Predominant Role of Host Proteases in Myocardial Damage Associated with Infectious Endocarditis Induced by Enterococcus faecalis in a Rat Model

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Infective endocarditis (IE) remains a life-threatening infectious disease with high morbidity and mortality. The objectives of the present study are to assess the host proteolytic activities of the vegetations and their cytotoxic potential in a rat model of experimental IE. Rats were infected with a strain of Enterococcus faecalis of particularly low virulence and weak protease expression. We tested the presence of proteases released by infiltrated leukocytes (matrix metalloproteinases and elastase) or produced in situ within the septic vegetation, such as those linked to the fibrinolytic system (plasmin and plasminogen activators). We also assessed the tissue damage induced by the infective thrombus in vitro and ex vivo. The model of IE was characterized by larger and more extensive vegetations in infected than in nonseptic rats and by an intense neutrophil infiltrate interfacing with the injured underlying tissue. Neutrophil extracellular DNA was shown to trap bacteria and to produce increased levels of cell-free DNA in plasma. Matrix metalloproteinase-9, elastase, and plasminogen activators were increased in septic versus nonseptic vegetations (as shown by zymography and immunohistology). Finally, proteolysis of the extracellular matrix and apoptosis were shown to be associated with host proteases. Bacteria exhibited no detectable proteolytic activity or direct cytotoxic effects. Bacterial membranes/dead bacteria were sufficient to induce leukocyte recruitment and activation that could promote vegetation formation and growth. Our results suggest that, despite the lack of bacterial proteases, the continuous attractant signals coming from bacterial colonies may lead to a chronic and deleterious aggression toward myocardial/valvular tissues by host proteases.

Infective endocarditis (IE) still affects numerous patients and remains a life-threatening infectious disease with high morbidity and mortality. The fibrinoid vegetation that constitutes the pathogenic lesion of IE is a thrombus containing proliferating bacteria and is responsible for damage to the underlying tissue and local complications, including acute heart failure, valvular regurgitation, and myocardial abscess (1). Vegetations (V) also produce septic emboli. The first step of the process is endothelial damage, leading to the formation of a nonbacterial fibrin/platelet thrombus due to activation of coagulation (1, 2). The second essential step is the adhesion of circulating bacteria to this thrombus. Colonizing bacteria, via their chemoattractant properties, represent a trigger for a massive infiltration of leukocytes into the vegetation, a process which in turn further activates the host coagulation system. Thus, this amplification of fibrin formation is enhanced, leading to vegetation growth and protrusion. This bacterial thrombus is not simply a passive reservoir of bacteria but is a biologically active neotissue formed by an accumulation of platelets, fibrin, bacteria, and the chronic recruitment and activation of neutrophils (3, 4). The biological activities of the septic vegetation (SV) can be separated into bacterial and host biological activities.

The biological activities of the fibrinoid vegetations are involved in two processes, (i) the formation and continuous growth of vegetations (5) and (ii) the ability of this neotissue to convey bacterial and endogenous proteases responsible for the destruction of the surrounding tissue and for the production of emboli (6).

Neutrophils, which represent the first line of defense against bacteria, have recently been reported to play a major role in thrombus formation via a concerted action of serine proteases and externalized nucleosomes (also known as neutrophil extracellular traps, or NETs) (2). NETs are extracellular DNA/histone structures associated with neutrophil granule contents (elastase, cathepsin G, myeloperoxidase, etc.) that can trap pathogens and exert bactericidal action (7).

We recently reported in a different context that the persistence of an infected thrombus in contact with the underlying tissue represents a source of blood-conveyed proteases, including both bacterial and host leukocyte proteases released upon activation (4, 8). For example, neutrophil elastase has been shown to induce cell death subsequent to pericellular proteolysis and to be deleterious for vascular tissues (9, 10). Similarly, the generation of plasmin (11) and the presence of thrombin (12) may represent effectors of tissue damage. Furthermore, because it contributes to thrombus destabilization, fibrinolytic activity has been shown to be correlated with thromboembolic events. We hypothesize that host components of the vegetation (leukocytes and proteases) con-
tained in the thrombus may play a major role in the physiopathology of tissue damage occurring in IE.

