Accessibility of Epitopes on Fibrin Clots and Fibrinogen Gels

By Roman Procyk, Bohdan Kudryk, Stephen Callender, and Birger Blombäck

Radiolabeled antibodies were perfused into fibrin clots and fibrinogen gels formed in vitro to assess the reactivity of selected epitopes. An antifibrin monoclonal antibody (MoAb) (antibody 1D4/xI-f), directed against an epitope in the Aα-chain C-terminal region (Aα241-476), bound to 35% of the epitope in crosslinked fibrin clots and 37% of the same epitope in factor XIII-induced fibrinogen gel networks. A different MoAb (4-2/xI-f, anti-392-406) bound to only 7% of the epitope in both fibrin and fibrinogen gels. As expected, an antifibrin MoAb (antibody T2G1, antiBβ15-21) did not bind to fibrinogen gels, but bound to fibrin, although to only 14% of the available T2G1-reactive epitopes. An antibody that does not recognize fibrin (antibody 1-8C6, antiββ1-21) did not bind to fibrin clots and bound to 35% of the 1-8C6 epitope present in fibrinogen gels, a level of binding also observed with antibody T2G1 and fibrinogen gels only after the latter were treated with thrombin. T2G1 epitope expression was affected much more than 1D4/xI-f epitope expression in clots formed in buffers of high or low ionic strength, conditions known to influence clot structure. Studies on the availability, in quantitative terms, of the T2G1-reactive epitope in fibrin clots is of particular importance because this antibody is currently being used in clinical trials as a clot imaging agent.

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Fibrin IS the main component of the fibrous network of blood clots and is formed by the polymerization of thrombin-activated fibrinogen molecules. Activation occurs at the N-terminal of fibrinogen through cleavage of the Aα15Arg-16Gly bonds, which releases the N-terminal fibrinopeptides A, and the Bβ14Arg-15Gly bonds, which releases the N-terminal fibrinopeptides B. Polymerization sites at the C-terminal region of neighboring fibrin molecules, thus enabling activated fibrin monomers to align in a double-stranded protofibril. The monomers arrange end-to-end and half staggered with respect to the other strand of the protofibril. Protofibrils grow in length and associate laterally to form thicker fibrin fibers of about 84 nm in width. These fibers also associate to form thicker fibrin strands that are evenly distributed throughout the clot volume.

Studies with purified fibrinogen show that the conditions during clot formation (eg, concentration of fibrinogen, thrombin, calcium, buffer ionic strength, etc) influence the final clot structure. In addition, fibrin clot structure is stabilized by plasma factor XIII through the formation of covalent cross-linkages formed between particular glutamine and lysine residues in adjacent fibrin monomers in the fibrin fiber. Factor XIII can also catalyze the polymerization and gelation of fibrinogen in a process that does not require thrombin-mediated exposure of polymerization sites on fibrinogen. The gels contain both fibrinopeptides A and B, and confocal laser three-dimensional microscopy has shown that they consist of irregularly distributed masses of polymerized material held together by thin fibrin strands.

The present study was conducted to determine how differences in clot structure affected the interaction of several antifibrin(ogen) monoclonal antibodies (MoAbs) with fully hydrated fibrin clots. The antifibrin MoAb T2G1 (antiBβ15-21), antifibrinogen MoAb 1-8C6 (antiBβ1-21), and new antibodies 1D4/xI-f (antiAα241-476) and 4-2/xI-f (antiγ392-406) directed against epitopes in the C-terminal regions of the Aα- and γ-chains of fibrinogen were used as probes. Fibrinogen gels were also used to study the expression of epitopes in a gel structure very different from that of fibrin. Results show that all of the MoAbs recognize only a part of the total number of the epitopes present. For some antibodies, epitope expression was also affected by changes in fibrin fiber structure. In quantitative terms, the assessment of reactive epitopes in fibrin clots is of particular importance for studies evaluating applications of antifibrin antibodies as clot-imaging agents.

