Vitronectin (VN, complement S-protein) is a multifunctional protein which participates in cell adhesion, coagulation, fibrinolysis, and protection against complement lysis. VN is incorporated into several complexes, such as the terminal complement complex and thrombin-antithrombin III, and is bound to plasminogen activator inhibitor 1. The present study showed that purified VN spontaneously forms polymers of approximately 1600 kDa with a Stokes radius of 10 nm. The polymers are to a varying extent stabilized by disulfide bonds, but are quite stable even after reduction and alkylation, indicating the importance of non-covalent bonds. Plasma VN circulates mainly as a 65/75-kDa monomer containing a cryptic heparin-binding site which is exposed upon a conformational change induced by different stimuli, such as coagulation, heating, adsorption to surfaces, or exposure to acids, urea, or other denaturating agents. In the present study, VN was demonstrated to expose its heparin-binding site and its conformationally dependent 8E6 epitope when incorporated into the terminal complement complex. We suggest that exposure of the heparin-binding site and a putative hydrophobic binding site of VN are linked events dependent upon the same conformational change. In vivo, complex formation probably induces the heparin-binding site. Such a link might also explain why purified heparin-binding VN spontaneously forms polymers. The heparin-binding site may be involved in the elimination of multimolecular complexes containing VN.

VN is a multifunctional protein which participates in cell adhesion and in regulation of the complement and hemostatic systems. It is an adhesive protein which binds to glass, plastic, and a variety of organic molecules including collagen, elastin, and many bacteria. Furthermore, it forms complexes with thrombin-antithrombin III (1) and plasminogen activator inhibitor 1 (2) whereby it is regarded to play a procoagulant role. It also binds to integrins (e.g. platelet gpIIb/IIIa and the VN receptor) by the sequence RGD (3), such as fibrinogen, fibronectin, thrombospondin, and von Willebrand factor, and is reported to play a role in platelet adhesion and aggregation (4, 5). VN inhibits complement-mediated cytolyis at least at two stages during assembly of the TCC, first by binding to the amphiphilic site of the C5b-7 complex, thereby blocking its insertion into cell membranes (6), and second by inhibiting C9 polymerization of the membrane TCC (7). Clusterin, another plasma protein, is also able to inhibit the insertion of C5b-7 into membranes by a similar mechanism and is a constituent of fluid-phase TCC as well (8). The relative importance of VN and clusterin in inhibition of complement lysis is not yet established.

The VN gene is polymorphic, coding for polypeptide chains with different sensitivity to proteolytic cleavage, thereby causing a characteristic mixture of 65- and 75-kDa proteins in plasma (9). VN contains a cryptic heparin-binding site which is exposed upon conformational change (10, 11), effectively induced by denaturation with 8 M urea, 6 M guanidine hydrochloride, 0.05 M HCl, or heating at 100 °C for 5 min. Such treatment also leads to exposure of the conformationally dependent epitope for the monoclonal antibody 8E6 (12, 13). While only 2% of VN in plasma was reported to bind heparin, this fraction increases 3.5-fold during coagulation (14). The heparin-binding form was in that study found enriched in the 65-kDa component. Its Stokes radius was estimated to be 5.6 nm in contrast to 3.9 nm of the non-heparin-binding form. It was therefore suggested that the heparin-binding form consists of aggregates and exhibits an unfolded conformation. Since the heparin-binding property of VN appears to be physiologically inducible, it is reasonable to assume that this property has functional implications.

