Secretion of Tissue-Type Plasminogen Activator and Plasminogen Activator Inhibitor by *Rickettsia conorii-* and *Rickettsia rickettsii-*Infected Cultured Endothelial Cells

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Hemostasis abnormalities have been described in patients with Mediterranean spotted fever and Rocky Mountain spotted fever. Evidence of the activation of the fibrinolytic system has been obtained in both diseases. After experimental Rocky Mountain spotted fever, an elevated level of fibrinogen was found in parallel with the activation of the fibrinolytic system and transient elevation of the tissue-type plasminogen activator. Later protein is mainly synthesized by endothelial cells. The ability to culture human endothelial cells in vitro provides a unique system to study the secretion of tissue-type plasminogen activator and of plasminogen activator inhibitor after rickettsial infection. Human vascular endothelial cells derived from the umbilical vein, when infected with *Rickettsia conorii* or *Rickettsia rickettsii*, secreted as much tissue-type plasminogen activator as control cells. The activity of plasminogen activator inhibitor however, was higher in the supernatants of infected cells than in those of control cells. This rickettsia-induced imbalance of the tissue-type plasminogen activator-inhibitor pair was a very early event after in vitro infection. The involvement of this system during Mediterranean spotted fever and Rocky Mountain spotted fever remains to be demonstrated.

Rickettsia conorii and *Rickettsia rickettsii* organisms are obligate intracellular bacteria (23). Their ultrastructure and biochemical composition are similar to those of gram-negative bacteria. DNA-DNA annealing was reported to show 91 to 94% homology between both species (20). *R. conorii* and *R. rickettsii* are respectively the etiologic agents of Mediterranean spotted fever (MSF) and Rocky Mountain spotted fever (RMSF). From the skin, the portal of entry, rickettsiae spread via the lymphatics and bloodstream to all parts of the body. In each site, the rickettsiae enter and proliferate within endothelial cells (22).

Hemostatic impairment has been described in RMSF (4) and in the malignant form of MSF (13). Thrombocytopenia has been reported in 32 to 52% of patients with RMSF (4, 7) and in 35% of MSF patients (12). The kinetics of the platelet count was determined for 412 patients with MSF through the first month of disease (M. Drancourt et al., Ann. N.Y. Acad. Sci., in press). The mean value dropped from 240×10^9/liter at the time of admission to 170×10^9/liter on day 5 of disease. This decrease was followed by a rebound increase up to 330×10^9/liter on day 20 of disease. In vitro-enhanced adhesion of platelets to *R. rickettsii*-infected endothelial cells has been demonstrated (15). Evidence of platelet activation has been reported during the early stage of human experimental RMSF (10). In both diseases, the coagulation pathways are quickly activated; a drop in the plasma level of antithrombin III and a rise in the plasma level of fibrinopeptide A, which are very suggestive of thrombin formation, have been described in experimental RMSF (10).

In MSF, however, it still remains unclear; during the first three weeks of MSF, stability of antithrombin III levels and a drop of protein C levels have been observed (18). Otherwise, no differences in the measurements of immunochemical and biological activities of high-molecular-weight kino-

gen, of prekallikrein, and of factor IX have been found in patients with MSF and controls (19). Finally, evidence of the activation of the fibrinolytic system has been reported in both diseases. Elevated levels of fibrin degradation products were found in 10 patients among 31 with MSF (18). After human challenge with *R. rickettsii*, the elevations of fibrin degradation products and alpha-2-antiplasmin-plasmin complexes and the declines in the level of plasminogen and alpha-2-antiplasmin provided strong evidence of activation of the fibrinolytic system. In this study (10), elevated levels of tissue-type plasminogen activator (t-PA) were noted 24 h after the onset of fever. The median values of t-PA antigen rose from 24.7 and 21.6 ng/ml before challenge to 34.8 and 29.1 ng/ml after 24 h of fever in, respectively, five controls and nine vaccinated subjects. An unexpected elevated level of fibrinogen was found in parallel with the activation of the fibrinolytic system and with the rise of t-PA. No data concerning the secretion of the plasminogen activator inhibitor (PAI) were available. t-PA and PAI are mainly synthesized by endothelial cells (8). These cells can be propagated in vitro, and experimental infection of cultured endothelial cells is a suitable model for rickettsial diseases. This model was used in the present work to study the secretion of t-PA and PAI, apart from the other hemostatic elements involved in vivo.