The main objectives of the present study were to assess host proteolytic activities (gelatinases, elastase, and plasmin) contained in the vegetation and their cytotoxic potential in a rat model of experimental infective endocarditis (13, 14). In particular, we aimed at separating the respective involvement of the bacterial and nonbacterial biological components of the infective endocarditis thrombus in myocardial/valvular tissue degradation and fibrinolytic activity and thereby providing evidence of a pivotal role of host proteases. We also tested the hypothesis that the material derived from dead bacteria would be sufficient to trigger myocardial/valvular tissue damage. For this purpose, rats were infected with a clinical strain of Enterococcus faecalis of particularly low intrinsic virulence and weak protease expression, either alive or dead (bacterial membranes).

**MATERIALS AND METHODS**

**Experimental models and microbial strain.** Aortic valve endocarditis was induced in 16 male Wistar rats as described by Durack et al. in rabbits (15), following a protocol adapted to rats by our group (13). The study was designed with 80% power to detect a relative 50% difference in vegetation area between groups. Statistical testing was performed at the 2-tailed α level of 0.05 using a t test. In brief, a polyethylene catheter (PE 10; Clay Adams) was connected to an invasive blood pressure monitor and inserted into the left ventricle via the right carotid artery under ketamine-xylazine anesthesia. The left-ventricular location of the catheter was checked on the pressure curve as previously described (13). The catheter remained indwelling throughout the experiment in order to induce mechanical injury that would lead to the formation of a nonbacterial endocardial vegetation (vegetation). Twenty-four hours after catheterization, 8 rats underwent bacterial inoculation (Enterococcus faecalis JH2-2) (16, 17) via the jugular vein in order to induce bacterial colonization of the thrombotic vegetation, so as to obtain an infectious endocardial thrombus (septic vegetation). A suspension of E. faecalis JH2-2 (0.5 ml) was inoculated at a concentration of 10⁶ CFU/ml saline. The mean bacterial count in vegetations was estimated at 8 log₁₀ CFU/g. Eight rats were injected with sterile saline (0.9% NaCl), and 8 more rats were sham-operated to serve as controls. The procedure and the animal care complied with the principles of animal care formulated by the National Society for Medical Research. An additional set of experiments was performed using 5 rats per group injected with bacteria, saline, and bacterial membranes in order to assess the efficacy of amoxicillin during incubation of the vegetations and the effects of dead bacterial material on vegetation formation. For this purpose, bacteria were sonicated (2 times for 45 s), ultracentrifuged (50,000 × g for 15 min at 10°C), and washed as described previously (18). Membrane material corresponding to 0.5 ml of bacteria (10⁶ CFU/ml) was injected into 5 rats.

**Animal euthanasia.** Sacrifice was performed at day 4 following bacterial infection. After thiopental anesthesia, hearts were explanted and macroscopically examined. Aortic valve endocarditis was confirmed by visual inspection of the aortic valves and left ventricle, which were fixed in glutaraldehyde for histological analysis. Five-micrometer-thick serial sections were routinely stained with Masson’s trichrome, hematoxylin and eosin, and Alcian blue with nuclear red counterstaining to visualize areas of mucoid accumulation.

The Apostain method was used to detect apoptotic cells according to the manufacturer’s instructions (IgG monoclonal F-7-26 at 10 μg/ml; AbCys), with visualization by an anti-mouse antibody conjugated with horseradish peroxidase (HPR), followed by the Histogreen (AbCys) reagent and nuclear red counterstaining as described previously (14). Macrophages were stained by using a rabbit polyclonal anti-ED1 antibody (Abcam) at 0.5 μg/ml (nuclei were counterstained with hematoxylin). Enterococcus bacteria and neutrophils were detected by immunofluorescence using polyclonal rabbit anti-Enterococcus IgGs (Abcam) at 15 μg/ml and anti-neutrophil IgGs (diluted at 1:3,000; Cedarlane), revealed by anti-rabbit antibodies conjugated with Alexa 488, with 100 ng/ml DAPI (4′,6′-diamidino-2-phenylindole) for nuclear counterstaining.

**Biochemical analysis and proteolytic activities.** (i) Chemicals and reagents. Plasmin was from American Diagnostica, and leukocyte elastase and the plasmin fluorometric substrate from Calbiochem. All other products were from Sigma except for products for electrophoresis, which were from Bio-Rad.