EXPERIMENTAL PROCEDURES

Materials

The following equipment and reagents were obtained from Pharmacia LKB Biotechnology Inc.: HPLC equipment, Superose 6 HR 10/30, Mono Q HR 5/5, fast desalting column HR 10/10, Sepharose CL-6B, Sepharose 4B, heparin-Sepharose CL-6B, Protein A-Sepharose CL-4B, molecular weight standards for gel filtration, Multiphor II electrophoresis unit, Multiphor II NovaBlot electrophoretic transfer unit, Excel gel SDS gradient 8–18%, and high and low molecular weight standards; from Sigma: EDTA; 2-mercaptoethanol, glycine, guanidine hydrochloride, zymosan A, Coomassie Brilliant Blue R, 3,3'-diaminobenzidine, Tris (Trizma base), and polyoxyethylene sorbitan monolaurate (Tween 20); from Merck, Darmstadt, Germany;

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disodium hydrogen phosphate, 1-butanol; from Fluka: iodoacetamide; from Amersham, UK: peroxidase-linked polyclonal anti-mouse Ig F(ab')2-fragment, peroxidase-linked polyclonal anti-rabbit IgG; from Boehringer Mannheim: 2,2'-azino-di-[3-ethylbenzthiazolin-sulfonate(6)]; from Amtec Diagnostics: bovine serum albumin; from Southern Biotechnology Associates: peroxidase-linked goat anti-mouse IgG1; from Norwegian Red Cross Blood Centre, Oslo, Norway: outdated citrate plasma.

**MAb** 8E6 was a kind gift from Prof. Erkki Rouslashi and Dr. David Schranck, La Jolla Cancer Research Foundation.

### Purification of VN

VN was purified by three different methods, A, B, and C.

**Method A: Heparin Affinity Chromatography Using 8 M Urea (15)**—This method is based on activation of the heparin-binding site of VN by 8 M urea. Briefly, serum was obtained from 2 liters of mixed plasma from eight blood donors by adding 40 ml of 1 M CaCl2, incubation at room temperature for 1 h, and centrifugation. Serum was passed successively through columns containing Sepharose 6B and heparin-Sepharose 6B to remove serum proteins with affinity for Sepharose or heparin. The heparin-binding site of the remaining serum VN was then activated by adding urea to a final concentration of 8 M. The serum was again passed through a heparin-Sepharose column and the column was washed with 10 mM sodium phosphate buffer containing 5 mM EDTA and 8 M urea, pH 7.7. The column was subsequently washed with this buffer containing 0.13 M NaCl and then with the same buffer containing 0.19 M NaCl and 10 mM 2-mercaptoethanol. Pure VN was eluted in a subsequent step with the buffer containing 0.5 M NaCl and finally dialedyzed against PBS (6.7 mM phosphate buffer, pH 7.4, 140 mM NaCl). The recovery was 17 mg of VN per liter of serum estimated by absorbance measurement at 280 nm using an E 1%1 cm value of 1.38 (7). All chromatographic procedures in this work were performed at room temperature if not defined otherwise.

**Method B: A Combination of Heparin Affinity Chromatography without Urea and Mono Q Ion Exchange Chromatography**—Serum was applied undiluted to heparin-Sepharose 6B which was equilibrated with a 10 mM phosphate buffer, pH 7.7, containing 5 mM EDTA and 0.13 M NaCl. After washing out unbound serum proteins, the column was eluted with a subsequent step with the buffer containing 0.5 M NaCl and finally dialedyzed against PBS (6.7 mM phosphate buffer, pH 7.4, 140 mM NaCl). The eluate was diluted 1:3 in distilled water and applied to a Mono Q HR 5/5 column equilibrated with 30 mM Tris-HCl, pH 8.7, containing 2%—1-butanol. Proteins were eluted by a 0—0.8 M NaCl gradient in the same buffer. The VN elution peak appeared at 0.55 M NaCl. VN was subsequently dialyzed against PBS.

**Method C: A Combination of Immunosorbent and Mono Q**—One rabbit was initially immunized with 50 µg of VN (method A) in Freund's complete adjuvant and then boosted with the same amount of protein every 4th week. IgG was isolated from the rabbit serum by Protein A affinity chromatography and was found to be monospecific to VN using a human serum in Western blotting. Fifteen mg of IgG were coupled to 5 ml of CNBr-activated Sepharose 4B. Ten ml of undiluted serum were applied. The column was washed with PBS before VN was eluted with a 0.1 M glycine buffer, pH 2.8, containing 0.15 M NaCl, and then dialyzed against PBS. The further purification procedure using Mono Q was as described under method B.