MATERIALS AND METHODS

Cell culture methods. Endothelial cells were harvested from fresh human umbilical cord veins by using 0.1% collagenase (Boehringer Mannheim France SA, Meylan, France) by the method of Jaffe et al. (5). Cells from four umbilical cords were pooled and plated in T-25 tissue culture flasks (CEB SA, Angers, France) previously coated with 1% gelatin (Difco Laboratories, Detroit, Mich.). Cells were grown to confluency at 37°C with atmospheric air and 5% CO_2 in medium 199 with Earle salts containing 20% decom-

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plemented fetal calf serum. No antibiotics were added to the medium. Endothelial cell monolayers demonstrated the typical cobblestone morphology and stained positively for factor VIII antigen by indirect immunofluorescence. Primary cell cultures reached confluency in an average of 6 days. The cells were washed three times with phosphate-buffered saline (PBS), harvested with 0.05% trypsin, and resuspended into the same medium to an average of 2×10^4 cells per ml. A 250-μl portion of this solution was placed into each well of a 96-well microdilution plate (Costar Data Packaging, Cambridge, Mass.) previously coated with 1% gelatin. All experiments were performed on cells after only one passage. The next day, culture medium was removed, and the confluent monolayers were washed twice with PBS and infected with *R. conorii* (ATCC Moroccan strain) or *R. rickettsii* (Sheila Smith strain). Rickettsiae grown on Vero cells were purified by differential centrifugation. The inoculum infectivity was determined by plaque assay (20) and adjusted to 4×10^4 PFU/ml (stock solution). The experimental concentration of 4×10^3 PFU/ml was obtained by dilution with cell culture medium. Lanes 1 to 6 on the microdilution plate were used for negative controls and filled with culture medium (50 μl per well); lanes 7 to 12 were used for infected cells and filled with rickettsial solution (50 μl per well). The plate was incubated at room temperature for 1 h to allow cell infection. Finally, 200 μl of culture medium was added to each well. Supernatants from six infected wells and six control wells were removed 0, 1, 2, 4, 8, 12, 24, and 48 h after the end of the incubation time and frozen at $-20°C$ until use. Sampling times were chosen to study the early secretion of t-PA and PAI, as a preliminary study (11) showed that both proteins were detectable in the supernatant of *R. conorii*-infected cells 24 to 72 h postinfection (p.i.).

Assay techniques. (i) **Assay of t-PA.** t-PA antigen levels were determined by an enzyme-linked immunosorbent assay. The immunoglobulin G fractions of two monoclonal antibodies (62E8 and 3B6; provided by D. Collen, Leuven, Belgium) were diluted with 0.04 M phosphate buffer (pH 7.4) containing 0.14 M NaCl, 10 g of polyethylene glycol 6000, and 60 g of saccharose per liter (coating buffer).

Samples of 200 μl of a 4-μg/ml solution were incubated for 48 h at 4°C in a 96-well microdilution plate (Costar). The wells were treated for 2 h at room temperature with 200 μl of coating buffer containing 10 g of bovine serum albumin per liter and then washed with 200 μl of coating buffer and finally with a solution of 10 g of mannitol and 20 g of saccharose per liter. The plates were stored at $-20°C$. Immediately before use, the plates were washed twice with 0.04 M phosphate buffer (pH 7.4) containing 0.14 M NaCl. Wells were filled with 180 μl of samples. After incubation for about 18 h at 4°C in a moist chamber, the wells were emptied and washed four times with PBS containing 0.002% Tween 80 (PBS-Tween). The plates were filled with 160-μl samples of a horseradish peroxidase–anti-t-PA immunoglobulin G conjugate (29B9 HRP; furnished by D. Collen) diluted 1:2,000 with PBS-Tween containing 1 mg of bovine serum albumin per ml and incubated for 2 h at room temperature. Peroxidase reaction was performed by the addition of 160-μl portions of 0.1 M citrate–0.2 M sodium phosphate buffer (pH 5.0) containing 200 μg of *ortho*-phenylenediamine per ml and 0.003% hydrogen peroxide. After 1 h at room temperature, the reaction was stopped with 50 μl of 4 M H_2SO_4. The A_{492} was measured with a multiscan spectrophotometer (Titertek; Flow Laboratories, Inc., McLean, Va.). The results were reported in nanograms per milliliter by reference to a standard of purified human t-PA (international reference 83/517

obtained from the National Institute for Biological Standard and Control, London). A standard curve was constructed between 0.1 and 4 ng/ml.

