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J Clin Invest. 1981;**67**(2):575-580. <https://doi.org/10.1172/JCI110069>.

Research Article

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Effect of Fibrinolytic Products D and E on Fibrinogen and Albumin Synthesis in the Rat

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ABSTRACT Previous studies are in conflict over the effect of infusing mixed fibrinogen-fibrin degradation products on fibrinogen synthesis, as determined by changes in fibrinogen concentration or by incorporation of labeled amino acids into fibrinogen. We have injected purified homologous fragments D₁ and E into rats and measured their fibrinogen and albumin synthetic rates by the [¹⁴C]carbonate technique, a method that provides quantitative estimates of hepatic secretory protein synthesis. Fibrinogen fractional synthetic rates were increased 2.5 times in animals injected with fragment D₁, compared with saline-injected controls. No increases were observed in fragment E-injected animals. Neither fragment produced changes in albumin synthesis. Fragment D increased plasma fibrinogen concentration, but did not raise plasma haptoglobin levels. These results suggest that fragment D is a regulator of fibrinogen synthesis.

INTRODUCTION

Regulation of fibrinogen synthesis is complex and not well understood (1). So far, no evidence has been obtained for feedback regulation of synthesis by plasma fibrinogen level (2). Plasmin degradation products of fibrinogen might exercise such control, and several groups of investigators have made studies of the effect of infusing fibrinogen/fibrin degradation products on plasma fibrinogen levels. Three groups found that these products caused significant increases in plasma fibrinogen levels in dogs (3, 4) or rabbits (5), whereas two reported no change in rabbits (6, 7). Otis and Rapaport (7) found that fibrinogen degradation products did not increase incorporation of ⁷⁵Se-methionine into fibrinogen, whereas Kessler and Bell (8) reported that enhanced incorporation of this label into fibrinogen did

occur after infusion of "stage two and stage three" products of fibrinogen digestion. Recently, Ittyerah et al. (9) found that degradation products derived from noncross-linked fibrin had no effect on radiolysine incorporation into fibrinogen.

The infusates used by all these workers consisted of mixtures of early (X and Y) and/or late (D₁, D₂, D₃, and E) fibrinogen or noncross-linked fibrin products, or cross-linked fibrin degradation products (D-dimer and E); and their methods for evaluating changes in fibrinogen synthetic rates were indirect. We have injected rats with purified, homologous fragments D₁ and E, and have measured their rates of fibrinogen and albumin synthesis by the [¹⁴C]carbonate method (10, 11). We find that fragment D₁, but not E, stimulates fibrinogen synthesis.

METHODS

Some of the methods have been described previously (12, 13). Rat fibrinogen was separated from pooled heparinized rat plasma by repeated ammonium sulfate precipitation (14). The product obtained was >95% clottable and yielded a single band without (Fig. 1) and separate α A and β B bands with (Fig. 2) thiol-reducing agents (15) on polyacrylamide gel electrophoresis (16). Plasminogen was prepared by affinity chromatography (17) and activated by human urokinase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). Fragments D and E were isolated from fibrinogen digests by controlled plasminolysis (18) in the presence of physiologic concentrations of calcium (19). Lysis was stopped by the addition of 0.8 M epsilon aminocaproic acid. Residual plasmin and low molecular weight peptides were removed by passage through a 2.5 × 48-cm lysine-agarose column (17). D and E were separated by ion-exchange chromatography (20). The final products were dialyzed against 0.15 M saline. Polyacrylamide gel electrophoresis revealed E and a D fragment with the characteristic mobility of D₁ (Figs. 1 and 2), the only D product of physiologic fibrinogenolysis (19, 21). Four batches of fragments were used for injection into animals. Solutions of D and E were sterilized using a Millipore filter (0.45 μ m, Millipore Corp., Bedford, Mass.) and stored at 4°C in sealed sterile containers for no more than 1 wk before use. Plasma fibrinogen concentration was measured by

Received for publication 24 March 1980 and in revised form 22 October 1980.

