Activation of the contact system has been documented in severe sepsis and hereditary angioedema, but a sensitive, specific, and quantitative assay for assessing the degree of involvement of this proteolytic enzyme cascade is not yet available. We have developed a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) for the $\alpha_M$-macroglobulin-kallikrein ($\alpha_M$-Kal) complex using an F(ab')2 derivative of a monospecific polyclonal antibody against $\alpha_M$ as the capture antibody and a unique murine monoclonal antibody, 13G11, against the heavy chain of kallikrein as the detector antibody. The assay does not detect complexes in normal plasma but reacts with complexes generated by activating normal plasma with dextran sulfate at 4°C in a range of 5 to 375 nmol/L. A close correlation of the ELISA with an amidolytic assay for $\alpha_M$-Kal was documented.

A CTIVATION OF the contact system in disease states, notably sepsis and hereditary angioedema, has been reported but has been difficult to quantify. Measurement of the plasma concentration of the zymogens, factor XII, prekallikrein (PK), and the procofactor high molecular weight kininogen (HK), are difficult to interpret because of changes in synthesis or distribution in vivo. Simultaneous measurement of activity and antigen and examination of their ratios has been more useful. In adult respiratory distress syndrome1 associated with sepsis or trauma, reduction in functional PK, HK, and C1 inhibitor (C1-INH) were shown, whereas the corresponding antigens were normal or even increased. However, measurement of small differences in these ratios may limit the sensitivity. HK cleavage has been shown by Schmaier et al2 to be present in plasma from sepsis syndrome patients, but it did not correlate with the extent of contact system activation. Contact system activation in sepsis was deduced from reduction in PK and HK activity without significant changes in PK antigen levels. In hereditary angioedema (HAE), attacks of the disease1 were associated with a decrease in PK activity while PK antigen levels remained within normal limits, whereas both HK activity and antigen levels diminished. Cleaved HK was described on immunoblots of plasma obtained during attacks of HAE patients.6

A second approach is to measure enzyme-inhibitor complexes. Using in vitro methods, Schapira et al6 found that the inactivation of purified kallikrein (Kal) in normal plasma was mainly divided between the two inhibitors, C1-INH and $\alpha_M$-macroglobulin ($\alpha_M$). Van der Graaf et al6 found that in plasma activated by exposure to an anionic surface, C1 inhibitor-kallikrein (C1-INH-Kal) complex formation predominates (52%), while $\alpha_M$-Kal complex (35%) is responsible for a smaller inhibitor contribution. In patients with typhoid fever, Colman et al described that the reduction of functional PK and C1-INH in the presence of normal antigenic levels as well as the formation of C1-INH-Kal complexes correlated with the progress of the disease. They concluded that contact activation of PK had occurred.

Lewin et al first described a specific enzyme-linked immunosorbent assay (ELISA) to quantitate C1-INH-Kal complexes in plasma. In a study of Rocky Mountain Spotted Fever,9 mild activation of the contact system could be documented by ELISA. These studies suggested that the ELISA to measure complex formation might be a sensitive and useful tool for the detection of contact system activation. Unfortunately, complexes of C1-INH-Kal did not correlate with disease severity in recent sepsis studies.10,11 Complexes of $\alpha_M$-Kal are cleared in vivo more slowly by means of a different mechanism12 than C1-INH-Kal complexes; therefore, quantitation of $\alpha_M$-Kal complexes formed in plasma of diseased patients may be a better indicator of contact...

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Submitted July 6, 1990; accepted February 12, 1991.

Supported in part by Contract No. N00014-88-K-0606 from the Office of Naval Research (R.W.C.); National Institutes of Health (NIH) Grant HL24365 (R.W.C.); NIH Grant HL 35553 (A.H.S.); National American Heart Association Grant-in-Aid No. 891247 (A.H.S.); a grant from the Southeastern Pennsylvania Affiliate of the American Heart Association (A.H.S.); and Research Career Development Award HL 01615 (A.H.S.). Also supported by Grant-in-Aid No. 891231 from the Southeastern Pennsylvania Affiliate of the American Heart Association, National Program (R.A.P.); Grant-in-Aid No. 890156 from the Southeastern Pennsylvania Affiliate of the American Heart Association (J.D.P.); and Biomedical Research Support Grant No. 507 RR05417, Division of Research Resources, NIH (J.D.P.).