MATERIALS AND METHODS

Proteins and reagents. Human fibrinogen (Imco, Stockholm, Sweden), prepared as previously described, was stored at 70°C as a stock solution of about 14 mg/mL in 0.05 mol/L Tris-HCl (pH 7.4) buffer containing 0.1 mol/L NaCl and 0.001 mol/L EDTA (TNE buffer). The fibronectin present in this preparation was removed by affinity chromatography on gelatin-Sepharose. Fibrinogen concentration was measured spectrophotometrically in alkaline area using E (1%, 1 cm) = 16.5 at 282 nm. Human factor XIII, thrombin, and plasmin were obtained from the Department of Blood Coagulation Research, Karolinska Institutet, Stockholm, Sweden. Hirudin was from Pentapharm (Basel, Switzerland) and aprotinin was from Mobay Chemical Corp (New York, NY). Bovine serum albumin (BSA) was from Sigma (St Louis, MO).

MoAbs. The preparation and characterization of the antifibrin MoAb designated T2G1 was reported previously. Antibody T2G1 is an IgG1, κ-isotype. (T)N-DSK [(Aα16-51, Bβ15-118, γ1-78),] the thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fails to bind fibrinogen and fibrin I (desAA-fibrin), but reacts with thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fails to bind fibrinogen and fibrin I (desAA-fibrin), but reacts with thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fails to bind fibrinogen and fibrin I (desAA-fibrin), but reacts with thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fails to bind fibrinogen and fibrin I (desAA-fibrin), but reacts with thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fails to bind fibrinogen and fibrin I (desAA-fibrin), but reacts with thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fails to bind fibrinogen and fibrin I (desAA-fibrin), but reacts with thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fails to bind fibrinogen and fibrin I (desAA-fibrin), but reacts with thrombin-treated N-terminal CNBr-fragment of fibrinogen, was used as immunogen for preparation of this antibody. The antibody fail...
1-8C6 were obtained from ascites by chromatography on Baker-bond ABx (JT Baker, Phillipsburg, NJ) by a method described previously. Antibody 1D4/xl-f (IgG1 κ) is directed to an epitope located in the C-terminal region of the α-chain. It reacts completely with fibrinogen, Aα1-610, and intact as well as trypsin-digested fragment Aα241-476, the so-called H12-DSK. It was obtained from a fusion experiment using the Ig nonsecretion myeloma P3X63Ag8.653 and spleen cells of an animal immunized with reduced and carboxymethylated cross-linked human fibrinogen. Details of the immunization, fusion, and screening protocols were similar to those reported previously.

MoAb C4 (IgG2, κ-isotype) directed against myosin was used as a nonspecific control reagent and was obtained from Dr John M. Brown.

Radiolabeling of antibodies. Approximately 4 mg of MoAb (about 8 mg/mL) in phosphate-buffered saline (pH 7.0) was labeled with 35S (DuPont Co, Wilmington, DE) using the Enzymobead radiiodination reagent (Bio-Rad, Richmond, CA). Unbound iodine was removed by gel filtration on PD-10 columns (Pharmacia, Piscataway, NJ) and the labeled antibodies dialyzed against 200 vol of TNE buffer during 3 hours with three changes of outer fluid. After dialysis, 90% to 93% of the total radioactivity could be precipitated with trichloroacetic acid. The specific radioactivity ranged from 100 to 300 cpm/μg protein and was determined from spectrophotometric measurement of protein concentration using E (1%, 1 cm) = 14 at 280 nm and measurement of the radioactivity in the γ-counter.