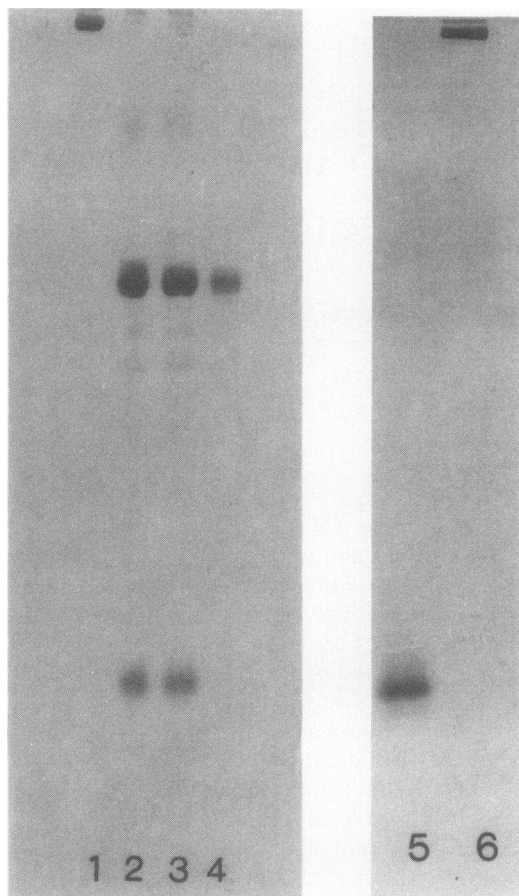


FIGURE 1 Sodium dodecylsulfate-polyacrylamide (7.5%) slab-gel electrophoresis of nonreduced fibrinogen (lanes 1 and 6), plasmic digests of fibrinogen (lanes 2 and 3), fragment D₁ (lane 4), and fragment E (lane 5). Fibrinogen was separated from pooled rat plasma by repeated ammonium sulfate precipitation (14). Fragments D₁ and E were prepared by controlled plasminolysis in the presence of calcium (19). Residual plasmin and low molecular weight peptides were removed by passage through a lysine agarose column (17). D₁ was separated from E by ion-exchange chromatography (20). Early plasmic digests in lanes 2 and 3, prepared without calcium, show traces of D₂ and D₃ located between the predominant D₁ and E bands. The amounts of protein applied were 10 μ g for lanes 1 and 4, and 20 μ g for lanes 2, 3, 5, and 6.

