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Abnormal fibrinogens IJmuiden (BβArg$_{14}$→Cys) and Nijmegen (BβArg$_{44}$→Cys) form disulfide-linked fibrinogen–albumin complexes

(dysfibrinogenemias/thrombophilia/genomic DNA sequence)

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ABSTRACT **The molecular defects in two congenital abnormal fibrinogens, IJmuiden and Nijmegen, were determined by sequence analysis of genomic DNA amplified by the polymerase chain reaction. Both fibrinogens were heterozygous, IJmuiden having a BβArg$_{14}$→Cys substitution and Nijmegen having a BβArg$_{44}$→Cys substitution. Clotting induced by thrombin or Reptilase was impaired in both fibrinogens, indicating defective fibrin polymerization. Immunoblot analysis of both purified fibrinogens demonstrated that some of the abnormal molecules were linked by disulfide bonds to albumin. In addition, abnormal high molecular weight fibrinogen complexes with M_rs between 600,000 and 700,000 were present. Fibrinogen–albumin and high molecular weight complexes were also detected in the patients' plasmas. Quantative analysis demonstrated that of the total plasma fibrinogen in the IJmuiden patient, 20% was linked to albumin and 10% was present as high molecular weight complexes. In plasma Nijmegen, 13% was linked to albumin and 15% was present as high molecular weight complexes. These results demonstrate that the additional abnormal cysteine in fibrinogens IJmuiden and Nijmegen resulted in the formation of disulfide-linked complexes with other proteins, predominantly albumin. We also found that a significant fraction of the abnormal fibrinogen molecules contained free sulfhydryl groups. These findings complicate interpretation of functional studies of these altered fibrinogens.**

The fibrinogen molecule is a dimer of three polypeptides, Aα, Bβ, and γ, linked together by 29 disulfide bonds (1). During coagulation fibrinogen is converted to an insoluble fibrin matrix by thrombin-catalyzed removal of fibrinopeptides from the Aα and the Bβ chains. Congenital dysfibrinogenemia is a disorder in which a fibrinogen structural abnormality results in altered functional characteristics. A number of abnormal fibrinogens have been described (2), some associated with bleeding disorders and some associated with thrombosis. The structural defects for ≈75 cases are known, with 21 cases of arginine substituted by cysteine—14 cases of AαArg$_{16}$→Cys, 2 cases of BβArg$_{14}$→Cys, and 5 cases of γArg$_{275}$→Cys. For a few cases the consequences of an additional cysteine have been studied. For homozygous fibrinogen Metz (3) and heterozygous fibrinogens Kawaguchi and Osaka I (4), it has been reported that AαCys$_{16}$ participates in an intramolecular disulfide bond between two abnormal Aα chains. Consequently, in heterozygous individuals, abnormal molecules are homodimers—that is, both Aα chains in one fibrinogen molecule are abnormal. In contrast, analysis of another heterozygous AαCys$_{16}$ mutant, Stony Brook I (5), indicated that heterodimers are present. Homodimers of abnormal chains have been postulated for the two heterozygous cases of BβArg$_{14}$→Cys, Christchurch II (6) and Seattle I (7). The additional cysteine in heterozygous fibrinogen Osaka II (8), γArg$_{275}$→Cys, was linked to a cysteine molecule. Free sulfhydryl groups have not been detected in these abnormal fibrinogens (3–5, 7, 8).

This report describes the structural defects in two fibrinogens isolated from patients suffering from thrombophilia. These are fibrinogen IJmuiden, BβArg$_{14}$→Cys, and fibrinogen Nijmegen, BβArg$_{44}$→Cys. We found that these abnormal fibrinogens circulate as disulfide-linked fibrinogen–albumin complexes, as disulfide-linked high molecular weight fibrinogen complexes, and as molecules with free sulfhydryl groups.