The immunoreactivity of the radiolabeled antibodies was determined according to Lindmo et al using fibrinogen- or fibrin-Sepharose, as described by Rosebough et al, except that Sepharose 4B (Pharmacia) was used and the separation of supernatant and Sepharose beads was accomplished in Centrex disposable centrifugal microfilters (Schleicher & Schuell, Keene, NH). The immunoreactivities generally ranged between 77% and 85%, values similar to those reported for iodination of T2G1. The radiolabeled antibodies were also evaluated by direct enzyme-linked immunosorbent assay (ELISA). Serially diluted unlabeled and radiolabeled antibodies were bound to pin to plastic wells coated with antigen (10% acetic acid solubilized fibrin monomer or fibrinogen) and specifically bound antibody was detected by a peroxidase-conjugated rabbit antibody directed against mouse λg (DAKO Corp, Santa Barbara, CA). Binding of the radiolabeled antibodies was very similar if not identical to that of the binding of unlabeled antibodies.

Fibrinogen gel formation. Fibrinogen gels were formed in the absence of thrombin essentially as described. A mixture of fibrinogen (final concentration of about 1.3 mg/mL in TNE buffer), hirudin (8 antithrombin units [ATU]/mL), and CaCl2 (0.02 mol/L) was incubated for 5 minutes at room temperature and then dibuthiothreitol was added to a concentration of 0.0005 mol/L from a 0.22 mol/L stock solution in water. Polymerization was initiated by the addition of activated factor XIII to a concentration of 0.4 U/mL. Activated factor XIII was prepared as described earlier.

Gel formation took place in small 0.1-mL vessels constructed from polystyrene pipets and in cuvettes, as described for fibrin, except that gel formation was allowed to proceed overnight. About 80% to 85% of the fibrinogen in the gelatin sample was incorporated into the fibrinogen gel network and the turbidity of the gel at 350 nm after 22 hours was about 1.3.

Flow studies. Vessels containing fibrin or fibrinogen gels were attached to a reservoir holding buffer (TNE buffer or TNE buffer containing 1 mg/mL BSA and 0.1% NaHCO3). Flow of buffer through the fibrin was controlled by adjusting the hydrostatic pressure to between 5 and 25 cm to obtain a flow rate of about 2.4 mL/h for fibrin clots and 0.6 mL/h for fibrinogen gels. About 10 vessel volumes (1 mL) were collected initially to remove any unclotted fibrinogen and establish a stable flow rate. The flow was uniform throughout the clot/gel as judged by the elution of bromphenol blue dyed buffer through the vessel. The flow rate was determined for each vessel containing fibrin or fibrinogen gel by weighing the eluates collected in two to three consecutive spans of 10 to 30 minutes. The amount of protein in the vessels was measured spectrophotometrically after removing and hydrolyzing the clot/gel in alkaline urea.

Antibody binding studies. Radiolabeled antibodies were diluted to the desired concentration (50 to 5,500 nmol/L) with percolation buffer (TNE buffer containing 1 mg/mL BSA and 0.1% NaHCO3), and 0.2 mol of the solution was injected into the buffer stream immediately above the fibrin or fibrinogen gel at room temperature. After 0.15 mL had passed through, the buffer flow was stopped for 50 to 60 minutes to allow for antibody binding. Longer periods of incubation (up to 2 hours) led to only a slightly greater uptake (not shown). For determination of the amount of antibody binding to fibrinogen, the fibrin gels were radioautographed and the amount of radioactivity bound was determined from a linear plot of radioactivity bound versus concentration of antibody.
bound, the tubes were percolated with buffer until no further radioactivity eluted (about 20 column volumes). Overnight percolation, using buffer or buffer with detergent (0.05% [vol/vol] Tween 20), did not significantly increase the removal of any more antibody from the clots/gels. The radioactivity of the total eluate and of the tube containing the clot/gel was determined. Typically, better than 95% of counts applied were recovered. The amount of antibody retained on the clot/gel was estimated from the radioactivity of the tube containing the clot/gel and the specific radioactivity of the antibodies. Binding data is presented without corrections for nonspecific interactions (which were found to be negligible, see Results) or percent immunoreactivity.

