Evidence of Activation of the Protein C Pathway During Acute Vascular Damage Induced by Mediterranean Spotted Fever

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Mediterranean spotted fever (MSF) is a rickettsiosis belonging to the spotted fever group of infections and is caused by tick-borne *Rickettsia conorii*. MSF shares with other rickettsioses one dominant feature, widespread microvascular injury. Rickettsiae infect human endothelial cells, proliferate intracellularly by binary fission, and spread contiguously to infect numerous adjacent endothelial cells. Rickettsial vasculitis, in fact, comprises endothelial injury (swelling and necrosis) and the immune and phagocytic host responses (lymphocytes and macrophages). The pathophysiologic effects of vascular injury include increased vascular permeability, edema, hypervolemia, and activation of humoral inflammatory and coagulation mechanisms. As discussed previously, endothelial damage could be the principal mechanism responsible for triggering the hypercoagulation state that occurs in the acute phase of MSF.

Intact vascular endothelium provides several anticoagulant mechanisms for the maintenance of blood fluidity and the prevention of thrombosis. One of the several systems in which these cells participate is the protein C anticoagulant pathway. The cell surface possesses a thrombin-binding protein, thrombomodulin (TM). Thrombin bound to TM initiates protein C activation on endothelial surfaces. Activated protein C (APC) then catalyzes the proteolytic inactivation of factors Va and VIIIa with its cofactor protein S. APC has also been reported to have profibrinolytic activity, possibly by neutralizing plasminogen activator inhibitors. In vivo studies have shown that APC is antithrombotic in a baboon model of platelet-dependent thrombosis and in a dog model of venous thrombosis.

APC is inhibited by a heparin-dependent plasma inhibitor named protein C inhibitor (PCI), which has been purified and characterized and the sequence of its cDNA reported. Plasma contains another major inhibitor of APC that is heparin-independent, which has been shown to be a-antitrypsin (a,AT). We demonstrated that APC forms complexes in vivo with each of these inhibitors in several clinical situations where activation of coagulation has occurred, and have suggested that the measurement of APC complexes may provide sensitive parameters for specific detection of activation of the clotting and protein C pathways.

As indicated, any injury of the endothelial cells that affects the appropriate expression of the protein C system could potentially lead to a hypercoagulable state and result in thrombotic complications. The acute phase of MSF fulfills the criteria of endothelial cell perturbation that may lead to such a state.

This study was undertaken to obtain qualitative and quantitative information on the activation and inactivation of protein C in MSF and its distribution between its known inhibitors, PCI and a,AT, during the acute and subacute phases of this vascular infectious disease.

MATERIALS AND METHODS

Patient data. Twenty-eight consecutively admitted patients (age range 5 to 86 years, mean 59) from the 1988-1989 epidemic in Salamanca (Spain) and 30 normal subjects (age range 18 to 65 years, mean 39) were examined. Diagnosis of MSF was confirmed...
by clinical findings and by serologically measuring titters of antibody specific for this illness using indirect immunofluorescence. Blood samples were obtained on admission, ie, during the acute phase of the disease (day 1) and 30 days after the start of treatment (day 30). The elapsed time from the onset of symptoms (fever) to diagnosis ranged between 3 and 12 days (mean, 7 days).

In the acute phase of the disease, two patients presented clinical signs and evident symptoms of deep vein thrombosis in their legs. The diagnosis was confirmed by ascending venography. However, all patients recovered favorably and did not exhibit any other clinical complications.

Informed consent was obtained from all patients and control subjects before blood collection.

Blood samples: Blood collection was made by venipuncture with a 19-gauge needle. Nine parts of blood were added to plastic tubes containing one part of 0.13 mol/L trisodium citrate with or without 50 mmol/L benzamidine. Blood samples were rapidly centrifuged at 2,500g for 20 minutes. The plasma was used fresh or snap-frozen in small portions and stored at -70°C until the assays were performed in series (less than 6 months). Pooled normal human plasma was made from 24 healthy donors and used as a reference.