(ii) **PAI activity assay.** PAI activity was measured by an indirect spectrophotometer assay described by Verheijen et al. (17) involving both plasminogen activator and the measurement of plasmin activity with a synthetic peptide substrate, H-D-Val-Leu-Lys-pNA (S-2251; obtained from Kabi, Stockholm, Sweden) in the presence of fibrinogen fragments. Supernatants (25 μl) and various amounts of t-PA were added to wells of a 96-well polystyrene flat-bottomed microdilution plate (Costar) containing 0.1 M Tris hydrochloride (pH 7.5), 0.1% (vol/vol) Tween 80, 0.12 mg of cyanogen bromide digest of fibrinogen per ml, 0.13 μM plasminogen (human Lys-plasminogen), and 0.30 mmol of S-2251 per liter in a final volume of 250 μl per well. The plate was incubated at 25°C for between 1 and 2 h, and the absorbance change in the wells was read with a multiscan photometer (Titertek). The inhibition level was determined by extrapolation as indicated above and expressed in units of t-PA inhibited per milliliter of supernatant.

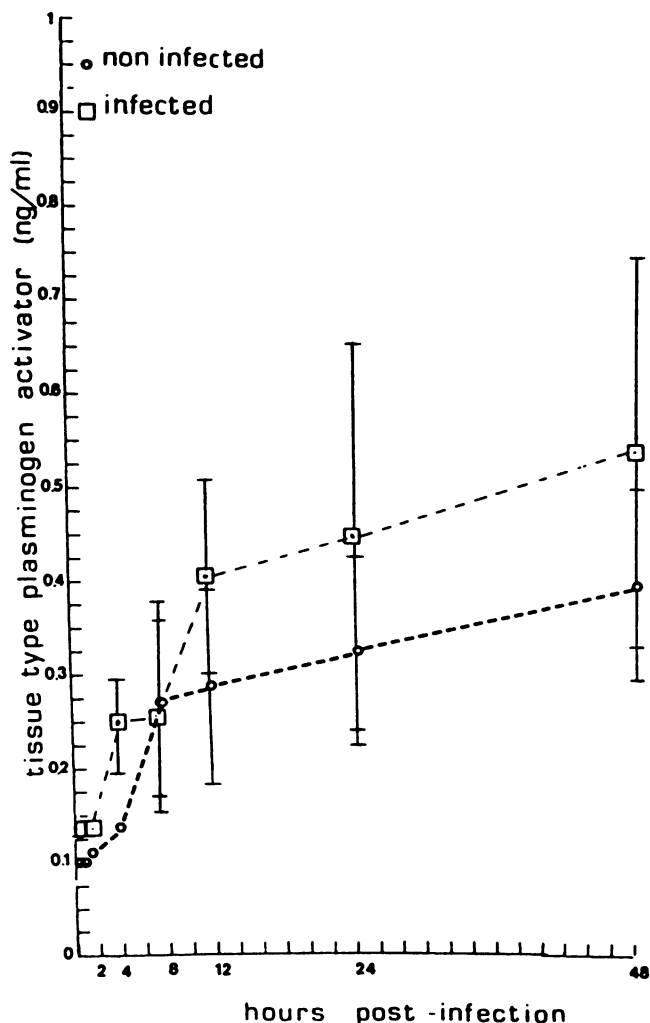**Statistical analysis.** Comparisons of t-PA and PAI levels