MATERIALS AND METHODS

Materials. Unspecified materials were obtained from sources previously described (9). *Crotalus atrox* venom, DNA grade (A-6013), and ultra-low-gelling (A-5030) agarose were from Sigma. CH-activated Sepharose 4B, gelatin-Sepharose 4B, precast polyacrylamide gradient gels, SDS/PAGE molecular weight calibration kit, and an FPLC system with a Superose 12 column were obtained from Pharmacia. Rabbit anti-human albumin antiserum was purchased from Behringwerke AG, horseradish peroxidase (HRP) conjugated-goat anti-human albumin and goat anti-rabbit immunoglobulins were from Nordic, and rabbit anti-human fibrinogen Bβ 1–42 was from IMCO (Stockholm). A pool of rabbit anti-human fibrinogen IgGs was prepared as described (10). The monoclonal anti-fibrinogen antibody (11) conjugated with HRP (Y18/HRP) was a generous gift of W. Nieuwenhuizen (IVVO-TNO, Gaubius Laboratory). Nitrocellulose was from Schleicher & Schüll (Dassel, F.R.G.). Polystyrene microtiter plates (Immulon) were from Greiner (Alphen a/d Rijn, The Netherlands). Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)] and 4-chloro-1-naphthol were from Aldrich. Phosphate-buffered saline (PBS, pH 7.4) contained 0.15 M NaCl, 0.01 M Na_2HPO_4, and 1.6 mM KH_2PO_4.

Coagulation Studies. Blood was collected and plasma was prepared as described (12). Thrombin and Reptilase (Boehringer Mannheim) clotting times were performed as described (13). The plasma concentration of fibrinogen was determined functionally (14) and immunologically (15). Release of FpA and FpB from purified fibrinogen was determined as de-

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Abbreviations: HRP, horseradish peroxidase; HMW, high molecular weight; LMW, low molecular weight; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

scribed (13). Coagulation profiles of purified fibrinogen dialyzed against 0.1 M Tris·HCl (pH 7.5) were determined by measuring the increase in absorbance at 350 nm (9).

Protein Purification. Fibrinogen was purified from plasma (16), contaminating fibronectin was removed by adsorption to a gelatin-Sepharose column (19), and the fibrinogen was concentrated by precipitation in 50% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 0.15 M NaCl, dialyzed against this solution for 24 hr at 4°C, and stored at −20°C. Rabbit anti-human albumin IgGs were purified from serum by precipitation with 18% Na_2SO_4 (17), and the precipitate was dissolved in and dialyzed against distilled water for 24 hr at 4°C and lyophilized.

SDS/PAGE. SDS/PAGE was performed on 5–25% gradient gels according to Laemmli (18) or on 2–16% precast gels using an electrophoresis buffer of 0.04 M Tris/0.02 M sodium acetate/0.002 M EDTA/0.02% SDS, pH 7.4, and a sample buffer of 0.01 M Tris·HCl/0.001 M EDTA/1% SDS, pH 8.0. Molecular weights were estimated using a low molecular weight (LMW) calibration kit and a mixture of monoclonal IgM, fibronectin [eluted from gelatin-Sepharose (19)], and fibrinogen with molecular weights of 900,000, 450,000, and 340,000, respectively.

Immunoblot Analysis. SDS/PAGE gels were electroblotted onto nitrocellulose (20). Intact proteins and *C. atrox* venom digests were transferred to 0.45-μm and 0.1-μm nitrocellulose sheets, respectively. The sheets were incubated with 0.01 M Tris·HCl, pH 7.4/0.15 M NaCl/0.5% gelatin/0.05% Tween 80 for 2 hr. Antibodies were diluted in 0.01 M Tris·HCl, pH 7.4/0.15 M NaCl/0.05% Tween 80 and cross-reacting bands were visualized with 4-chloro-1-naphthol (21).

***C. atrox* Venom Digestions.** Fibrinogen was dialyzed against 0.05 M Tris·HCl, pH 7.5/0.1 M NaCl/5 mM EDTA, and the concentration was adjusted to 2 mg/ml. Lyophilized venom was dissolved in this buffer to 0.1 mg/ml and 5 μl was added to 0.5 ml of fibrinogen. The sample was incubated for 1 hr at 37°C and the reaction was stopped by adding an equal volume of 0.1 M Tris·HCl, pH 6.8/2% SDS/8 M urea, followed by boiling for 5 min. When reducing disulfide bonds, 5% (vol/vol) 2-mercaptoethanol was added before boiling.

