Monoclonal Antibodies Against Bovine Tissue Factor, Which Block Interaction With Factor VII_a

By Steven D. Carson, Ronald Bach, and Sharon M. Carson

Two monoclonal antibodies that recognize bovine tissue factor (coagulation factor III) have been obtained following the fusion of hyperimmune mouse spleen cells with NS-1 plasmacytoma cells. Both antibodies, TF1-E2 and TF1-F7, have γ1 heavy chains and λ light chains. TF1-E2 and TF1-F7 have each been used to purify bovine tissue factor from a crude detergent extract of bovine brain by immunoaffinity chromatography. Both antibodies inhibit tissue factor procoagulant activity and block the association of factor VII_a with tissue factor. The association of TF1-F7 and tissue factor solubilized in Triton X-100 was measured under equilibrium conditions. The K_d for this antibody–antigen interaction was 2.1 ± 0.2 nmol/L. TF1-E2 effectively competes with TF1-F7 for tissue factor binding, indicating that the monoclonal antibodies recognize overlapping sites on the protein. These antibodies will be useful reagents for large-scale purification and for structure-function studies of bovine tissue factor. In particular, since they appear to bind to the same region of the tissue factor molecule as factor VII_a, they will be useful as specific probes for studying the kinetics of tissue factor-initiated coagulation and for immunocytochemical localization of tissue factor in bovine cells.

TISSUE FACTOR (tissue thromboplastin, coagulation factor III) is a membrane protein that initiates coagulation by serving as an essential cofactor for the activation of factors IX and X by factor VII (reviewed by Nemerson and Bach[1] and Carson[2]). Tissue factor (TF) from bovine brain was first purified in 1981 by immunoadsorbent affinity chromatography.[3] The efficiency of this method is less than optimum because the specific antibodies constitute only a fraction of the total rabbit immunoglobulin used to prepare the antibody–agarose conjugate. Development of the monoclonal antibodies described in this report has increased the recovery of tissue factor from immunoaffinity columns and provided an antibody source from which all of the immunoglobulin bound to the agarose is directed against the antigen of interest. Since these monoclonal antibodies presumably recognize a single determinant on the tissue factor molecule, they will be useful probes of structural and functional features of this membrane protein.

MATERIALS AND METHODS

BALB/c mice were immunized using purified bovine tissue factor as described by Carson et al.[4] The first intraperitoneal injection contained 40 μg of protein emulsified with Freund's complete adjuvant and 1 μg of cholera toxin (provided by Dr Alex Kurosky, University of Texas Medical Branch at Galveston). The intraperitoneal booster of 20 μg protein in Freund's incomplete adjuvant was administered after two weeks. An intravenous injection of 5 μg of apoprotein was given three days before cell fusion. The mouse spleen cells were fused with P3-NSI-1-Ag4-1 (NS-1) cells and cultured in selective medium as previously described in detail.[4]

Culture supernatants were screened for antibodies to tissue factor using an enzyme-linked immunosorbent assay (ELISA) as described by Carson et al.[4] Immulon II flat-bottom plates (Dynatech, Alexandria, Va) were coated with rabbit IgG directed against mouse immunoglobulin (Dako Corp, Santa Barbara, Calif) diluted 1:500 in 0.1 mol/L $NaHCO_3$. After the plates were washed free of unbound antisera, 100 μL of spent medium from cultures to be tested were added to each well. After 3 hours at 4 °C, the wells were blocked with bovine serum albumin (BSA) and then washed. Bovine tissue factor (100 μL of a 0.2 μg/mL solution in Tris-saline with 1 mg/mL BSA and 0.1% Triton X-100 [New England Nuclear, Boston]) was added to each well and allowed to bind to any anti-tissue factor antibodies present from the culture supernatants. Bound tissue factor was detected with monospecific rabbit antibodies conjugated to horseradish peroxidase.[5] Specificity was established by conducting the ELISA with tissue factor omitted. Desirable cultures were cloned at least twice in agarose.[6]

