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EVIDENCE FOR TWO CLASSES OF RAT PLASMA FIBRINOGEN γ CHAINS DIFFERING BY THEIR COOH-TERMINAL AMINO ACID SEQUENCES

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Human fibrinogen molecules contain two classes of functionally equivalent γ chains (termed γ and γ') differing by their COOH-terminal amino acid sequences. We investigated rat plasma fibrinogen for the presence of this heterogeneity using DEAE-cellulose chromatography to separate reduced S-carboxymethylated chains. Like human γ' chains, rat γ' chains were more negatively charged, somewhat larger (~1000 daltons), had a different COOH-terminal acid than γ chains, and were functionally equivalent to other γ chains. The γ' chain population from normal and turpentine-stimulated animals amounted to 28 and 30% of all γ chains, respectively. suggesting that regulation of their production is not sensitive to stimulation of fibrinogen synthesis.

Normal human fibrinogen molecules, each of which consists of 3 pairs of polypeptide chains (Aα, Bβ and γ, respectively), contain two classes of functionally equivalent γ chains (termed γ and γ') differing from one another by their COOH-terminal amino acid sequences (1,2). The γ' chains amount to ~7% of the total γ chain population, are more negatively charged than γ chains (3,4), and are distinguishable from γ chains by gel electrophoresis under alkaline or acidic conditions (3), by isoelectric focusing (4) or by ion exchange column chromatography on DEAE-cellulose (4). Available evidence suggests that this heterogeneity is common among animal species (5,6).

The human γ' chain has the same amino acid sequence as the γ chain as far as the final four amino acid residues, following which it terminates with a unique 20-residue sequence rich in aspartic and glutamic acids (2). We have

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postulated that both types of chains are transcribed from a single gene by differential RNA splicing (2) although other explanations remain viable.

Studies of rat fibrinogen indicate that it is structurally and functionally analogous to human fibrinogen (7-9). Several investigators have chosen this species for in vitro study of hepatic fibrinogen biosynthesis and assembly (7, 9-12), and have made important observations concerning the nature of mRNAs directing its polypeptide chain synthesis (10,12), the existence of NH_2-terminal "signal" peptides in nascent molecules (9), the timing and mechanism of Bβ and γ chain glycosylation (9,11), and the preparation of cDNA clones for each of the chains (12). The rat also serves as a useful model for studying in vivo aspects of fibrinogen metabolism (13-16). The recognized experimental value of this animal, plus our desire to evaluate whether this species might be useful for studying the γ/γ' heterogeneity, led to the present investigation.

Materials and Methods

Rat fibrinogen was prepared from female Wistar rats (CF strain from CNRS, France) weighing 180-200g or from females that had been treated to induce a hyperfibrinogenemic state by subdermal injection with 1 ml sterile turpentine 48h prior to exanguination (17). Citrated plasma was obtained by collecting blood from decapitated animals into a tube containing 1/6 volume 3.8% Na citrate and 0.1% ε-ACA* solution. Blood was centrifuged at room temperature at 4000x g for 20 min and fibrinogen was purified from the supernatant plasma as described by Capet-Antonini (18) except that all buffers contained 0.02% ε-ACA. The fibrinogen level in normal plasma was 2 to 3 g/l (14) and 5 to 6 g/l in plasma from turpentine-treated animals. The coagulability of the final material (19) was 98%.

Factor XIIIa catalyzed crosslinking of rat fibrin in the presence or absence of DNS-cad was carried out as recently described (1). Reduction and S-carboxymethylation of this material or of rat fibrinogen was carried as previously outlined (20).

DEAE-cellulose gradient elution chromatography of unmodified fibrinogen in a tris-phosphate buffer system was carried out by a modification (3) of an earlier system (21). Chromatography of S-carboxymethyl fibrinogen derivatives was carried out on DEAE-cellulose in 8M urea at pH 7.0 with a tris-phosphate gradient from 0.01M to 0.2M phosphate (4,20). Chromatographic fractions from DNS-cad labelled samples were monitored by their fluorescence (excitation wavelength 355 nm; emission wavelength 525 nm) as well as by their absorbance at 280 nm. Gradient elution chromatography of these derivatives on CM-cellulose in 8M urea was performed as previously described (3).