Purification of Fibrinogen–Albumin Complexes. Purified rabbit anti-human albumin IgGs were immobilized to CH-activated Sepharose 4B following the manufacturer's guidelines. The amount of IgG coupled was ≈3 mg/ml of wet gel. The immunoadsorbent was packed in a column (bed volume, 2 ml) and equilibrated with 6 mM disodium tetraborate/0.2 M boric acid/5 mM EDTA, pH 7.4. Purified fibrinogen was diluted to 0.5 mg/ml with borate buffer, and 5 ml was applied to the column. The column was washed with 25 ml of borate buffer, followed by 10 ml of borate buffer containing 1 M NaCl to elute nonspecifically bound protein. Albumin complexes were eluted with borate buffer containing 8 M urea, and fractions containing protein were pooled, dialyzed against distilled water for 16 hr at 4°C, lyophilized, and dissolved in PBS to 0.1 mg/ml.

Enzyme Immunoassays. Microtiter plate wells were incubated with 0.12 ml of rabbit anti-fibrinogen IgGs (20 μg/ml) for 16 hr at 4°C and washed three times with PBS and 0.05% Tween 20 (PBS/Tween). Samples (0.1 ml) diluted in PBS/Tween were added, incubated for 1 hr at room temperature, and the wells were washed as above. Bound fibrinogen was measured by incubation for 1 hr at room temperature with 0.1 ml of rabbit anti-fibrinogen/HRP (10). Bound fibrinogen–albumin complexes were measured by incubating with 0.1 ml of goat anti-albumin/HRP for 1 hr at room temperature. The wells were washed and bound HRP was quantitated by incubation with 0.2 ml of substrate solution containing H_2O_2 and 3,3′,5,5′-tetramethylbenzidine (22). After 30 min 50 μl of 2 M H_2SO_4 was added and the absorbance at 450 nm measured on a Titertek Multiscan.

Gel Filtration of Plasma. Plasma (0.25 ml) was diluted with PBS (1:5) and applied to a FPLC Superose 12 column equilibrated with PBS. Fractions were assayed for fibrinogen and/or fibrinogen–albumin complexes using the enzyme immunoassay described above.

Titration of Free Sulfhydryl Groups. Free sulfhydryl groups in fibrinogen were determined using DTNB (23) in the presence of urea (24). Purified fibrinogen was dissolved to 10 mg/ml in 0.05 M Tris·HCl, pH 8.2/1 mM EDTA/8 M urea. Five microliters of 10 mM DTNB was added to 0.5 ml of fibrinogen and the sample was incubated for 10 min at room temperature. The absorbance at 412 nm was measured and reactive sulfhydryls were determined from the molar extinction coefficient = 13,600 $M^{-1}\cdot cm^{-1}$ (23).

DNA Amplification and Sequence Analysis. Genomic DNA was isolated from blood cells (25). Primers β1a (5′-GGTGTTGGAATAGTTACATTCC-3′) and β1b (5′-ATCAGTGCACCCACCAAGTCTGGG-3′) amplified the DNA coding for amino acids Bβ 9–72. Amplification by PCR (26) was performed in 100 μl containing 1 μg of genomic DNA, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 0.3 μg (each) of primer in 10 mM Tris·HCl (pH 8.3) at 25°C, 50 mM KCl, 3.0 mM $MgCl_2$, and 0.001% gelatin. The samples were heated at 94°C for 4 min, 2 units of Taq DNA polymerase (Perkin–Elmer/Cetus) was added, and the samples were incubated for 30 cycles of 1 min at 94°C, 0.5 min at 58°C, and 1.5 min at 72°C. Amplified DNA was precipitated with ethanol, dried, dissolved in 20 μl of distilled water, and run on a 1.0% (wt/vol) ultra-low-gelling agarose gel. The band with the size predicted by the genomic sequence (27) was excised in ≈20 μl and heated to 55°C. One microliter of the melted agarose was mixed with 1 μl of primer, β1a (60 ng), and 2 μl of 5× annealing buffer (T7 sequence kit, Pharmacia), and the volume was brought up to 14 μl with distilled water. The mixture was heated to 95°C for 3 min and immediately put on ice. Labeling (5 min) and termination (10 min) reactions were performed using the T7 DNA sequencing kit as specified by the manufacturer.