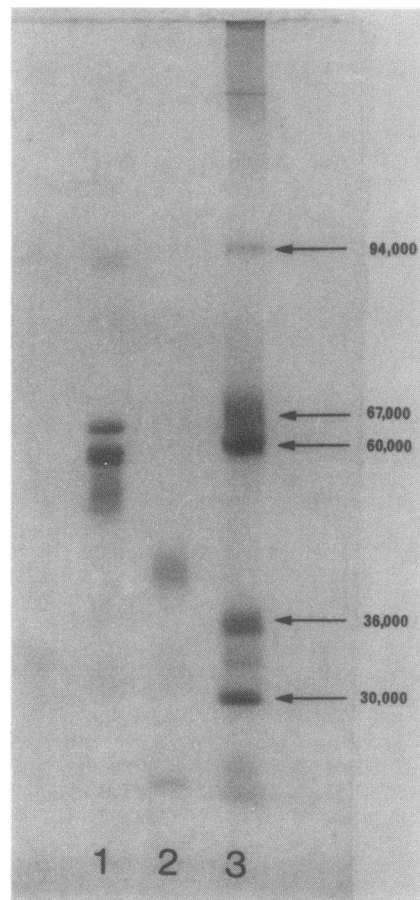


FIGURE 2 Sodium dodecylsulfate-polyacrylamide (7.5%) slab-gel electrophoresis of thiol-reduced rat fibrinogen (lane 1) and fragment D₁ (lane 2), prepared as described in Fig. 1, and compared with protein standards of indicated molecular weight (lane 3). Molecular weight estimates (lane 1) for fibrinogen A α - and B β - chains are 63,000 and 58,000, compared with 61,000 and 58,000 reported by Van Rujven-Vermeer et al. (15). Two γ -chain species were seen with estimated molecular weights of 53,000 and 50,000, compared with a single γ -chain of 51,000 (15). Molecular weight estimates for fragment D peptide chains, γ -, β -, and α -chains (lane 2, in descending order), were 41,000, 38,000 and 18,000, compared with 42,000, 38,000, and 12,000 reported for human D₁ (35). 10 μ g of protein was applied to lanes 1 and 2.

isotope dilution (14). Radioiodination of fibrinogen and albumin was done by the ICl method of McFarlane (22).

Black-hooded rats of the Long-Evans strain, housed under controlled conditions of temperature and humidity, were allowed free access to food and water during the study. They were given drinking water containing 0.008 M NaI for at least 24 h before the study. Generally, eight rats were used in an experiment, one group of four receiving one of the fragments and the other group receiving saline. 2 mg of fragment D, 1 mg of fragment E, or an equal volume (1 ml) of 0.15 M saline was injected intraperitoneally at 5 p.m. on day 0. At 9 a.m. on day 1 (16 h later), 200 μ Ci of sodium [¹⁴C]carbonate, 10 μ Ci of [¹³¹I]-fibrinogen, and 10 μ Ci of [¹²⁵I]-albumin were administered by intravenous injection at the base of the tail. The rats were

lightly anesthetized with ether during this procedure. They were subsequently bled from the tip of the tail at 0.25, 4, 4.5, 5, and 5.5 h after injection of the label. At 6 h the rats were anesthetized with ether and bled by cardiac puncture. [¹³¹I]-fibrinogen and [¹²⁵I]-albumin radioactivities were measured in the 0.25- and 6-h samples for determination of plasma volume and to provide correction factors for loss of fibrinogen and albumin by catabolism and to interstitial fluids. Plasma volumes (counts injected divided by counts per milliliter in 0.25-h sample) and plasma-loss correction factors (counts per milliliter 0.25-h sample divided by counts per milliliter in 6-h sample) from each group of four rats were averaged. The 4 to 5.5-h samples from each four rat group were pooled for each sampling time. Urea specific radioactivity

was determined by treating deproteinized plasma with urease. The CO₂ released was measured manometrically (23) and then condensed into phenylethylamine after which its ¹⁴C radioactivity was determined by liquid scintillation counting. The specific radioactivities of urea carbon in the pooled samples were plotted against time and fitted by a single exponential function. The required specific radioactivity, S_u, was taken as that extrapolated to zero time (23). F_u, the fraction of total urea synthesized per day, assuming steady-state conditions for urea, was taken as equal to the slope of the exponential function. After removal of aliquots for radioiodine counting, the remaining plasma from the 6-h samples for each four-rat group was also pooled. Fibrinogen and albumin guanidine carbon specific radioactivities and fibrinogen, albumin, and urea concentrations were determined in this final pooled sample. For measurement of guanidine carbon specific radioactivity, fibrinogen was isolated by double precipitation with ammonium sulfate and clotted with thrombin. The clot was thoroughly washed with saline. Albumin was separated by trichloroacetic acid-ethanol precipitation (24). Fibrin and albumin were digested in 6 N HCl for 22 h at 110°C. The acid was removed by vacuum absorption and the arginine-6-carbon in the amino acid residues was released as CO₂ by treatment with arginase and urease. Assay of CO₂ was as described for urea. The guanidine carbon specific radioactivity of fibrinogen (or albumin) thus obtained was corrected to zero time by multiplying by the fibrinogen (or albumin) plasma-loss correction factor.