### Purification of TCC

TCC was purified by immunosorption using MAb aE11 which recognizes the C9 monopeptide exposed in TCC, but not in native C9 (16). 30 mg of aE11 IgG were coupled to 8 ml of CNBr-activated Sepharose 4B. Ten ml of ZAS were applied, and further procedure was as described above (method C). The recovery was 1.5 mg of TCC as measured by the method of Bradford using bovine serum albumin as a standard. Gel filtration of purified TCC using Superose 6 indicated at minimum 80% purity. Contaminants were demonstrated by EIA to be IgG, IgA, albumin, and IgM (data not shown). All the main protein bands from SDS-PAGE were identified as known components of fluid-phase TCC by Western blotting (C5b, C6, C7, C8, C9, VN, and clusterin). Western blotting of purified TCC and purified VN (method A) indicated that the VN content of TCC was about 6% w/w, consistent with 1 VN molecule per complex (Fig. 1).

### Serum and Plasma Pools

Serum and EDTA plasma were simultaneously collected from nine healthy subjects and pooled. Some of the pooled serum and plasma were activated with zymosan A (see below). All pools were divided into aliquots and stored at ~70 °C. These samples were used in all experiments where identical VN content was mandatory.

### Preparation of VN-depleted Serum and Zymosan Activation

The anti-VN column described under method C was used. The column was equilibrated with ice-cold 10 mM phosphate buffer, pH 7.7, containing 5 mM EDTA and 0.13 M NaCl before 1 ml of serum containing 5 mM EDTA was applied. The fractions containing unbound serum proteins were collected. For the recovery of the column, bound protein was eluted with PBS containing 4 mM guanidine hydrochloride, pH 5.5, before the column was equilibrated with the former buffer. The processed serum sample was again applied, and the procedure was repeated 10 times before the serum was concentrated to its original volume, and the degree of depletion was determined by EIA. Dilutions of native serum and VN-depleted serum were coated on Nunc Maxisorp microtiter plate. The VN content in depleted serum was 10% of native serum as detected by MAb to VN.

After addition of Mg2+ to EDTA-plasma and VN-depleted serum, serum, VN-depleted serum, and EDTA-plasma were activated with 10 mg/ml zymosan for 1 h at 37 °C. There was no sign of coagulation in the plasma sample. The samples were then spun at 10,000 × g for 10 min.

### Enzyme Immunoassay

TCC was quantitated by EIA essentially as described previously (17), using our own anti-C6 MAb (9C4) and peroxidase-linked goat anti-mouse IgG1 as detecting system. This was feasible since the primary antibody of the assay, aE11, is an IgG2a while 9C4 is an IgG1. The assay showed that the TCC content in the zymosan-activated samples was similar. The relative VN contents of TCC from the different sources were estimated by exchanging the MAb to C6 with MAb to VN which also is an IgG1. By this assay we found the VN content of TCC in the zymosan-activated VN-depleted serum to be only 5% compared to TCC in ZAS and purified TCC.

### Production of Fab Fragments

IgG fractions were prepared from rabbit anti-VN serum and normal rabbit serum (nonimmune) by Protein A affinity chromatography. Fab fragments were prepared by papain digestion. Fc fragments and uncleaved IgG were removed by Protein A. Affinity-purified anti-VN Fab fragments were prepared by applying anti-VN Fab to a VN-Sepharose column (2.2 mg of VN purified by method A coupled to 1.8 ml of Sepharose 4B). Bound Fab fragments were eluted by PBS containing 4 mM guanidine hydrochloride, pH 5.5, and subsequently dialyzed against PBS. The anti-VN activities of the affinity-purified Fab fragments were increased 4-fold compared to the starting Fab fragments measured by EIA, while Fab fragments of normal rabbits showed no anti-VN activity.