(ii) Determination of cf-DNA concentrations in plasma. The cell-free DNA (cfDNA) concentrations in plasma of rats were determined by using Quant-it Picogreen double-stranded DNA (dsDNA) reagent (Invitrogen, France). Briefly, 10-μl amounts of samples and Lambda DNA standard (1 ng/ml to 1 μg/ml) were diluted in Tris EDTA buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5, 100 μl final amount) before the addition of 100 μl Picogreen dsDNA reagent. After mixing and then incubation for 5 min at room temperature in the dark, the fluorescence was measured using a microplate reader (excitation, 480 nm, and emission, 520 nm).

(iii) Endocarditis thrombus-associated plasmin activity. Twenty microliters of sample (E. faecalis JH2-2, or conditioned medium) was diluted in 1 ml of buffer (0.05 M HEPES, pH 7.4, 0.75 M NaCl), and a plasmin-selective fluorescent substrate was added (MeOSuc-Ala-Phe-Lys-AMC [trifluoroacetate salt], 40 μM; Bachem). Purified plasmin was used as a positive control (10 to 20 nM). Fluorescence emission kinetics were measured at 460 nm after 380-nm excitation with a spectrofluorometer (Hitachi F 2000) during 2 h. Plasmin concentrations were calculated from the rates of fluorescence emission/minute of the standard curve of purified plasmin.

(iv) Plasminogen activators. Purified plasminogen (10 μg/ml, Sigma) was incubated for 1 h at 37°C with tissue extracts of vegetations and healthy myocardium. Enterococcus faecalis (JH2-2 strains, 8 × 10⁵ CFU/ml) was also tested for its capacity to convert plasminogen into plasmin. Samples were added to fibrin-coated 96-well plates in the presence of 0.5 μM plasminogen (final volume, 100 μl). Purified tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) were used as
positive controls, and amiloride was used as an inhibitor for u-PA. Plasmin activity was then measured as described above.

(v) Gelatin zymography. Electrophoresis of conditioned medium (for matrix metalloproteinase-2 [MMP-2] and MMP-9) and extracts (for leukocyte elastase) was performed under nonreducing conditions in a 7.5% (for MMP-2 and MMP-9) or 12.5% (leukocyte elastase) polyacrylamide gel containing 1 mg/ml gelatin. Non-MMP gelatinolytic activity was detected using the above-described method with the addition of 2.5 mg/ml fucoidan (Sigma-Aldrich) to the resolving gel, as previously described (3).

The gels were renatured in 12.5 mM Tris-HCl, pH 7.4, 2.5% Triton X-100 and incubated at 37°C in 50 mM Tris-HCl, pH 7.8, 10 mM CaCl2, buffer for 24 h before staining with Coomassie blue. Molecular mass determinations were made with reference to prestained protein standards. Finally, the gels were scanned with a densitometer for subsequent quantification with Scion Image software.

(vi) Detection of laminin by Western blotting. Proteins from tissue extracts were separated by electrophoresis (SDS-PAGE 10% acrylamide) in denaturing and reducing conditions (SDS, heating at 95°C for 5 min in Laemmli buffer containing 5% beta-mercaptoethanol). Proteins were electrotransferred onto a nitrocellulose membrane. After saturation of nonspecific binding sites by 5% nonfat milk in TBS-T (10 mM Tris-HCl, pH 7.4, 155 mM NaCl, and 0.1% Tween 20), the membrane was incubated with a polyclonal rabbit antilaminin antibody at 0.1 µg/ml (Novus biologicals); after five 5-min washes with TBS-T, the membrane was incubated for 1 h at room temperature with anti-rabbit peroxidase-conjugated secondary antibody. Visualization was performed using chemiluminescence (Amerham ECL).

Cell culture. (i) Protocol. Fibroblasts were isolated from aortic adventitia (19) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum until cell confluence in 96-well plates. Experiments were performed before the 5th passage.

