Monoclonal IgG Anticoagulants Delaying Fibrin Aggregation in Two Patients With Systemic Lupus Erythematosus (SLE)

By Dennis K. Galanakis, Ellen M. Ginzler, and Senih M. Fikrig

There is paucity of information regarding the prolonged plasma thrombin time known to occur in some patients with systemic lupus erythematosus. Detailed investigations of plasma from two such patients disclosed that IgG accounted for this defect in each case. IgG isolated from plasma of either patient possessed the property of delaying fibrin aggregation and prolonging the clotting times of fibrinogen. Preincubation of IgG from either patient with anti-IgG or anti-Fab (rabbit) serum abolished this anticoagulant property. Moreover, the anticoagulant IgG from the first patient was neutralized with anti-κ chain and anti-IgG$_3$, that from the second patient with anti-λ chain and anti-IgG$_1$ serum. These anticoagulants were also dissimilar with respect to their interactions with fibrin(ogen). IgG from the first patient had no anticoagulant activity against fibrin(ogen) species lacking intact Aα chains. IgG from the second patient displayed undiminished anticoagulant effect on such fibrin(ogen) species. We conclude that each anticoagulant interacted with a distinct region(s) on the fibrinogen molecule and that these interactions affect or involve sites that participate in the fibrin self-assembly process.

INVESTIGATIONS on circulating anticoagulants associated with systemic lupus erythematosus (SLE) led to the discovery that a prolonged plasma thrombin time attributable to such anticoagulants was present in some SLE patients.[1][4] Early evidence indicating that plasma from such patients had the property of prolonging the thrombin time of normal plasma when other defects were absent[2,3] indicated that this defect reflected circulating anticoagulants that were distinct from the commonly termed "lupus anticoagulant." Moreover, their presence in defibrinated plasma[1,4] implied that they were not fibrin(ogen) derivatives. Evidence for this interpretation was provided by Struzik et al.,[5] who reported that immunoglobulins in the plasma from one SLE patient possessed antibody (or immunoprecipitating) properties against, as well as the capacity to inhibit the biologic activity of, thrombin. This finding accounted for the prolonged thrombin time in their patient and suggested that immunoglobulins play a role when this defect is present in patients with SLE.

Aside from these important developments and from the fact that the prolonged thrombin time seems to occur at least as frequently[6,7] as the "lupus anticoagulant," there is a paucity of information regarding other possible causes of this abnormality in SLE. Particularly lacking are studies on the role of fibrino-(geno)lysis, the functional behavior of fibrinogen, and the possible occurrence of anticoagulants directed against fibrinogen. During our investigations of the pro-

longed thrombin time of plasma from two patients with SLE, circulating IgG anticoagulants possessing the property of prolonging the clotting time of fibrinogen were found. The present report concerns these studies and focuses on the functional behavior of these anticoagulants.

MATERIALS AND METHODS

Patients. Two female patients with the diagnosis of SLE, C.F. (patient A) and B.M. (patient B), 14 and 38 yrs old, respectively, were followed at this institution. Throughout several years of observation, both patients invariably had a prolonged plasma thrombin time with no other coagulation abnormality demonstrable in plasma studies. The serum fibrinogen-fibrin--related antigen[8] levels always remained less than 5 μg/ml in both patients. Both patients had positive LE preparations and evidence of renal (mesangial) lesions typical of SLE by light microscopy and histologic immunofluorescence studies. Each patient experienced one episode of nephrotic syndrome, and in patient B.M. this was accompanied by venographic evidence of bilateral renal vein thrombosis and lung scan signs of pulmonary emboli.