Blood samples for determination of protein C antigen, total and free protein S, PCI antigen, antithrombin III (AT-III), αAT, C14b-binding protein, and enzyme-inhibitor complexes were collected in citrate containing 50 mmol/L benzamidine. Protein S functional activity and protein C amidolytic activity were determined in samples collected with citrate alone.

Quantitative assay of the proteins C system components. Protein C antigen and amidolytic activity,28 anticoagulant protein S,28 and PCI antigen and activity5 were assayed as indicated before. Total and free protein S antigen were assayed by enzyme-linked immunosorbent assays (ELISAs), as indicated by Deutz-Terlouw et al,26 using specific anti-protein S polyclonal antibodies.26 Complexes of APC with PCI (APC:PCI)20 or with αAT (APCαAT),23 and complexes of protein C with PCI (KK:PCI) were determined by using sandwich ELISAs, as reported. Briefly, microplates were coated with monoclonal antibody (MoAb) to protein C (for APC:PCI and APCαAT complexes) or to PCI (for KK:PCI complexes), and complexes were detected with peroxidase-labeled polyclonal antibodies to either PCI or αAT or KK, respectively. Reference curves were constructed with known amounts of in vitro preformed complexes. The detection limit of the assays in plasma was 1,3, and 15 ng/mL, respectively.

Determination of other blood coagulation components. Platelets were counted with an electronic hematology analyzer (Hemalog-8; Technicon, Tarrytown, NY). Factor VIIIC procoagulant activity (VIII:C) was assayed in a one-stage test using platelet-poor plasma (PPP) from a patient with severe hemophilia A as substrate, as previously reported.3 Quantitative immunologic determinations of von Willebrand factor antigen (vWF:Ag), AT-III, αAT, and C14b-binding protein were performed by a electroimmunoassay technique using rabbit antiserum to these proteins (Behringwerke, Marburg, Germany). Factors II, VII, IX, X, XI, and XII were assayed in one-stage tests using PPP obtained from patients congenitally deficient in the respective factors as substrate (Behringwerke), as previously reported.13 Fibrinogen was assayed by the Clauss method27 and fibrin degradation products (FDP) by agglutination of particles coated with specific antibodies (Diagnostica Stago, Asnieres, France). Complexes of thrombin with AT-III (T:AT-III) were assayed by a sandwich ELISA (Enzygnost-TAT, Behringwerke AG) that uses a polyclonal antibody specific for neoantigeneic determinants on thrombin as the capture antibody and peroxidase-labeled polyclonal antibody to AT-III as the tag.

Statistical analysis. A normal (Gaussian) distribution of levels of each parameter in each group was checked using the Kolmogorov-Smirnov test. Variations in parameter levels in patient groups compared with levels in the normal control group were investigated using the Student’s t-test with two-tailed P for significance. Comparisons between patients upon admission (day 1) and 30 days later were calculated with the Student’s r-paired test. All assay variables are represented by their mean and standard deviation.

RESULTS

Coagulation parameters. Table 1 shows the mean values of several blood coagulation parameters studied in 30 healthy subjects and in 28 MSF patients. Compared with the control group, MSF patients in the acute phase of disease (day 1) showed a significant decrease in plaatlet count and VIII:C/vWF:Ag ratio (P < .001) and a significant increase in vWF:Ag and fibrinogen (P < .001). At the time of diagnosis (day 1), nine patients were detected as FDP positive (> 10 μg/mL). After 30 days of treatment (day 30), a significant increase in platelet number was observed when compared with the values at day 1 (P < .001). VIII:C/vWF:Ag ratio increased significantly (P < .001) at day 30 when compared with that at day 1, but it remained decreased compared with normal values (P < .01). vWF:Ag decreased during the course of the disease, but it remained increased compared with normal values (P < .001). Coagulation factors II, VII, IX, X, XI, XII, and prekallikrein were in the normal range both before and after treatment.

Table 2 shows the mean values of several components of the protein C pathway and other related parameters studied in normal subjects and in MSF patients. Compared with the control group, MSF patients in the acute phase of disease (day 1) showed a significant decrease in protein C amidolytic activity and antigen, anticoagulant protein S activity, and PCI antigen (P < .001). In contrast, free protein S antigen and AT-III levels at day 1 were normal. At day 1, a significant increase in total protein S antigen,
when compared with those at day 1, these values remained slightly decreased compared with normal values ($P < .05$). α:AT, total protein S, and C4b-binding protein decreased steadily towards normal values as the disease progressed (Table 2). AT-III and free protein S did not show any significant change during the evolution of the disease.

