complement and coagulation enzymes to prevent potential non-complement enzymes from causing C activation.

In the absence of EDTA, neither benzamidine nor Trasylol, either individually or in combination, could fully stabilize C3 (i.e., prevent C activation through the alternative pathway) vs time (Fig. 2A). It should be noted that in Fig. 1, the concentrations of C3a generated in EDTA whole blood at room temperature after 60 min were 600 mg/L, whereas in Fig. 2A, the C3a values were 200 mg/L under the same conditions. The differences between these two results reflect variations in in vitro C activation that are commonly observed between individual normal donors. The blood donor in Fig. 1 was not the same as the donor in Fig. 2A. From these data it is clear that inhibitors of coagulation proteinases were incapable of preventing C3 conversion. When EDTA was added to the blood, there was a remarkable increase in C4 conversion, but virtually no C3 was converted. The enzymes responsible for C4 conversion in the presence of EDTA remain unknown. We hypothesized the enzyme to be MASP-2 of the lectin pathway (37, 38). It is not C1s of the classical pathway because C1 is effectively inactivated by the removal of calcium ions (40) and any C1s activated would be under the rigorous control of C1 inhibitor (41).

Futhan, with or without EDTA added, was effective in stabilizing both C3 and C4 in normal whole blood for up to 1 h at room temperature (Fig. 2A). When blood samples were drawn and immediately placed on ice, the samples were stabilized for much longer (Fig. 2B). Note that EDTA was sufficient to prevent C3a generation in the control blood at 4 °C over a 27-h period. However, even at 4 °C, significant quantities of C4a can be generated in EDTA blood unless Futhan is added. These results define a suitable window of opportunity for collecting stabilized plasma from blood samples drawn into Futhan/EDTA, and this protocol can be applied to both research and clinical samples.

We examined the C3a and C4a concentrations in EDTA blood obtained from healthy donors, processed immediately with and without Futhan present. These data helped to establish a baseline for circulating concentrations of these factors in healthy individuals. The mean values for C3a and C4a in EDTA plasma and in EDTA/Futhan plasma were obtained from a total of 20 individuals. The plasma was recovered immediately from the blood samples, and analysis gave the data shown in Table 1. This study involved 11 male and 9 female donors between the ages of 27 and 48 years. Although on inspection the EDTA/Futhan values for C4a appeared to be only marginally lower than the EDTA-plasma values when a careful blood processing procedure was used, there was a statistically significant protective effect of the Futhan. When the values in Table 1 (i.e., immediate processing to collect plasma) are compared with values obtained from EDTA whole blood held on ice for only a few hours (see
Blood samples were drawn in EDTA (1 g/L) or Futhan (0.2 g/L) plus EDTA, and the range of values are also reported (in parentheses) for these samples. EDTA and Futhan/EDTA plasma in II was significant (P < 0.001). The difference between the C4a values obtained from patient plasma vs time, even at 4 °C.

C3a and C4a concentrations in normal EDTA plasma usually rose rapidly even in normal blood.

The plasma was analyzed for C3a (■) and C4a (■) vs time at 37 °C. The C3a and C4a concentrations at time 0 were markedly increased in this patient’s Futhan/EDTA plasma, indicating that in vivo activation had occurred. The C3a concentration vs time was no higher in EDTA plasma than in the Futhan/EDTA (F/EDTA) sample. The C4a concentration in EDTA plasma was higher than the concentration in Futhan/EDTA at time 0 and continued to rise over the entire 60 min at 37 °C. The C4a concentration of 11.2 mg/L at 60 min in the SLE patient’s EDTA plasma was 10 mg/L after 60 min at 37 °C compared with 8–9 mg C4a/L in two normal plasmas (data not shown). Although the rate of C4 activation in the SLE patient’s EDTA plasma at 37 °C was very high, this appears to be a typical rate of in vitro C activation at this temperature. These 37 °C results for C4 conversion illustrate that if blood is not chilled immediately after it is drawn and/or Futhan is not added to the stabilized samples, the background concentration of C4a will rise rapidly even in normal blood.