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0006-4971(91)7712-001633.00/0

activation. In a patient studied serially between and during attacks of HAE, α,M-Kal complexes and cleaved HK were both detected by immunoblots.4 Harpel et al4 described a quantitative immunoimmobilization-enzyme assay for α,M-Kal complexes using an agarose immobilized anti-α,M antibody capture system and a small fluorogenic peptide substrate as a Kal detector to discover the formation of complexes in contact-activated plasma. To detect α,M-Kal complex formation for use as a reflection of contact activation in diseased patients, we developed a specific sandwich ELISA for detection of α,M-Kal complexes in plasma, and we have used the assay to examine the plasma from individuals in which the contact system is suspected to be activated.

MATERIALS AND METHODS

Materials. Immulon-2 flat-bottom 96-well microtiter plates were purchased from Dynatech Lab, Inc (Chantilly, VA). The chromogenic substrate for Kal H-D-prolyl-L-phenylalanine L-arginine p-nitroanilide dihydrochloride (S-2302) was supplied by Kabi Diagnostica (Stockholm, Sweden). Polybrene (hexadimethrin bromide), bovine serum albumin (BSA) (RIA grade), Tween-20, and dextran sulfate (500 Kd) were purchased from Sigma. α,M-Kal complexes were formed by reacting purified α,M (generously provided by Dr Steven Gonias, University of California at Davis, Sacramento) with the anti-α,M antibody against human protein-A agarose obtained from BioRad Lab (Richmond, CA). The Ig was cleaved with pepsin to yield F(ab')2 fragments, and the Fc fragments were removed by adsorption on protein-A agarose according to methods already described.19,20 These F(ab')2 fragments served as capture antibody. The second (detecting) antibody was a purified mouse IgGκ monoclonal antibody (MoAb) 13G11,19 an antibody specific to the heavy-chain region of human PK and recently localized to the 28-Kd C-terminal portion. Alkaline phosphatase was covalently linked to MoAb 13G11 using the glutaraldehyde method described by Voller et al.19 The substrate for alkaline phosphatase was 5-nitrophenyl phosphate (PNPP) purchased from Sigma. α,M-Kal complexes were formed by reacting purified α,M (generously provided by Dr Steven Gonias, University of Virginia at Charlottesville) with a fourfold molar excess of Kal for 30 minutes at room temperature. PK was purified by immunoaffinity from fresh frozen plasma.19 PK was then converted to Kal by incubation with factor XII fragment (100:1 molar ratio) for 30 minutes at 23°C. The α,M-Kal complex was then separated from excess Kal by gel filtration on a Superose-12 column using FPLC (Pharmacia, Piscataway, NJ). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)21 was performed on the reaction mixture and on the fractions from the column to confirm that the complex was separated from free Kal.

Immunoblot analysis. A Western blot analysis was performed using the 13G11 antibody. Samples were run on a 5% to 20% gradient SDS gel and transferred to an Immobilon-P membrane (Millipore, Milford, MA) by means of a semidyblotter (American Bioeteins, Inc; Hayward, CA) following the manufacturer’s procedures. The Immobilon-P membrane was blocked with 10% nonfat dry milk in 0.09 mol/L Na phosphate, 0.15 mol/L NaCl, pH 7.4, containing 0.05% Tween-20 (PBS-Tween), incubated with the alkaline phosphatase-conjugated 13G11, and subsequently incubated with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) in 0.05 mol/L Na2CO3, pH 9.8, 1 mmol/L MgCl2.