Plasma was obtained by centrifugation from citrate-collected venous blood and tested before or after storing at $-20°C$. Fibrinogen subfractions from patient or normal plasma[9,10] or from plasmic digests[9] were prepared as previously described. Thrombin was of human origin (lot H-1, a gift from Dr. D. L. Aronson), reptilase was obtained from Abbott Laboratories (North Chicago, Ill.), and ancrod (Venicil) was a generous gift from Grant Barlow. Preparation of fibrin and the fibrin aggregation experiments were carried out as previously outlined.[11] IgG (prepared as outlined below) was incorporated in the buffer used to dilute the fibrin solution and permit fibrin polymerization.[11]

Electrophoresis of fibrinogen or of (noncrosslinked) fibrin clots obtained from plasma (Fig. 1) was performed according to the method of Weber and Osborn.[12] The amounts of plasma fibrin(ogen) molecules enriched with catabolic derivatives[10] (i.e., electrophoretic bands anodal to band I; Fig. 1) were assessed by gel scanning densitometry.[11] The amounts of these catabolic derivatives in control samples from nine single normal donors varied from $20°_0$ to $36°_0$. The possible role of these variations on the thrombin time of normal plasma was excluded by certain mixing experiments in which the band I:non band I (i.e., fraction I-6, Fig. 1) ratio was varied. The results (not shown) established that only when non-band I fibrinogen species amounted to $>60°_0$ of the total did significant prolongations of the clotting times of plasma or of fibrinogen solutions occur.

IgG was isolated from serum by DEAE-cellulose chromatography[13] using a Tris-PO_4 buffer, pH 8.6, 0.005 M with respect to PO_4. Residual bound proteins were eluted from the column with 0.2 M buffer and dialyzed against PO_4 buffer (pH 7; see below) prior to testing for the presence of anticoagulant activity. Alternatively, IgG was removed from plasma by immunoaffinity chromatography[13] in the following way: Rabbit anti-human IgG serum was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's procedure. A 0.9 × 15 cm Sepharose-antibody column was equilibrated with 0.135 M NaCl 0.01 M PO_4, pH 7, at room temperature. Then 5 ml of patient serum (previously heated, 56°C, 15 min) were applied to the column (flow rate 40-50 ml/hr), which was washed with buffer until all unbound proteins (monitored at 280 nm) had been removed. The bound protein was eluted by either 0.1 M sodium acetate (pH 4) 8 M deionized urea or 0.2 M glycine pH 2, buffered to pH 7 with a concentrated Tris solution, dialyzed extensively in distilled water, and lyophilized. Isolated IgG displayed a single precipitin line against rabbit anti human IgG serum (Boehring) in agarose double immunodiffusion experiments and a single (broad) protein band in dodecyl sulfate electrophoresis. Protein concentrations were determined spectrophotometrically, assuming $A_{1cm}^{1°}$ of 13 and 15.5 for IgG[14] and fibrinogen,[15] respectively, at 280 nm. Thrombin, reptilase, and ancrod clotting times were performed as previously described.[11]

