Demonstration of Rickettsia conorii-Induced Endothelial Injury In Vivo by Measuring Circulating Endothelial Cells, Thrombomodulin, and von Willebrand Factor in Patients With Mediterranean Spotted Fever

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The endothelial cell (EC) is the primary target for Rickettsia conorii (RC) in Mediterranean spotted fever (MSF). Clinical manifestations such as thrombosis and vasculitis are mediated by pathologic changes localized in blood vessels. To study the in vivo endothelial injury induced by RC, markers of endothelial damage, including circulating EC (CEC), plasmatic thrombomodulin (TM), and von Willebrand factor (vWF), were investigated in 12 patients with MSF. CEC were counted in whole blood by a new immunomagnetic separation assay using a specific anti-EC antibody, S-Endo 1. Plasmatic TM and vWF antigens were measured by enzyme-linked immunosorbent assay. High levels of CEC and soluble TM is regarded as a molecular marker of EC injury because it is not secreted by stimulated EC but is released from the cell surface upon cellular damage.

The endothelial cell (EC) is the primary target in humans infected with Rickettsia conorii (RC), the etiologic agent of Mediterranean spotted fever (MSF). Rickettsia penetration and replication in EC cause vascular injury that appears to be the principal mechanism leading to the hypercoagulable state observed during the acute phase of the disease. Clinical manifestations of MSF, such as vasculitis and microthrombus formation, are mediated by pathologic changes in blood vessels, and strongly suggest that damage to EC occurs in vivo.

Morphologically detectable EC injury has been shown in vitro by electron microscopic studies of cultured EC infected by Rickettsia rickettsii. Within 2 to 3 days postinfection, the injury is manifested by cytosol rarefaction, nuclear degeneration, and release of Rickettsiae often still contained within cells or cell fragments. Cytopathic changes and detachment of injured EC have also been observed in vivo in guinea pigs infected by Rickettsia rickettsii. Vascular injury has been reported to lead to the appearance of EC or cell remnants in the blood of patients with various vascular disorders, but the endothelial nature of these “cell clumps and carcases” has not been proven unequivocally, because sensitive immunodetection methods using EC-specific monoclonal antibodies (MoAbs) have only recently become available.

In the present study, a new immunomagnetic separation assay based on specific antiendothelial MoAb S-Endo 1 was used to investigate the presence of circulating EC (CEC) potentially desquamated from infected vessels in 12 patients suffering from MSF.

Two proteins synthesized by vascular endothelium, the von Willebrand factor (vWF) and thrombomodulin (TM), have been found increased in diseases involving vascular injury. vWF is stored in Weibel-Palade bodies in EC and is released on stimulation of the cell. Secreted vWF is stored in Weibel-Palade bodies in EC and is released on stimulation of the cell. TM is a transmembrane glycoprotein (GP) that interacts with thrombin, thereby serving as a cofactor in the activation of protein C, a major physiologic anticoagulant. A soluble form of TM is found in plasma and presumably represents several degraded forms of vascular TM. This cell fragments were found in patients with a severe or malignant form of MSF. Sequential studies of CEC showed a decrease from 162 ± 454 cells/mL before treatment to 6 ± 7 cells/mL during treatment and recovery. Mean plasma TM and vWF levels that were also elevated before therapy (TM, 106 ± 27 ng/mL; vWF, 420% ± 164%) decrease progressively (TM, 55 ± 43 ng/mL; vWF, 148% ± 26%) during treatment. The measurement of cellular and molecular markers of vascular damage such as CEC, plasmatic TM, and vWF contributes to the definition of the Rickettsia-induced endothelial injury in vivo.

MATERIALS AND METHODS

Selection of patients and blood collection. Twelve patients with clinical manifestations of MSF were included in this study. The malignant form of the disease was defined in patients presenting two laboratory and two clinical criteria among the following: (1) laboratory data include thrombocytopenia (<100 × 10^9/L), renal failure, hyponatremia (<130 mmol/L), hypocalcemia (<2.1 mmol/L), and hypoxemia; (2) clinical data are purpuric rash, stupor, pneumonia, bradycardia, coma, jaundice, and gastrointestinal bleeding. In such cases, lethality is 50-80%. The presence of two laboratory criteria and one clinical criterion defined a severe form for which prognosis is usually better.

Before appropriate antibiotic therapy, 5-mL blood samples were drawn with a sterile 19-gauge butterfly needle into siliconized tubes, containing either EDTA (1 mg/mL) for CEC isolation and TM and vWF measurements, or heparin for rickettsia culture. Blood collection was performed on the day of admission to the hospital before treatment (D1), then twice during treatment: once between day 2 and day 5 (D2-5), and once between day 6 and day 14 (D6-14). For CEC measurement the first 1 mL of blood was discarded to avoid contamination.