α-M-Kal complex assay. This assay is a “sandwich” ELISA type, in which the coating antibody is an F(ab')2 fragment of polyclonal antibody against human α,M, applied directly to the wells. The second layer is the sample to be examined; the third layer is the alkaline phosphatase-conjugated monoclonal detector antibody, 13G11, directed to the Kal heavy chain. Microtiter wells are coated with 2 µg/mL of F(ab')2, in coupling buffer (0.16 mol/L H3BO3, 0.125 mol/L NaCl, pH 8.5), 100 µL/well. The plate is then incubated at 37°C for 4 hours, then washed with PBS-Tween, pH 7.4, by means of an automated microplate washer EL-402 (Biotek Instruments Inc, Winooski, VT). This washing procedure is repeated after each subsequent step. The wells are then blocked by incubation with PBS-Tween containing 0.5% BSA (PBS-Tween BSA), 150 µL/well, for 1 hour at 37°C. Samples are added (normal or patient plasma), diluted in PBS-Tween BSA, 100 µL/well, and incubated for 2 hours at 37°C. One hundred microliters of a 3 µg/mL solution of alkaline phosphatase-conjugated 13G11 in PBS-Tween BSA is then added to the wells and incubated at 37°C for 2 hours. Substrate (PNPP), 1 mg/mL, is prepared in buffer (0.05 mol/L Na2CO3, 1 mmol/L MgCl2, pH 9.8), and 100 µL/well added. The plate is read when the yellow color reaction is apparent. Because the color reaction is slow, usually appearing after 1 hour, stopping the reaction with 30% acetic acid (100 µL/well) is optional. The absorbance in the wells with PBS-Tween alone was subtracted from those with samples. Samples were read by a microplate reader with a 405-nm filter, model EL-402 (BioTek).

Initially, we developed a readily available, quantifiable standard for the assay. PK-deficient plasma (Fletcher plasma) was reconstituted with a known amount of purified α,M-Kal complex (375 nmol/L). The amount of purified α,M-Kal preformed complexes was quantified two ways: amidolytic activity and protein assay. When α,M-Kal complex activity is compared with that of Kal, the complex has been shown to retain 25% of its amidolytic activity toward the small peptide substrate, S-2302. The purified α,M-Kal complexes were also measured for protein using a modified Bradford assay.22 We assayed the α,M-Kal reconstituted PK-deficient plasma in parallel with dextran sulfate (DS)-activated pooled normal plasma (see below) in the ELISA. Using identical serial dilutions for both, the absorbance curves were seen as superimposable (data not shown), allowing us to correlate the amount of complexes in the DS-activated pooled normal plasma with the purified standard.

For every experiment, a standard curve was generated using dilutions of DS-activated PNP. Activation of PNP was performed in the cold (4°C) to decrease Kal binding to Cl-INH and augment its binding to α,M.24 PNP was diluted with an equal volume of 30 µg/mL DS, dissolved in H2O to obtain a final concentration of 15 µg/mL, and incubated at 4°C for 10 minutes. The activated plasma is finally diluted to 1/10, 1/50, 1/100, 1/250, and 1/500. DS-activated PNP was defined to contain 375 nmol/L of α,M-Kal complex (based on reconstitution experiments). Unknown samples were usually diluted 1/10 initially in PBS-Tween BSA, but when appropriate they were adjusted to fit within the limits of the standard curve. All samples and standards were performed in triplicate. Standard curves for the assay were generated by plotting the absorption values of the standards against the log 1/dilution of the DS-activated PNP. The plot was fitted to a third-order polynomial equation by an iterated procedure using Sigma Plot (Jandel Scientific, CA). The polynomial equation was then used to interpolate the value of the diluted unknown sample, and to account for dilution, the value was factored to its true value.

Blood obtained from all patients was drawn into polypropylene tubes containing 3.8% sodium citrate anticoagulant. The ratio of
anticoagulant to whole blood was 1/10. Plasma was separated from blood cells within 30 minutes at room temperature and was quickly frozen at −70°C. Only blood samples that had never been thawed since collection were used for the complex and functional assays.

**PK assays.** Functional PK was measured in a chromogenic assay using substrate H-D-Pro-Phe-Arg para-nitroanilide.\(^{22,23}\) PK antigen was measured by radioimmunodiffusion with a monospecific polyclonal antiserum produced in goats.\(^{24}\)

**Patient characteristics.** Eleven patients were studied, all of whom fulfilled the criteria for the diagnosis of sepsis syndrome and had at least five of eight criteria within a 12-hour period, as previously reported.\(^{25}\) Five of the patients had documented bacterial growth from various sites of infection and negative blood cultures (nonbacteremic). Three of the six patients with positive blood cultures grew *Staphylococcus aureus*: one patient was positive for *Escherichia coli*, one individual was positive for β hemolytic streptococcus, and one patient was positive for *Klebsiella pneumoniae*. Of these 11 patients, one nonbacteremic patient, one *S. aureus*-positive patient, and the *K. pneumoniae*-positive patient were previously described by Schmaier et al\(^{26}\) (labeled in that report as type 3, 5, and 6). These patients were assayed for their PK and HK antigen and function levels, and for the molecular forms of HK by immunoblotting.