Clotting times on solutions of fibrinogen-IgG mixtures were performed by the Fibrometer (BBL, Cockeysville, Md.); fibrinogen and IgG final concentrations were 0.5-1.0 mg and 1.4-2 mg/ml, respectively, in pH 7 buffer (see above). Rabbit antisera to IgG subtypes were kindly provided by Dr. Julian Rosenthal. For clotting times in the presence of antibody-treated IgG the following procedure was employed: Heat-treated (56°C 15 min) horse or rabbit anti-human (IgG, Fab, or light chain) serum (Cappel, Cochranville, Pa.) treated with 0.02 mM EDTA and dialyzed extensively against pH 7 buffer was incubated with equal volumes of isolated IgG
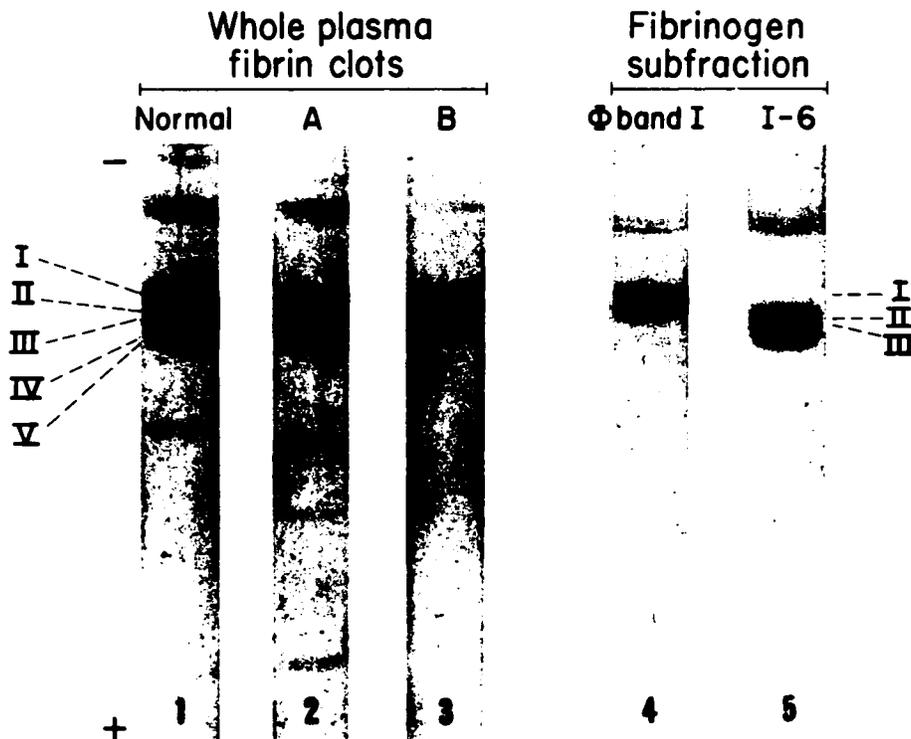
Fig. 1. Electrophoretic band pattern in sodium dodecyl sulfate (3.5% polyacrylamide) gels of normal and patient plasma clot samples (gels 1–3) and of purified normal band I and non-band I (subfraction 1-6) fibrinogen (gels 4 and 5, respectively). Relatively heavier protein load was applied to gel 1 to show minor anodol bands in normal plasma clots. [Note with regard to subfraction I-6 and ϕ band 1: When unreduced fibrinogen subfractions that are separable on the basis of solubility are examined by this technique, at least three major (I–III) and two minor (IV and V) bands can be distinguished.[17] Electrophoretic bands II and III, when present together in mixtures made from subfractions or in unfractionated plasma samples, overlap to such an extent that they cannot be resolved from one another.[10] Aα chain composition of the various dimeric fibrinogen molecules occupying each band, as reported elsewhere,[10] was as follows: Band I molecules possessed intact Aα chains or a slightly smaller chain termed A$\alpha/2$ (mol wt 67,300 daltons). Most band II molecules contained two A$\alpha/4$ remnants but minor species containing an A$\alpha/4$ remnant plus an Aα or A$\alpha/2$ chain were also found. The molecule most representative of band III was one with an A$\alpha/4$ remnant plus a smaller remnant (e.g., A$\alpha/6$, mol wt 46,500; A$\alpha/9$, mol wt 31,800). The exact composition of species within minor bands IV and V was less certain, but it is clear that they consisted of combinations of remnants smaller than A$\alpha/4$ (i.e., A$\alpha/6$ to A$\alpha/12$).]

(7 mg/ml) at 37°C for several hours or overnight at room temperature in the presence of Kunitz pancreatic trypsin inhibitor (200 U/ml). Immunoprecipitates (when present) were removed by centrifugation and either heparin (Upjohn) or hirudin (Pentapharm) were added (to 4 and 10 U/ml, respectively) prior to adding fibrinogen and determining the reptilase clotting times. Under these conditions antiserum alone had no effect on fibrinogen reptilase times. Fibrin clots from those mixtures showing a prolonged clotting time were washed and electrophoretically examined before and after reduction with dithiothreitol (in dodecyl sulfate 9°$_0$ gels) to ascertain that the prolongation was not due to proteolytic degradation.

RESULTS

Analyses of the prolonged plasma thrombin time. Plasma clotting times were prolonged whether thrombin, reptilase, or ancrod were employed for clot for-

Table 1. Clotting Times of Patient Plasma With Thrombin, Reptilase, and Ancrod

	Plasma Clotting Times		
Clotting Enzyme	Control ($\pm$ 1 sec)	Patient A ($\pm$ 2 sec)	Patient B ($\pm$ 2 sec)
Thrombin	19	34	29
Thrombin + CaCl	22	38	34
Reptilase	19	48	32
Ancrod	18	59	66

