

High levels of catalytic antibodies correlate with favorable outcome in sepsis

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Sepsis is the leading cause of death in intensive care units and results from a deleterious systemic host response to infection. Although initially perceived as potentially deleterious, catalytic antibodies have been proposed to participate in removal of metabolic wastes and protection against infection. Here we show that the presence in plasma of IgG endowed with serine protease-like hydrolytic activity strongly correlates with survival from sepsis. Variances of catalytic rates of IgG were greater in the case of patients with severe sepsis than healthy donors ($P < 0.001$), indicating that sepsis is associated with alterations in plasma levels of hydrolytic IgG. The catalytic rates of IgG from patients who survived were significantly greater than those of IgG from deceased patients ($P < 0.05$). The cumulative rate of survival was higher among patients exhibiting high rates of IgG-mediated hydrolysis as compared with patients with low hydrolytic rates ($P < 0.05$). An inverse correlation was also observed between the markers of severity of disseminated intravascular coagulation and rates of hydrolysis of patients' IgG. Furthermore, IgG from three surviving patients hydrolyzed factor VIII, one of which also hydrolyzed factor IX, suggesting that, in some patients, catalytic IgG may participate in the control of disseminated microvascular thrombosis. Our observations provide the first evidence that hydrolytic antibodies might play a role in recovery from a disease.

The initial host response to infection relies on cellular and molecular effectors of the innate immune system. The recognition of pathogen-associated molecular patterns stimulates the production of proinflammatory cytokines such as tumor necrosis factor α , and activates components of the complement cascade (1). Natural antibodies, which represent the spontaneous repertoire of circulating immunoglobulins in healthy unimmunized individuals, are part of the innate immune system; they promote the clearance of pathogenic substances from the circulation and prevent pathogen dissemination (2, 3). The inability to regulate the inflammatory response initiated upon infection leads to severe sepsis that is characterized by widespread microvascular injury and thrombosis, organ ischemia, and dysfunction (4).

Catalytic antibodies are immunoglobulins endowed with a capacity to hydrolyze an antigenic substrate. Catalytic antibodies have mostly been reported in diverse pathological states, including autoimmunity, alloimmune and inflammatory disorders, and viral infections (5–9). Although there is evidence supporting a pathogenic role for factor VIII-hydrolyzing antibodies in inhibitor-positive patients with hemophilia A and for a subset of platelet-fragmenting antibodies in HIV infection (8, 10), the deleterious role of catalytic antibodies in the other disorders remains debated. Catalytic antibodies of the IgG and IgM isotypes are also part of naturally occurring antibodies (11, 12) and are suggested to participate in the removal of metabolic wastes and protect from bacterial infections (13–15). We hypothesized that catalytic antibodies may limit infection and

inflammation in patients with sepsis, and, conversely, that the lack of a catalytic antibody response may hasten pathogenesis.

Materials and Methods

Patients. Plasma from 34 consecutive patients diagnosed with severe sepsis (9 of 34) or septic shock (25 of 34) for <24 h, was obtained from the Department of Medical Intensive Care, Hôpital Cochin, Paris, under approval by the local ethic board on human subject research. Patients were 17–81 years old (median, 53.5 years) with a 16:18 male/female ratio. Seventeen patients had no underlying disease. Twenty-two patients presented with pneumonia, three presented with urosepsis, two presented with osteoarthritis, two presented with intraabdominal infection, two presented with liver abscess, two presented with bloodborne infection, and one presented with meningitis. The simplified acute physiology score II (SAPS II) and the sequential organ failure assessment (SOFA) score were recorded on the day of diagnosis. The median SAPS II was 40.5, and the median SOFA was 7. Twenty-three patients had at least two dysfunctional organs or systems, and 18 required mechanical ventilation. Patients had equal incidence of Gram-positive and -negative infections. All patients received standard medical care and daily clinical and laboratory data were recorded. Ten patients (29.4%) died within the 28 days after admission. The activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined as described (16). aPTT is expressed as the ratio of the scored value to a reference value. PT is expressed as the percentage of the clotting time measured with reference to a standard plasma. Plasma from 10 healthy blood donors and a therapeutic preparation of i.v. immunoglobulins (IVIg, ZLB Behring, Bern, Switzerland) were used as sources of control IgG.