Immunoglobulin G was purified from rabbit serum, from culture supernatants, and from mouse ascites fluids by chromatography on protein A-agarose.[7] Rabbit antiserum against homogeneous bovine tissue factor was prepared as previously described.[3] Purified IgG was dialyzed against 0.05 mol/L Tris, 0.10 mol/L NaCl, pH 7.5 (Tris-saline), and aliquots of 3 to 7 mg IgG/mL were stored at −20 °C. The monoclonal IgG were characterized by electrophoresis[8] and immunodiffusion.[9] For electrophoretic analyses, hybridomas were grown in medium supplemented with ^{35}S-methionine. The culture supernatants were heated with sodium dodecylsulfate and mercaptoethanol. After electrophoresis, the 12.5% acrylamide gels were subjected to fluorography.[10] Specific antisera (Bionetics Laboratory Products, Kensington, Md) against γ chains and classes γ1, γ2a, γ2b, and γ3 and against κ and λ chains were used for immunodiffusion.

Inhibition of tissue factor procoagulant activity by purified antibodies was determined using a two-stage clotting assay.[3] Tissue factor was reconstituted into phospholipid vesicles composed of 30% phosphatidylserine and 70% phosphatidylcholine using octyl glucoside.[11] Factor VII-deficient plasma was prepared using a factor VII-specific immunoaffinity column.[12] In the first stage of the assay, the concentration of factor VII_a was 1.1 nmol/L, and the concentration of factor X was 250 nmol/L. The standard curve for the assay was established using a serial dilution of the reconstituted tissue factor; 1 pg of tissue factor protein was defined as 1 unit of procoagulant activity. A log–log plot of clotting time *v* tissue factor U/mL was linear. The dilutions of the tissue factor standard (and clot times) used to construct the standard curve were: 50 U/mL (20.4 seconds), 25 U/mL (24.1 seconds), 12.5 U/mL (33.8 seconds), 6.25 U/mL (44.8 seconds), and 3.125 U/mL (60 seconds). The reconstituted tissue factor and antibodies were diluted into Tris-saline containing 0.1% BSA and combined. The final concentration of tissue factor in the mixture was 25 pg/mL, and the IgG concentrations varied from 0 to 10 μg/mL as indicated in Fig 2.

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Following incubation at room temperature for two hours, each sample was assayed at least twice without further dilution. The percentage of inhibition of tissue factor activity was calculated as follows: % inhibition = (1 − [test activity/control activity]) × 100. The calculation of I_{max} and $K_{1/2}$ for each inhibition isotherm was performed as described in the legend in Fig 2.

To determine if the observed inhibition of tissue factor procoagulant activity by the antibodies was mediated by displacing the enzyme (factor VII_a) from its essential activator (tissue factor), direct binding measurements were performed in the presence of varying concentrations of IgG. The binding of factor VII_a to tissue factor inserted into phospholipid vesicles was monitored by first constructing a reaction mixture composed of ^{3}H-factor VII_a (10 nmol/L), tissue factor (10 nmol/L), IgG (0 to 1,100 μg/mL), and 5 mmol/L $CaCl_2$ in Tris-saline with 20% sucrose and 0.1% BSA. Following incubation for 30 minutes at 37 °C, each sample was centrifuged for ten minutes at 95,000 rpm in a Beckman airfuge to separate the tissue factor-containing vesicles and bound enzyme from unbound enzyme. The concentration of ^{3}H-factor VII_a bound to tissue factor in the vesicle fraction and unbound ^{3}H-factor VII_a remaining in the solvent fraction was determined by liquid scintillation counting. The calculations for the percentage of inhibition of ^{3}H-factor VII_a binding and the I_{max} and $K_{1/2}$ for the displacement isotherms were performed following the same procedures described for inhibition of procoagulant activity.