Clotting times were performed with a fibrometer (37°C). All values reflect the mean of at least five determinations; SD is given in parentheses. Thrombin and ancrod were employed in 0.135 M NaCl Tris-Cl pH 7.4 buffer. Thrombin concentrations were 0.6 and 0.3 U/ml in thrombin and thrombin + CaCl solutions, respectively. Final fibrinogen concentrations were 1.2–1.5 mg/ml, and those of ancrod were 4 U/ml.[33]

mation (Table 1) and whether or not CaCl (0.02 M) was present in the clotting mixture. Repeated freeze-thawing did not alter the prolonged thrombin time. This abnormality could be attributed neither to the plasma fibrinogen concentration nor to the serum fibrinogen-fibrin antigen levels, both of which were consistently within normal values in each patient. Equal mixtures of patient and normal plasma always resulted in clotting times intermediate to those of the patient and those of normal plasma, suggesting the presence of circulating anticoagulants. This finding was also consistent with a qualitatively abnormal fibrinogen,[16] a possibility particularly suggested by the persistence of the clotting time defect in diluted plasma from patient A (not shown). Accordingly, these possibilities were examined. First, under similar conditions there was no difference between the thrombin times of isolated patient (subfraction 1-2) fibrinogen (mean 28 sec, range 27-30) and those of fibrinogen from normal plasma (27 sec). Second, coagulable circulating fibrinogen catabolites (which are known to display prolonged thrombin time[15]) were assessed by dodecyl sulfate polyacrylamide electrophoresis of noncrosslinked plasma clots (Fig. 1). This analysis permits assessment of the amounts of these catabolites in plasma by utilizing their migration rates, which are distinct (i.e., more anodal) from that of more intact fibrinogen (i.e., band I, Fig. 1). There was no increase in the amounts of these catabolites; that is, gel scanning densitometry disclosed that 32% (patient A) and 18% (patient B) of the total material, respectively, was anodal to band I (normal range 20%-36%). Thus the prolonged plasma thrombin times could be attributed neither to an abnormal fibrinogen nor to the amounts of coagulable fibrinogen catabolites in plasma.

Identification of the anticoagulants. In preliminary experiments, the presence of thrombin- or heat-defibrinated patient plasma (56°C, 15 min) prolonged the thrombin and reptilase time of fibrinogen solutions. Moreover, defibrinated plasma from which IgG had been removed (by DEAE-cellulose chromatography) displayed no anticoagulant activity against the thrombin or reptilase times of fibrinogen. These observations led to further analyses of the clotting times of fibrinogen in the presence of IgG; the results (not shown) showed that IgG isolated from either patient A or B prolonged the thrombin, reptilase, and ancrod clotting times of fibrinogen.

Preincubation of patient IgG with anti-IgG sera followed by addition of hirudin or heparin, mixing with fibrinogen, and performing reptilase times (Table 2) permitted further analyses of each anticoagulant. Anti-IgG and

Table 2. Reptilase Clotting Times of Fibrinogen Incubated With Untreated or Antiserum-treated
IgG (30 min, 37°C) and Kunitz Pancreatic Trypsin Inhibitor (KPTI) (200 U/ml)

	Reptilase Times (Sec.)				
	+ Buffer	+ Anti-IgG	+ Anti-Fab	+ Anti-κ	+ Anti-λ
Normal	29	28	29	30	29
Patient A	38	30	30	29	38
Patient B	46	31	28	46	28

Each value reflects the mean of at least six determinations, the range varying 1–2 seconds from this mean. Clotting times were performed at 37°C in 0.15 M NaCl–0.01 M Tris-HCl, pH 7.4. Final fibrinogen (subfraction "band 1") and IgG concentrations were 1 and 2 mg/ml, respectively. IgG (12 mg/ml) was incubated with an equal volume of undiluted rabbit antiserum (containing EDTA 2 mM) at room temperature overnight. Prior to addition of fibrinogen, hirudin or heparin (final concentrations 0.5 and 1 U/ml, respectively) had been added to the mixture.

anti-Fab sera consistently abolished the anticoagulant effect (i.e., corrected the prolongation of the reptilase clotting time) of IgG from either patient. The two anticoagulants were distinct, however, when antisera to light chains and to IgG subtypes were employed. That is, the anticoagulant property of IgG from patient A was abolished by anti-IgG$_3$ and anti-κ chain serum; that of IgG from patient B was abolished by anti-IgG$_1$ and anti-λ chain serum. Antisera to other IgG subtypes (e.g., IgG$_4$) had no effect on the anticoagulant property of either IgG. Thus the anticoagulant effect of both patients' plasma was clearly a property of the IgG fraction, and the two anticoagulants differed with respect to their IgG types.