Purification of IgG. IgG was isolated from plasma by chromatography on protein G-Sepharose, followed by immediate size-exclusion chromatography on a superose-12 column equilibrated with 50 mM Tris, 8 M urea, and 0.02% NaN₃, pH 7.7. IgG-containing fractions were pooled and dialyzed against 50 mM Tris/100 mM glycine/0.02% NaN₃, pH 7.7, at 4°C. The purity of IgG preparations was assessed by SDS/PAGE and immunoblot. IgG was quantified by ELISA. The purification procedure makes it unlikely that the hydrolysis is caused by contaminating proteases.

Hydrolysis of Proline-Phenylalanine-Arginine-Methylcoumarinamide (PFR-MCA). To assess their catalytic activity, IgG antibodies (13.3 nM) were mixed with proline-phenylalanine-arginine-methyl-

Abbreviations: SAPS II, simplified acute physiology score II; SOFA, sequential organ failure assessment; PT, prothrombin time; aPTT, activated partial thromboplastin time; PFR-MCA, proline-phenylalanine-arginine-methylcoumarinamide; IVIg, i.v. immunoglobulins; DIC, disseminated intravascular coagulation.

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coumarinamide (PFR-MCA, 100 μ M, Peptide Institute, Osaka) in 40 μ l of 50 mM Tris-HCl/100 mM glycine/0.025% Tween 20/0.02% NaN₃, pH 7.7, in white 96-well U-bottomed plates and incubated in the dark for 24 h at 37°C. Hydrolysis of the PFR-MCA substrate was determined as the fluorescence of the leaving group (aminomethylcoumarin; μ_{em} , 460 nm, μ_{ex} , 370 nm) using a fluoroscan. Fluorescence values were compared to a standard curve of free MCA, and the corresponding quantities of released MCA were computed. At each time point, background release of MCA, measured for each PFR-MCA concentration in wells containing the substrate alone, was subtracted from the value observed in the presence of the antibodies. Data are expressed as the quantity of released MCA computed at time 0 subtracted from the quantity of released MCA computed at a given time point, per amount of time per amount of IgG (i.e., μ mol per min per mol). Data presented summarize three to five separate experiments (interclass correlation coefficient, 0.73).

Hydrolysis of Biotinylated Antigens. Recombinant human factor VIII (3 nmol, Kogenate II, Bayer), plasma-derived human albumin (HSA, 5 nmol, Laboratoire Français de Fractionnement et de Biotechnologie, Les Ulis, France), factor IIa (13 nmol, LFB), and factor IX (2 nmol, BeneFix, Baxter, Deerfield, IL) were desalted and recovered in 3 ml of borate buffer (100 mM borate, pH 7.0/150 mM NaCl/5 mM CaCl₂). Biotin (11 μ g, 25 μ g/ml) was allowed to react with each antigen with gentle agitation in the dark for 2 h at 4°C. Biotinylated antigens were dialyzed against borate buffer for 2 h at 4°C, aliquoted, and stored at -20°C until use.

Biotinylated antigens (385 nM) were incubated in 40 μ l of catalytic buffer (50 mM Tris-HCl, pH 7.7/100 mM glycine/0.025% Tween 20/0.02% NaN₃) with the IgG samples (10 μ g/ml, 66.7 nM) to be tested for 24 h at 37°C. Samples were mixed with Laemmli's buffer without mercaptoethanol (1:1 vol/vol), and 20 μ l of each sample was subjected to SDS/10% PAGE. Protein fragments were then transferred onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). After overnight blocking in PBS, 1% BSA/0.1% Tween 20 at 4°C, membranes were incubated with streptavidin-coupled horseradish-peroxidase (Amersham Pharmacia) for 30 min at room temperature. After washing in PBS containing 0.1% Tween 20, labeled proteins were revealed by using the ECL kit (Amersham Pharmacia) and BIOMAX ML films (Kodak). Films were scanned by using a SnapScan 600 (Agfa, Mortsel, Belgium) scanner.

Results

Hydrolytic Activity of IgG from Septic Patients. Plasma was collected from 34 consecutive septic patients diagnosed for <24 h with severe sepsis (9 patients) or septic shock (25 patients) (Table 1) (17). IgG was purified from plasma by affinity chromatography on protein G, followed by size-exclusion chromatography in the presence of 8 M urea to ensure the removal of putative contaminating proteases. The hydrolytic activity of IgG was measured upon incubation with PFR-MCA, a generic peptidic substrate for serine proteases. IgG from septic patients was found to hydrolyze PFR-MCA with a mean hydrolytic activity of 119.6 \pm 175.5 μ mol/min per mol (ranging from -16.3 to 893.1 μ mol/min per mol). The mean hydrolytic activity of IgG of 10 healthy donors was 60.7 \pm 54.4 μ mol/min per mol (ranging from 19.5 to 199.2 μ mol/min per mol). It was 62.6 \pm 25.4 μ mol/min per mol in the case of pooled normal IgG for therapeutic use (IVIg). Hydrolysis of the PFR-MCA substrate by IgG of patients and controls was dose- and time-dependent (data not shown).