RESULTS

Coagulation Studies. The thrombin clotting times for plasma IJmuiden (25.0 s) and Nijmegen (20.2 s) and the Reptilase clotting times (26.0 s and 22.7 s, respectively) were slightly prolonged compared to normal plasma (18.5 s for thrombin and 20.0 s for Reptilase). The functionally determined fibrinogen concentration in plasma IJmuiden (0.9 mg/ml) and Nijmegen (1.2 mg/ml) was less than half that determined immunologically (2.7 mg/ml and 3.5 mg/ml, respectively). Clotting of purified fibrinogen induced by either enzyme was impaired for both abnormal fibrinogens. Thrombin-catalyzed release of FpB (Bβ chain residues 1–14) from fibrinogen IJmuiden was half that of normal fibrinogen. All other FpA and FpB release from either abnormal fibrinogen with either protease was normal.

DNA Sequence Analysis. Since FpB release from fibrinogen IJmuiden was abnormal, we amplified the DNA from exon II of the Bβ chain, which codes for amino acids 9–72. In addition to this segment, we also amplified Nijmegen DNA encoding domains associated with abnormal clotting. DNA sequence analysis (Fig. 1) demonstrated that the amplified Bβ fragment from fibrinogen IJmuiden had a single base substitution in the codon for Arg_{14} (CGT), changing this to the codon for Cys (TGT). The corresponding Bβ gene fragment from fibrinogen Nijmegen had a similar mutation, changing the codon for Arg_{44} (CGT) to Cys (TGT). No other changes were found. Direct sequence determination of the amplified fragments showed the normal and the abnormal sequence, indicating that these patients are heterozygous.

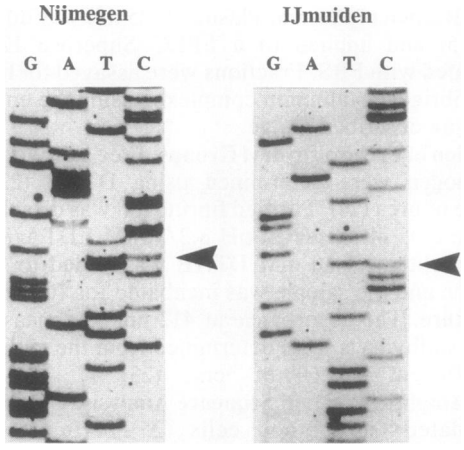

FIG. 1. DNA sequence analysis of the amplified Bβ gene fragments. Direct sequence analysis of amplified fragments demonstrated heterozygous mutations at the positions indicated by the arrowheads. In both cases C was substituted by T.

Analysis of Fibrinogen Cleavage with *C. atrox* Venom. *C. atrox* venom cleaves the Bβ chain of fibrinogen between amino acids 42 and 43 (28). When purified normal fibrinogen was digested with venom and analyzed on immunoblots developed with a rabbit antibody to Bβ 1–42, two crossreacting bands were seen, one at the top of the gel and a second with $M_r \approx 6000$ (Fig. 2*C*, lane 3). As undigested samples contained only a crossreacting band at the top of the gel (Fig. 2*D*, lane 3), the two bands found after venom digestion are probably residual intact fibrinogen and Bβ 1–42. Immunoblot analysis of purified fibrinogen Nijmegen ($B\beta Arg_{44} \rightarrow Cys$) with anti-Bβ 1–42 demonstrated that after incubation with *C. atrox* venom, an increased amount of fibrinogen Nijmegen remained intact (Fig. 2*C*, lane 1), indicating that the venom did not efficiently cleave the abnormal chain. A reduced amount of the species with $M_r \approx 6000$ was present as expected if only the normal Bβ chain was cleaved. SDS/PAGE analysis of reduced samples confirmed that approximately half of the Bβ chains in fibrinogen Nijmegen remained intact after incubation with *C. atrox* venom (data not shown).