Although AT-III levels were normal on day 1, a significant increase ($P < .001$) in T:AT-III complexes could be detected. However, a trend of T:AT-III towards normal values was seen on day 30 (Table 2).

At the time of diagnosis (day 1), two patients had deep vein thrombosis, but there were no relevant laboratory differences compared with the other 26 patients (data not shown).

Quantification of protein C activation and inactivation. Because of the remarkable decrease in protein C activity and its specific inhibitor, PCI, which suggests activation of the protein C system, we determined the levels of the complexes of APC with two physiologic inhibitors, PCI and α:AT, as well as of KK:PCI complexes. Figure 1 shows the results obtained. All healthy subjects had levels of APC:PCI complex below the detection limit of the assay used (<3 ng/mL) (Fig 1B). On the other hand, in the acute phase of the disease at day 1, 16 of the 28 patients studied had detectable APC:PCI complex levels, ranging from 3 to 32 ng/mL, and a mean value of 11 ng/mL (Fig 1B). These complexes decreased significantly ($P < .001$) with the evolution of the disease and, on day 30, only eight patients had detectable APC:PCI complexes, ranging from 3 to 8 ng/mL.

All healthy subjects had detectable levels of APC:α:AT complexes, ranging from 3 to 12 ng/mL, with a mean value of 7 ng/mL (Fig 1A). As seen in Fig 1A, MSF patients at day 1 had significantly increased levels of APC:α:AT complex (mean 27 ± 13 ng/mL, range 8 to 53 ng/mL) as compared with controls ($P < .001$). APC:α:AT complex levels were significantly decreased on day 30 (mean 14 ± 7 ng/mL, range 3 to 37 ng/mL) compared with day 1 ($P < .001$), but remained significantly increased ($P < .001$) compared with normal controls (Fig 1A).

PCI is a good inhibitor of KK in vitro studies. Because this enzyme may be formed in the course of infection and vascular damage, we investigated the presence of KK:PCI complexes in MSF patients. Detectable KK:PCI complex levels (<15 ng/mL) were not found

### Table 2. Protein C Pathway Components and Other Related Parameters in 28 Patients With MSF During the Acute Phase of the Disease (day 1) and After Remission (day 30) and 30 Healthy Subjects

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patients (n = 28)</th>
<th>Healthy Subjects (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 30</td>
</tr>
<tr>
<td>Protein C antigen</td>
<td>68 ± 19*</td>
<td>99 ± 20</td>
</tr>
<tr>
<td>amidolytic (%)</td>
<td>60 ± 18*</td>
<td>103 ± 19</td>
</tr>
<tr>
<td>Protein C inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>antigen (%)</td>
<td>41 ± 20*</td>
<td>99 ± 21</td>
</tr>
<tr>
<td>activity (%)</td>
<td>37 ± 20*</td>
<td>97 ± 19</td>
</tr>
<tr>
<td>α:AT (%)</td>
<td>159 ± 36*</td>
<td>102 ± 16</td>
</tr>
<tr>
<td>T:AT-II complex (ng/mL)</td>
<td>11 ± 7*</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>T:AT-II complex (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4b-binding protein</td>
<td>135 ± 31*</td>
<td>102 ± 18</td>
</tr>
<tr>
<td>AT-III (%)</td>
<td>96 ± 24</td>
<td>101 ± 12</td>
</tr>
<tr>
<td>Data for all parameters are expressed as mean ± SD.</td>
<td></td>
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</tr>
</tbody>
</table>

*P < .001 with respect to healthy subjects.  
†P < .001 with respect to patients at day 1.  
‡P < .001 with respect to patients at day 1.
in the healthy normal subjects, whereas 21 of the 28 MSF patients had detectable levels of KK:PCI (mean 46 ± 16 ng/mL, range 21 to 86 ng/mL) (Fig 1C) during the acute phase of the disease (day 1). After remission of the disease (day 30), only five patients had detectable KK:PCI complex levels (range 24 to 33 ng/mL) (Fig 1C).