S-carboxymethyl peptides separated by DEAE-cellulose chromatography were subjected to COOH-terminal analysis by incubation with carboxypeptidase A and/or B (22) as recently detailed (1). NH_2-terminal analyses of rat

*Abbreviations used:
DEAE, diethylaminoethyl; DNS-cad, dansylcadaverine; ε-ACA, epsilon amino caproic acid; CM, carboxylmethyl; tris, (tris)hydroxymethylaminomethane

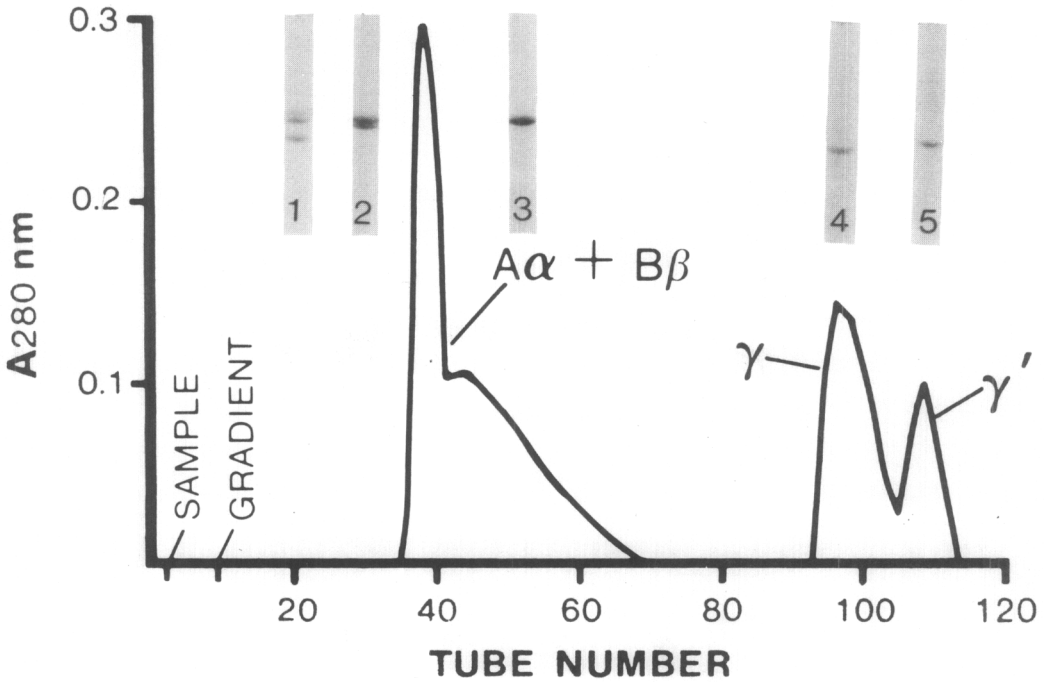

Fig. 1 DEAE-cellulose chromatography of 22 mg S-carboxymethyl rat fibrinogen using a gradient (9 chambers, 75 ml each) from 0.01M to 0.2M tris-phosphate in 8M urea at pH 7.0 The times of sample and gradient application are indicated; fractions of 6 ± 0.2 ml were collected. The types of chains contained within each peak are indicated Weber and Osborn gels are shown: Starting material, 1; first peak, 2; second peak, 3; third peak, 4; fourth peak , 5.