The plasmas from the organ transplant patients, whose mean C3a and C4a concentrations are shown in Fig. 3B, gave even more interesting patterns when each individual sample was examined. The patterns observed in Fig. 3B for EDTA vs Futhan/EDTA plasma C activation indicated that these patients had high concentrations of active plasma enzyme. When Futhan and EDTA were used to stabilize the plasma samples, there was no significant C3a or C4a generation in any individual patient’s plasma vs time at room temperature (Fig. 5). However, the concentrations of both C3a and C4a in Futhan-stabilized plasma, which should represent the true circulating concentrations, were increased in all patients compared with healthy, nontransplant individuals. One sample (patient 15) gave a markedly increased C3a value after 1 h of incubation, and we concluded that mishandling during the assay procedure was responsible for this artifactual result. The incremental increases in Futhan/EDTA plasma C3a or C4a after 1 h at room temperature were minimal in all patients except patient 15.

A more dramatic result was observed when the EDTA plasmas from these patients were evaluated. The scale in

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<th>Table 1. C3a and C4a* in plasma.</th>
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*Values given for n = 20 donors, 11 males and 9 females, ages 27–48 years. Blood samples were drawn in EDTA (1 g/L) or Futhan (0.2 g/L) plus EDTA, and then processed immediately at room temperature. The standard deviations (±) and range of values are also reported (in parentheses) for these samples.

o Total data set containing several apparent outlying values.

s Test indicated that mean values for C3a in EDTA vs Futhan/EDTA plasma were not significantly different. The difference between the C4a values obtained from EDTA and Futhan/EDTA plasma in II was significant (P = 0.002).

Fig. 2B), it is clear that C4 is not protected in blood from healthy donors without the addition of Futhan.

These data also demonstrated that some donors who present themselves as normal can have background C4a concentrations >2 SD higher than the mean value, as shown in row I of Table 1. The fact that C4a was significantly lower in Futhan/EDTA samples than in samples with EDTA alone indicates that low levels of in vitro activation also commonly occur in normal plasma.

C activation in EDTA plasma was compared to activation in EDTA/Futhan plasma at room temperature (22–24 °C) over a period of 1 h (Fig. 3A). The data are reported as mean plasma concentrations (m) from a separate group of donors than those reported in Table 1, and the only values that changed during this period of time were the C4a concentrations. C4a was increased approximately three- to fourfold on average over 60 min in EDTA plasma for this group of 11 normal blood donors. C4a generation varies significantly even between normal individuals, and C4a concentrations in normal EDTA plasma usually rose significantly vs time, even at 4 °C.

A dramatically different pattern of C4 activation was observed in the plasmas of liver transplant patients (42) compared with normal individuals (Fig. 3B). These subjects were stable transplant patients not undergoing a clinically detectable rejection episode. The background plasma C3a and C4a concentrations at time 0 were significantly higher than those in plasma from the normal population. After 60 min at room temperature, the mean C4a concentration in the patient EDTA plasma had risen to 25- to 50-fold higher than in normal EDTA plasma.

We examined activation of C4 in a patient with SLE and found that the circulating concentrations of C3a and C4a (i.e., in EDTA/Futhan plasma) were much higher than in normal EDTA/Futhan plasma, which is consistent with classical pathway activation (Fig. 4). The C4a concentration in EDTA plasma from the SLE patient exceeded that in EDTA/Futhan plasma by a factor of 2 to 2.5. This difference was significant by Student’s t-test (P < 0.05). The incremental increases in Futhan/EDTA plasma C3a or C4a after 1 h at room temperature were minimal in all patients except patient 15.
Fig. 6 is more than 10-fold higher to accommodate the large increases in C3a and C4a occurring after 1 h at room temperature. Although the mean values for C3a and C4a were markedly increased, there were extreme differences between individual patients. Patients 4, 6, 8, 9, 11, 12, 15, and 16 showed extensive in vitro C activation vs time, which we speculate to be caused by the MASP enzymes of the lectin pathway. The fact that only some of these patients demonstrated marked increases in C3a and C4a generation in vitro indicates that a discriminating method exists to detect or evaluate these patients once the underlying cause for activating the converting enzyme(s) is understood. Because these transplant outpatients demonstrated no clinical signs of organ rejection or infection when these plasma samples were taken, no biopsies were obtained to further evaluate the status of the organ.

