A Subfraction of Human Fibrinogen with High Sialic Acid Content and Elongated γ Chains*

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Fibrinogen isolated from normal human single donor or pool plasma was fractionated by DEAE-cellulose chromatography. Three different fibrinogen subfractions were obtained. The most acidic fraction comprising 22% of the whole fibrinogen pool was prominent by two special features: 1) its sialic acid content was significantly higher than that of bulk fibrinogen, namely 8 mol of sialic acid/mol of fibrinogen versus 6 mol in bulk fibrinogen. 2) Two-dimensional electrophoresis of the polypeptide chains obtained after reduction revealed a preferential accumulation in this subfraction of the elongated γ chains previously described as γ chain heterogeneity of whole human fibrinogen.

Human fibrinogen is a glycoprotein with a sialic acid content of 6 mol/mol of protein (1). It consists of 2 sets of 3 nonidentical (Aα, Bβ, γγ) polypeptide chains. The Bβ and γ chains are glycosylated (2), whereas the Aα chains do not contain carbohydrate residues (3). Human fibrinogen and its polypeptide chains are heterogeneous upon DEAE- and CM-cellulose fractionation and by isoelectric focusing analysis (4-11). The isoelectric focusing pattern of human fibrinogen shows 3 isoelectrically different γ, 3 Bβ, and 6 Aα chains (11). Part of these heterogeneities is due to differences in sialic acid in the Bβ and γ polypeptide chains (10, 11). In order to evaluate whether these heterogeneities in respect to sialic acid were of intramolecular or intermolecular nature or both, we fractionated native human fibrinogen from single donors or pool plasma by ion-exchange chromatography. We thus obtained a fibrinogen fraction differing by its sialic acid content and revealing elongated γ chains.

MATERIALS AND METHODS

Chromatography of Fibrinogen on DEAE-52-Cellulose—Human fibrinogen was obtained from AB Kabi, Stockholm (Grade L) or was isolated by ethanol precipitation (12). The clottability was 95%. All reagents used were analytical grade, and the urea solutions were equilibrated with the starting buffer. The column was eluted with 2 columns of the starting buffer to eliminate any nonadsorbed protein. Elution was carried out at room temperature at a flow rate of 28 ml/h and was monitored by the continuous measurement of the absorbance of the effluent at 280 nm (Dual path monitor UV-2, Pharmacia). 3-ml fractions were collected. Fibrinogen was eluted from the DEAE column by stepwise elution with 3 different buffers: Buffer 1, 0.03 M phosphate/0.06 M Tris, pH 7.6; buffer 2, 0.05 M phosphate/0.08 M Tris, pH 6.8; and buffer 3, 0.5 M phosphate/0.5 M Tris, pH 4.4. The protein-containing fractions were pooled and dialyzed against 0.15 M Na-citrate, pH 7.4, and were finally concentrated by ultrafiltration (Amicon YM membranes) to 4 mg/ml prior to further analysis.

Chemical Measurements—Sialic acid was determined by the thiobarbituric acid assay according to Aminoff (13) after hydrolysis of bound sialic acid with 0.1 M H2SO4 at 80 °C for 60 min. N-Acetyl neuraminic acid (98% purity, Sigma) was used as standard. Results are expressed as moles of sialic acid/mol of protein using M, = 340,000 for fibrinogen. Protein concentrations were determined either by absorbance measurement at 280 nm, using an extinction coefficient of 15.1, or by a modified Lowry assay (14), using fibrinogen as a standard.

Electrophoretic Analysis—Prior to the electrophoresis, the samples were extensively dialyzed at 4 °C against 0.05 M Tris/HCl, pH 8.5, in 8 M urea. The fibrinogen samples were reduced with a 60-fold excess of dithiothreitol (Calbiochem) for 60 min at 20 °C. 25-75 μg of protein were applied per gel.

Isoelectric focusing was carried out in highly cross-linked gels. DATD' was used as cross-linking agent (15). The final concentration of the gels was T = 7%, C = 25%. The isoelectric focusing gels contained 7 M urea, 1% Triton X-100. A pH gradient 4-9 was used by mixing equal amounts of Pharmalyte (Pharmacia), pH 4-6.5, and with pH 6.5-9. The anode was 10 mM glutamic acid and the cathode was 10 mM ethanolamine. The focusing runs were carried out in gel rods (0.5 × 8 cm) with 30 V/gel, for 12 h at 8 °C. The Pharmalyte concentration was 2.7%. The gels were chemically polymerized with K-peroxidoxin-fate. Before sample application (75 μg), the gels were prerun until a constant voltage was obtained. After the run, the gels were fixed for at least 6 h in 12.5% trichloroacetic acid, 5% sulfosalicylic acid to remove Pharmalyte. The gels were stained with 0.02% Coomassie brilliant blue R-250 overnight. Diffusion destaining was accelerated by overlaying 1-butanol on the destaining solution. This allowed reduction of the background to a minimum within 12 h.