Purified IgG from 9 of the 34 septic patients exhibited catalytic activities greater than the mean catalytic activity of IVIg \pm 2 SD (i.e., 113.5 μ mol/min per mol), whereas IgG from only 1 of 10 healthy controls exhibited such high catalytic activity (Fig. 1). The variances of catalytic rates of IgG were greater in the case

Table 1. Clinical characteristics of patients with sepsis (n = 34)

Age, median (25th, 75th percentile)	53.5 (47, 72.5)
Male sex, n (%)	16 (47.1)
Prior or preexisting conditions, n (%)	
Hypertension	5 (14.7)
Congestive cardiomyopathy	2 (5.9)
Diabetes	3 (8.8)
Cirrhosis	5 (14.7)
Chronic obstructive pulmonary disease	5 (14.7)
Cancer	1 (2.9)
Immunosuppression	7 (20.6)
Type of patient, n (%)	
Medical	32 (94.1)
Elective surgery	1 (2.9)
Emergency surgery	1 (2.9)
Infecting organism, n (%)	
Gram-positive	12 (35.3)
Gram-negative	10 (29.4)
Mixed	4 (8.9)
Undiagnosed	8 (11.8)
SAPS II, median (25th, 75th percentile)	40.5 (32.8, 53.3)
SOFA, median (25th, 75th percentile)	7 (6, 10)
No. of dysfunctional organs or systems (%)	
0	0
1	11 (32.4)
2	14 (41.2)
3	5 (14.7)
\geq 4	4 (11.7)
Mechanical ventilation, n (%)	18 (52.9)
Use of any vasopressor, n (%)	25 (73.5)
Use of epinephrine or norepinephrine, n (%)	13 (38.2)

The SAPS II ranges from 0 to 174 with higher scores indicating more severe disease. The SOFA ranges from 0 to 24.

of patients than healthy controls (*F* test, *P* < 0.001), indicating that sepsis is associated with alterations in plasma levels of hydrolytic IgG. Rates of IgG-mediated hydrolysis were not related to the nature of the infecting organism nor to that of the underlying medical condition.

High Rates of IgG-Mediated Hydrolysis Correlate with Survival. Twenty-four of the 34 patients included in the study (70.6%) were alive 28 days after admission. The rates of PFR-MCA hydrolysis by IgG of patients who survived sepsis were significantly greater than those of IgG of deceased patients (Fig. 1, Mann-Whitney *U* test, *P* < 0.05). The 34 patients of the study were then ranked according to IgG hydrolysis rates and divided into two groups based on the median catalytic activity of IgG. The cumulative rate of survival over a period of 28 days was significantly higher among patients exhibiting high rates of IgG-mediated hydrolysis as compared with patients with low hydrolytic rates of IgG (Fig. 2, Kaplan-Meier analysis, *P* < 0.05). Dividing the patients into quartiles and calculating the odd risk (OR) ratio for each quartile, confirmed that high rates of PFR-MCA hydrolysis correlate with increased survival from sepsis (Fig. 3). In line with these observations, there was an inverse relationship between the rates of PFR-MCA hydrolysis by patients' IgG and the severity of the disease as assessed by the SAPS II score (Spearman's rank correlation test, *P* < 0.002), the requirement for vasopressor drugs (*P* < 0.05) and the levels of plasma lactate (*P* < 0.05), as well as organ dysfunction assessed by means of the SOFA score (*P* < 0.001).