(ii) Viability tests. After 24 h of serum deprivation in DMEM, fibroblasts were incubated for 24 h with the vegetation or with healthy cardiac tissue placed in contact with the cell layer. After tissue removal, the remaining viable adherent cells were assayed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (tetrazolium salt)] test: cells were incubated with MTT at 0.5 mg/ml for 1 h at 37°C. The enzymatic reaction resulted in the formation of violet formazan crystals in viable cells, which allowed the measurement of areas of cell disappearance. A microphotograph was taken, and the surface area of dead cells was expressed as the percentage of the total well surface area. Formazan crystals were then solubilized with dimethyl sulfoxide (DMSO) (100 µl/well), and the optical density measured by spectrophotometry at 540 nm.

(iii) Induction of apoptosis by proteases ex vivo. Intact parts of myocardium taken from healthy rats were incubated at 37°C for 24 h in RPMI medium alone or supplemented with plasmin (500 nM), leukocyte elastase (50 nM), or E. faecalis JH2-2 at 106 CFU/ml. After incubation, samples were fixed in 3.7% paraformaldehyde for 24 h and paraffin embedded for immunostaining and detection of single-stranded DNA (ssDNA) (Apostain; AbCys France), following the manufacturer’s instructions.

Statistical analysis. Results were analyzed using Prism software (GraphPad Prism software, version 5.0b) and expressed as means ± standard deviations (SD). Analysis was performed using the paired nonparametric (Wilcoxon) test for comparison of the results for septic vegetation (SV) versus vegetation (V) samples. A P value of <0.05 was considered statistically significant.

RESULTS

Endocarditis rat model. All animals had the catheter correctly positioned within the left ventricle and developed endocardial thrombi (Fig. 1A, V), which were especially visible after the inoculation of bacteria (Fig. 1B, SV). Macrosopic examination after sacrifice demonstrated that left-sided vegetations were mainly localized on the aortic valves (Fig. 1B, arrows) and that infective endocarditis resulting from inoculation of bacteria was characterized by larger and more-widespread vegetations (on the ascending aorta and in the left ventricle) than were observed in nonseptic rats (5.2 ± 3.1 mm² versus 1.5 ± 1.3 mm², P < 0.05) (Fig. 1A and B). Alcian blue staining revealed the presence of large positive areas of mucoid substance (blue) at the interface between the vegetation and the myocardial/aortic tissue (Fig. 1C). These mucoid areas reflect the destruction of the myocardium by the infective thrombus. Nuclear red counterstaining showed the presence of an intense inflammatory cell infiltrate, predominantly within the SV area interfacing with the injured underlying tissue (Fig. 1C and D). The mucoid substance contained many lacunae, as well as condensed and fragmented nuclei (Fig. 1E), as a consequence of cell disappearance and death. Among the inflammatory cells, macrophages, detected by immunohistochemistry (Fig. 1F), were less numerous than polymorphonuclear cells. At a higher magnification, indications of phagocytosis could be observed within the myocardium (Fig. 1F, inset, arrow), suggesting an intense clearance activity. Interestingly, injection of dead bacterial material following the mechanical damage mediated by the catheter was sufficient to produce vegetations enriched in polymorphonuclear cells, as shown by myeloperoxidase-positive immunostaining (Fig. 2A to C). Bacterial antigens could be detected in the vegetation, suggesting a persistent capacity for dead bacteria/mem-
branes to adhere to the thrombus (see Fig. S1 in the supplemental material). The nonseptic vegetations obtained by injection of saline contained only a few neutrophils and displayed signs of cica-
trization (Fig. 2D to F).

In SV, the presence and localization of *E. faecalis* JH2-2 and neutrophils were determined by immunofluorescence (Fig. 3). Bacteria were present within the vegetation, forming colonies that resulted in an intense staining of delineated areas similar to microabscesses (Fig. 3A, arrows). At the myocardial interface, bacteria were localized in neutrophil-rich areas (shown by immunostaining for myeloperoxidase) (Fig. 3B). DAPI staining showed the presence of extracellular DNA known as NETs (neutrophil extracellular traps) (Fig. 3C), indicating an overactivation of neutrophils. Confocal microscopic observation after double immu-
nostaining for histone H1 (green) and JH2-2 bacteria (red) confirmed the presence of bacteria trapped by NETs (Fig. 3D to G). In plasma, the concentration of cell-free DNA, reflecting the solubi-
ization of NETs in the circulation, was significantly increased in infected versus noninfected rat groups (*P* < 0.006) (Fig. 3H).