FIG. 1. Comparative Western blotting of purified VN (method A) and purified TCC to determine the VN content of TCC. SDS-PAGE was performed under reducing conditions with the following samples: VN 25 ng (A), 12.5 ng (B), 6.3 ng (C), or TCC 200 ng (D). The blots were stained with rabbit anti-VN.
Heparin Affinity Chromatography

EDTA-plasma, serum, ZAS, zymosan-activated VN-depleted serum, purified VN, and purified TCC were investigated. The buffer used was PBS or PBS containing 0.05% Tween 20. Bound protein was eluted by the same buffer adjusted to contain 0.5 M NaCl. The VN and TCC content of the effluent and the eluate was determined by specific double antibody ELAs. The ELA for VN also partly recognizes VN in TCC. This assay will be described elsewhere.

To inhibit the binding of TCC to heparin-Sepharose, anti-VN Fab was added to the TCC and the mixture incubated for 1 h at room temperature before application to the heparin-Sepharose column. Normal rabbit Fab fragments were used as control.

Electrophoresis

SDS-PAGE was performed using 8–18% Excel gel SDS gradient. High and low mass protein markers were used. The gels were stained with Coomassie Brilliant Blue R or blotted to 0.45-μm pore nitrocellulose. The nitrocellulose was blocked with 3% bovine serum albumin overnight. The blots were washed with PBS containing 0.1% Tween 20 or PBS containing 0.05% Tween 20 before application to the heparin-Sepharose 6B equilibrated with PBS. Bound protein was eluted by the same buffer adjusted to contain 0.5 M serum, purified VN, and purified TCC were investigated. The buffer used was PBS or PBS containing 0.05% Tween 20. Bound protein was eluted by the same buffer adjusted to contain 0.5 M NaCl. The VN and TCC content of the effluent and the eluate was determined by specific double antibody ELAs. The ELA for VN also partly recognizes VN in TCC. This assay will be described elsewhere.

To inhibit the binding of TCC to heparin-Sepharose, anti-VN Fab was added to the TCC and the mixture incubated for 1 h at room temperature before application to the heparin-Sepharose column. Normal rabbit Fab fragments were used as control.

Western Blotting—Western blots stained with MAb to VN confirmed that all observed bands contained VN (Fig. 2). The 75- and 65-kDa bands corresponded to the two forms of VN found in serum (7). The 60-kDa band of purified VN was not observed in serum; it probably represents a degradation product.

Gel Filtration—The occurrence of VN polymers was confirmed by HPLC gel filtration (Fig. 3). The average M\textsubscript{r} was 1,000,000 corresponding to polymers containing about 15 VN subunits. Stokes radius was estimated to 10 nm. The complex could not be dissociated using 8 M urea in the buffer. Reduction of the sample by boiling in 5% mercaptoethanol changed the elution profile of the protein only slightly indicating that disulfide bonds were reformed when mercaptoethanol and VN were separated on the column. After reduction with dithiothreitol and alklylation with iodoacetamide, the protein eluted as a polymer in 0.5 M NaCl, 2 M NaCl, and 4 M urea but as a monomer in 8 M urea, suggesting that the polymers were stabilized by strong non-covalent bonds.

When examined immediately after elution from the heparin-Sepharose column the preparation contained monomers only. The polymerization occurred during dialysis against PBS and was time-dependent. If dialysis against PBS was performed rapidly using an HPLC desalting column, polymerization was only partial, with an average M\textsubscript{r} of 400,000.

Three purifications were performed. The average M\textsubscript{r} of the polymers was virtually identical each time. When formed, the polymers showed a stable size since their elution profile was identical after 3-month storage at 4 °C. The polymers were