**Determination of avidity coefficients.** Measurement of the dissociation constant of antigen-antibody equilibria in solution was performed according to Friguet et al. using classical indirect ELISA, except that incubation to reach equilibrium was done for 4 hours at room temperature. For the T2G1 antibody, plastic plates were coated with acid-solubilized fibrin monomer. The antigen used for equilibrium binding was serially diluted soluble fibrinlike monomer prepared from fibrinogen by limited reduction, alkylation, and treatment with thrombin. Fibrinogen was used as antigen for equilibrium binding in solution for the 1-8C6 and 4-2/xl-f antibodies and also to coat the plastic plates. For the 1D4/xl-f antibody, Aa241-476 (kindly provided by Dr Birgit Hessel, Karolinska Institute) was used as antigen and also to coat plastic plates. Specifically bound antibody on the plastic wells was detected with a peroxidase-conjugated rabbit antibody directed against mouse IgG (DAKO Corp, Santa Barbara, CA).

Measurement of the dissociation constant of antigen-antibody equilibria using matrix-bound fibrin or fibrinogen was performed according to Rosebrough et al., except that Sepharose 4B was used.

**RESULTS**

The binding of MoAbs directed against epitopes in fibrinogen was examined in native fibrin clots formed from purified fibrinogen and thrombin (Fig 1). Each MoAb bound in a specific manner. The antibody 1D4/xl-f, directed against an epitope (Aa241-476) in the C-terminal region of the Aα-chain, showed stoichiometric binding for applications up to about 250 pmol of antibody. Application of more 1D4/xl-f antibody did not result in a significant increase in the amount of antibody retained, indicating that saturation of binding probably occurred at a level of about 0.7 mol of antibody bound per mole of fibrin. The antibody T2G1 (antiBβ15-42) showed completely different properties, with binding to fibrin increasing at a much slower rate than for the 1D4/xl-f antibody. Saturation of binding occurred at a level of about 0.3 mol of antibody bound per mole of fibrin. The smaller F(ab)2 fragment of T2G1 gave a similar binding profile (not shown). The antibody 4-2/xl-f, directed against γ392-406, showed little specific binding to fibrin and saturation occurred at a level of about 0.14 mol of antibody bound per mole of fibrin.

Nonspecific interactions involving antibodies and fibrin were found to be negligible. Only 0.01 mol of the antmyosin antibody C4 bound per mole of fibrin, less than 1% of the amount applied (Table 1). The uptake of antibody 1-8C6 (antiBβ1-21) was also less than 1% of the amount applied. Because antibody 1-8C6 is directed against an epitope not present in fibrin its negligible binding also indicated that all of the fibrinogen pipeted into the tube for clot formation was either converted to fibrin or washed out during the initial percolation step before introduction of antibodies (see Materials and Methods).

The data in Fig 1 indicated that even after exposure of clots to excess antibody, the amount retained never reached proportions equimolar to the number of epitope sites in fibrin. The low amount of binding did not result from a diminished immunoreactivity caused by radiolabeling of antibodies (see Materials and Methods), and it was not related to loss of epitope sites during fibrin clot formation. The latter was verified in experiments where fibrin from the tubes was digested by plasmin (0.05 mg plasmin, in TNE buffer overnight at 37°C) and the digest assayed by indirect ELISA. In these ELISA experiments the expected amount of T2G1 and 1D4/xl-f epitopes was recovered (not shown).

The specificity of antibody binding was assessed in experiments with fibrinogen gels. The latter were formed through covalent ligation of fibrinogen catalyzed by factor XIII and do not require the removal of fibrinopeptides by thrombin (see Materials and Methods). The results in Table 2, obtained at saturating levels of antibody (ie, 2.5 molar excess over the number of moles of fibrinogen), show
that antibody T2G1 did not bind to the fibrinogen gel because it is directed to a cleavage-specific epitope that requires Bβ15G5 as a free N-terminal. The control antmyosin antibody also did not bind these gels. By contrast, both antibody 1-8C6 and 1D4/xf-f showed a high level of binding, ie, about 0.7 mol antibody bound per mole of fibrinogen gel.