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contamination by EC from the vessel wall. Blood from patients undergoing coronary angioplasty served as a positive control for CEC.13 TM and vWF Ag were measured in platelet-poor plasma obtained after centrifugation at 3,000g for 20 minutes and filtration at 0.22 μm to eliminate eventual cellular debris. Pooled normal plasma was similarly prepared from 20 healthy donors and used as a reference.

Diagnosis of MSF. MSF was specifically confirmed by isolation of Rickettsia conorii from blood specimens, using the centrifugation shell-vial technique39,40 or by demonstration of seroconversion with a microimmunofluorescence assay (MIF).41 Sera were analyzed by a Western blot test.42,43

Antibodies. We have previously presented a series of MoAbs raised against human umbilical vein EC (HUVEC). The S-Endol MoAb was selected for the present study because of its specificity and high affinity for EC.14 Flow cytometry analysis showed that S-Endol was highly reactive with HUVEC and nonreactive with hematopoietic cells, mesothelial cells,15 or fibroblasts. In addition, immunohistochemical staining using S-Endol showed a strong staining of endothelium from different vascular beds but not with other cell types.14 The 6D1 MoAb that binds to bladder cancer cells, but not to blood cells or EC, was used as a negative isotype-matched control.44 A rabbit polyclonal antibody directed against human vWF45 and another directed against placental human TM46 were used as EC-associated reagents. A rabbit antiserum specific for human T lymphocytes47 was used as negative control.

Preparation of immunomagnetic beads. Monodispersed magnetizable particles (Dynabeads-M-450) were obtained from Dynal A.S. (Oslo, Norway). These 4.5-μm diameter polystyrene beads were coated with affinity-purified sheep antimouse IgG, covalently bound on the surface (Dynal-SHAM-M450; Biosys, Compiègne, France). They were coated with S-Endol MoAb as second layer. Typically, 350 μL of the SHAM-M450 (4 × 10^10 particles/mL) was washed according to the manufacturer's instructions, using a strong magnet (magnetic particle concentrator 6 [MPC6]; Dynal) and then incubated overnight at 4°C under head-over-head agitation with 1 mL of a 10 μg/mL solution of S-Endol MoAb in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide (PBS-BSA-A). After three washes with PBS-BSA-A, S-Endol–coupled immunomagnetic beads were resuspended in 1 mL PBS-BSA-A to obtain a final concentration of 1.4 × 10^8 immunomagnetic beads/mL. Particles coated with 6D1 MoAb were used as negative controls (nonrelevant [NR]-immunomagnetic beads).

Immunomagnetic separation and counting of circulating EC. Separation and quantitation of CEC were performed as previously described.15,46 Briefly, 2 mL of whole blood diluted 1 to 4 in PBS-BSA-A were mixed with 40 μL of S-Endo 1–coated beads and submitted to gentle agitation for 20 minutes on a rock and roller apparatus (Robbins Scientific, Bio Techgen, Les Ulis, France). All operations were performed on ice to minimize nonspecific binding of beads. Magnetic beads and rosetted cells were separated from blood using the MPC6 concentrator. After two washes to eliminate unbound cells, the magnet was removed and the rosetted cells were washed from the tube wall using 100 μL PBS-BSA-A and divided into two equal fractions. One was stained with acridine orange (3 μg/mL in PBS) for cell counting. Analyses were performed under an optical fluorescence microscope (λ = 490 nm) using a Nageotte hemocytometer for accurate quantification. For each sample, the rosetted cells present in the total volume of the hemocytometer were scored optically. The quantitation of rosetted EC was performed in duplicate. The criteria retained for the identification of an EC were rosettes bearing more than 10 beads and a cell size in the

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**Figure 1.** Mean levels ± SD of (A) CEC, (B) vWF antigen, and (C) TM antigen in 12 patients with a diagnosis of MSF, just before treatment (D1), from days 2 to 5 (D2-5), and from days 6 to 14 of treatment (D6-14).
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Table 1. CEC, Plasmatic vWF, and TM Antigen in 12 Patients With MSF

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>vWF (%)</th>
<th>TM (ng/mL)</th>
<th>CEC (cells/mL)</th>
<th>Platelets (10^5/μL)</th>
<th>HT</th>
<th>Complications</th>
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<tr>
<td></td>
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<td>D2o</td>
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For each parameter, the values were determined just before treatment (D1), from days 2 to 5 (D2o), and from days 6 to 14 of treatment (D3o).

Platelet (10^5/μL) count at D1, addition of heparin therapy, and clinical complications are also shown.