In the present study, we have measured PK function and antigen in the remaining eight patients as well as α\(_2\)M-Kal complex in all 11 patients. The five nonbacteremic patients are identified as A through E, and the bacteremic patients as F, G, H (*S. aureus*); I (*E. coli*); J (*B. streplococcus*); and K (*K. pneumoniae*). The previously studied patients were E, H, and K (3, 5, and 6, respectively, in reference 2). Of the six bacteremic patients, only the *K. pneumoniae*-positive patient (K) had documented hypotension (80/50 mm Hg). Additionally, six healthy controls and six patients with liver disease had blood collected and processed the same way. The patients with liver disease had samples collected when stable and when they were seen in an outpatient liver disease clinic (Miami Veterans Administration Hospital, Miami, FL). All of these patients had total bilirubin ≥5.0 mg/dL and albumin ≤3.0 g/dL, and elevations of their alkaline phosphatase and/or transaminase. Blood samples were also collected from HAE patients while well or during attacks of HAE.\(^{4}\) Each of the HAE patients was characterized to have type 1 HAE with C1-INH activity and antigen levels ≤60 μg/mL.

**RESULTS**

**Specificity of the assay.** A key to the development of our assay was the use of the MoAb 13G11, which recognizes an epitope on the heavy chain of PK.\(^{18}\) This antibody is unique in that it recognizes Kal while it is complexed with α\(_2\)M,\(^{4,25}\) whereas previously used monospecific polyclonal antibodies did not.\(^{8}\) The specificity of the antibody 13G11 is demonstrated in Fig 1. A Western blot with purified samples showed that the MoAb detects PK (lane 4) as well as Kal-α\(_2\)M complexes (lane 3). In plasma activated with DS (lane 2), the antibody detected PK (a doublet at 86 and 88 Kd), C1-INH-Kal complexes (190 Kd), and multiple bands of α\(_2\)M-Kal complexes, while in unactivated plasma (lane 1) only a PK doublet was visualized. The higher molecular weight diffuse bands in lane 1 are also seen when PK-deficient plasma is run on a Western blot under the same conditions and thus represent nonspecific binding of the MoAb to other plasma proteins.

**Sensitivity and reproducibility of the assay.** The DS-activated plasma quantified for α\(_2\)M-Kal complexes served as the working reference to calculate the amount of complexes in each patient sample (see Materials and Methods). Plasma activated with DS at 4°C showed a decreasing concentration of complexes over a 100-fold dilution (Fig 2). The sensitivity of the standard curve ranged between 0.55 and 37.5 nmol/L of complexes, which, when unknowns are corrected for a 10-fold dilution, give a working range of 5 to 375 nmol/L. The lower detection limit of the assay was 5 nmol/L of complexes; concentrations below 5 nmol/L are indistinguishable from background. The horizontal curve produced by normal plasma indicated that there was no interference by plasma constituents and no detectable α\(_2\)M-Kal complexes in unactivated plasma (Fig 2). The average value (±1 SD) for the intra-assay coefficient of variation (CV) is 6.7% ± 2.9% (n = 9). In three experiments comparing 12 different samples, an average (±SD) interassay CV of 6.4% ± 2.7% was obtained.

To optimize the assay we examined the influence of several reagents on the formation of complexes. DS itself did not interfere with detection of preformed complexes over the range of concentrations used in the standard curve.
The amidolytic assay is less specific than the ELISA because the S-2302 is not a specific substrate for Kal. S-2302 is digested by plasmin and activated factor XII as well, and thus measures complexes of these two proteases with $\alpha_M$ as well as $\alpha_M$-Kal complexes.

Studies in HAE. HAE is an appropriate human disease model to study the diagnostic potential of this assay. As a consequence of C1-INH deficiency in this disease, the proportion of potential complexes with $\alpha_M$ is increased. Figure 4 shows $\alpha_M$-Kal formation during the quiescent phase of the disease and in the course of an attack in the same patient. Both plasma samples were tested directly preactivation and postactivation with DS and were compared with normal plasma and activated plasma. The quiescent sample behaved similarly to PNP, and the $\alpha_M$ complexes increased after exposure to DS (left panel, Fig 4). Alternatively, the sample obtained during an attack contained the maximal amount of $\alpha_M$-Kal complexes because DS did not further increase the concentration of complexes in this plasma.