**Recruitment of the 8E6 Epitope**

Recognition of the conformationally dependent 8E6 epitope in VN from different sources was determined by a competitive ELISA similar to that described by Tomasi and Mosher (13). Dynatech Immunol. 2 polystyrene microtiter plates were coated with purified VN (method A) 1 mg/liter overnight. Samples assumed to contain equal amounts of VN were compared. The VN content of serum and plasma was measured by our own ELISA and was assumed to be unchanged after zymosan activation of the samples. The VN content of purified TCC was assumed to be 10% w/w. The samples were diluted in PBS, pH 6.0, containing 0.2% Tween 20, 10 mM EDTA, and either 8E6 ascites diluted 1/100, 000 or the Quidel MAb to VN diluted 1/40,000. The dilution of the MAbs had been determined by titration at the level at which a further 2-fold dilution resulted in about 50% reduction of the OD signal. The diluted samples were incubated for 1 h at 37 °C before they were applied to the microtiter plate which had been washed with PBS containing 0.1% Tween 20. The plates were then incubated for 30 min at 37 °C, washed again, and incubated with peroxidase-linked PAb to mouse IgG. Wells containing no VN defined a 100% signal; whereas wells containing no MAb defined zero.

**FIG. 2. SDS-PAGE (lanes B and C) and Western blots (lanes D and E) of VN purified by method A, and Western blot of serum (lane F).** A 8–18% gradient gel was used. The gel was stained with Coomassie Brilliant Blue R, the blots with MAb to VN A, SDS-PAGE of molecular weight markers, M\textsubscript{r} 94,000–14,400; B, SDS-PAGE of 3 μg of VN, reducing conditions; C, SDS-PAGE of 3 μg of VN, nonreducing conditions; D, Western blot of 30 ng of VN, reducing conditions; E, Western blot of 30 ng of VN, nonreducing conditions; F, Western blot of 0.1 μl of serum, reducing conditions.

**RESULTS**

**SDS-PAGE, Western Blotting, and Gel Filtration of Purified VN**

**Method A:** VN Purified by Heparin-Sepharose Using 8 M Urea

**SDS-PAGE**—The SDS-PAGE pattern was strikingly different under reducing and nonreducing conditions (Fig. 2). When 1% 2-mercaptoethanol was added to the sample, we observed three bands at 60, 65, and 75 kDa, respectively. By contrast, under nonreducing conditions the protein hardly entered the gel, indicating that the protein consists of disulfide-linked polymers with M\textsubscript{r} minimally 300,000.

**Western Blotting**—Western blots stained with MAb to VN confirmed that all observed bands contained VN (Fig. 2). The 75- and 65-kDa bands corresponded to the two forms of VN found in serum (7). The 60-kDa band of purified VN was not observed in serum; it probably represents a degradation product.

**Gel Filtration**—The occurrence of VN polymers was confirmed by HPLC gel filtration (Fig. 3). The average M\textsubscript{r} was 1,000,000 corresponding to polymers containing about 15 VN subunits. Stokes radius was estimated at 10 nm. The complex could not be dissociated using 8 M urea in the buffer. Reduction of the sample by boiling in 5% mercaptoethanol changed the elution profile of the protein only slightly indicating that disulfide bonds were reformed when mercaptoethanol and VN were separated on the column. After reduction with dithiothreitol and alklylation with iodoacetamide, the protein eluted as a polymer in 0.5 M NaCl, 2 M NaCl, and 4 M urea but as a monomer in 8 M urea, suggesting that the polymers were stabilized by strong non-covalent bonds.

When examined immediately after elution from the heparin-Sepharose column the polymerization contained monomers only. The polymerization occurred during dialysis against PBS and was time-dependent. If dialysis against PBS was performed rapidly using an HPLC desalting column, polymerization was only partial, with an average M\textsubscript{r} of 400,000.

Three purifications were performed. The average M\textsubscript{r} of the polymers was virtually identical each time. When formed, the polymers showed a stable size since their elution profile was identical after 3-month storage at 4 °C. The polymers were

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Vitronectin in Complexes Binds Heparin

FIG. 3. HPLC gel filtration of purified VN using Superose 6 under dissociating conditions (buffer containing 8 M urea). Abscissa scale, fraction number; left ordinate, OD at 280 nm of HPLC fractions (O); right ordinate, OD at 405 nm in EIA of individual HPLC fractions using rabbit PAb to VN (■). A, freshly eluted purified VN from the heparin-Sepharose column (method A, monomer); B, purified VN after dialysis against PBS (method A, polymer); C, VN after reduction and alkylation of the polymeric form (method A, monomer); D, freshly purified VN from the immunosorbent (method C, monomer).

demonstrated to be slightly heterogeneous by the following experiment (Fig. 4). When each of three fractions of the protein elution peak were reapplied to the column, they eluted as sharper peaks corresponding to their respective original positions.