**Vegetation-associated proteases.** We then tested the presence of proteases released by infiltrated leukocytes (matrix metallopro-
teinases and elastase) or produced *in situ* within the SV, such as those linked to the fibrinolytic system (plasmin and plasminogen activators).

(i) **MMP-2 and MMP-9 activity.** Gelatinolytic activity corre-
sponding to MMP-9 (both active and pro forms) was exclusively found in the medium conditioned by SV (Fig. 4A and B). This MMP-9 activity is likely to be released by polymorphonuclear leu-
kocytes (PMNs), as attested by the presence of complex neutro-
phil gelatinase-associated lipocalin–MMP-9 (Fig. 4A) (20). The pro form of MMP-2 was released equivalently under the three conditions, whereas active MMP-2 was essentially found in the case of infection (Fig. 4A and B). Interestingly, the bands corre-
sponding to MMP-9 and active MMP-2 were only detected in SV but could not be attributed to the *E. faecalis* JH2-2 strain, which is devoid of detectable gelatinase activity, at least in its planktonic form (confirming previously reported results [17]) (see Fig. S2 in the supplemental material). This result indicates that bacteria may trigger leukocyte recruitment and activation, leading to MMP-9 release. The presence of active MMP-2 implies proteolytic activation by enzymes like plasmin or elastase.

(ii) **Leukocyte elastase.** Gelatin zymography performed on tis-
sue extracts revealed the presence of leukocyte elastase activity only in SV (Fig. 4C). Densitometric quantification confirmed that significantly more elastase activity was found in septic than in noninfected vegetations (Fig. 4D). The *E. faecalis* JH2-2 strain did not exhibit any detectable elastase activity (see Fig. S2 in the sup-
plemental material). When SVs were incubated without antibiot-
ics, an increased bacterial proliferation was observed that was as-
associated with a more-important release of elastase into the conditioned medium (see Fig. S2 and S3).

(iii) **Fibrinolytic system activity.** Plasmin activity was assessed by incubating tissue extracts from SV, V, or control myocardium with a plasmin-selective fluorogenic substrate. SV exhibited higher plasmin activity than noninfected vegetations or the con-

![FIG 2](https://example.com/figure2.png) **Histological analysis of vegetations resulting from injection of dead bacteria and saline.** (A, B, C) Tissue after injection of dead bacteria. (D, E, F) Tissue after injection of saline. Representative sections were stained with hematoxylin and eosin (A, D) and immunostained for myeloperoxidase (anti-MPO) (B, E). DAPI was used to visualize nuclei (100 ng/ml) (C, F).
not reveal any plasmin activity linked to bacteria (data not shown). This result suggests that bacteria probably do not directly enhance the conversion of plasminogen into plasmin but, potentially, do this via the recruitment of leukocytes (expressing plasminogen activators). To further investigate the origin and the mechanisms leading to increased plasmin activity in SV, we searched for the source of plasminogen activators. Plasmin activity was monitored after the addition of purified plasminogen to SV and V extracts in the presence of fluorogenic substrate (Fig. 5B). Compared to V, SV were able to convert significantly more plasminogen into plasmin, indicating that the recruitment of plasminogen activators was correlated with infection by *E. faecalis* JH2-2. The addition of amiloride, a urokinase inhibitor, provided complete inhibition of plasmin generation, suggesting that u-PA is the main plasminogen activator associated with SV (Fig. 5B). *E. faecalis* JH2-2 bacteria (10^8 CFU/ml) were unable to activate plasminogen (data not shown), demonstrating that these bacteria are not the direct source of plasminogen activators.

**Induction of tissue damage by infected endocarditis thrombus.** (i) Cell survival tests. In order to test the potential cytotoxicity associated with SV, primary cultures of rat vascular fibroblasts were incubated with infected or noninfected vegetations (same wet weight). After 16 h of incubation, cell viability was evaluated by the MTT test. We found marked areas of cell detachment and cell disappearance in wells in which fibroblasts had been incubated with SV, whereas fibroblast layers incubated with healthy cardiac tissue were intact (Fig. 6A). The quantification of areas devoid of cells showed that they were significantly larger in wells incubated with SV than in those incubated with V or control myocardium (*P* < 0.05) (Fig. 6B) (between 15 and 25% decreased viability in wells with SV relative to the viability in wells with V and myocardium).