Three HAE patients were studied (Fig 5). Four plasma samples were added to plasma before activation, the activation by DS was prevented, and when PB was added to fully activated plasma, no effect was observed on the ELISA (data not shown). Thus, plasma could be drawn with or without PB for this assay.

Correlation of chromogenic assay and ELISA for $\alpha_M$-Kal complexes. $\alpha_M$ differs from serpins such as C1-INH both in its structure and in its mechanism of protease inhibition. (After formation of Kal from PK, the light chain of Kal containing the active serine group, interacts with C1-INH and forms an inactive covalent complex.) In contrast, after Kal cleaves $\alpha_M$ in the "bait region," it traps Kal, partially preserving its activity on small substrates. The complex retains 25% of free Kal activity toward S-2302. This unique behavior of the $\alpha_M$-Kal complex is shown in Fig 3. The upper panel of Fig 3 illustrates a normal activated PNP standard curve in the sandwich ELISA. The lower panel shows the results of an amidolytic assay performed under the same conditions as the ELISA, i.e., at the same time, with the same plasma preparation in equal dilutions, in a parallel microtiter plate. The only difference between these two assays is in the last step of the procedure. While in the ELISA the second conjugated antibody was added as the last component, in the amidolytic assay the substrate S-2302 was added last. The two curves were almost superimposable, indicating that the amidolytic activity of $\alpha_M$-Kal complex parallels its antigenicity. The same results were obtained when the substrate S-2302 was added in addition to the second antibody (data not shown), indicating that the antibody did not prevent access of the substrate to the complex.
Fig 4. Plasma levels of αM-Kal complexes in HAE during quiescent and attack phases. Effect of in vitro activation. Both plasma samples were obtained from the same patient during different phases of the disease. The dotted lines represent the standard response of the complex ELISA using DS-activated PNP (upper line) and unactivated PNP (lower line). Left panel, quiescent phase; right panel, attack phase. DS activation of the samples was similar to the method described for activating PNP (see Materials and Methods). (●), DS-activated patient plasma sample; (○), unactivated patient plasma sample.

Studies on patients with sepsis syndrome. Observations were made on 11 patients with sepsis syndrome, six bacteremic (positive blood cultures) and five nonbacteremic (Fig 6). Four of six bacteremic patients had values of αM-Kal complex elevated above the lower detection limit. None of the nonbacteremic patients had detectable complexes, similar to the group of healthy individuals and patients with liver disease but without infection. The patient with the highest concentration of complexes (over 100 nmol/L) was the only one to have documented hypotension (80/50 mm Hg).

These sepsis syndrome patients were further examined for evidence of contact activation by measuring PK function and antigen and comparing these values with their αM-Kal complex concentrations (Fig 7). As seen in Fig 7, the blood culture-negative patients had no detectable elevation of αM-Kal complexes and did not show marked dissociation of PK function and antigen. Only patient C had levels of PK below 50% of normal. Four of the six culture-positive patients had an elevation of greater than 5 nmol/L in the concentration of αM-Kal complexes. One
patient, I, had a marked decrease in both PK function and antigen. Two patients, F and K, the latter showing hypotension, had convincing decreases in their functional PK levels, with preservation of antigen levels. It should be noted that in patient K the complexes accounted quantitatively for the difference between PK antigen level and function.

**DISCUSSION**

Septic shock is still a major cause of morbidity in every part of the world, with mortality remaining at about 50%. Contact system activation can be detected both in septicemic hypotension and in HAE, and is a potentially important mechanism in the pathogenesis of both syndromes. Therefore, there is a need for a sensitive and specific assay to enable detection of the onset of septic shock before irreversible changes and for prognostic purposes.