Two-dimensional electrophoresis was performed according to O'Farrell (16) with some minor modifications. Isoelectric focusing was performed in gel rods (0.25 × 7 cm) (T = 7%, C = 25% DATD) in the presence of 8 M urea and 1% Triton X-100. The concentration of the Pharmalyte (pH 4-9) being 2.7%. The focusing time was 12 h with 12 V/gel and the temperature was 10 °C. Immediately after isoelectric focusing, the gel rod was placed on a SDS-polyacrylamide slab gel (8 × 6.5 × 0.3 cm) (T = 10%, C = 4%, the cross-linking agent being N,N'-diallyltartardiamide). The gel rod was covered with 1% agarose membranes to 4 mg/ml prior to further analysis.

RESULTS AND DISCUSSION

Human fibrinogen has been known for several years to demonstrate a reproducible heterogeneity on DEAE-cellulose (17). Thus, using a concave gradient elution, human fibrinogen could be separated into two populations with different electrophoretic mobilities. Recently, Wolfenstein-Todel and Mos-
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Esson (17) demonstrated that this heterogeneity is partially due to the presence of elongated $\gamma$ chains, called $\gamma'$. Elongated $\gamma$ chains were also described by Francis et al. (18), who suggested that the $\gamma$ chain variant has $M_r = 53,100$ instead of 50,100. We have recently shown by two-dimensional electrophoresis that the elongated $\gamma$ chain variant shows a heterogeneity with respect to sialic acid content similar to that observed in normal $\gamma$ chains (19).

In an attempt to clarify whether the sialic acid-dependent heterogeneity of human fibrinogen is of intra- or intermolecular nature, we have tried to better separate the fibrinogen subfractions using stepwise elution on DEAE-cellulose. Fibrinogen could thus be fractionated into three instead of two different populations (Fig. 1). The proportions of the subfractions in order of their elution were 13, 65, and 22% of the whole protein adsorbed on the ion-exchange cellulose. The total recovery of fibrinogen was 95%. The sialic acid content of the 3 different subfractions is shown in Table I. The most acidic fibrinogen fraction, subfraction 3, always contained at least 2 mol more sialic acid/mol of fibrinogen as compared with pool fibrinogen. The same elution pattern could subsequently be demonstrated in fractionated fibrinogen from plasma of 2 healthy single donors and from 4 different plasma pools.

These findings are in disagreement with the results of Martinez (20) who found an identical carbohydrate content in the 2 fibrinogen fractions separated by DEAE-cellulose chromatography. In the present experiments, we have achieved a separation of fibrinogen into 3 peaks. Peak 3 probably corresponds to peak 2 of Martinez but has been much better resolved, allowing differences in sialic acid to be established.

The 3 fibrinogen subfractions were analyzed by isoelectric focusing after reduction of the disulfide bridges with dithiothreitol. The first 2 subfractions differed from pool fibrinogen to some extent, but only with regard to the Aa chains (Fig. 2), whereas the B$\beta$ and $\gamma$ band pattern appeared to be identical when compared with pool fibrinogen. However, a group of acidic bands, which are faintly stained on the bottom of the gel of pool fibrinogen (Fig. 2, gel 1) are missing on the gels of the first 2 subfractions. They are concentrated in the third most acidic fibrinogen fraction (Fig. 2, gel 4). The two-dimensional gel slab (Fig. 3) shows that these acidic bands correspond most likely to the $\gamma'$ bands described by Mosesson and

![Fig. 1. Stepwise DEAE-cellulose chromatography of human pool fibrinogen.](image)

![Fig. 2. Isoelectric focusing on polyacrylamide gel.](image)

![Fig. 3. Two-dimensional separation of reduced pool fibrinogen (left) and reduced fibrinogen subfraction 3 (right).](image)
co-workers (5, 17). Indeed, 50% of the γ bands from this sialic acid-rich fibrinogen fraction appears to be of the γ' type. The Aα, Bβ, and normal γ bands of this sialic acid-rich fibrinogen fraction do not show any significant differences when compared to the electrophoretic pattern of pool fibrinogen, either with regard to the band number or with respect to the isoelectric points, indicating that the extra sialic acid residues with regard to the band number or with respect to the isoelectric points, indicating that the extra sialic acid residues ought to be located on the γ' chains.

Preliminary studies (21) of the carbohydrate moiety of the γ and Bβ chains showed that these oligosaccharides may be branched. Complex oligosaccharide moieties with 3 or 4 branching points have recently been reported for plasma glycoproteins like α-1 acidic glycoprotein and fibronectin (22, 23). It may thus be possible that the γ' chains contain a more complex oligosaccharide than the normal γ chains. On the other hand, it is conceivable that the extra polypeptide tail of the γ' chain may carry an additional glycosylation site. The polypeptide tail of the γ' chain is located at the carboxyl terminus and its amino acid composition is known. Although part of it has recently been sequenced (24), it is so far not possible to locate an additional possible glycosylation site.

It may be concluded from our results that normal human fibrinogen, both pooled and from single donors, contains a distinct subfraction with unusual features: 1) its sialic acid content is significantly higher than that of the bulk fibrinogen, and 2) it contains approximately 50% of elongated γ chains (γ' chains).

Although the nature and location of the additional carbohydrate are not yet known, it appears likely that the 2 features are interdependent. However, it can safely be concluded that the sialic acid-dependent heterogeneity of human fibrinogen is not only of intramolecular but also of intermolecular nature.

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