(ii) Apoptosis induction by proteases. To determine which components of the infected vegetation could be directly involved in the myocardial injury, we incubated slices of fresh cardiac tissue with plasmin, leukocyte elastase, or *E. faecalis* JH2-2 (10^8 CFU/ml) over a 24-h period at 37°C (5% CO₂). Apoptain immunostaining was used to detect apoptosis. We found that plasmin and elastase were able, *ex vivo*, to induce apoptosis of myocardial cells (Fig. 7C and D). Plasmin induced more apoptosis than elastase, whereas treating tissue with control culture medium (DMEM) and *E. faecalis* JH2-2 did not induce any detectable apoptosis (Fig. 7A and B). The results of this experiment further support the role of nonbacterial proteases, such as neutrophil elastase and plasmin, in inducing tissue damage.

(iii) Proteolysis of extracellular matrix. To assess pericellular proteolysis, which may be a trigger for detachment-induced apoptosis (anoikis), we evaluated the proteolysis of laminin, one of the main proteins of the myocardial extracellular matrix. Western blot analysis of tissue extracts revealed the disappearance of the...
120-kDa band (laminin γ chain) in septic and in noninfected vegetations but not in controls (Fig. 8). In addition, an ~50-kDa band was also degraded in all septic vegetations and in some noninfected vegetations. Pericellular proteolysis of laminin could represent a trigger for apoptosis, leading to myocardial injury in this model of infective endocarditis.

DISCUSSION

Taken together, our data suggest that host proteases concentrated in the vegetation may be involved in cell and tissue damage. This is the first study providing evidence for a major involvement of host proteases resulting from host-pathogen interactions in the pathophysiology of IE. Infected vegetations contained more proteolytic activities than noninfected ones due to the presence of a markedly increased number of leukocytes attracted by the pathogens, including neutrophils and macrophages. Although it cannot be excluded that bacterial proteases participate in tissue destruction and subsequent clinical complications, the fact that the bacterial strain used here did not exhibit any detectable protease activity and direct cytotoxic effects is a major point of this study. Other microorganisms, such as Staphylococcus aureus, have been tested previously in our laboratory, but these strains, which do possess intrinsic protease activities, induced important mortality in rats (unpublished results). The present choice of E. faecalis JH2-2 allowed us to demonstrate that host protease activities could be involved in the pathological process. Despite its well-known lack of intrinsic virulence, Enterococcus faecalis JH2-2 was still able to induce authentic IE. This bacterial strain accounts for approximately 5% to 20% of all cases of clinical endocarditis (21) but does not require gelatinase expression to cause infection. Of 235 strains

![Graph](image-url)
of *E. faecalis*, including 14 endocarditis isolates, Roberts et al. reported that more than 40% lacked gelatinase activity (22), showing that gelatinase production is not necessary for infection and the severity of the disease (inducing infective endocarditis). Here, we used the JH2-2 bacterial strain, in which we have verified the absence of gelatinase, plasmin activity, and intrinsic cytotoxic effects. The accumulation of neutrophils and their subsequent activation was induced by bacterial membranes/dead bacteria that reached and adhered to the thrombus, as shown by the presence of diffuse DNA networks (likely to be neutrophil extracellular traps). Thus, in our IE model, JH2-2 may represent a trigger for the recruitment and activation of leukocytes. We have shown that the host response to *E. faecalis* infection involves neutrophil attraction and associated elastase release, MMP expression and activation, and plasmin generation. The capacity of bacterial membranes to recruit PMNs in spite of the absence of detectable protease activity could be an explanation for the variations in virulence of the different strains of *E. faecalis*.

Elastase is a potent protease that can induce adherent cell death by apoptosis subsequent to pericellular proteolysis (10, 23, 24). MMPs have been demonstrated to be involved in tissue damage, i.e., mucosal lesions of inflammatory bowel diseases (24) or myocardial injury subsequent to myocardial infarction (25). Here, we report that septic vegetations release large amounts of matrix metalloproteinase-2 and -9 in both pro and active forms. Both elastase and plasmin are known inducers of MMP activation. In fact, soluble factors released by neutrophils, particularly elastase, have been shown to activate metalloproteinase-2 (26, 27). Elastase was also reported to activate MMP-9 *in vivo* (28). Moreover, neutrophils are the main source of urokinase (u-PA), able to convert

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**FIG 6** Endocarditis thrombus induces *in vitro* cell detachment. (A) Photograph of fibroblast culture wells after the MTT reaction. The living cell monolayer is gray, and dead areas are defined by a black line. Untreated control cells represent 100% of viable adherent cells, and the results are expressed as the percentage of decreased viability relative to wells incubated with noninfected vegetations (V) (mean ± SD of three separate experiments performed in triplicate; *, P < 0.05).