Discussion

It is generally recognized that measurements of C activation in human blood samples are at best cumbersome and procedurally difficult to perform, and at worst are unreliable. Complement is a system of >20 plasma proteins, and these components represent three known activation pathways or cascades. The unreliability of C measurements among test systems is related in part to the various tests themselves. Measurements of the blood concentrations of the various intact complement components have been used to determine whether concentrations are in the...
normal or abnormal range, but this type of data fails to indicate whether ongoing C activation is occurring. Measurements of the various breakdown or activation products, such as C3c, C3d, C4d, or the C5b-9 complex, have all been utilized to monitor ongoing C activation. These measurements become difficult to interpret if in vitro activation occurs, and there are various drawbacks, such as distribution problems (i.e., cell bound vs free) and catabolism or metabolism issues that also arise. The fragments C3a and C4a are smaller and more stable than most other molecules released during C activation and are prime candidates for monitoring the C-activation process. It is generally accepted that accurate measurement of circulating concentrations of the C3a and C4a molecules could have important clinical applications in monitoring immune diseases.

The primary advantages of measuring these two activation fragments of components C3 and C4 are (a) C3a and C4a concentrations are direct indicators of the type and extent of activation because C4a is generated only during classical or lectin pathway activation and C3a generation in the absence of C4a confirms alternative pathway activation; (b) The desArg forms of C3a or C4a no longer bind to their specific cellular receptors and thus circulate freely without further degradation, and once C3a and C4a are released into the blood, they are rapidly converted to the desArg forms (C3a desArg and C4a desArg) by serum carboxypeptidase N (43); (c) C3 and C4 are abundant blood proteins, and even minor C activation can be detected by sensitive assays for these breakdown products; (d) the factors C3a desArg and C4a desArg are extremely stable and soluble proteins and are not denatured by handling; and (e) generation of C3a or C4a signals the production of numerous other physiologically important breakdown products of the C cascade, namely C3b, C4b, C3bi, C4bi, C3d, C4d, and C5b-9, each having known biologic functions and activities.

Complement is not stable in EDTA plasma vs time, and numerous earlier attempts to stabilize complement in blood or plasma have been reported. The most successful procedure to date involved the use of a broad-spectrum serine proteinase inhibitor called Futhan (31–35). A variety of inhibitor and stability studies using Futhan have been reported in the literature; unfortunately, many of these reports are published in Japanese journals, and these results are not widely known. Our goal was to design a protocol for blood or plasma collection and handling that can effectively stabilize C, even in patient blood samples in which in vitro C activation is extensive. Previous studies using Futhan to prevent C activation were focused mainly on normal blood samples. In normal EDTA blood or plasma held at 4 °C, we observed significant activation of C4 vs time. There was little conversion of C3 in normal EDTA blood or plasma at 4 °C up to 24 h, indicating that the proteinase responsible for C4a generation selectively cleaves C4 over C3. The addition of 0.01 g/L Futhan to the EDTA plasma produced a measurable reduction in C4 conversion vs time, and 0.10 g/L Futhan virtually eliminated C4 cleavage over a 24-h period. Because 0.10 g/L Futhan is the concentration of the inhibitor used in commercial Futhan/EDTA blood-drawing tubes, many of our experiments were performed using this concentrations of the inhibitor. It was important to test whether the combination of Futhan and EDTA in blood or plasma would be adequate to stabilize the C4 over a 24-h period. This would indicate that blood or plasma samples could be drawn at one site and shipped for analysis, either on ice or frozen, to another site. It appears that Futhan/EDTA plasma, but not whole blood, can be collected and safely stored at 4 °C for at least 24 h, based on the data shown in Fig. 2B. The EDTA/Futhan plasma appears to provide an appropriate sample for obtaining reliable circulating concentrations of C3a and C4a (or C3 and C4) while avoiding in vitro activation that could render the data invalid.