Method B: VN Purified by Heparin-Sepharose without Urea

SDS-PAGE—Under reducing conditions two strong bands at 60 and 65 kDa and a weak band at 85 kDa were seen (Fig. 5). Under nonreducing conditions these bands were weak, but diffuse bands corresponding to higher molecular masses were seen, probably representing oligomers of different size. These oligomers were apparently split when disulfide bonds were broken.

Western Blotting—The 60-kDa, the 65-kDa, and the diffuse high molecular mass bands all contained VN, whereas the 85-kDa band did not.

Gel Filtration—Freshly purified protein eluted at low M, fractions, but after storage at 4 °C it gradually eluted at higher M, fractions corresponding to polymers of approximately the same size as described above (method A). When formed, these polymers were not dissociated in 2 M NaCl, but partially dissociated in 4 M urea.

Method C: VN Purified by Immunosorbent

SDS-PAGE—A similar pattern as described above for method B was seen even though the 60-kDa band dominated both under reducing and nonreducing conditions and the diffuse high molecular weight bands were less apparent (Fig. 5).

Western Blotting—The pattern was entirely as described under method B above.

Gel Filtration—Freshly purified protein eluted as a monomer of M, 70,000, but after storage it gradually eluted at higher molecular weight fractions with an elution peak located at maximum M, 900,000. These polymers were also stable in 2 M NaCl, but partially dissociated in 4 M urea.

FIG. 4. HPLC gel filtration of polymeric VN using Superose 6. One mg of purified VN (method A) was applied, and 1-ml fractions were collected. Fractions 10, 12, and 14 were reapplied on the column (0.2 ml of each) and subsequently eluted as peaks corresponding to their original positions. Abscissa scale, fraction number; ordinate, OD 280 nm. A, original preparation; B, fraction 10 reapplied; C, fraction 12 reapplied; D, fraction 14 reapplied.

FIG. 5. SDS-PAGE of VN purified by methods B and C, 5 µg of protein per lane. The gel was stained with Coomassie Brilliant Blue R. A, method C, reducing conditions; B, method C, nonreducing conditions; C, method B, reducing conditions; D, method B, nonreducing conditions.

Gel Filtration of Plasma and ZAS

In plasma VN appeared mainly in the albumin-containing fractions corresponding to a monomer with Stokes radius of 3.6 nm (Fig. 6). In ZAS there was a high molecular weight fraction of VN consistent with its incorporation into the TCC. Between the two main peaks a continuum of VN was observed which most likely represented complexes, either of VN alone, or of VN bound to other proteins.
Vitronectin in Complexes Binds Heparin

**FIG. 6.** HPLC gel filtration of plasma (A) and ZAS (B) on Superose 6. **Abscissa scale,** fraction number; **left ordinate,** OD 280 nm; **right ordinate,** OD 405 nm in EIA using MAb to VN (■) or MAb aE11 recognizing TCC (□).

**TABLE I**

<table>
<thead>
<tr>
<th>Source</th>
<th>Bound VN (%)</th>
<th>Bound TCC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>19</td>
<td>85</td>
</tr>
<tr>
<td>ZAS</td>
<td>46</td>
<td>85</td>
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<tr>
<td>VN method A</td>
<td>98</td>
<td>85</td>
</tr>
<tr>
<td>VN method B</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>VN method C</td>
<td>88</td>
<td>10</td>
</tr>
<tr>
<td>TCC</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>ZAVNDS*</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*Zymosan-activated VN-depleted serum.