Abbreviations: ND, not determined; HT, heparin therapy.

RESULTS

Isolation and characterization of circulating EC in MSF patients. Blood samples from 12 patients with symptoms and serologic confirmation of MSF were studied. Mean levels ranged from 162 ± 454 cells/mL before treatment to 6 ± 7 cells/mL during the last 9 days of therapy (Fig 1A). Among a total number of 31 CEC measurements, 23 were found to be above normal (>1 EC/mL) (Table 1). Between patients a large scattering in CEC number (ranging from 0 to 1,600/mL) was observed. No CEC were detectable for patients no. 1 and 2. For the others, a decrease in the number of CEC was shown in the course of MSF. In patients who did not develop complications, the level of CEC was below 10 cells/mL, except for patient no. 4, who had a mild form of MSF and a number of CEC close to 50 cells/mL. In contrast, elevated values of CEC, ranging from 18 to 1,600 cells/mL, were detectable in patients with malignant or severe forms of the disease who developed complications and sometimes required anticoagulant therapy. The elevated level observed for patient no. 3, a 66-year-old woman, under anticoagulant therapy for a mitral prosthetic valve could also be explained by mechanical injury induced by the valve. The highest number of CEC was detected in a patient with a severe form followed by a rapid recovery (patient no. 12).

After May-Grünwald staining of cytocentrifuge preparations, the isolated cells were large, 30 to 50 μm in diameter, often with a pyknotic nucleus, an irregular contour, and a pale blue cytoplasm outlined by attached beads (Fig 2A). Because no CEC were detectable in normal subjects, they were compared with EC isolated from blood of patients submitted to coronary angioplasty (Figs 2B, D, F). In this situation, which represented a positive control of "endothelial mia," we have shown the presence of whole nucleated cells...
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at very low concentrations (from 1 to 20 cells/mL) in the blood of catheterized patients. In MSF patients, detection of CEC was sometimes difficult, primarily because cellular boundaries were not easy to observe despite counterstaining. In addition, the necrotic or degenerated aspect of many of the cells isolated from blood samples made their identification difficult without complementary characterization using endothelial-associated probes. The endothelial nature of such isolated cells was confirmed by the expression of both vWF (Fig 2C) and TM (Fig 2E) antigens, as seen by immunofluorescence labeling. Interestingly, in all CEC from MSF patients, the vWF staining pattern was more diffuse and fainter (Fig 2C) than the typical granular staining observed in CEC detected during angioplasty (Fig 2D). In addition to whole cells and cell clumps, cell fragments of various sizes (Fig 2G) were also observed in each sample.

Measurement of plasmatic vWF and TM antigens: Patients diagnosed as having MSF showed higher overall plasma vWF Ag (Table 1) than did normal controls (94.9% ± 18.5%). Figure 1B shows the chronicologic evolution of vWF Ag during RC therapy: mean Ag level decreased progressively from 420% ± 164% before treatment to 148% ± 26% during the last 9 days. There was no clear-cut relation between vWF Ag and disease severity, because the highest levels (more than 400%) were found in patients with both mild or severe forms of MSF.

Mean plasma TM was elevated before treatment (106 ± 27 ng/mL compared with 40 ± 25 ng/mL for normal subjects). Its evolution during therapy is shown in Fig 1C: it decreased progressively, decreasing close to control values during the last 9 days (55 ± 43 ng/mL). In all the patients, disease improvement was accompanied by a gradual return towards normal TM values, but there was no clear-cut relationship between the magnitude of the disease and the TM level (Table 1). This is illustrated by the data for patients no. 5, 9, and 10, which show a severe form of MSF, but less marked elevations of plasmatic TM. Inversely, important increases of TM (more than 100 ng/mL) were found in patients no. 1, 2, and 6, who had a mild form of the disease, with CEC less than 2 cells/mL. The fact that patients with the greatest number of CEC did not have the highest levels of TM strongly suggested that the plasmatic TM measured by ELISA is soluble and not cell-associated.

As seen in Table 1, CEC were correlated neither with TM, nor with vWF. In addition, TM and vWF were not correlated. In patients no. 1, 2, 3, and 12, both markers were clearly high, whereas they were moderately elevated in patients no. 8 and 10 and dissociated in the last 6 patients. This dissociation was particularly striking for patients no. 5, 9, and 10, who developed a severe form of the disease, with delayed recovery and prolonged fever, requiring anticoagulant therapy. In these 3 patients, platelet counts before treatment were less than 100 x 10^3/μL and CEC above 20 cells/mL. As seen in Table 1, the platelet count was slightly low in all patients, but it correlated neither with TM, nor with vWF antigen levels.