Correlation of IgG-Mediated Hydrolysis with Markers of Disseminated Intravascular Coagulation (DIC). Sepsis is characterized by DIC that involves the generation of intravascular fibrin and the consump-

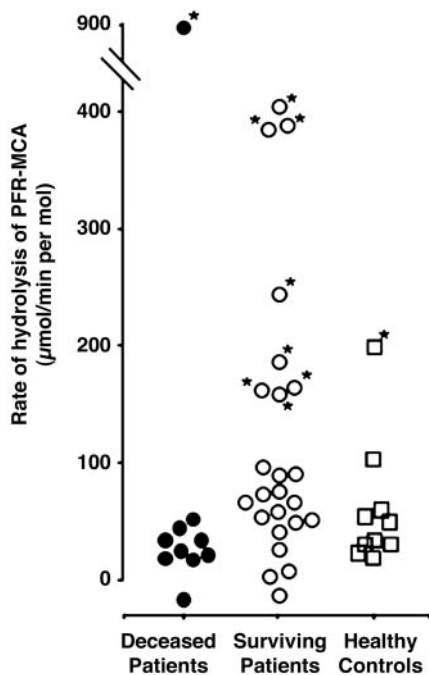


Fig. 1. Hydrolytic activity of IgG from septic patients. IgG was purified from plasma of 34 septic patients (circles) and 10 healthy donors (squares). Ten patients died within 28 days after diagnosis (filled circles), and 24 patients survived (open circles). To measure hydrolytic activity, IgG was incubated with PFR-MCA and fluorescence of released MCA quantitated. Spontaneous hydrolysis that occurred upon incubation of PFR-MCA in buffer alone was subtracted in all cases, thus yielding some negative values. *, Patients' IgG with rates greater than the mean catalytic activity of IVIg \pm 2 SD (i.e., 113.5 μ mol/min per mol).

tion of procoagulants and platelets. Interestingly, the PT, determined for each patient at the time of diagnosis of sepsis, exhibited a significant correlation with the hydrolysis rates of patients' IgG ($P < 0.05$, Fig. 3), whereas the aPTT and platelet counts displayed tendencies for significant correlation with rates of PFR-MCA hydrolysis ($P < 0.1$ in both cases; Fig. 3 and data not shown). We and others have demonstrated that catalytic IgG may inactivate factor VIII in patients with hemophilia A and

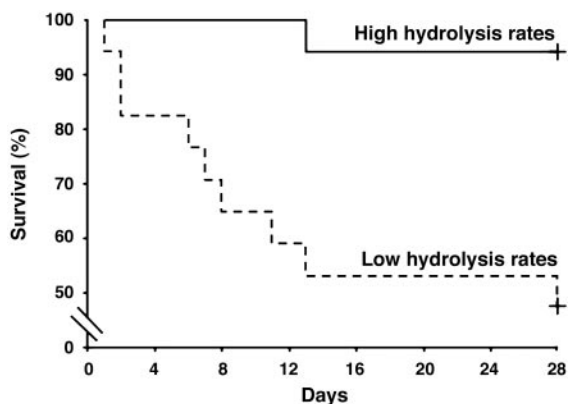


Fig. 2. High rates of IgG-mediated hydrolysis correlate with survival. Patients with sepsis were ranked according to the rates of hydrolysis of PFR-MCA exhibited by IgG purified from their plasma and divided into two groups based on the median catalytic activity of IgG. Cumulative rates of survival of patients with low catalytic rates (dotted line curve) differed significantly from those of patients with high catalytic rates (full line curve), as assessed in a Kaplan-Meier analysis (log-rank test, $P < 0.05$).

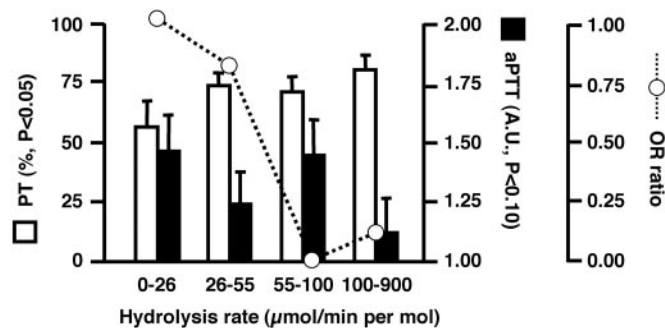


Fig. 3. Correlation of rates of IgG-mediated hydrolysis with markers of disseminated intravascular coagulation. The patients were ranked according to the rates of IgG-mediated PFR-MCA hydrolysis and grouped into quartiles. Odd risk (OR) ratios were calculated for each quartile as the number of deceased patients over that of surviving patients, and are indicated as empty circles. Mean \pm SEM of the prothrombin time (PT, expressed as the percentage of the clotting time measured with reference to a standard plasma, empty bars) and the activated partial thromboplastin time (aPTT, expressed as the ratio of the scored value to a reference value, full bars) as determined at the time of diagnosis of sepsis, are represented (eight or nine patients per quartile). PT values exhibited a significant correlation with hydrolysis rates of patients' IgG ($P < 0.05$, Spearman's rank correlation test using the ungrouped data). aPTT values displayed a tendency for significant correlation with rates of hydrolysis of patients' IgG ($P < 0.1$).