Direct binding of tissue factor to the purified antibodies was demonstrated using tissue factor-specific affinity columns prepared from rabbit or mouse IgG coupled to Affi-Gel 10 (Bio-Rad, Richmond, Calif). Coupling efficiency was determined by measuring the absorbance (280 nm) of the dialyzed supernatants following reaction. Unreacted sites on Affi-Gel were blocked with 1:10 vol of 1 mol/L glycine ethylester, pH 8.0, for one hour at 24 °C. The IgG–Affi-Gel columns contained coupled immunoglobulins as follows: rabbit (polyclonal) IgG (6.35 mg/mL), monoclonal TF1-E2 (1.84 mg/mL), and monoclonal TF1-F7 (2.70 mg/mL). Each column was prewashed with two-column volumes of each buffer used during immunoaffinity purification and stored at 4 °C in Tris-saline with 0.1% Triton X-100. The 2% Triton X-100 extract of bovine brain acetone powder was prepared as previously described,[3] and ^{3}H-tissue factor was added as an internal standard (≈2,500 cpm/mL) to monitor the tissue factor protein. Purified apoprotein, in Triton X-100, was labeled with tritium by reductive tritation as described for radiolabeling factor IX.[13] ^{3}H-tissue factor reconstituted into lipid vesicles had the same procoagulant activity as the unlabeled molecule. Each affinity gel (4 mL bed vol) was combined with 200 mL of 2% Triton X-100 brain extract and stirred for 48 hours at 4 °C. The gels were collected on a sintered glass funnel and washed at 4 °C with 200 mL Tris-saline, 0.1% Triton X-100 and 200 mL 1 mol/L NaCl, 0.05 mol/L Tris, 0.1% Triton X-100 before repouring into columns with Tris-saline, 0.1% Triton X-100. Each column was then eluted with 0.1 mol/L glycine, pH 2.0, 0.1 mol/L NaCl, and 0.1% Triton X-100, and elution of the ^{3}H-tissue factor was monitored by liquid scintillation counting.

The binding of TF1-F7 to tissue factor was measured directly by first coating polyvinyl microtiter plates (Costar No. 2595) with the monoclonal antibody. One hundred microliters of monoclonal IgG, 20 μg/mL in Tris-saline, were added to each well and incubated overnight at 4 °C. The plate was then washed with buffer, and unreacted sites were blocked with 3% gelatin in Tris-saline for three hours at 37 °C. The gelatin was removed, and the wells were again washed with Tris-saline. A constant amount of ^{3}H-tissue factor (749,000 cpm/μg) was added to each well along with varying amounts of unlabeled tissue factor in Tris-saline with 0.1% Triton X-100 and 0.1% BSA. The final reaction volume was 100 μL per well and the concentration of tissue factor, labeled plus unlabeled, ranged from 1.6 nmol/L to 16.6 nmol/L. The plate was incubated for 4½ hours at 37 °C. Following equilibration the unbound material in each well was removed, and the plate was rapidly washed six times with cold Tris-saline containing 0.1% Triton X-100. Each well was then cut from the plate and placed in a scintillation vial along with 3 mL Aquasol 2. Bound tritium was determined by liquid scintillation counting. Background binding of labeled material to wells with monoclonal IgG was less than 0.5% of the added ^{3}H-tissue factor. Fractional binding of tissue factor to each microtiter well was calculated as tritium bound/tritium added. The fractional binding at each concentration of antigen was repeated six times, and the average was used to calculate the concentration of bound and free tissue factor at the 15 concentrations of tissue factor tested. The K_d and B_{max} for this equilibrium binding isotherm were calculated as described in Fig 5.

The ability of monoclonal antibody free in solution to displace ^{3}H-tissue factor from solid phase monoclonal antibody TF1-F7 was assessed as descibed above except that unlabeled tissue factor was replaced by monoclonal IgG. The antibodies tested included the two anti-tissue factor preparations, TF1-E2 and TF1-F7, and a control monoclonal antibody against rabbit IgG, RIg9C.[4] The IgG concentrations in the reaction mixture ranged from 0 to 500 μg/mL. Displacement of ^{3}H-tissue factor from the microtiter wells was monitored by liquid scintillation counting as described above.