When purified fibrinogen IJmuiden was digested with venom and analyzed by immunoblotting with antibody to Bβ 1–42, four crossreacting species were found, as shown in Fig. 2*C*, lane 2. Two of these were present in normal fibrinogen (Fig. 2*C*, lane 3), although the band representing residual fibrinogen was more intense in fibrinogen IJmuiden than in normal fibrinogen, indicating that the snake venom activity was somewhat inhibited by the IJmuiden mutation. The remaining two species with M_rs $\approx$ 14,000 and 68,000 (Fig. 2*C*, lane 2) were not present in normal fibrinogen digests. SDS/PAGE analysis of reduced fibrinogen IJmuiden samples showed no bands in addition to those seen with normal reduced fibrinogen (data not shown). These results suggest that some of the Bβ 1–42 peptide in fibrinogen IJmuiden was linked to other proteins or peptides by disulfide bonds. The species with $M_r \approx 14,000$ is probably Bβ 1–42 dimer, a product previously suggested for two other $B\beta Arg_{14} \rightarrow Cys$ substitutions in abnormal fibrinogens Christchurch II (6) and Seattle I (7).

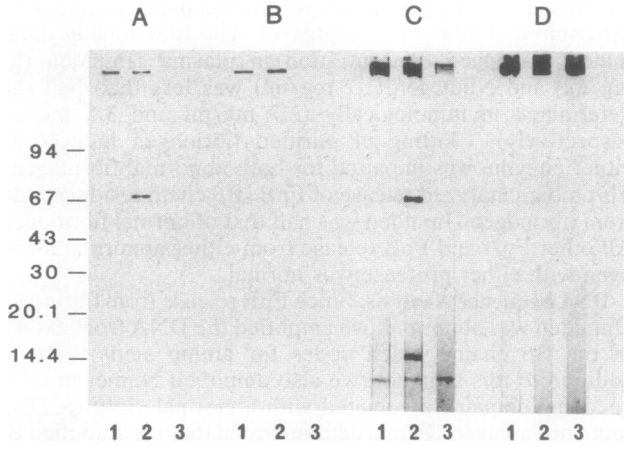

FIG. 2. Immunoblot analysis of *C. atrox* digests of fibrinogen (*A* and *C*) and intact fibrinogen (*B* and *D*). Samples of fibrinogen Nijmegen (lanes 1), fibrinogen IJmuiden (lanes 2), and normal fibrinogen (lanes 3) were electrophoresed on a 5–25% polyacrylamide gradient/SDS gel. Proteins were blotted onto nitrocellulose and developed with goat anti-albumin/HRP (*A* and *B*) or with rabbit anti-Bβ 1–42 followed by goat anti-rabbit IgG/HRP (*C* and *D*). Molecular weights are given as $M_r \times 10^{-3}$.

Immunoblot Analysis with Antibodies to Human Albumin. To determine whether the additional IJmuiden band with $M_r \approx 68,000$ was due to complex formation with albumin, as described for an antithrombin III variant (29), we prepared blots with unreduced samples and developed these with anti-albumin/HRP. We also examined fibrinogen Nijmegen for the presence of albumin complexes. Venom digests of fibrinogen IJmuiden (Fig. 2*A*, lane 2) contained two anti-albumin crossreacting species. One that remained at the top of the gel was also present with undigested fibrinogen IJmuiden (Fig. 2*B*, lane 2). The second had $M_r \approx 68,000$. Since this species also reacted with anti-Bβ 1–42, it is most likely a complex of albumin and Bβ 1–42. Venom digests of fibrinogen Nijmegen contained one species at the top of the gel that reacted with the anti-albumin antibodies (Fig. 2 *A* and *B*, lane 1), indicating that fibrinogen Nijmegen is also linked to albumin. Normal fibrinogen samples did not react with the anti-albumin antibodies (Fig. 2 *A* and *B*, lane 3). Immunoblot analysis of reduced fibrinogen IJmuiden and fibrinogen Nijmegen samples showed a single anti-albumin crossreacting species with $M_r \approx 67,000$, the same as the albumin band in the molecular weight calibration markers; reduced normal fibrinogen did not react with the anti-albumin antibodies (data not shown). These results are consistent with the presence of disulfide-linked albumin–fibrinogen complexes in fibrinogen Nijmegen and fibrinogen IJmuiden. This is also consistent with the observation that SDS/PAGE analysis of reduced abnormal fibrinogen samples contained no abnormal bands because albumin and the Aα chain of fibrinogen comigrate under these conditions.