S-carboxymethyl γ and γ' chains, human S-carboxymethyl γ chains (1) (NH_2-terminal tyrosine) and bovine serum albumin (NH_2-terminal aspartic acid) were performed by the dansyl chloride method as described by Gray (23). Neuraminidase (Type X, Sigma Chem. Co., St. Louis, MO) treatment of rat γ chains was done as outlined by Coller (24). When human S-carboxymethyl γ chains were digested by this method, 72% of the sialic acid (25) was removed. Polyacrylamide gel electrophoresis was performed in the Weber and Osborn (26) or Laemmli system (27) as detailed (1). Following electrophoresis in the Laemmli system, fluorescent bands were detected and photographed under a long wave UV lamp prior to staining with Coomassie brilliant blue (1).

Results

Gradient elution CM-cellulose chromatography of S-carboxymethyl rat fibrinogen from turpentine-stimulated animals revealed that γ chains emerged in a single peak prior to the Aα and Bβ chains, which were eluted subsequently

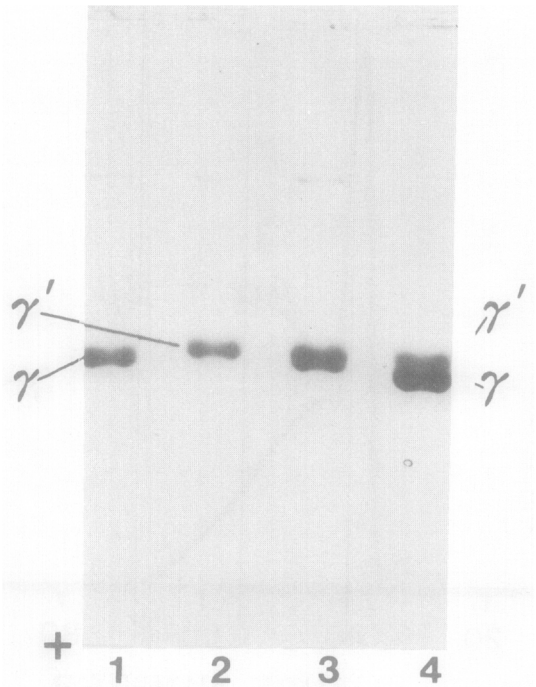

Fig. 2 Electrophoresis (Laemmli system) of S-carboxymethyl rat or human and chains. Rat chains, 1; rat chains, 2; mixture of rat and chains, 3; mixture of human and chains, 4.

in a second asymmetrical peak (data not shown). This elution pattern is similar to that found in the case of human fibrinogen (3).

Upon DEAE-cellulose chromatography of the same material (Fig. 1) the γ chains emerged after Aα and Bβ chains in two prominent peaks, labelled γ and γ' in order of their elution (70% γ; 30% γ'). Electrophoresis of chromatographic fractions in the Weber and Osborn system revealed that material in the γ' peak migrated somewhat less anodally than that in the γ chain peak, corresponding to a somewhat larger size (~1000 daltons) than rat γ chains (mol. wt. ~49,000, refs. 7,8).

Electrophoresis of S-carboxymethyl peak fractions in the Laemmli system gave better resolution of γ and γ' chains than in the Weber and Osborn system and confirmed that the size difference between these two chains was less than that between corresponding human chains (Fig. 2). Desialation of γ and γ' chains did not alter their electrophoretic behavior in this system.