RESULTS

Following the fusion of NS-1 and hyperimmune spleen cells, only four of the cell cultures gave positive results in the ELISA. One of the monoclonal antibodies produced a positive ELISA result even when tissue factor was omitted from the assay. It was subsequently shown to bind rabbit IgG that was probably introduced into the tissue factor during immunoaffinity purification.[3,4] The other three monoclonal antibodies recognized bovine tissue factor, and two of these, TF1-F7 and TF1-E2, cloned easily and produced ample ascites fluid in mice. Electrophoretic analysis of ^{35}S-labeled supernatants from these cultures revealed that both hybridomas produced heavy and light chains consistent with IgG (Fig 1). The slight difference in heavy chain mobilities suggested that these cultures produce different immunoglobulins, even though immunodiffusion showed that the immunoglobulins from both were composed of γ1 heavy chains and κ light chains.

Experiments to assess the effects of TF1-F7 and TF1-E2 on the functional properties of tissue factor showed that both antibodies prolonged the clotting time of the two-stage tissue factor assay. As shown in Fig 2, both monoclonal antibodies diminished TF activity in a dose-dependent manner. Although neither antibody inhibited the procoagulant activity completely (Fig 2, panels B and C), tissue factor activity could be essentially eliminated by specific antibodies, as demonstrated with the polyclonal rabbit IgG (Fig 2, panel A).

The inhibition of tissue factor expression in the two-stage clotting assay suggested that the polyclonal and monoclonal antibodies may have blocked the formation of an enzymatically active complex created when factor VII_a binds to tissue factor. To test this directly, determination of factor VII_a binding to tissue factor was conducted in the presence of competing IgG (Fig 3). As with inhibition of tissue factor activity, each immunoglobulin inhibited the binding of factor

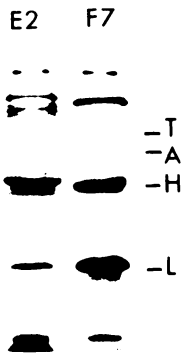

Fig 1. Autoradiogram of polyacrylamide gels following electrophoretic analysis of monoclonal antibodies. Hybridoma cultures TF1-E2 and TF1-F7 were maintained in the presence of [^{35}S]-methionine, and secreted proteins were analyzed in SDS after reduction of disulfide bonds. The positions of calibration proteins (transferrin [T, mol wt = 80,000], BSA [A, mol wt = 68,000], and IgG heavy [H, mol wt = 50,000] and light [L, mol wt = 23,000] chains) were determined by placing the autoradiogram over the stained and dried gel.

VII_a in a dose-dependent fashion. This clearly demonstrated that the immunoglobulin preparations competed with factor VII_a for binding to tissue factor. As with inhibition of tissue factor activity, neither monoclonal IgG completely inhibited factor VII_a binding to tissue factor. The I_{max} for the polyclonal IgG (99.7% ± 5.4%) suggests that the binding of

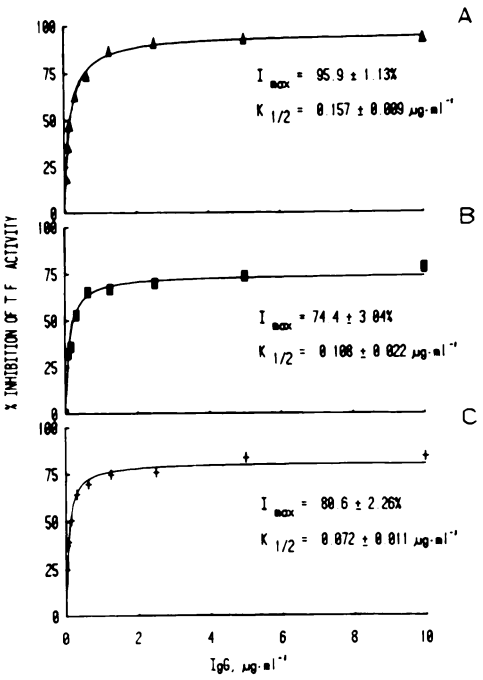

Fig 2. The inhibition of tissue factor procoagulant activity by polyclonal and monoclonal IgG was quantified. The data were fitted to the following equation: % inhibition = I_{max} [IgG]/($K_{1/2}$ + [IgG]). Marquardt's nonlinear least squares program[14] was used to calculate the maximum inhibition (I_{max}) and the IgG concentration that gave half-maximal inhibition ($K_{1/2}$). Experiments were conducted with polyclonal IgG (A), TF1-E2 IgG (B), and TF1-F7 IgG (C).