DISCUSSION

Microvascular injury is central to the pathogenesis of MSF-associated hemostatic alterations. The main finding of this study was the demonstration of above normal levels of CEC or EC fragments and increased concentrations of plasma TM and vWF in the blood of patients with MSF, clearly reflecting the vascular lesional process associated with this pathology.

Normal intact endothelium is thromboresistant. Damage to EC can result in their detachment from blood vessels, exposing the thrombogenic subendothelium. Infection of vascular EC by RC results, in most patients, in desquamation of endothelial injury objectivized by an increased number of CEC. Demonstration of RC in these circulating cells has recently been made by Drancourt et al. This confirmed an hypothesis raised 20 years ago by Walker et al suggesting that entry into the circulation of EC detached from infected vessels may result in "intraendothelial cell Rickettsemia." In most MSF patients, CEC numbers rarely exceeded 100 cells/mL, and decreased in parallel to recovery. Comparable CEC levels have been reported in other clinical situations, including cytomegalovirus (CMV) infections with circulating infected cells, thrombotic thrombocytopenic purpura, sickle cell disease (SCD), or coronary angioplasty. The presence of RC in CEC isolated from MSF patients may explain their necrotic aspect compared with EC mechanically desquamated during angioplasty, indicating that the morphology of the cells detected depends on the nature of the lesional process. The endothelial origin of the circulating cells and cell fragments isolated by S-Endo1 was confirmed using an antibody against TM. Although these fragments are currently difficult to quantitate, they should be considered because they certainly contribute, together with whole cells, to what could be called "endothelemia." This endothelial fragmentation has also been observed in patients undergoing coronary angioplasty.

The mechanism of EC desquamation in MSF remains to be clarified. Vascular EC lie on a basement membrane constituted by a thick network of adhesive proteins. It is likely that EC lose some of their adhesive properties as a result of infection. Both the previously described absence of CD51 (the vitronectin receptor β chain) from CEC of CMV-infected patients and the results of in vitro experiments showing a relation between Herpes virus infection and reduced cellular adhesion support this hypothesis. The mechanism of EC desquamation in MSF has also been attributed by different investigators to a direct cytopathic action, effect
of a toxin, immunopathologic mechanisms, or oxidative stress.

As shown in circulating CMV-infected EC, CEC from MSF patients did not display the typical granular vWF immunostaining pattern. This could be attributed to the release of vWF from Weibel-Palade bodies after cell infection, as we observed in a previous study on cultured EC infected by RC. Similarly, different in vitro models have shown the stimulated release of vWF after experimental infection of EC by Rickettsia rickettsii or CMV. This phenomenon could contribute to explain the high level of plasma vWF found in most MSF patients.

vWF is an established indicator of the stimulation and/or injury of ECs and many studies have shown that plasma vWF is increased in a broad spectrum of infectious and noninfectious diseases. In MSF patients, the elevated levels of plasmatic vWF and the parallelism between vWF decrease and patient recovery have already been reported. However, vWF is also released by activated platelets. Therefore, TM is more relevant to assess EC lesions, because it specifically originates from EC, and it is released on EC damage, but not EC stimulation. It has recently been shown that TM release from EC is increased both in vitro, in response to various injurious substances (H2O2, prostaglandin, lipopolysaccharide, elastase), and in vivo in different pathologies associated with vascular damage. Demonstration of increased level of TM in MSF is made in the present study. In most patients, mean TM levels decreased as the patient recovered from the disease. However, our data showed a large scattering in TM plasma levels, with no evident correlation with the magnitude of the disease nor with vWF values, probably because of the different release pattern of these proteins.

The mechanism that causes TM release still remains unclear. One possibility is that TM circulates in blood after being proteolyzed from the cell surface by enzymes, such as elastase, cathepsin, or kallikrein. Another possibility could be linked to cell infection because we showed a decreased membrane expression of TM in RC-infected cultured EC. This could be caused by an increase in TM release. Shedding of proteins from the cell surface is a well-documented phenomenon, because in addition to a number of cytokines and growth factor receptors, soluble forms of adhesive molecules can also be released.

A decrease in TM expression on the EC surface would be responsible for hypercoagulable state, because protein C-TM system plays an important role in the regulation of intravascular thrombogenesis. From our observation, severe forms of the disease are probably associated with the activation of the coagulation system, as thrombocytopenia suggested. This activation seems to be a relevant aspect of MSF. This vascular alteration consists in both desquamative EC injury reflected by CEC and nondenuading endothelial damage associated with increased release of stored molecules, such as vWF, or membrane proteins, such as TM. Monitoring of these cellular and molecular markers may provide useful information on endothelial activation or damage in a number of infectious and noninfectious diseases, including Rickettsiae.

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