Analysis of Intact Fibrinogen and Larger Complexes. Purified fibrinogen samples were analyzed on 2–16% gradient SDS/PAGE gels stained with Coomassie blue. Normal fibrinogen (Fig. 3*A*, lane 3) contained two species previously shown (30, 31) to be intact fibrinogen [high molecular weight (HMW) Fbg, M_r = 340,000] and fibrinogen with a partially degraded Aα chain (LMW Fbg, M_r = 300,000). These two species were present in both abnormal fibrinogens. In addition, each abnormal fibrinogen contained three minor species—one with $M_r \approx 400,000$ and two with M_rs between 600,000 and 700,000 (Fig. 3*A*, lanes 1 and 2). Immunoblots prepared from similar gels and developed with a monoclonal anti-fibrinogen antibody (Fig. 3*B*) demonstrated that these minor species contained fibrinogen. Two additional crossreacting species of M_r = 360,000 and M_r between 600,000 and 700,000 were present. Similar blots developed with anti-albumin antibodies (Fig. 3*C*) showed that the species with M_rs = 360,000 and 400,000 contained albumin. These results demonstrate that fibrinogens Nijmegen and IJmuiden contain two fibrinogen–albumin complexes with M_rs expected for albumin linked to LMW Fbg and HMW Fbg, respectively. The larger species (M_r 600,000–700,000) may be dimers

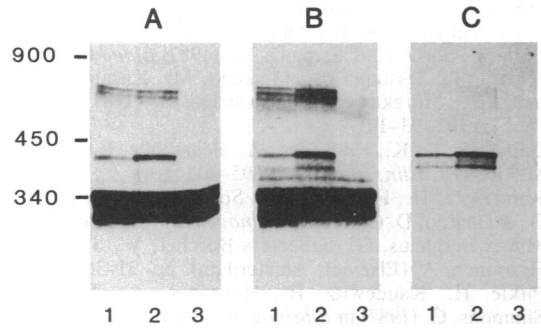

FIG. 3. SDS/PAGE and immunoblot analysis of purified fibrinogen. Samples of fibrinogen Nijmegen (lanes 1), fibrinogen IJmuiden (lanes 2), and normal fibrinogen (lanes 3) were electrophoresed on 2–16% polyacrylamide gradient/SDS gels and either stained with Coomassie blue (*A*) or transferred to nitrocellulose and developed with a monoclonal antibody to fibrinogen (Y18/HRP, *B*) or with goat anti-albumin/HRP (*C*). Molecular weights are given as $M_r \times 10^{-3}$.

formed from HMW Fbg and LMW Fbg, or they may be complexes of fibrinogen with other proteins.

Determination of Free Sulfhydryl Groups. The number of sulfhydryl groups present in these fibrinogens was determined by titration of the denatured purified proteins with DTNB (23, 24). Less than 0.05 mol of SH per mol of fibrinogen was found with normal protein, whereas fibrinogen IJmuiden contained 0.18 ± 0.05 mol of SH per mol of fibrinogen, and fibrinogen Nijmegen contained 0.13 ± 0.05 mol of SH per mol of fibrinogen. Thus, both abnormal proteins contained new free sulfhydryl groups.