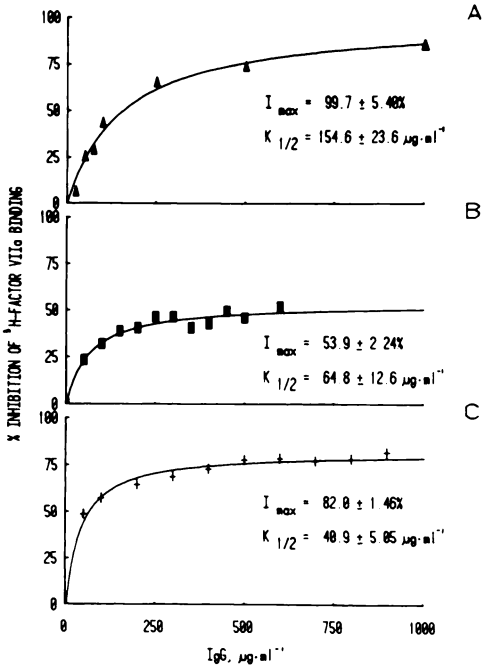

Fig 3. The inhibition of ^{3}H-factor VII_a binding to the tissue factor was determined, and the data were fitted to a rectangular hyperbola as described in Fig 2. Experiments used polyclonal IgG (A), TF1-E2 IgG (B), and TF1-F7 IgG (C).

enzyme to tissue factor can be completely blocked by polyclonal rabbit IgG.

To assess the usefulness of the monoclonal antibodies for purification of tissue factor, affinity columns were constructed from both monoclonal and polyclonal immunoglobulins. Tissue factor was purified from the 2% Triton X-100 extract of bovine brain, and recovery from each affinity gel was monitored by adding a trace amount of ^{3}H-tissue factor as an internal standard. The material eluted from each affinity column was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig 4). The 43,000-dalton tissue factor polypeptide was a major component of the eluates from all three columns. The unreduced lanes from all three columns also contained a polypeptide of 85,000 daltons as well as stained bands that appeared to be due to IgG leached from the columns. Identification of the IgG-derived bands was based on their apparent mol wt (± mercaptoethanol) and immunoblotting (not shown). After reduction, only IgG heavy and light chains, along with the tissue factor 43,000-dalton monomer, were observed. The results of the immunoaffinity purifications (Fig 4) clearly demonstrated that each affinity column bound the tissue factor apoprotein. To determine if all the tissue factor molecules purified on the polyclonal column were recognized by the monoclonal antibodies, tissue factor purified on the polyclonal antibody column was recycled over the TF1-F7 monoclonal affinity column. Measurement of tissue factor procoagulant activity in the bound and unbound pools following relipidation indicated that >95% of the activity bound to the monoclonal affinity column. Electrophoresis of the bound and unbound pools confirmed that the