activate prothrombin (factor II) in patients with Bence-Jones proteins (9, 18). Therefore, we tested the hydrolytic activity of patients' IgG toward several proteins of potential relevance to DIC in sepsis. IgG of three of the surviving patients exhibited hydrolyzing activity toward factor VIII, and IgG from one patient in this group also cleaved factor IX (Fig. 4). None of the IgG from deceased patients hydrolyzed procoagulant molecules. Factor II and HSA were not hydrolyzed by patients' IgG (data not shown).

Discussion

We investigated the prevalence of catalytic antibodies in patients with severe sepsis and septic shock. We observed a high prevalence of catalytic IgG in the plasma of septic patients. Antibodies with serine protease-like activity were associated with a favorable outcome. This finding indicates that catalytic antibodies may be associated with the recovery from a disease.

The catalytic activity of IgG was detected as the cleavage of the peptide PFR-MCA, a generic synthetic substrate for kallikrein-like serine proteases. PFR-MCA and related synthetic substrates have extensively been used in the past to screen for the presence of human catalytic antibodies because the latter display selectivity for cleavage at bonds containing basic amino acids (11, 18–21). We and others have documented that the catalytic activity of IgG is mediated by the $F(ab')_2$ fragments of the antibodies and that it is not inhibited by a broad range of generic protease inhibitors (7, 9, 18). In the present study, the purification of IgG on protein G-Sepharose immediately followed by size-exclusion chromatography in 8 M urea further excluded the possibility that PFR-MCA hydrolysis was mediated by contaminating proteases.

The pathophysiological relevance of catalytic antibodies remains unclear. Catalytic antibodies have mostly been reported in pathological states, including asthma, alloimmune immunization to factor VIII in hemophilia A, Hashimoto's thyroiditis, systemic lupus erythematosus, scleroderma, rheumatoid arthritis, multiple sclerosis, and HIV-related immune thrombocytopenia (5–9, 22–26). However, with the exceptions of factor VIII-hydrolyzing IgG in hemophilia and platelet fragmentation caused by antibody-induced generation of H_2O_2 in HIV infection, the deleterious role of catalytic antibodies has not been convincingly