Measurement of Fibrinogen–Albumin Complexes. To detect the presence of fibrinogen–albumin complexes in plasma, we developed an enzyme immunoassay specific for these complexes. To obtain reference standards, we isolated complexes from purified abnormal fibrinogens by affinity chromatography on a rabbit anti-albumin IgG Sepharose column as described in *Materials and Methods*. Purified complexes were added to normal plasma to create reference samples with different fibrinogen/fibrinogen–albumin ratios. Dose–response curves of the patients' plasmas were compared to reference curves (Fig. 4). Complexes with plasma IJmuiden were estimated as 20% of total fibrinogen (Fig. 4*B*) and complexes with plasma Nijmegen were estimated as 13% of total fibrinogen (Fig. 4*A*).

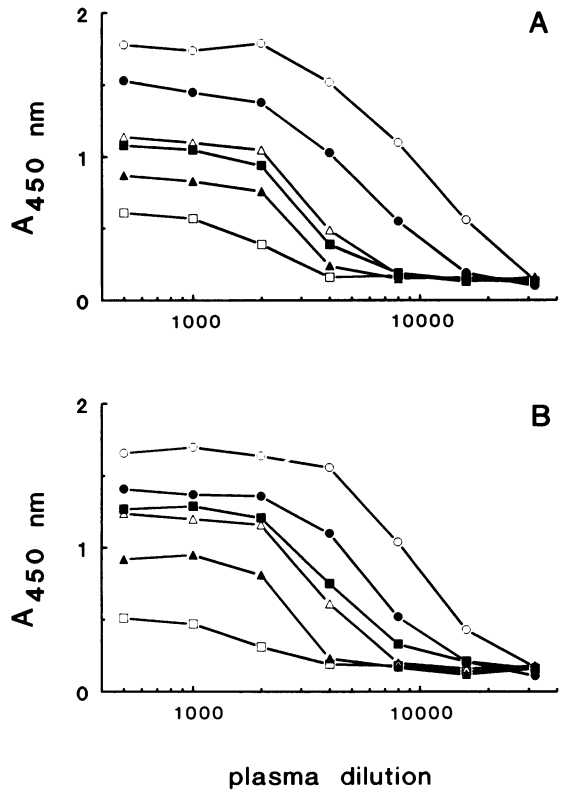

FIG. 4. Enzyme immunoassays for fibrinogen–albumin complexes in plasma. Dose–response curves were determined for plasmas Nijmegen (■, *A*) and IJmuiden (■, *B*). Standard curves were prepared by mixing normal plasma with 50% (○), 25% (●), 15% (△), 10% (▲), and 5% (□) of purified fibrinogen Nijmegen–albumin complexes (*A*) or fibrinogen IJmuiden–albumin complexes (*B*).

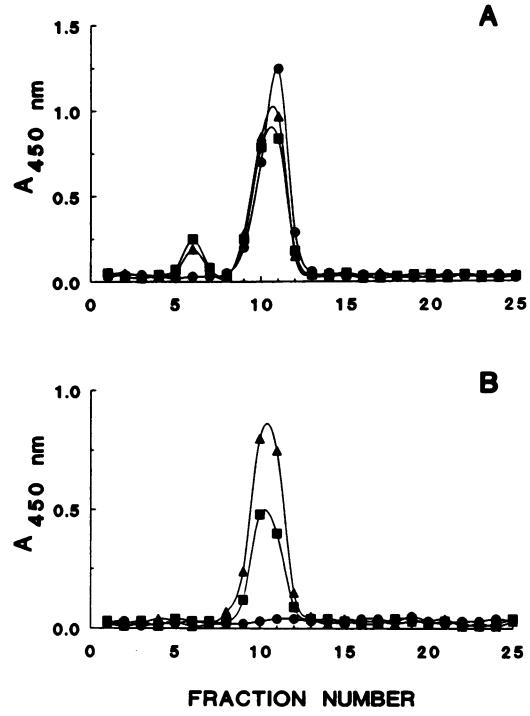

FIG. 5. FPLC analysis of plasma samples. Normal (●), Nijmegen (■), and IJmuiden (▲) samples were run over a Superose 12 column and the collected fractions were analyzed by immunoassay for fibrinogen (*A*) or fibrinogen–albumin complexes (*B*).