**Correlation between APC:inhibitor complexes and T:AT-III complexes.** Figure 2 shows the correlation between APC:α,AT and T:AT-III complex levels (A), APC:PCI and T:AT-III complex levels (B), and APC:α,AT and APC:PCI complex levels (C) in MSF patients during the acute phase of the disease at day 1. There was a positive correlation (r = .504, P < .005) between APC:α,AT complex levels and T:AT-III complex levels (Fig 2A). However, no significant correlation was observed between T:AT-III and APC:PCI complex levels (r = .138, P > .5) (Fig 2B) nor between APC:PCI levels and APC:α,AT levels (r = .309, P > .1) (Fig 2C).

**Correlation between KK:PCI complexes and APC:inhibitor and T:AT-III complexes.** Figure 3 shows the correlation between the KK:PCI complex levels and APC:α,AT (A), APC:PCI (B), or T:AT-III complex levels in acute MSF patients (n = 28) at day 1. There was a positive correlation (r = .438, P < .05) between KK:PCI complex levels and APC:α,AT complex levels and between KK:PCI and APC:PCI complex levels (r = .459, P < .05) but not between KK:PCI and T:AT-III complex levels (r = .314, P > .1).

**Normalization of APC:α,AT complex levels.** Because α,AT is an acute phase reactant and is elevated in MSF patients (Table 2), we “normalized” the values for APC:α,AT by dividing the observed level of APC:α,AT by the observed level of α,AT, and then calculated the correlation of the other complexes measured versus the “normalized” APC:α,AT levels. Again, a positive correlation was found between “normalized” APC:α,AT levels and T:AT-III (r = .551, P < .01) and between “normalized” APC:α,AT and KK:PCI levels (r = .510, P < .01), and no significant correlation was found between “normalized” APC:α,AT and APC:PCI levels (r = .281, P > .1). Although there was a significant increase in the α,AT levels in MSF patients at admission (Table 2), no correlation was found between the levels of APC:α,AT and those of α,AT (data not shown).

**DISCUSSION**

In the acute phase of MSF, widespread inflammatory microvasculitis is a dominant feature. A pronounced decrease in the VIII:C/vWF:Ag ratio secondary to endothelial damage and laboratory and clinical indications of activation of the hemostatic system, such as a significantly reduced numbers of circulating platelets and decreased protein C

Fig 2. (A) Correlation between the levels of APC:α,AT complex and T:AT-III complex in MSF patients at day 1. (B) Correlation between the levels of APC:PCI complex and T:AT-III complex in MSF patients at day 1. (C) Correlation between the levels of APC:PCI complex and APC:α,AT complex in MSF patients at day 1. For calculation of the coefficients of correlation, APC:PCI complex levels below the detection limit (<3 ng/mL) were considered to be 1 ng/mL.
levels, were found in this study, and FDP was detected in some patients as well as clinical evidence of deep vein thrombosis in two of them. On the other hand, and as previously reported,1 coagulation factors II, VII, IX, X, XI, and XII did not show significant variations, and fibrinogen levels were strikingly elevated, reflecting the behavior of fibrinogen as an acute-phase reactant (Table 1). This finding suggests that the abnormalities in the hemostatic system seen in MSF patients are due to local endothelial alterations and are not secondary to a disseminated intravascular coagulation (DIC). In fact, after rickettsial infection of endothelial cells, fourfold increased platelet attachment to the vessel wall29 and signs of platelet activation30 have been demonstrated.

In the presence of an endothelial cell surface protein, TM, the rate of protein C activation increases approximately 20,000-fold.31 Thus, the small amounts of thrombin generated by the platelet activation that occur in the acute phase of rickettsial infection might accumulate on the endothelial cell surface, and the resulting thrombin-TM complex formed could trigger the activation of the anticoagulant protein C system. In fact, during the acute phase of MSF, activation of protein C system is directly demonstrated by the reduced protein C and PCI levels as well as by the appearance or increase of circulating APC:PCI and APC:α2AT complexes.