Antibody binding was also studied in fibrinogen gels in which fibrinopeptides A and B were removed by percolation with thrombin before application of antibody (Table 2). Antibody T2G1 bound to the thrombin-treated fibrinogen gel, thus verifying that it required fibrinopeptide B removal for epitope recognition and binding. The 1D4/xf-f antibody bound to the thrombin-treated fibrinogen gel to the same extent as before thrombin treatment. This was expected because thrombin should not affect the C-terminal end of the fibrinogen Aα-chain and it should also not lead to the release of any fibrinogen from the gel matrix. However, treatment with thrombin destroyed the 1-8C6-reactive epitope and led to a dramatic decrease of 1-8C6 binding (a drop from 0.7 to 0.01 mol 1-8C6 bound per mole of thrombin-treated fibrinogen gel). The antmyosin antibody showed little binding, indicating that entrapment of antibodies in the gel network was negligible. The previously described experiments confirmed the specificity of the antibodies and the binding reactions.

Avidity coefficients for the different MoAbs were calculated by Scatchard analysis of the binding data from the experiments with fibrin clots and fibrinogen gels (Table 3).

All of the values fell between $10^{-7}$ and $10^{-8}$ mol/L, with the 1D4/xf-f antibody showing the strongest association value to native fibrin and the T2G1 antibody the weakest. The T2G1 antibody appeared to bind somewhat more tightly to the thrombin-treated fibrinogen gel than to fibrin.

The dissociation constants of antigen-antibody equilibria in solution obtained from indirect ELISA and from binding studies using antigens immobilized to Sepharose beads are also shown in Table 3 for comparison. The values obtained from ELISA were generally between 5 to 100 times smaller, the most significant difference occurring for the T2G1 and 4-2/xf-f antibodies. The values from the Sepharose-fibrinogen and Sepharose-fibrin binding experiments indicated slightly stronger binding constants than those obtained from the clots and gels for the T2G1 and 1-8C6 antibodies; however, the 1D4/xf-f antibody did not show this trend. The number of binding sites per mole of antigen deduced from ELISA binding data by Scatchard analysis (not shown) indicated that 1 molecule of T2G1 antibody bound to a molecule of antigen and about 1 to 1.5 molecules of 1D4/xf-f antibody bound to a molecule of antigen. As already mentioned, the clot/gel binding data showed that binding occurred at about 0.7 molecules antibody per molecule of antigen for antibody 1D4/xf-f and also for antibody T2G1 in experiments with thrombin-treated fibrinogen gels. These values approach 1 molecule antibody bound per molecule of antigen when correcting for the 77% to 85% immunoreactivity obtained after radiolabeling (see Materials and Methods). However, in the case of antibody T2G1 and 4-2/xf-f binding to fibrin clots, the values still remain below 1, even after correcting for immunoreactivity.

The relationship between clot structure and antibody binding was investigated in experiments where the conditions during clot formation were modified to produce fibrin clots with different structural features (eg, larger or smaller pore size, fiber length and diameter, etc; see Materials and Methods). Antibody binding was not significantly changed in fibrin clots formed at lower fibrinogen concentrations or at different thrombin concentrations (not shown). However, as seen in Table 4, antibody T2G1 binding increased in clots formed at successively higher ionic strength, with binding to clots formed at the highest ionic strength (0.25) rising to more than twice the level obtained with clots formed at the lowest ionic strength (0.13). By contrast, antibody 1D4/xf-f showed little change in the extent of