Measurement of Larger Fibrinogen Complexes. To measure the concentration of larger fibrinogen complexes (M_r = 600,000–700,000), samples were separated by gel filtration and fractions were assayed for fibrinogen (Fig. 5*A*) and fibrinogen–albumin complexes (Fig. 5*B*), as described in *Materials and Methods*. Fibrinogen–albumin complexes were present in fractions 9–12 for both abnormal plasmas. In addition, both plasmas contained a fibrinogen crossreactive peak at fraction 6, indicating the presence of larger fibrinogen complexes. Analysis of the peak areas demonstrated that these larger complexes were approximately 10% and 15% of total fibrinogen in the IJmuiden and Nijmegen plasmas, respectively.

DISCUSSION

We have identified the structural defect in two congenital abnormal fibrinogens, designated IJmuiden and Nijmegen. Both were heterozygous for a single base substitution (C → T) changing codons for Arg to Cys. The defect in fibrinogen IJmuiden, $B\beta Arg_{14} \rightarrow Cys$, has also been found in fibrinogens Christchurch II (6) and Seattle I (7). The defect in Nijmegen, $B\beta_{44} Arg \rightarrow Cys$, has not been previously reported. We examined the fate of these abnormal Cys residues and found that abnormal fibrinogens circulate as disulfide-linked complexes with albumin. The disulfide bonds presumably form between

the additional cysteine introduced by the mutation and the free sulfhydryl group present in albumin (32). Both abnormal fibrinogens contained two complexes, presumably albumin linked to HMW fibrinogen and to LMW fibrinogen. It is unknown whether fibrinogen becomes linked to albumin during or after synthesis of these proteins, for albumin and fibrinogen are synthesized in hepatocytes. Disulfide-linked albumin complexes have also been detected with a variant of antithrombin III [Northwick Park, Arg_{393}→Cys (29)], which is also synthesized in hepatocytes.

We also identified three larger fibrinogen complexes in both abnormal fibrinogens. These could be disulfide-linked fibrinogen dimers with bonds between the two forms of fibrinogen giving three species, HMW–HMW, HMW–LMW, and LMW–LMW dimers. *C. atrox* venom digestion of purified fibrinogen IJmuiden demonstrated the presence of disulfide-linked Bβ 1–42 dimers ($M_r \approx 14{,}000$). This product could arise from cleavage of fibrinogen dimers, but the $M_r \approx 14{,}000$ band is more intense than expected from the fibrinogen dimer species. This suggests that intramolecular disulfide bonds between the Bβ chains of fibrinogen IJmuiden are also present, in agreement with the reports on fibrinogen Christchurch II (6).

We did not determine whether fibrinogens IJmuiden and Nijmegen are linked to small molecules such as cysteine, as was reported for fibrinogen Osaka II (8), but we did find that a substantial fraction of the mutant molecules contained free sulfhydryl groups. If we assume that half of the Bβ chains are mutated in these heterozygous samples, then 36% of the IJmuiden mutant chains and 26% of the Nijmegen mutant chains contain free sulfhydryls. (This also assumes that the new cysteine is that being measured as a free sulfhydryl.) Similarly, albumin complexes account for 40% of the IJmuiden mutant chains and 26% of the Nijmegen mutant chains, and larger fibrinogen complexes account for 20% of the IJmuiden and 30% of the Nijmegen mutant chains. These forms of the altered fibrinogens complicate interpretation of functional studies. For example, the previously described defective tissue-type plasminogen activator binding to fibrin Nijmegen (12) could arise either directly from the loss of an essential residue, $B\beta Arg_{44}$, or indirectly from the presence of disulfide-linked complexes or free sulfhydryl groups.

In summary, we have used the PCR to determine the structural defects in abnormal fibrinogens IJmuiden, $B\beta Arg_{14}$→Cys, and Nijmegen, $B\beta Arg_{44}$→Cys. Analysis of the purified abnormal fibrinogens and the corresponding plasmas demonstrated that the additional mutant cysteine residues formed disulfide-linked complexes with albumin. This finding severely complicates structure–function analysis, whereby amino acid substitutions are associated with altered functions of the fibrinogen molecule.

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