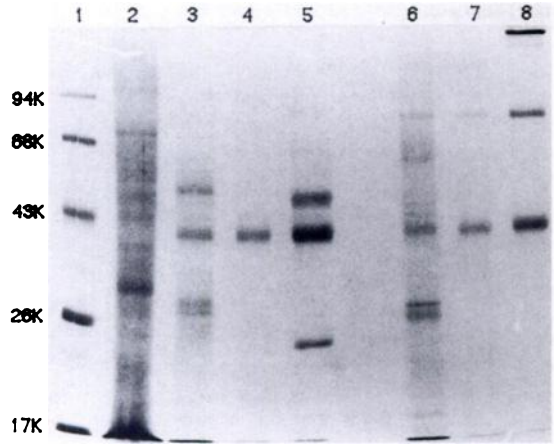

Fig 4. Electrophoretic analysis of tissue factor purified on immunoaffinity columns. SDS-PAGE was performed according to the procedure of Laemmli.[8] The samples follow: Lane 1, mol wt standards (phosphorylase B, 94 K; bovine serum albumin, 68 K; ovalbumin, 43 K; chymotrypsinogen, 26 K; and myoglobin 17 K. Lane 2, 150 μL 2% Triton X-100 brain extract. Lanes 3 and 6, pH 2 eluate for the polyclonal affinity column. Lanes 4 and 7, pH 2 eluate from the TF1-E2 affinity column. Lanes 5 and 8, pH 2 eluate from the TF1-F7 affinity column. Lanes 1 through 5 were reduced with 2% mercaptoethanol. Lanes 6 through 8 were unreduced. Each affinity column eluate lane represents 25% of the total protein eluted from the columns. The gel was stained with Coomassie brilliant blue R-250.

43,000-dalton tissue factor polypeptide purified on the polyclonal antibody column was fully retained by the monoclonal antibody column.

The equilibrium binding of solid-phase monoclonal antibody TF1-F7 and tissue factor solubilized in 0.1% Triton X-100 was performed as described in Materials and Methods. The binding isotherm (Fig 5) yields a K_d of 2.1 ± 0.2 nmol/L for the antigen–antibody interaction. The B_{max} for the equilibrium-binding isotherm, 1.36 ± 0.03 nmol/L, suggests that 0.5% of the added IgG was absorbed to the microtiter plate and available for binding to tissue factor, assuming two receptor sites per IgG molecule.

Displacement of ^{3}H-tissue factor from solid phase TF1-F7 by monoclonal IgG free in solution is shown in Fig 6. Both anti-tissue factor monoclonals TF1-F7 and TF1-E2 inhibited the binding of tissue factor to the microtiter plate, while an irrelevant monoclonal IgG, RIg9C, had no effect on the binding.

DISCUSSION

The development of monoclonal antibodies that recognize bovine tissue factor is a significant advance in studies of this membrane protein. Using an affinity column constructed from monoclonal antibody TF1-F7, tissue factor from bovine brain has been purified on a large scale with a significant increase in yield (twofold) over the previous affinity preparations in which polyclonal antibodies were used.[3] This improved performance is largely due to the greater capacity of the monoclonal affinity column for tissue factor, and reduction of contaminant proteins present in the eluate from the polyclonal antibody column. Fewer steps following the immunoaffinity chromatography are therefore required to achieve tissue factor homogeneity. The monoclonal-purified tissue factor had the same specific activity following relipidation as the previously characterized material purified using polyclonal antibodies. Since tissue factor is a minor component of the brain homogenates from which it is obtained,[3] this increased efficiency in purification will facilitate preparation of sufficient amounts of the apoprotein for further structural as well as functional studies.

We have presented data demonstrating that two monoclonal antibodies that bind bovine tissue factor have been prepared. We have shown that these antibodies inhibit tissue factor procoagulant activity (Fig 2) and that this inhibition is probably the result of the displacement of factor VII_a from tissue factor by the monoclonal IgG (Fig 3). This strengthens the hypothesis that the enzyme-activator complex is the

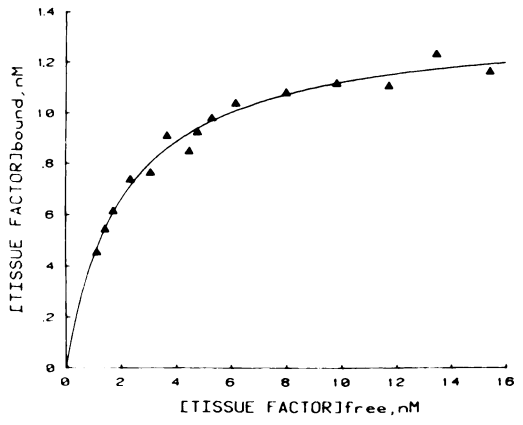

Fig 5. The binding of tissue factor and TF1-F7 was performed as described in Materials and Methods. The data were fitted to the binding equation: $[TF]_{bound} = B_{max}[TF]_{free}/(K_d + [TF]_{free})$. K_d and B_{max} were calculated using Marquardt's nonlinear least squares program.