### Table 2. Binding of MoAbs to Fibrinogen Gels at Saturation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody Specificity</th>
<th>Antibody Bound (mol/mol FBRg gel)</th>
<th>Antibody Bound (mol/mol ThFBG gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2G1</td>
<td>AntiBβ15-21</td>
<td>0.01</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>1D4/xf-f</td>
<td>AntiAa234-476</td>
<td>0.73 ± 0.09</td>
<td>0.75 ± 0.01</td>
</tr>
<tr>
<td>4-2/xf-f</td>
<td>Antiγ392-406</td>
<td>0.12 ± 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>1-8C6</td>
<td>AntiBγ1-21</td>
<td>0.70 ± 0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>C4</td>
<td>Antimyosin</td>
<td>0.04</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Antibody binding determined by applying about 870 pmol of antibody to a vessel containing fibrinogen gel (297 ± 20 pmol fibrinogen). Values not corrected for nonspecific binding. Average of two to seven gels with standard deviation of the mean.

†Vessels containing fibrinogen gels were sequentially treated with thrombin (6 NIH U/gel) and antibody binding determined by applying about 870 pmol of antibody. Values not corrected for nonspecific binding and change of immunoreactivity following radiolabeling. Average of two to seven vessels with standard deviation of the mean.

### Table 3. Dissociation Constants for MoAbs and Several Forms of Ligand

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody Specificity</th>
<th>Fibrin clots (mol/L)</th>
<th>FBG/ThFBG Gels (mol/L)</th>
<th>Competition ELISA (mol/L)</th>
<th>FBG/FN-Sepharose (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2G1</td>
<td>AntiBβ15-21</td>
<td>$3.5 \times 10^{-5}$</td>
<td>$1.4 \times 10^{-7}$</td>
<td>$3.0 \times 10^{-5}$</td>
<td>$1.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>1D4/xf-f</td>
<td>AntiAa234-476</td>
<td>$5.3 \times 10^{-4}$</td>
<td>$6.8 \times 10^{-4}$</td>
<td>$1.4 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-7}$</td>
</tr>
<tr>
<td>4-2/xf-f</td>
<td>Antiγ392-406</td>
<td>$1.3 \times 10^{-1}$</td>
<td>$1.1 \times 10^{-7}$</td>
<td>$5.8 \times 10^{-4}$</td>
<td>$6.0 \times 10^{-4}$</td>
</tr>
<tr>
<td>1-8C6</td>
<td>AntiBγ1-21</td>
<td>$1.1 \times 10^{-7}$</td>
<td>$5.8 \times 10^{-4}$</td>
<td>$6.0 \times 10^{-4}$</td>
<td></td>
</tr>
</tbody>
</table>

Values obtained from Scatchard analysis of binding data. The ligands for binding were presented in different forms: fibrin clots; FBG gels; ThFBG gels (thrombin-treated fibrinogen gels) for antibody T2G1; competition ELISA (liquid phase competition, see Materials and Methods); FBG-Sepharose (fibrinogen-Sepharose beads) for antibody 1D4/xf-f; and FN-Sepharose (thrombin-treated fibrinogen-Sepharose beads) for antibody T2G1.
Table 4. Binding of MoAbs to Fibrin Formed at Different Ionic Strengths

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>Antibody Bound (pmol/pmol fibrin)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T2G1 (Bp15-21)</td>
</tr>
<tr>
<td>0.13</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>0.21</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>0.23</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>0.25</td>
<td>0.52 ± 0.07</td>
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</table>

*Antibody binding determination by applying about 870 pmol of antibody to vessels containing fibrin (378 ± 16 pmol fibrin). Values not corrected for nonspecific binding and for change of immunoreactivity after radiolabelling. Average of two to eight vessels with standard deviation of the mean.

†Clots formed in buffers containing NaCl to give the specified ionic strengths. Clots were percolated with buffer of 0.15 ionic strength before use for antibody binding experiments (see Materials and Methods).

binding, except for a small decrease in binding to clots formed at the highest ionic strength.