Because Futhan is a serine proteinase inhibitor that inhibits coagulation proteinases as well as complement proteinases, we examined several other proteinase inhibitors that are known to inhibit proteinases in both blood systems (Fig. 2A). We were surprised to find that both benzamidine and Trasylol (Kunitz inhibitor) were relatively ineffective in preventing C3 conversion in whole blood over a 60-min period. This is presumably because these inhibitors more effectively block the complement serine proteinase factor I than factor B. Factor I is a control proteinase that inactivates C3 convertase (C3bBb) by cleaving the essential cofactor C3b to C3bi, destroying the active enzyme complex. Therefore, if factor I is inhibited, small quantities of the C3bBb complex will form in the blood and permit the alternative pathway to progress unimpeded. When EDTA was added to these same blood samples, C3 conversion by the Ca2+-dependent classical and alternative pathways was prevented.

C4 conversion in the presence of EDTA indicated activation of a plasma proteinase in the absence of calcium. Neither of the coagulation proteinase inhibitors used here were effective in blocking C4 conversion; however, Futhan appears to be fully effective in inhibiting this proteinase. We hypothesize that the proteinase responsible for the in vitro activation of C4 may be the MASP-2 enzyme of the lectin pathway (38). The activation or induction of MASP-2 activity in plasma by EDTA has not yet been demonstrated, and this mechanism is currently being explored. Consequently, we suggest that the MASP-2 enzyme of the lectin pathway may be primarily responsible for in vitro C activation; however, the proteinase(s) in EDTA plasma have not been identified (see Table 2).

In Fig. 3A, the C4a values in EDTA plasma were the only ones to increase vs time, and these concentrations remained quite low. These data matched published data for best estimates of the minimal background or baseline circulating concentrations of C3a or C4a in the general population (35). The C4 conversion values in a group of liver transplant patients shown in Fig. 3B were remark-
ably high (20-fold higher than the control group) even at time 0 (i.e., the actual time it takes to spin down the blood, recover the plasma, and add precipitating agent to stop the activation). After 60 min, the conversion of both C3 and C4 was extensive in EDTA plasmas from some of the organ transplant patients. These data indicated that, although the plasma proteinases appeared to prefer C4 over C3, at high concentrations, these enzymes were capable of cleaving both C3 and C4. In fact, there are reports that MASP-1 preferentially cleaves C3 (44). The key information provided by these studies, in terms of potential clinical applications for the C3a/C4a assay system, was that Futhan remains protective even when the proteinase concentrations are markedly higher than in normal plasma.

A recent report describing the C-activation profiles of C vs time in liver transplant patients suggested that pathologic events such as acute rejection episodes, hepatitis C, or cytomegalovirus infections as well as bacterial infections can be detected by monitoring the circulating concentrations of C3a and C4a (42). Many of the events monitored in these liver transplant patients appeared to involve primarily the classical pathway (i.e., only C4a concentrations were increased). Considering the results shown in Figs. 5 and 6 in this study, we hypothesize that components C3 and C4 are being activated in vitro either by a non-complement proteinase or by MASP enzymes of the lectin pathway. It is clear that the pathology leading to expression of the enzyme activity in EDTA plasma has some specificity because only 8 of 19 patients showed marked increases in C activation. The condition or process responsible for this high enzyme activity remains unknown in this patient population that exhibited no overt clinical signs of organ malfunction or rejection. Consequently, many such complications in organ transplant patients that lead to increased enzyme activity in EDTA plasma have remained undetected and undiagnosed. Many other immune diseases, particularly autoimmune diseases such as SLE (Fig. 4) and rheumatoid arthritis, have been associated with C4 conversion solely by the classical pathway and not the lectin pathway. Futhan/EDTA plasma samples are clearly recommended as a means to monitor these diseases. The circulating C3a and C4a concentrations can be accurately determined if Futhan/EDTA plasma samples are used as described in this report. Independent evaluations of C-activation products either in vivo or in vitro promise to provide a means to separate, characterize, and identify involvement of the various C-activation pathways in human disease.

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