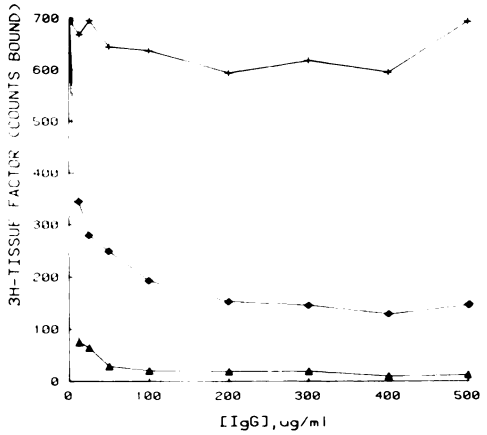

Fig 6. Displacement of ^{3}H-tissue factor from solid phase TF1-F7 by IgG in solution was performed by utilizing the microtiter plate binding assay. The data are presented as counts bound to the microtiter well (^{3}H-tissue factor) versus added (IgG) in solution. Three monoclonal antibodies were tested: TF1-F7 (▲), TF1-E2 (♦), and RIg9C (+).

enzymatically active species responsible for tissue factor-initiated coagulation.[15] In addition to the inhibition data, binding of tissue factor to the antibodies was directly demonstrated by ELISA and confirmed by immunoaffinity purification of the Triton X-100–solubilized tissue factor apoprotein (Fig 4).

We have measured the binding of tissue factor in 0.1% Triton X-100 to TF1-F7 under equilibrium conditions (Fig 5). The K_d for this association is 2.1 ± 0.2 nmol/L. In addition, we have shown that both anti-tissue factor monoclonals, TF1-F7 and TF1-E2, block the association of tissue factor with solid-phase TF1-F7 in the microtiter plate assay (Fig 6). A monoclonal antibody that recognized rabbit IgG[3,4] has no effect on the binding of tissue factor to TF1-F7. These results suggest that TF1-F7 and TF1-E2 recognize overlapping sites on the protein. Together with the inhibition data (Figs 2 and 3), 3e conclude that both monoclonal antibodies recognize epitopes on a region of the protein surface at or near the factor VII binding site.

The results from experiments to characterize these monoclonal antibodies have already provided some information relevant to the properties of tissue factor. First, the appearance of the 85,000-dalton protein in polyacrylamide gel analysis of preparations from the immunoaffinity columns suggests that at least some of the tissue factor in detergent extracts may exist in a dimeric form stabilized by disulfide bonds. Whether this hypothesized higher mol wt form of tissue factor exists in nature or was created during purification remains to be determined. The 85,000-dalton band has been observed in previous tissue factor-affinity preparations. It copurifies with the 43,000-dalton tissue factor polypeptide on concanavalin A-Sepharose, and similar to tissue factor, is not digested by chymotrypsin. Since the 85,000-dalton protein is bound by tissue factor-specific polyclonal and monoclonal affinity columns, it may be antigenically related to the 43,000-dalton tissue factor monomer or tightly bound to it. Since the two molecules have been separated by gel filtration, the latter possibility seems unlikely. An as yet untested possibility is that the 85,000-dalton protein binds to the columns by a mechanism other than antigen–antibody association.

It should be noted that, because the assays to measure inhibition of tissue factor activity were performed in the presence of competing ligand (factor VII_a), the parameters (I_{max} and $K_{1/2}$) determined from the inhibition isotherms (Figs 2 and 3) are not equivalent to the equilibrium binding parameters (B_{max} and K_d) for the association of tissue factor and anti-tissue factor IgG. Despite this qualification, TF1-F7 and TF1-E2 were each able to block factor VII_a binding to tissue factor-containing vesicles and inhibit the procoagulant activity. These results show that the antigenic determinant(s) recognized by these antibodies are on the portion of tissue factor exposed outside of the phospholipid vesicles. Furthermore, the determinant(s) are probably at or near the factor VII binding site of tissue factor. These antibodies will clearly be useful for isolating and probing functional domains of tissue factor.

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