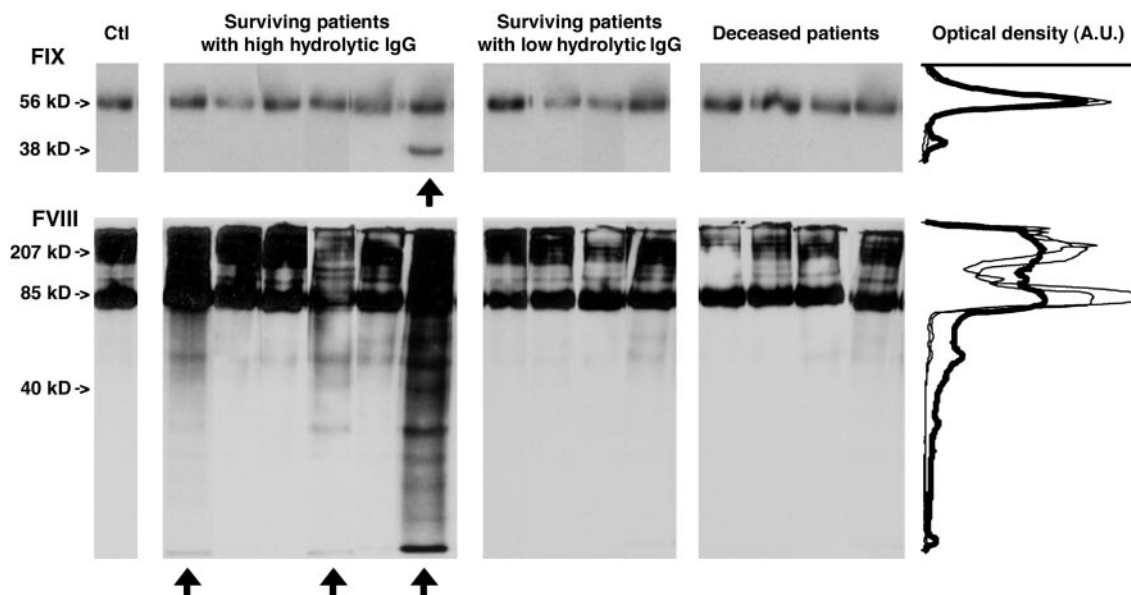


Fig. 4. Hydrolysis of factors IX and VIII by patients' IgG. Human recombinant factor IX (FIX) and VIII (FVIII) were incubated in the presence of purified IgG for 24 h at 37°C. Migration profiles are shown for 14 representative patients. Mean optical densities of the migration profiles are indicated as arbitrary units (A.U.) for six surviving patients with high rates of IgG-mediated hydrolysis (thick line curve), four surviving patients with low rates, and four deceased patients (thin line curves). Coagulation proteins incubated in buffer alone (Ctl) or in the presence of IVIg (data not shown) were used as negative controls. Patients' IgG hydrolyzed neither HSA nor prothrombin (data not shown).

demonstrated (8, 10). Thus, it remains unclear whether catalytic antibodies detected in pathological conditions are actual effectors of the pathogenic immune response or whether their expression is secondary to the dysregulated homeostasis of the immune network. Catalytic IgG have been documented in healthy individuals (21). Antibodies in the milk of healthy mothers are endowed with protein kinase and DNase activities (27, 28). Furthermore, as recently reported, the intrinsic ability of antibodies to convert molecular oxygen into hydrogen peroxide and ozone, imparts them with a bactericidal potency (14, 29). In this respect, our data are in line with the hypothesis that catalytic antibodies play a protective role against infection (13).

Our data suggest that a lack of catalytic antibodies during sepsis and septic shock may be deleterious. Indeed, 9 of 10 deceased patients had IgG with low catalytic rates at the time of diagnosis, and the only deceased patient who presented with high catalytic rates of IgG passed away 13 days after enrollment from invasive aspergillosis, a fungal infection in which neutrophils and macrophages represent major lines of host defense and in which antibody-mediated immunity is known to play a secondary role (30). The inability of some patients to express higher levels of catalytic antibodies may be related to the genetic polymorphism of the repertoire of Ig variable region-encoding genes that characterizes the human population. This finding is reminiscent of findings in BALB/c and MRL/lpr mice, that have different abilities to produce catalytic antibodies and that use different V genes, after immunization with phosphonate haptens (31, 32). Alternatively, lower levels of IgG-mediated hydrolysis may relate to an increased prevalence of anti-idiotypic antibodies that neutralize autologous IgG catalysts. Among the 25 patients who survived, several had rates of IgG-mediated PFR-MCA hydrolysis at the time of enrollment that were not significantly higher than that of healthy donors and IVIg. This finding suggests that IgG-mediated hydrolysis is one among different mechanisms that may confer protection in sepsis. Indeed, the initial host response to infection is known to rely on effectors of the innate immune system, including molecules from the family of Toll-

like receptors (1), from the complement cascade and natural antibodies (2, 33).

Sepsis is associated with DIC that results in organ ischemia and multiorgan dysfunction (4). The administration of activated protein C, which functions as a proteolytic inhibitor of the clotting factors V and VIII and is endowed with antithrombotic, profibrinolytic, and antiinflammatory properties (34), reduces mortality in patients with severe sepsis (35). Here, we report an inverse correlation between the markers of severity of DIC (i.e., PT) and rates of hydrolysis of PFR-MCA by patients' IgG, which points toward a potential antithrombotic implication of catalytic antibodies in sepsis. Our finding that IgG from three of the surviving patients hydrolyzed FVIII and/or FIX is in line with this hypothesis. Interestingly, antibodies that hydrolyze procoagulant factors have been described in patients with multiple myeloma and hemophilia A, with a possible outcome on the hemostatic status of the patients (9, 18).

It is classically admitted that the beneficial effect of antibodies in sepsis relates to their capacity to activate complement and opsonize infectious particles (3). We speculate that the catalytic activity of IgG synergizes with these properties. Whether catalytic antibodies play a direct bactericidal role, participate in the control of disseminated microvascular thrombosis, and/or regulate inflammation remains to be uncovered. The data further suggest that manipulation of the catalytic antibody response may represent a therapeutic approach of sepsis.

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