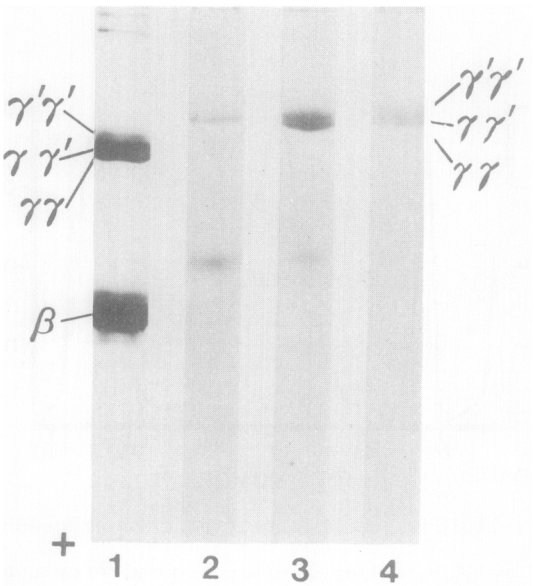

Fig. 3 Electrophoresis (Laemmli system) of reduced, corsslinked rat fibrin (gel 1) and S-carboxymethylated material (separate analysis) from the -chain peak of the colum shown in Fig. 4 (chain peak, ascending limb, 4). The band in gels 2 and 3 anodal to the -dimer position represents monomeric DNS-cad labelled chains.

Functional evaluation of the γ chains was obtained by analyzing reduced, crosslinked rat fibrin in the Laemmli system (Fig. 3); this analysis revealed bands corresponding to the three types of dimers which result from non-selective covalent crosslinking of γ and γ' chains (1). Additional evidence that rat γ chains were functionally like those from other animals was obtained by incubating the fluorescent amine donor DNS-cad with fibrin in the presence of factor XIIIa. Following reduction and S-carboxymethylation, the material was subjected to DEAE-cellulose chromatography (Fig. 4). Electrophoretic analysis of the fluorescent γ chain peak in the Laemmli system followed by visualization of fluorescent bands under UV light revealed that DNS-cad had been incorporated into both the γ-dimer and γ-monomer positions.

The relatively heavy sample Load required for adequately visualizing fluorescence obviated the possibility of resolving the γ-dimer position into

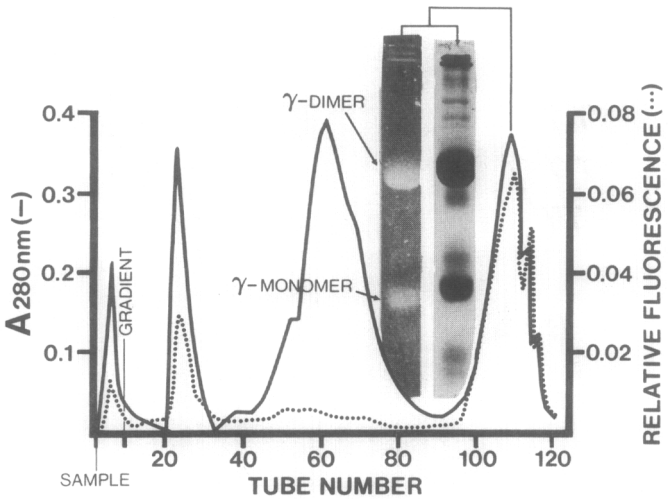

Fig. 4 DEAE-cellulose gradient elution chromatography in 8M urea of 23 mg DNS-cad labelled S-carboxylmethylated fibrin. See legend Fig. 1 for general conditions. Fractions of 2.2 + 0.1 ml were collected (total gradient volume, 225 ml); at the completion of the gradient, the column was flushed with limit buffer. An electrophoretic gel (Laemmli system) is shown of material from the chain peak photographed under UV light, left, and subsequently stained with Coomassie blue, right.

the expected three bands. However, electrophoresis of appropriate loads from the γ-dimer/γ-monomer peak of this column (ascending, middle and descending portions) showed that the order of elution of γ dimers (i.e., γγ, γγ', γ'γ') corresponded to the net charge conferred by each form (Fig. 3, gels 2-4).

To examine further whether the structural difference between rat γ and γ' chains is analogous to that in human γ and γ' chains, S-carboxymethyl chains were subjected to COOH-terminal analysis. Upon incubation with carboxypeptidase A, the γ chain yielded 0.65 methionine residues per 50,000 g. Small amounts (0.1 to 0.2 residues/$5x10^4$g) of other amino acids (Asp, Thr, Ser, Gly, Val, Leu, Tyr) were also found, consistent with their representing amino acids in the ultimate COOH-terminal sequence. Under the same digestive conditions, no amino acids were released from γ' chains with either carboxypeptidase A or B. NH_2-terminal analysis of rat γ or γ' chains revealed no identifiable NH_2-terminal group, although analysis of

human γ chain and bovine serum albumin controls gave the expected NH_2-tyrosine and aspartic acid, respectively.