FIG. 1. t-PA concentration in the supernatants of noninfected and *R. conorii*-infected cultured endothelial cells. Mean values and standard deviations of three experiments are shown.
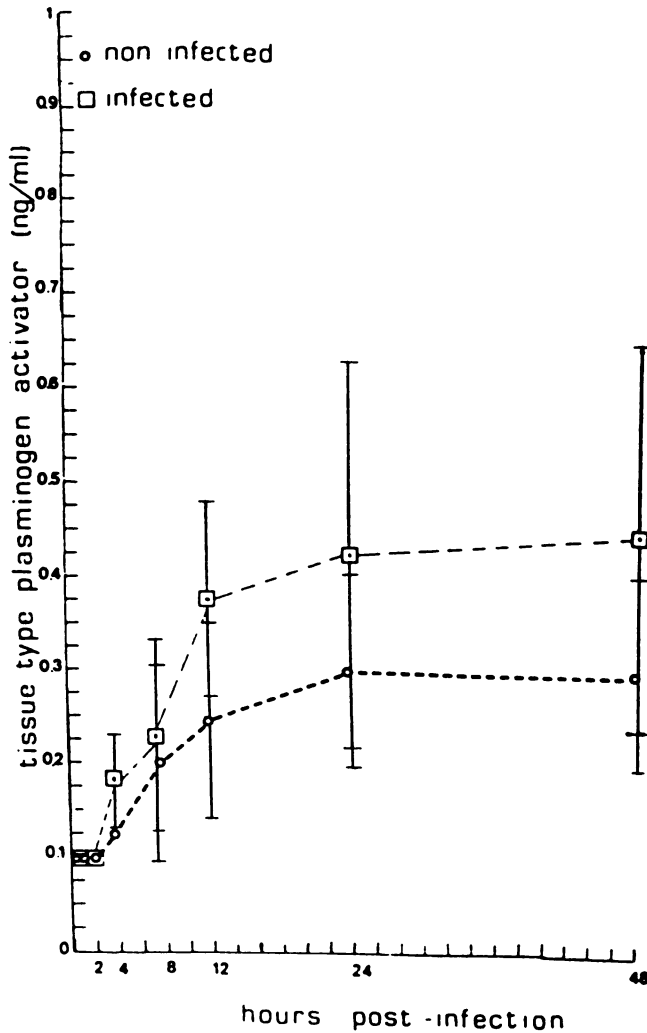
FIG. 2. t-PA concentration in the supernatants of noninfected and *R. rickettsii*-infected cultured endothelial cells. Mean values and standard deviations of three experiments are shown.

between noninfected and infected cells and between *R. conorii*- and *R. rickettsii*-infected cells were done by the Student's *t* test applied to the means for each variable. Use of the term significant in this report indicates a probability of <0.05.

RESULTS

Morphology of infected endothelial cells. Microscopic examination of rickettsia-infected endothelial cell monolayers before supernatant extraction did not disclose morphological changes until 48 h p.i. Cytopathic effects were seen in approximately 5% of *R. conorii*- and 10% of *R. rickettsii*-infected cells 48 h p.i. No extensive cellular damage was observed. The infection rate observed by Gimenez staining (2) ranged from 0 to 10 rickettsiae per cell 48 h p.i.

t-PA secretion. Slight accumulation of t-PA was measured in the supernatant of noninfected cells. t-PA was undetectable at t_0, t_1, t_2, and t_4 and rose 0.3 ng/ml at t_{48}. This concentration range is about 10-fold lower than concentrations obtained when human serum is used in culture medium (3). A mild time-dependent increase of t-PA was measured in

the supernatants of *R. conorii*-infected (Fig. 1) and *R. rickettsii*-infected (Fig. 2) cells. The kinetics of t-PA in the supernatants of infected cells was parallel to that of noninfected cells, except at t_{12}; the ratio of t-PA in infected cells versus noninfected cells was then 1.6. However, differences between infected and noninfected cells or between *R. conorii*- and *R. rickettsii*-infected cells were not significant.

PAI activity. PAI activity was present in the supernatant of noninfected endothelial cells, but the range of values was 10-fold lower than previously reported (3). The absence of human serum in our culture medium and the low number of cells per well might explain this discrepancy. PAI activity in the supernatants of *R. conorii*-infected (Fig. 3) and *R. rickettsii*-infected (Fig. 4) cells was significantly higher than in the supernatants of control cells, except at t_0. No significant difference was found between *R. conorii*- and *R. rickettsii*-infected cells.