Albumin and fibrinogen synthetic rates were determined by the method developed by Reeve et al. (10) and by McFarlane (11). The method uses the specific radioactivity of the carbon of newly synthesized urea, after the injection of [¹⁴C]carbonate, as a measure of the specific activity of the arginine-6-carbon entering liver-synthesized proteins. Measurement of this guanidine carbon specific radioactivity in the isolated proteins then allows calculation of the protein synthetic rate by McFarlane's formula (11). The method provides results expressed as fraction of intravascular protein pool synthesized per unit time.

In other experiments, plasma concentrations of fibrinogen and haptoglobin were measured 16 h after intravenous or intraperitoneal injection of D or saline and compared with the

known nonspecific response of these proteins to subcutaneously injected turpentine (1 ml) (25). Haptoglobin concentrations were measured by the electrophoretic method of Javid and Horowitz (26), and the colorimetric method of Owen et al. (27). Good reproducibility was obtained between these methods.

RESULTS

Table I shows mean synthetic rates and plasma concentrations for two hepatic secretory proteins, fibrinogen and albumin. The rats treated with intraperitoneal injections of fragment D had fibrinogen synthetic rates about two-and-a-half times greater than those injected with either fragment E or saline. Plasma fibrinogen levels were also elevated in D-treated animals. There were no significant differences in albumin synthetic rates or plasma levels among the three treatment groups. Plasma volumes (mean±SE) for D-, E-, and saline-injected animals were 13.13±0.73, 11.70±0.38, and 12.09±0.67 ml.

Table II shows fibrinogen and haptoglobin plasma concentrations in rats 16 h after intraperitoneal saline or fragment D or 16 h after subcutaneous turpentine. Two dosage levels of fragment D were used, 1 and 2 mg. Plasma concentrations of fibrinogen in D-injected animals differ significantly from those in saline-injected animals, with an incremental effect with increasing amounts of D. No effect on haptoglobin concentration was seen after D injection, but turpentine produced significant increases in both fibrinogen and haptoglobin plasma levels.

Six rats were injected intravenously with 2 mg of fragment D. The plasma fibrinogen concentrations were measured 16 h later and averaged 5.06±0.31 mg/ml. This mean is significantly higher (*P* < 0.01) than the

TABLE I
Fractional Synthetic Rates and Concentrations of Fibrinogen and Albumin
in Rats Injected with Fragment D, Fragment E, or Saline

Treatment	n ₁ *	Fractional synthetic rate		Concentration		n ₂ *
		fraction of intravascular pool/d		mg/ml plasma		
		Fibrinogen	Albumin	Fibrinogen	Albumin	
D	7	1.274±0.014‡	0.758±0.053	4.28±0.17‡	25.4±1.2	5
E	11	0.456±0.045	1.009±0.103	3.41±0.11	24.9±1.1	5
Saline	17	0.517±0.025	0.974±0.068	3.17±0.12	26.9±0.2	11

Rats were injected intraperitoneally with 2 mg of D, 1 mg of E, or 1 ml of .15M saline 16 hours before measurements. All values are mean±SE.

* Number of groups of four rats. Simultaneous fibrinogen and albumin measurements were made in the groups indicated in column n₂. Albumin measurements were not made in all groups, as indicated by column n₁.

‡ Mean fibrinogen concentrations and fractional synthetic rates differ significantly (*P* < 0.01) from corresponding values in E- and saline-injected animals. The three means in each column were compared by a one-way analysis of variance and the Student-Neuman-Keuls multiple comparison procedure modified for unequal samples sizes (28).