Inasmuch as fibrinogen γ' chains from turpentine-stimulated rats constituted a considerably higher proportion of the γ chain population compared with human γ' chains (~30% vs ~7%), we wondered whether stimulation of fibrinogen synthesis (i.e., turpentine treatment) might have resulted in large relative increases in the γ' chain population. For this reason we subjected S-carboxymethyl fibrinogen from unstimulated rats to DEAE-cellulose chromatography (data not shown), and found that the γ' peak amounted to ~28% of the total γ chain population.

In order to examine whether the γ/γ' heterogeneity in rats could be detected in the chromatographic elution pattern of unmodified fibrinogen molecules, material from turpentine-treated rats was subjected to DEAE-cellulose chromatography (3) (data not shown). In this system, human plasma fibrinogen typically emerges in two major peaks (3,21). In contrast, rat fibrinogen was eluted in a single relatively broad, nearly symmetrical peak emerging in a position between those usually occupied by human peaks 1 and 2.

Discussion

These studies indicate the presence of the same type of γ chain heterogeneity (i.e., γ/γ') in rat plasma as exists in human and other species' fibrinogen (3,5,6). Rat γ' chains are more negatively charged than γ chains and thus are readily separable on this basis by ion exchange chromatography on DEAE-cellulose (Fig. 1). These chains represent ~28 to 30% of the total γ chain population, a much higher proportion than is found in human fibrinogen (1,3,4). They are functionally normal in that they incorporate DNS-cad in the presence of factor XIIIa and are capable of crosslinking non-selectively with other γ chains.

Because of the high proportion of γ' chains in rat fibrinogen, this animal should serve as a useful experimental model for both qualitative and quantitative studies of this heterogeneity. For one thing, the $\gamma'\gamma'$ dimer is

easily detectable in crosslinked fibrin prepared from rat plasma. In contrast, the same dimer species from crosslinked human plasma fibrin is present in such small amounts ($\leq$0.5%) that it is not detected (1,28) unless the crosslinking mixture is specifically enriched with respect to γ' chains (i.e. peak 2 fibrinogen) (1).

Rat γ' chains have a greater molecular weight than γ chains although the difference is only about 1,000 daltons, compared with ~2000 in the case of human fibrinogen (1,2). In attempting to evaluate the location of the sequence accounting for this size difference, we found no NH_2-terminal acid in either γ or γ' chains, consistent with reports that the NH_2-terminus of rat γ chains is "blocked" (7,8). COOH-terminal analysis revealed methionine as the terminal acid in γ chains but γ' chains were resistant to carboxypeptidase A or B digestion. These findings provide good evidence that the region accounting for the difference between rat γ and γ' chains is located in their COOH-terminal sequences, a situation that is analogous to the findings in human γ chains (1,2).

As assessed by studies using DEAE-cellulose gradient elution chromatography of unmodified preparations (3,21), several species revealed the peak 1/peak 2 (i.e., γ/γ') heterogeneity although other specimens did not including bovine (5) and rat fibrinogen (this study). Nevertheless, the presence of the γ/γ' heterogeneity has subsequently been demonstrated in both bovine (6) and rat fibrinogen (this study), suggesting that appropriate analytical investigation may reveal this trait to be universal. Whatever the mechanism might be that regulates production of γ and γ' chains, it does not seem to be sensitive to stimulation of fibrinogen synthesis, since the relative proportion of γ and γ' chains in plasma molecules was not changed significantly by turpentine stimulation.

Acknowledgements

Since this report was submitted, Crabtree and Kant (29) have provided evidence that a single genomic DNA fragment from rats gives rise to two mRNAs evidently coding for γ and γ' chains.

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