Analyses pertaining to the mode of action of the anticoagulants.   The presence of the anticoagulant effect regardless of which clotting enzyme was employed (Table 1) suggested that the anticoagulants were directed against fibrin formation or aggregation. This was borne out in experiments in which patient IgG was shown to delay the onset of fibrin aggregation (Fig. 2); an additional effect was that both anticoagulants resulted in somewhat lower absorbance of the fibrin clots. The anticoagulant effect, moreover, was abolished when the IgG was mixed with equal amounts (w/w) of fibrinogen prior to its mixing with the fibrin monomer solution (not shown).

The effect of the anticoagulants on different molecular forms or derivatives of fibrinogen was also examined, and in these experiments the two anticoagulants again proved dissimilar. While both delayed the clotting times of "native" (i.e., band I) fibrinogen (Table 3), that from patient A had no anticoagulant effect on fibrinogen lacking the COOH-terminal region of its Aα chains (i.e., subfraction I-9) (Table 3, Fig. 3).[9] IgG from patient B differed in that it delayed not only the clotting times of subfraction I-9 but also those of a subfraction lacking an additional polypeptide, the NH$_2$-terminal region of its Bβ chains (subfraction I-9D^{50}) (Fig. 4).[17] These results showed that the two anticoagulants interacted with at least two different regions on the fibrin(ogen) molecule in exerting their delaying effect on fibrin aggregation.

DISCUSSION

The foregoing analyses established at the outset that circulating fibrinogen or its catabolic derivatives played no role in the prolonged thrombin time of our patients' plasmas and that a circulating IgG anticoagulant accounted for the
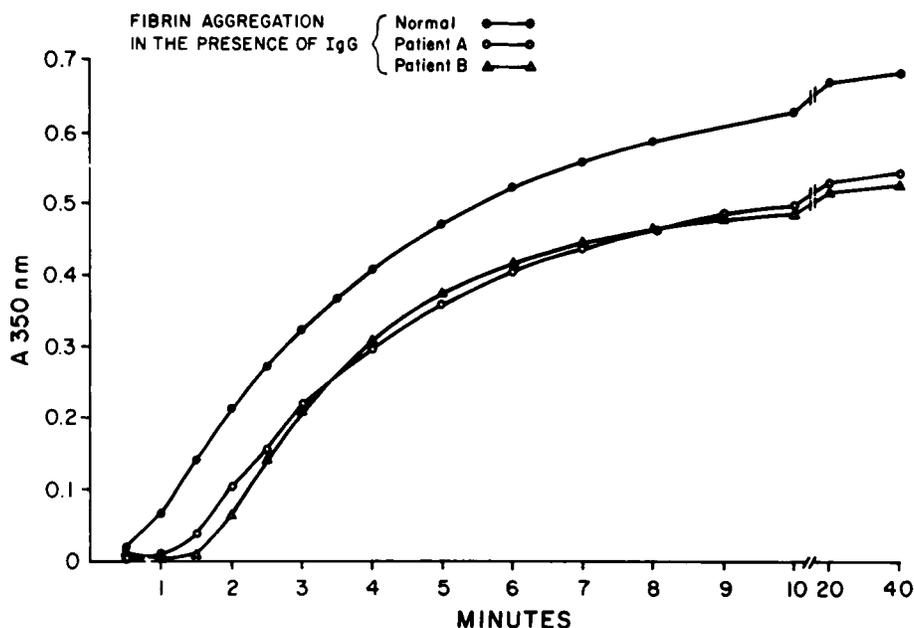
Fig. 2 Aggregation rates of fibrin monomer preparations (obtained from "band I fibrinogen" subfraction) in presence of normal and patient IgG. Final concentrations: fibrin 0.7 mg/ml, IgG 1.4 mg/ml, in phosphate buffer, pH 7 (ionic strength 0.18).