DISCUSSION

The concentration of t-PA and the activity of PAI were measured in the supernatants of noninfected and rickettsia-
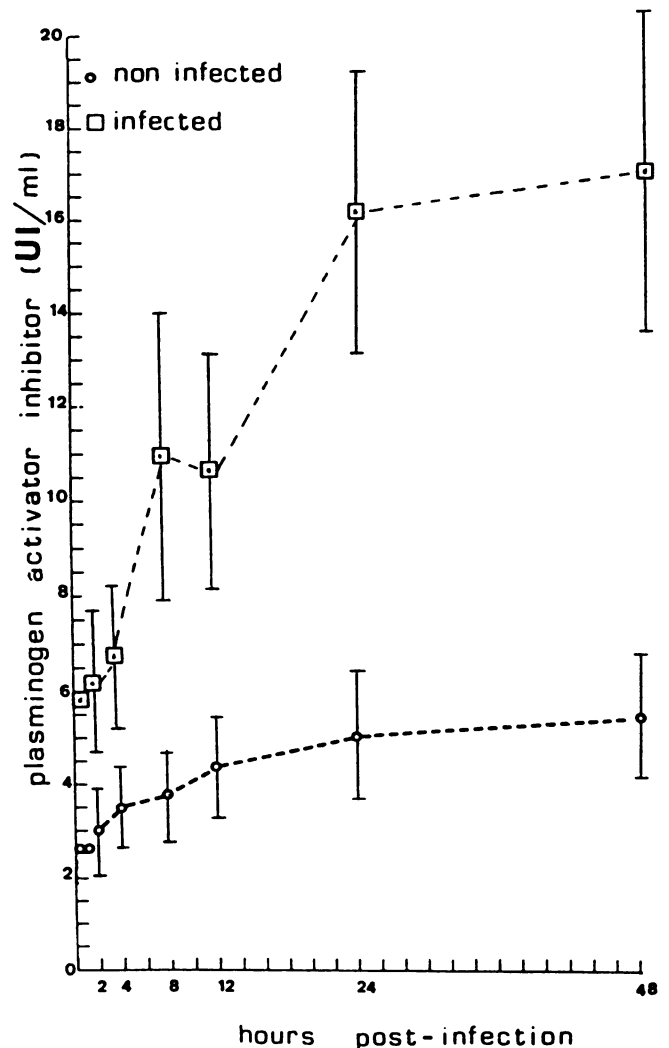

FIG. 3. PAI activity in the supernatants of noninfected and *R. conorii*-infected endothelial cells. Mean values and standard deviations of three experiments are shown.
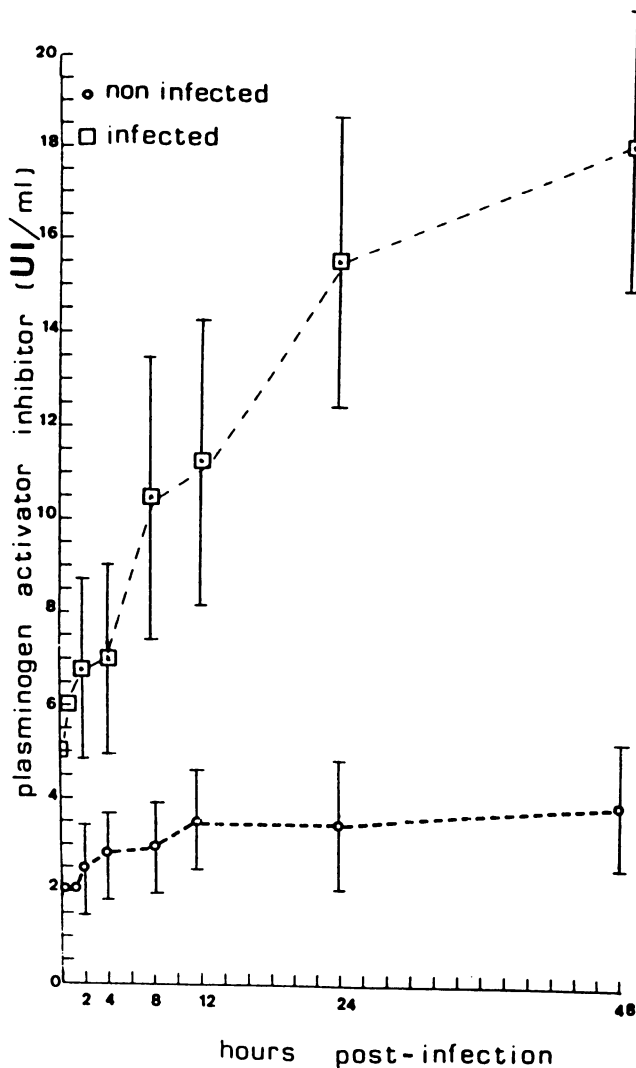
FIG. 4. PAI activity in the supernatants of noninfected and *R. rickettsii*-infected endothelial cells. Mean values and standard deviations of three experiments are shown.