**FIG. 7.** Heparin affinity chromatography of TCC. Purified TCC (0.5 mg) was applied to a column containing 4 ml of heparin-Sepharose (A) or Sepharose (B) equilibrated with PBS. The columns were eluted by PBS adjusted to 0.5 M NaCl. Fractions were tested in EIA diluted 1/100. **Abscissa scale,** fraction number; **left ordinate,** OD 280 nm (□); **right ordinate,** OD 405 nm in EIA using MAb aE11 (■) recognizing TCC, or MAb to VN (□). Marker a indicates application of TCC; marker e indicates change to elution buffer.

**Heparin-Sepharose Affinity Chromatography**

The binding of VN and TCC from different sources to heparin-Sepharose is summarized in Table I. The bulk of purified TCC preparations bound to heparin-Sepharose, but not to Sepharose (Fig. 7). A bound fraction of 84–96% was seen depending on the TCC concentration, the column size, the flow rate, and the presence of Tween. From a ZAS sample 85% of the TCC bound to heparin-Sepharose.

**FIG. 8.** Inhibition of the binding of 10 μg of purified TCC to heparin-Sepharose (2 ml) by 10 and 100 μg of rabbit anti-VN Fab fragments (○) or affinity-purified rabbit anti-VN Fab fragments (■). Fab fragments of normal rabbit IgG (▲) were used as control. The buffer used was PBS containing 0.05% Tween 20. Bound protein was eluted with the buffer adjusted to 0.5 M NaCl. Quantitation of bound and unbound fractions was done by EIA. Bound TCC fraction was: (TCC in the eluate)/(TCC in the eluate + TCC in the effluent).

**DISCUSSION**

The recognition of the epitope for 8E6 was highly dependent on the VN source (Fig. 9). The relative recognition was calculated as the reciprocal concentration of VN required for 50% reduction of maximum signal, relative to plasma (Table II). Quidel's MAb to VN also recognized VN from different sources to a different extent. However, even adjusting for this observation (A/B in Table II) the results demonstrated the following: 1) the 8E6 epitope is weakly expressed in plasma; 2) the 8E6 epitope is expressed during coagulation; 3) the 8E6 epitope is expressed during TCC formation; 4) there is an additive effect of TCC formation and coagulation on the expression of the 8E6 epitope; 5) VN in purified TCC expresses the 8E6 epitope equally well as in purified heparin-binding VN (method B); 6) the 8E6 epitope is even more strongly expressed after denaturation of VN (method A).

**FIG. 9.** Inhibition of the binding of 10 μg of purified TCC to heparin-Sepharose (2 ml) by 10 and 100 μg of rabbit anti-VN Fab fragments (○) or affinity-purified rabbit anti-VN Fab fragments (■). Fab fragments of normal rabbit IgG (▲) were used as control. The buffer used was PBS containing 0.05% Tween 20. Bound protein was eluted with the buffer adjusted to 0.5 M NaCl. Quantitation of bound and unbound fractions was done by EIA. Bound TCC fraction was: (TCC in the eluate)/(TCC in the eluate + TCC in the effluent).

**FIG. 10.** Inhibition of the binding of 10 μg of purified TCC to heparin-Sepharose (2 ml) by 10 and 100 μg of rabbit anti-VN Fab fragments (○) or affinity-purified rabbit anti-VN Fab fragments (■). Fab fragments of normal rabbit IgG (▲) were used as control. The buffer used was PBS containing 0.05% Tween 20. Bound protein was eluted with the buffer adjusted to 0.5 M NaCl. Quantitation of bound and unbound fractions was done by EIA. Bound TCC fraction was: (TCC in the eluate)/(TCC in the eluate + TCC in the effluent).