**FIG 7** Plasmin and leukocyte elastase but not bacteria induce myocyte apoptosis *ex vivo*, as shown by Apostain reaction with nuclear red counterstaining. Sections of myocardium were incubated in RPMI (control) (A) or solution containing 10⁵ CFU/ml *E. faecalis* JH2-2 (B), plasmin (0.5 μM) (C), or leukocyte elastase (50 nM) (D). This experiment was performed twice with similar results. Objective magnification, 20×.
plasminogen into plasmin (29). Pro-MMP-9 may be directly converted into its active form by u-PA (30) or via plasmin generation. The plasminogen activation system can activate both pro-MMP-2 and -9 (31). Here, we report that u-PA contained within infected vegetation is able to convert exogenous plasminogen into plasmin. Unlike bacterial strains that possess plasminogen-activating systems, such as Streptococcus mutans or Staphylococcus aureus (32, 33), the strain we used is devoid of any plasminogen activators. In our model, plasminogen activators are thus conveyed by leukocytes attracted to the site of the vegetation. Furthermore, plasmin is important for thrombolysis but may also favor the weakening of the thrombus, leading to the production of emboli. In addition to fibrin proteolysis, plasmin can also degrade proteins of the extracellular matrix, including fibronectin and laminin (34, 35). We have previously reported that pericellular plasmin generation induced apoptosis of smooth muscle cells and myofibroblasts (11, 36). Here, we have extended these concepts to fibroblasts in vitro and myocardial tissue ex vivo. Incubation of infected vegetations with a monolayer of rat adventitial fibroblasts induced cell death, suggesting a potential proapoptotic signal diverging from the septic vegetations, including MMPs, elastase, and plasmin. In addition, we report that purified elastase and plasmin, as well as activated neutrophil-conditioned medium, are able to induce apoptosis on intact myocardium ex vivo.

The disappearance of 120- and 50-kDa laminin bands in the conditioned medium from septic versus noninfected vegetations and control myocardium suggests that proteolysis of this important extracellular matrix protein has taken place. Proteolysis of the alpha-1 chain of laminin 1 by elastase was reported to unveil a cryptic domain that stimulates MMP-9 and u-PA expression by macrophages (37). Thus, in addition to the loss of survival signals mediated through the laminin-integrin pathway (38), proteolysis of laminin may also influence the expression of metalloproteases and the plasminogen activator urokinase.

In the vegetation, the bacteria appear to be densely packed in colonies encapsulated in a fibrous material, potentially representing a way to escape the immune system. As reported by McCormick et al., the formation of the vegetation could hinder host defenses from clearing bacteria (39). Neutrophils have recently been shown to promote thrombus formation via serine-proteases and externalized nucleosomes that they release upon activation (2). In our model, we have shown the presence of NETs that could participate in chronic thrombus renewal and vegetation growth. Cell-free DNA measured in plasma was also increased in plasma of infected rats compared to its level in plasma of noninfected rats, reflecting the formation of NETs in the septic vegetation, as previously reported in other pathological situations (4, 40).

Conclusion. Our results indicate that host-pathogen interactions represent an important contribution to the pathophysiology of thromboembolic events and tissue damage induced by vegetations of IE. In our model, the recruitment of host cell defense is a decisive step in the disease. The continuous attractant signals coming from bacterial material, even those devoid of intrinsic virulence, may lead to a chronic and deleterious proteolytic aggression toward myocardial/valvular tissues, originating from activated neutrophils via elastase and MMP release, as well as in situ plasmin generation. These proteolytic activities may represent a target for new therapies in IE. However, since in clinical situations, most microorganisms also possess proteolytic activities that can participate in the evolution of the pathology, new therapies should be combined with antibiotherapies in order to combat the deleterious effects of both the bacteria and the unregulated host response.

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