**DISCUSSION**

Several antifibrin MoAbs are presently in clinical trials and preliminary results on their effectiveness as clot-imaging agents have now been reported. 25-27 The usefulness of the antibodies as clinical probes has been tested through in vitro studies of antibody interaction with fibrin as well as in vivo studies in several animal models. 28-34 Screening studies are usually done by incubating purified fibrin clots in a solution containing antibodies and following the uptake of antibody for several hours or after overnight incubation. The experiments described in this report used both fibrin clots and fibrinogen gels formed from proteins in small vessels, and evaluation of interactions was performed by perfusing the vessels with radioactively labeled antibody. Antibody application and fibrinogen concentration were easily controlled, and binding equilibrium was reached in a short time in this in vitro system.

The MoAbs we studied recognized only a part of the total number of each epitope present in a fibrin clot. In the case of antibody 1D4/xl-f, binding occurred at a level of about 0.7 mol of antibody bound per mole of fibrin (Table 1). A similar level of binding occurred in fibrinogen gels (Table 2) and in fibrin clots formed at ionic strengths up to 0.23 (Table 4). This epitope (encompassing Aa241-476) is in a flexible region of the Aa-chain that includes a single chain segment that extends outward, folds back, and becomes part of a compact structure situated near the N-terminal central region of the molecule. 35-37 This feature of the Aa-chain may explain why the 1D4/xl-f epitope supported a high degree of interaction with antibody. The region of the Aa-chain with the 1D4/xl-f epitope is likely to be on the outer surface of a fibrin fiber, and this location probably permits epitope expression to remain high in different fibrin clot structures and fibrinogen gel networks. A readily accessible orientation in fibrin is consistent with the reported high plasmin susceptibility of this part of the Aa-chain in fibrin clots, its participation in fiber branching, and factor XIII crosslinking. 38 Furthermore, the only disulfide bridge to be reduced in fibrin clots under native conditions by the physiologic reducing reagent thioredoxin is also located in this region of the Aa-chain. 39 Yet both fibrinogen and fibrin molecules would be expected to have 1D4/xl-f epitopes per mole, while the binding data indicated that a single 1D4/xl-f antibody molecule bound per molecule of fibrin or fibrinogen. It is likely that greater amounts of binding were not achieved because of steric hindrance, i.e., antibody binding to one epitope site may preclude binding of another antibody molecule to the second one. Alternatively, it is possible that some fraction of 1D4/xl-f epitopes could have adopted a modified conformation in fibrin and in fibrinogen gels, one which the 1D4/xl-f antibody would no longer recognize.

Binding of antibody T2G1 to its epitope (Bp15-21) has been reported to occur through only one binding domain of the antibody, 39 and because there are two beta-chains in fibrin, maximum binding should occur when 2 mol of antibody are bound per mole of fibrin. However, the level of antibody binding was about 0.3 mol of antibody bound per mole of fibrin (Table 1), well below the theoretical maximum. The low level of binding was not caused by incomplete release of fibrinopeptide B that would prevent full expression of the T2G1 epitope in fibrin because antibody 1-8C6 (antiBP1-21) showed no binding to fibrin (Table 1). The level of antibody T2G1 binding to fibrin was also possibly not related to differences between antibody avidity because the values for all of the antibodies were in the range of 10^7 to 10^8 mol/L (Table 3). It is possible that binding of antibody T2G1 to one epitope site may preclude binding of another antibody T2G1 molecule to the second epitope site because both of the T2G1 epitopes in the amino-terminal end of the fibrin molecule are probably very close to each other. This appears to be the case with unpolymerized fibrin molecules. Only one T2G1 antibody molecule associates with a molecule of the soluble fibrin-like monomer used in the competition ELISA experiments (see Materials and Methods), as deduced from Scatchard analysis of the data (not shown). However, a ratio of less than one molecule of antibody bound per antigen molecule was observed for the binding of antibody T2G1 to fibrin clots. This appears to be the consequence of steric factors related to the way fibrin monomers are arranged in the fibrin clots. Several observations support this conclusion. The T2G1 epitope is likely found within the "B" polymerization site in fibrin that is thought to interact with a complementary "b" site located at the ends of neighboring fibrin monomer molecules during fibrin fiber formation. 40 In fibrinogen gels, the "B" polymerization site is activated when thrombin is passed through the gel network; however, it most likely remains "free," i.e., nonreactive with respect to its complementary site, because all of the fibrinogen molecules in the gel network are covalently cross-linked to each other by factor XIII and therefore immobile. Thus, the "B" polymerization site in fibrinogen gels fails to engage in the type of interactions that occur in fibrin fibers and the T2G1 epitope remains exposed and available for interaction with antibo-
ies. The data in Tables 1 and 2 support this explanation because they show that the amount of T2G1 antibody bound to the thrombin-treated fibrinogen gel is much greater than to an equivalent amount of fibrin and approaches a value of 1 mol of antibody bound per mole of antigen.