TABLE II
Plasma Concentrations of Fibrinogen and Haptoglobin in Rats Injected with Saline, Fragment D, or Turpentine

Treatment	Fibrinogen	Haptoglobin
	mg/ml	mg/ml
Saline	3.17±0.10 (21)	1.304±0.054 (16)
D(1 mg)	3.65±0.15 (7)*	1.162±0.049 (6)
D(2 mg)	4.16±0.13 (13)‡	1.267±0.085 (6)
Turpentine	5.62±0.13 (6)§	2.641±0.052 (7)§

Rats were injected intraperitoneally with 1 ml of 0.15 M saline, 1 mg of D, 2 mg of D, or subcutaneously with 1 ml of commercial turpentine 16 h before measurements. Values are mean±SE. The means in each column were compared as described in Table I. Numbers in parentheses indicate number of rats studied. Simultaneous measurements of fibrinogen and haptoglobin were made in most animals.

* Differs significantly from fibrinogen concentrations in rats treated with saline or 2 mg D, $P < 0.05$.

‡ Differs significantly from fibrinogen concentration in rats treated with saline, $P < 0.01$.

§ Differs significantly from corresponding protein measurements in all other treatment groups, $P < 0.01$.

mean of 4.16±0.13 mg/ml observed in 13 rats (Table II) receiving 2 mg of fragment D intraperitoneally.

DISCUSSION

We chose to study fibrinogen synthesis using the [¹⁴C]-carbonate technique because, in principle, this approach allows quantitative measurement of hepatic secretory protein synthesis over a brief period of time (10, 11). Assuming that urea carbon and fibrinogen (or albumin) arginine-6-carbon derive from the same intracellular pool of carbonate and that rapid labeling of the pool takes place after [¹⁴C]carbonate injection, it can be shown that $F_p = F_u \times S_p/S_u$, where F_p and F_u = the fractions of intravascular fibrinogen and total urea synthesized per day; and S_p and S_u = the specific radioactivity of fibrinogen arginine-6-carbon and urea carbon. Because the calculation requires only specific activities, quantitative recovery of urea carbon and fibrinogen arginine-6-carbon is unnecessary, allowing isolation of a protein fraction of greater purity.

The mean fractional synthetic rate for fibrinogen in saline-injected rats (Table I) is somewhat lower than the value of 0.662±0.036/d, $n = 14$, reported by Jeejeebhoy et al. (29). Albumin fractional synthetic rates in all treatment groups compare well with values obtained previously from this laboratory by both the [¹⁴C]carbonate method and by steady-state measurements with biologically screened ¹³¹I-albumin (12). On the other hand, and unlike fragment E-injected animals, the fractional synthetic rates in D-injected rats averaged about two-and-a-half times control values, and plasma fibrino-

gen concentrations in D-treated animals rose significantly as well. We do not give absolute fibrinogen or albumin synthetic rates in Table I because these are derived from several variables, i.e., the product of F_p , plasma volume, and plasma fibrinogen concentration, but the observed differences are even more marked. Using mean values for these variables, we find an absolute fibrinogen rate in D-treated animals of 72 mg/d, nearly four times the values of 18 and 20 mg/d for E- and saline-injected rats. The effect of D on fibrinogen metabolism seems to be dose related, since 2 mg produces a significantly higher plasma concentration than does 1 mg (Table II). The D effect does not seem to be due to a nonspecific acute phase response of fibrinogen, since D did not alter haptoglobin concentrations, in contrast to the rise in both fibrinogen and haptoglobin after turpentine. Neither does the increase appear to be associated with nonspecific effects resulting from intraperitoneal injection, since plasma levels after a single intravenous injection were even higher than after an intraperitoneal injection.