infected cultured endothelial cells. t-PA is the most potent fibrinolytic compound known to date and is used as therapy in myocardial infarction and in pulmonary embolism (9). PAI complexes t-PA stoichiometrically (16), and this results in t-PA inactivation. Both proteins are mainly synthesized by endothelial cells in a dynamic process so that PAI activity continuously balances t-PA secretion (8). The results we obtained with noninfected cells corroborate previous reports. Nonstimulated endothelial cells released both t-PA and PAI activity at low levels. Rickettsial infection induced an imbalance of the t-PA-PAI combination. Significant elevation of PAI activity was found in the supernatants of infected cells at the same time t-PA was not significantly rising. Therefore, the supernatants of rickettsia-infected cells shared an antifibrinolytic activity. The increase of PAI activity was detectable from the end of the 1-h incubation and was more striking with time. These events were therefore early events during in vitro rickettsial infection.

Their mechanisms are not yet known. Nonspecific release of t-PA and PAI due to extensive cell injury is questionable. No major cytopathic effect was noted within the first 48 h

p.i.; this fact corroborates results obtained after in vitro infection with *R. rickettsii* (14, 21). To date, the *Escherichia coli* lipopolysaccharide is the most potent stimulus for PAI secretion (3). Lipopolysaccharide stimulation of cultured endothelial cells yields a striking elevation of PAI secretion but little elevation of t-PA (1). The same results are obtained after endotoxin infusion in rabbits (1). *R. conorii* and *R. rickettsii* are gram-negative bacteria, and furthermore the rickettsial polysaccharide has been identified. The kinetics of t-PA and PAI activity that we observed mimic those obtained after lipopolysaccharide stimulation of cultured endothelial cells. These data are, however, inadequate to confirm the possible role of rickettsial lipopolysaccharide in PAI secretion by infected endothelial cells.

The model we used is a suitable one to study the relationship between rickettsiae and their target cells and the pathophysiology of rickettsial diseases (21). However, it did not contain all the subtle hemostatic elements involved in vivo. Particularly, clotting factors which are involved as feedback mechanisms of fibrinolysis were absent. In the same way, blood flow is known to be crucial in the pathogenesis of thrombosis but was not featured in our model.

Evidence of the activation of the fibrinolytic system has been obtained in natural MSF (18) and in experimental RMSF (10). In the latter study, an unexpected high level of fibrinogen was noted in parallel with fibrinolytic system activation and the rise of t-PA. No data concerning the PAI were available. Whether or not the t-PA-PAI system is involved during MSF and RMSF has still to be determined. However, this study and the experimental RMSF study (10) suggest that the secretions of both proteins are early events after endothelial cell infection. The chance of obtaining a specimen from a patient during the first hours after the onset of fever is small, and experimental data may not be confirmed by clinical studies, especially since the half-life of these proteins is in the minute range (9, 16). If present in patients with MSF or RMSF, the imbalance of the t-PA-PAI combination would lead to inefficient fibrinolysis and exacerbate venous thrombosis resulting from other clotting impairments (15, 18). A similar mechanism has been documented in patients with spontaneous or recurrent deep venous thrombosis (6).

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