defect in each patient. The characteristic delay of thrombin-, reptilase-, or ancrod-induced clot formation (Table 1) intimated that the anticoagulants did not act by inhibiting these enzymes. Conclusive support for this interpretation was provided by the fibrin aggregation experiments, showing that both IgG anticoagulants delayed the onset of clot formation. In addition, the prolonged clotting time effect could not be attributed to delayed release rates of fibrino-peptides by either anticoagulant. IgG from patient A had no effect on subfraction I-9, and that from patient B exerted its anticoagulant effect in the absence of peptide B (i.e., subfraction I-9D^{50}). Moreover, the two anticoagulants displayed similar clotting time prolongations (Tables 1 and 3) and a similar magnitude of delayed fibrin aggregation (Fig. 2). Thus the IgG from patient B played a minor role (if any) in influencing the thrombin time defect by affecting the release rate of peptide A.

The use of previously characterized coagulable fibrinogen derivatives per-

Table 3. Effect of Patient IgG on Thrombin Times of Fibrinogen With Intact (Band I) and Lacking Intact Aα Chains (I-9)

Fibrinogen Preparation	Thrombin Times (Range) in Presence of IgG From:		
	Normal Donor	Patient A	Patient B
Band 1	14 (12–15)	45 (44–46)	48 (46–50)
I-9	26 (24–27)	29 (28–30)	51 (49–53)

The clotting times were performed (at 37°C) in 0.135 M NaCl–0.01 M PO₄, pH 7; final fibrinogen, IgG, and thrombin concentrations were 1 mg, 2 mg, and 0.5 U/ml, respectively. Each value represents the mean of at least four determinations.
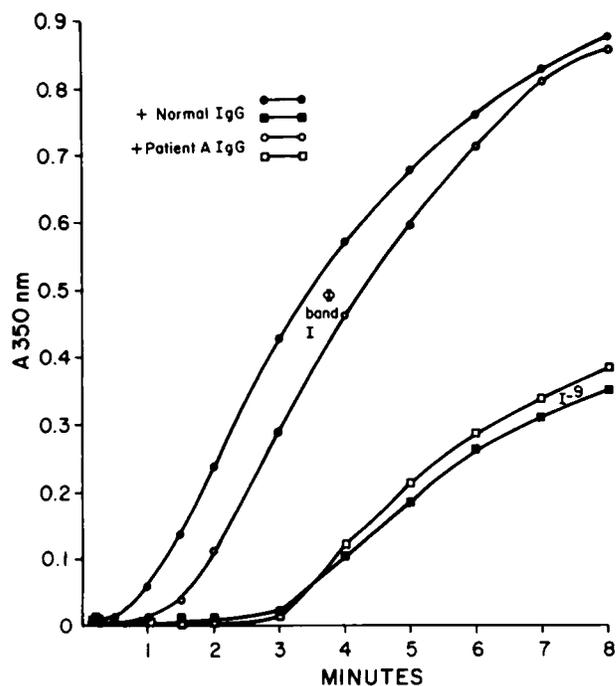
Fig. 3. Aggregation rates of fibrinogen fractions (ϕ band I and I-9) monitored by absorbance measurements at 350 nm following addition of human thrombin in presence of normal and patient A IgG. Final fibrinogen, IgG, and thrombin concentrations 0.5 and 1 mg/ml and 0.1 U/ml, respectively.

mitted analyses pertaining to sites on fibrin(ogen) that interacted with the anticoagulants. On one hand, IgG from patient A required the presence of the COOH-terminal region of the Aα chains for its anticoagulant effect. In contrast, the effect of IgG from patient B on (I-9 and) I-9D[50] indicated that another region(s) on the molecule interacted with this anticoagulant. The marked effect on I-9D[50] may relate to the absence of fibrin contact site(s) that when present (in I-9, for example) did not interact with the anticoagulant; thus interaction of the latter with the remaining site(s) resulted in a greatly prolonged clotting time.