Some confusion has existed regarding the appropriate D fragment to test, since fibrinogenolysis, as ordinarily carried out *in vitro*, produces three to five different D's (19, 30) varying from about 100,000 daltons down to 80,000 or less. It is clear now that plasminolysis of fibrinogen carried out in the presence of calcium yields only a single D product, D₁, with a molecular mass of about 100,000 daltons (19, 21), and D₁ was used in the experiments reported in this paper. However, smaller, nonphysiologic D fragments may stimulate fibrinogen synthesis, because Kessler and Bell (8) found that both a mixture of D and E from a 15-h fibrinogen digest and a mixture of X, Y, D, and E from a 40-min fibrinogen digest increased the incorporation of ⁷⁵Se-methionine into fibrinogen in rabbits 4 to 9 h after injection of fragments. (No change was seen after fragment X injection). Bocci et al. (31) noted increased fibrinogen levels, peaking at 24 h, after injection of as little as 4.3 mg of D prepared from 4-h digests of fibrinogen. (It is likely that their 4-h digest contained D₁ as well as smaller D fragments). In contrast, Kessler and Bell (32), using ⁷⁵Se-methionine, and Ittyerah et al. (9), using [¹⁴C]-lysine, found no increase in incorporation of label into fibrinogen after injection into rabbits of late stage digests of noncross-linked fibrin. Further, Otis and Rapaport (7) found no increased incorporation of ⁷⁵Se-methionine into fibrinogen after injection of 5-min and 2-h fibrinogen lysates into rabbits. These differences are difficult to reconcile. There may be a dose effect since Bocci et al. (31) noted that small doses of fragment D were more effective in raising plasma fibrinogen levels than doses one hundred times higher. We estimate from their data that the smallest effective dose used by Bocci et al. was equivalent to the D produced by lysis of ~2% of the total intravascular fibrino-

gen of their experimental animals, whereas Otis and Rapaport (7) used amounts equivalent to lysis of >30% of plasma fibrinogen. We observed increased fibrinogen synthesis after D injection equivalent to lysis of ~8% of our animals' intravascular fibrinogen and increased levels after injection of half that amount. Kessler and Bell (8) used somewhat larger amounts, estimated at 14%. Differences in results may also be due to the use of different lytic products. Small, late-stage fibrinogenolytic D products and D products derived from noncross-linked fibrin probably do not occur physiologically, and we chose, therefore, to use fragment D₁. Finally, a variety of methods have been used for estimating changes in fibrinogen synthesis. The [¹⁴C]carbonate technique offers several advantages over other procedures. It is specific for hepatic secretory proteins, there is little or no recycling of label (10, 11), and the method provides quantitative estimates of the rates of protein synthesis. Using this method, we obtained results which suggest that fragment D₁ is an important stimulator of fibrinogen synthesis.

Lipinski and Gurewich (33) have questioned whether fibrinogen (unlike fibrin) degradation products even occur in vivo except with thrombolytic therapy. However, Mersky et al. (34) have recently provided valuable information on this point. These investigators isolated fibrinogen-related antigen from human plasma by immunoaffinity chromatography. Starting with 2 ml or less of plasma from defibrinating patients, they found sufficient fibrinogen-related proteins to demonstrate by polyacrylamide gel electrophoresis the existence of components with electrophoretic mobilities the same as fibrinogen fragments X, Y, and D, as well as fibrin fragment D-dimer. It seems likely that lesser amounts of these fragments are present in less acute disorders associated with coagulation disturbances.

The failure of fragment E to increase fibrinogen production is of interest. Bocci et al. (31), examining the effect of 1–204 mg of fragment E on plasma fibrinogen levels in rabbits, found the lowest effective dose to be 20.4 mg (equivalent to lysis of >30% of plasma fibrinogen). They concluded that fragment D is at least ten times as effective as E as a stimulus. We found that 1 mg of E, equivalent to lysis of 16% of our animals' plasma fibrinogen, did not increase fibrinogen production. It is possible that higher doses of E might have an effect, but our data suggest that in the rat, as in the rabbit, E is not as effective a stimulator of fibrinogen synthesis as fragment D₁.

ACKNOWLEDGMENTS

We thank Dr. E. B. Reeve and Dr. N. M. Bass for their help and advice and Mr. M. Parker for his assistance with animal experiments. Dr. S. H. Walker kindly performed the statistical analyses.

This work was supported by the South African Medical Research Council, the University of Cape Town Staff Research Fund, the South African Atomic Energy Board, and the Medical Research Service of the United States Veterans Administration.

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