**FIG. 11.** Inhibition of the binding of 10 μg of purified TCC to heparin-Sepharose (2 ml) by 10 and 100 μg of rabbit anti-VN Fab fragments (○) or affinity-purified rabbit anti-VN Fab fragments (■). Fab fragments of normal rabbit IgG (▲) were used as control. The buffer used was PBS containing 0.05% Tween 20. Bound protein was eluted with the buffer adjusted to 0.5 M NaCl. Quantitation of bound and unbound fractions was done by EIA. Bound TCC fraction was: (TCC in the eluate)/(TCC in the eluate + TCC in the effluent).
procedures have been demonstrated to influence the properties of VN substantially. Dimer and oligomer formation of purified VN has been observed previously (19), but this phenomenon could be minimized following a careful purification procedure (7).

Since method A included treatment of the VN with 8 M urea and mercaptoethanol, it appears that polymerization of VN is restricted to denatured VN. However, VN purified without exposure to urea or mercaptoethanol also polymerized, though more slowly. These polymers were stabilized by disulfide bonds to a lesser degree and were partially disso-

**TABLE II**

Relative recognition of the epitopes for 8E6 and Quidel's MoAb to VN on VN from different sources

<table>
<thead>
<tr>
<th>Source</th>
<th>A (%)</th>
<th>B (%)</th>
<th>A/B</th>
</tr>
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<tbody>
<tr>
<td>Plasma</td>
<td>1</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>Serum</td>
<td>25</td>
<td>1.6</td>
<td>16</td>
</tr>
<tr>
<td>ZAP</td>
<td>33</td>
<td>1.6</td>
<td>21</td>
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<tr>
<td>ZAS</td>
<td>83</td>
<td>2.9</td>
<td>29</td>
</tr>
<tr>
<td>VN method A</td>
<td>2,350</td>
<td>11.5</td>
<td>205</td>
</tr>
<tr>
<td>VN method B</td>
<td>450</td>
<td>5.4</td>
<td>83</td>
</tr>
<tr>
<td>TCC</td>
<td>510</td>
<td>8.5</td>
<td>61</td>
</tr>
</tbody>
</table>

* Zymosan-activated plasma.

Fully explain why VN purified by method C polymerizes slowly and only to a limited degree.

The present observations are in accordance with data from Izumi et al. (14) who reported that heparin-binding VN has an increased Stokes radius (5.6 nm) as compared to non-heparin-binding VN (3.9 nm). They suggested this to be due to mainly an unfolded conformation of heparin-binding VN, but partially also to dimerization and complex formation with unknown, low molecular weight proteins in plasma. Their estimated Stokes radius for heparin-binding VN was distinctly lower than estimated for our polymers, indicating that the complexes observed in these two studies are different.

The differing tendency of disulfide bond stabilization in the observed polymers was probably due to different exposure to mercaptoethanol. VN is reported to contain a free, reactive thiol group causing spontaneous formation of disulfide-linked dimers (7). To form disulfide-linked polymers, at least two free thiol groups per molecule are needed; thus reduction is required to liberate sufficient free sulphydryl groups. However, the SDS-PAGE of VN purified by method B and C suggests the existence of disulfide-linked VN oligomers without previous exposure to reducing agents, indicating that there may be more than one free thiol group per VN molecule.

Plasma VN circulates mainly as a non-heparin-binding monomer, while in ZAS a large fraction is incorporated into TCC. We have demonstrated that VN in fluid phase TCC has an exposed heparin-binding site, as revealed by the binding to heparin-Sepharose of VN-containing TCC, but not of VN-deficient TCC. The 8E6 epitope is also expressed, supporting the view that VN is conformationally changed in TCC.

We suggest that the heparin-binding site of VN generally is exposed when VN enters into complexes. The binding of VN to C5b-7 is likely to be mediated by a more or less cryptic, inducible, hydrophobic site. Exposure of such a site may involve a conformational change also leading to exposure of the heparin-binding site and vice versa. We suggest the hydrophobic site to be sticky, mediating binding to other VN molecules as well as to some other biological molecules and plastic surfaces. This model would explain the strong tendency for heparin-affinity-purified VN to form polymers. Such a physicochemical change may be linked to a potential role of VN as a scavenger for complexes like TCC and thrombin-

**REFERENCES**

Vitronectin in Complexes Binds Heparin