During the acute phase of the disease all patients had detectable levels of APC:α2AT whereas only 16 of the 28 patients had moderate levels of APC:PCI complex. Moreover, there was a positive correlation between the APC:α2AT complex levels and the T:AT-III complex levels (r = 0.604, P < .005) (Fig 2A), which suggests that protein C activation and complexation increase parallel to thrombin generation. However, there was no significant correlation between APC:PCI and T:AT-III levels (Fig 2B) nor between the levels of APC:α2AT and the levels of APC:PCI (Fig 2C).

The relative proportion of APC:PCI and APC:α2AT complex levels seen here in MSF patients coincides with that found in patients with thrombotic disease,32,33 but contrasts with that detected in vitro following addition of purified APC to citrated normal plasma.34,35 In that in vitro study we observed that the two types of complexes are formed at approximately the same rate. The APC complex levels seen here in MSF patients also contrast with in vivo studies in a baboon thrombotic model in which infusion of APC into baboons generated APC:PCI complex levels twice as high as the APC:α2AT levels immediately following the APC infusion.32,33 A plausible explanation for the higher APC:α2AT complex/APC:PCI complex ratio seen in MSF patients as well as in patients with thrombotic disease is that the APC:PCI complexes are cleared faster than the APC:α2AT complexes. Indeed, we recently found that during APC infusions into baboons, APC:PCI complexes were cleared about three times more rapidly than APC:α2AT complexes, with a t1/2 of about 40 minutes versus 140 minutes, respectively.36 Another possibility is that enzymes generated during inflammatory vascular damage, such as thrombin and KK, could complex to and/or cleave PCI,1,12,23,26 thus reducing PCI levels, thereby making α2AT the major inhibitor of APC. In fact, in this study we found that 21 of the 28 MSF patients in the acute phase of the disease had detectable circulating levels of KK:PCI complex, whereas this complex was not detected in any of the
healthy normal subjects studied. These data provide the first evidence for in vivo occurrence of KK:PCI complexes. This evidence suggests that during the acute phase of vascular damage, or during any other situation in which KK is generated, this enzyme could also modulate the in vivo activity of PCI. Plasma prekallikrein activation in rickettsiosis has been demonstrated.28 M Rao et al.29 studied volunteers in whom Rocky Mountain spotted fever developed after challenge with *Rickettsia ricketttsii*, and observed an increase in plasma C1-inhibitor:KK complexes from 40 ng/mL before challenge to about 300 ng/mL 24 hours after the onset of the illness.

Twenty-seven of the 28 patients studied showed, at admission, APC:α,AT complex levels higher than T:AT-I11 complex levels, with ratios of APC:α,AT levels to that of T:AT-III ranging from 1.1 to 7.0. T:AT-III (t½ = 10 to 15 minutes)34 is probably cleared from the circulation more rapidly than APC:α,AT complex, and this difference in half-life would explain the lower T:AT-III levels detected in MSF patients.

Several observations suggest that APC may act beneficially at an early step in the chain of events involved in inflammation-induced coagulopathy.31 It has been shown that APC prevents the coagulopathy as well as the fatal outcome associated with *Escherichia coli*-induced shock in a baboon model of septicemia39 and that APC inhibits platelet-dependent thrombosis in a baboon arterial model.4 As indicated here, vasculitis seems to be an important trigger for activation of the anticoagulant protein C system. Initially, the activation of protein C could be considered a defense mechanism preventing local thrombosis, and this could be the reason for the absence of occlusive thrombosis at the level of the inflammatory vascular injury in fulminant rickettsiosis.38 One could speculate that when the vascular injury is more severe, it may lead to inadequate production of APC that then cannot prevent the severe hemostatic alterations seen in the severe forms of MSF.

In conclusion, our study performed in 28 MSF patients in the acute phase of the disease where a widespread microvascular injury (vasculitis) is a dominant feature has demonstrated the activation of the protein C anticoagulant system, suggesting that this pathway is intimately involved in vivo in the interaction of the coagulation and inflammatory system. These results also suggest the interest in screening the protein C system in clinical situations where the inflammatory process in a variety of vasculitides is involved.

REFERENCES

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