When the contact system is activated, complexes of Kal form with both C1-INH and αM. Such is the case in sepsis, while in HAE, because of C1-INH deficiency, most of the Kal is complexed with αM.4 A recent study11,12 failed to show a correlation of C1-INH-Kal complexes with the severity of sepsis. The tentative explanation offered was rapid clearance in vivo. C1-INH-Kal complexes are probably cleared by the liver because other complexes containing scapans bind to specific sites on hepatocytes.29 The clearance of αM-Kal complexes probably differs from that of C1-INH complexes. Complexes containing αM bind to macrophages as well as other cells in a calcium-dependent reaction, while the native αM does not.13 The in vivo receptor for the αM-protease complex has been shown to be different from the receptor that participates in the clearance of other protease-inhibitor-protein complexes.30 Although the clearance mechanism is not fully understood, one fact remains: while C1-INH-Kal complexes vanish rapidly from circulation, αM-Kal complexes are still present and readily detected. Gonias and Pizzo31 showed that among the various αM-protease complexes, αM-plasmin complexes were cleared slower. Because plasmin and Kal are both two-chain serine proteases of about the same molecular mass, we hypothesize that these two complexes share a similar clearance pattern. In addition, it is conceivable that monokines, such as tumor necrosis factor, released in sepsis downregulate receptors for clearance of αM-Kal complexes.

It is worthwhile to compare with this ELISA previous methods used for detection of contact activation. Measuring zymogen depletion alone31 usually is not sensitive enough, mainly because antigen levels do not decrease as a consequence of complex formation, and functional levels only decrease with septic shock but not with sepsis.32 In addition, PK levels may decrease due to decreased synthesis in the cirrhotic liver.33 One partial solution is the use of function to antigen ratios.14 This analysis, albeit more sensitive, does not always show significant differences between zymogen levels and function when activated. This point is illustrated clearly in Fig 7, where we can compare PK antigenicity and function to levels of αM-Kal complexes. In none of the five culture negative patients was the αM-Kal complex elevated, whereas four of the six bacteremic patients showed an elevation (Fig 6). Moreover, none of the five nonbacteremic patients show PK antigen activity dissociation, whereas this dissociation did occur in two of the six bacteremic patients. Patient K is interesting in three respects. First, he was the only one with documented shock (blood pressure 80/50); second, his plasma contained a very high concentration of complexes; and third, his plasma exhibited both a decrease in PK function and an increase in αM-Kal complexes. The increase in the concentration of αM-Kal complexes of bacteremic patients, H and I, who did not have any decrease in blood pressure, indicates that the αM-Kal complex is more discriminatory than measurements of PK activity to antigen activity ratios because the ratios are normal. It is important to note that the complexes become elevated in septic patients while they still are hemodynamically stable. These findings allow us to predict that the level of αM complexes may be correlated to severity of disease. A larger study of clinical sepsis is under way to test this hypothesis. In contrast, studies of HK cleavage as detected by Western blot (immunoblot) analysis on bacteremic and nonbacteremic patients did not distinguish culture-negative from culture-positive sepsis. In even patient K (Fig 7), who in fact was the sickest (in shock) and had markedly high levels of complexes (Figs 6 and 7), kininogen cleavage could not be detected on Western blot analysis.

We studied a group of HAE patients (Fig 5) during attacks of the disease and during the quiescent phase. Recent evidence indicates that bradykinin may contribute to angioedema44 and that contact activation may be important in initiating the attacks.45 As a result of C1-INH deficiency, when PK is activated to Kal the active enzyme will bind mostly to αM.4

We recognize that contact system activation is not the only contributing proteolytic system involved in HAE or sepsis. Other mechanisms of contact factor participation have been suggested. The classical complement system can be initiated by activated factor XII46 in an antibody-independent manner. In the local environment the assembly of contact system components on neutrophils,37 endothelial cells,38 and platelets39 may protect Kal from inhibition by αM.

The αM-Kal complex assay makes it possible to quantify significant elevations of complex formation during attacks and to appreciate the normalization of the elevated complexes while patients are in the quiescent phase of the disease. In related studies from our laboratory, two models of sepsis were examined by the complex ELISA. In one, human volunteers were injected with very low doses of endotoxin.40 In the second, baboons were injected with either lethal or sublethal doses of E coli.40 In these models, complex concentration correlated both with the decrease in blood pressure and the severity of the disease.

We conclude that measurement of αM-Kal complexes may provide a sensitive early warning of septic shock, and that the levels of complexes detected may correlate with disease severity. The assay should also be useful in observing HAE patients, as well as in diseases in which the involvement of the contact system is suspected.
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Alpha 2-macroglobulin-kallikrein complexes detect contact system activation in hereditary angioedema and human sepsis

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