Additional support for the conclusion that steric factors play an important role in antibody binding comes from experiments with fibrin clots formed at different ionic strengths. In structural terms, clotting in buffers of increasing ionic strength causes fibrin fibers to swell. Their water content increases and the concentration of protein in fibrin fibers decreases. Confocal laser three-dimensional microscopy shows fibrin fibers formed at increasing ionic strengths to be shorter, less compact, and have a somewhat increased strand width. In addition, high ionic strength conditions are likely to decrease protein-protein interactions during polymerization and perturb the interactions at the fibrin polymerization sites. In the case of the “B:b” sites, these effects appear to facilitate the interaction of antibody T2G1 with its epitope because almost twice the amount of antibody bound to the clots formed at high ionic strength (Table 4).

The lowest amount of binding to fibrin, and also to fibrinogen gels, was detected for the antibody 4-2/xl-f (antiv392-406). The C-terminal end of the γ-chain participates in diverse physiologic functions such as factor XIII catalyzed cross-link formation and contains sites recognized by receptors on platelets, neutrophils, and interacts with several strains of staphylococci. For these reasons the 4-2/xl-f epitope might be expected to be located in a relatively accessible part of the fibrin(ogen) molecule, as with the C-terminal Aα-chain 1D4/xl-f epitope. However, a high level of reactivity of the 4-2/xl-f epitope in fibrin(ogen) was not observed. It is possible that the conformation of the 4-2/xl-f epitope in fibrin(ogen) may be very different when compared with that in isolated γ-chain or polypeptides derived from the γ-chain because antibody 4-2/xl-f interacts very well with these polypeptides and with fibrinogen bound to plastic wells, but poorly with fibrinogen in solution (data not shown).

The overall binding properties of antibodies described here are consistent with the results of other studies. For example, in clots formed from purified fibrinogen, antibodies recognizing the C-terminal region of the γ-chain were reported to interact at a ratio of less than 0.1 mol of antibody per mole of fibrin, whereas the antibody with an epitope in an almost similar location (antibody 4-2/xl-f) reacted at a ratio of about 0.14 in our clot binding experiments (Table 1). Some epitopes were found to be more readily accessible in fibrin monomers than in a fibrin clot. An MoAb designated Y22, directed against an epitope comprising a three-dimensional discontinuous structure in the D-domain of fibrin(ogen), reacted more than three times better with aggregating fibrin (ie, antibody added to sample before initiation of clotting) than with preformed clots. Our results support the view that steric factors related to fibrin polymerization and fiber structure (ie, the arrangement of fibrin monomers in fibrin fibers) influence the binding ability of fibrin(ogen)-specific antibodies to clots. In clinical situations, fibrin clots formed in whole blood would be the targets of thrombus detection strategies using radiolabeled antifibrin antibodies or active antibody fragments. These clots will undoubtedly differ in structure from those formed in vitro using purified proteins as reported in this study. In addition, many factors in blood would affect the formation of the fibrin clot. It seems reasonable that the more massive, thicker fibrin fiber of clots formed in blood and plasma might bind less antibody on a per-mole basis. However, it is not yet possible to predict how changes in plasma or blood clot structure will affect particular antibody binding properties.

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