The demonstration that the anticoagulant activity was blocked by antisera to IgG, Fab, and a single IgG subtype implies that each anticoagulant IgG was of monoclonal origin. This contrasts to the heterogeneity (with respect to IgG subtypes) that has been reported[18] with IgG anticoagulants to factors VIII and IX in patients with SLE. Moreover, IgG of both λ and κ light chain determinants has been reported with antithrombin activity in a non-SLE patient.[19] As is frequently observed with other immunoglobulin anticoagulants,[18] those of our patients also lacked immunoprecipitating properties. Additional analyses also failed to provide evidence that these were precipitating antibodies to fibrinogen. That is, no complexes were shown by (Sepharose 6B) gel sieving chromatography of fibrinogen-IgG mixtures; furthermore, IgG present in plasma fibrin clots was easily separated by repetitive washing with physiologic buffer (Galanakis DK, et al: unpublished observations).

The occurrence in one of our patients of a circulating anticoagulant with bilateral renal vein thrombosis constituted what seems a paradoxic combination. This combination has been described in reports on the occurrence of other anticoagulants in patients with SLE[20-22] and thrombotic sequellae. Similar com-
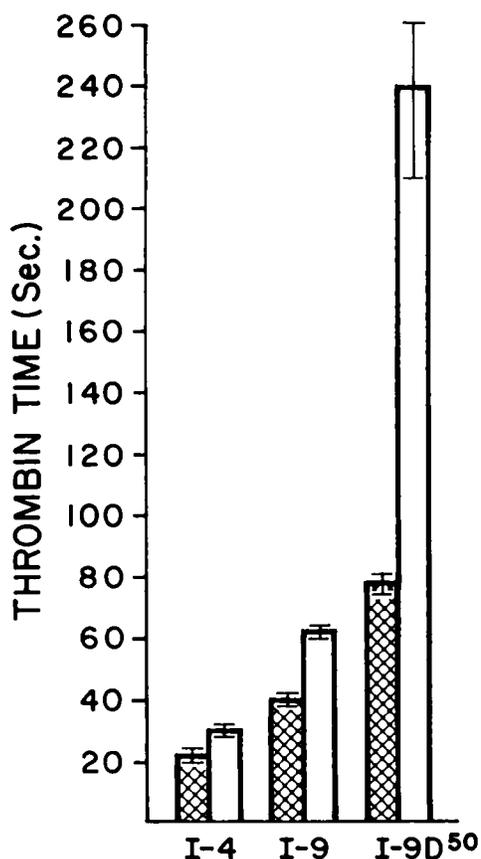
Fig. 4. Thrombin times of fibrinogen subfractions I-4, I-9, and plasma fibrinogen derivative I-9D^{50} in presence of IgG from normal (hatched columns) and patient B (open columns) plasma. Top of each column, mean, and vertical bars, range of five or more determinations. Final concentrations of thrombin, fibrinogen, and IgG 0.7 U/ml and 1.5 and 3 mg/ml, respectively, in 0.135 M NaCl–0.01 M PO$_4$, pH 7 (37°C).

binations have also been described in patients with other disorders and thrombotic complications.[23,24] These findings imply that the anticoagulants in these patients were ineffective in preventing thrombotic sequellae. Although as isolated hemostatic defects in SLE thrombin time anticoagulants are infrequently associated with serious bleeding,[6] their detection is important because their presence may require adjustment of anticoagulant therapy.[25] Thrombosis in such SLE patients has been interpreted as a host response to the presence of anticoagulants.[20] Formation of anticoagulants has also been attributed to local thrombosis related to the lesion(s) of SLE.[5] The occurrence of increased fibrinogen catabolism,[26] fibrin deposits in SLE lesions,[27] and the high incidence of antiplatelet immunoglobulins[28] seem to favor the latter interpretation.

Recent studies by Plow and Edgington[29] showed the presence of polyclonal antibodies to a cleavage-associated fibrinogen antigen (i.e., "neoantigen") in the plasma of a large number of normal individuals. The apparent binding of a small amount of IgG from normal plasma by fibrinogen[30] (covalently linked to Sepharose 6B) may relate to this phenomenon. Whether the anticoagulants described in this report and those reported in other disorders[24,31,32] [i.e., immunoglobulins possessing antifibrin(ogen) anticoagulant properties] reflect functional expressions of such phenomena or are of nonimmune origin remains to be established.

ACKNOWLEDGMENT

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