

## An Inhibitory Monoclonal Antibody Against Human Tissue Factor

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**We obtained a hybridoma using immune spleen cells from a mouse injected with human brain tissue factor that had been purified on a factor VII-agarose affinity column. This monoclonal IgG1, HTF1-7B8, inhibits tissue factor procoagulant activity. The concentration of HTF1-7B8 producing half-maximal inhibition is influenced by the concentration of factor VIIa, suggesting that the antibody and enzyme compete for the cofactor. The antibody was successfully used to detect both human and bovine tissue factor on nitrocellulose dot blots, indicating that the epitope recognized by this antibody is conserved in both species. This**

**antibody clearly reveals tissue factor on a Western blot. An HTF1-7B8 affinity column was used to purify tissue factor from both human brain and placenta. The electrophoretic mobilities in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and the amino acid compositions of the purified tissue factor from brain and placenta are indistinguishable, as are their specific procoagulant activities in reconstituted systems. This antibody will be useful for immunopurification and characterization of tissue factor structure and mechanism.**

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**T**ISSUE FACTOR (coagulation factor III, tissue thromboplastin) is the membrane glycoprotein that serves as the nonenzymatic cofactor for factor VII in the initiation of blood coagulation. Although pure tissue factor has been available from bovine sources for several years,<sup>1</sup> human tissue factor has only recently been purified.<sup>2,3</sup> This achievement was made possible by the discovery that immobilized human factor VII can serve as an affinity-matrix ligand for purification of detergent-solubilized tissue factor.<sup>2,3</sup> Affinity chromatography using factor VII as the coupled ligand is a highly specific protocol, but tens of liters of human plasma are required as a source of the factor VII necessary to construct the affinity column. Insofar as the immunoaffinity purification of bovine tissue factor provides a practical avenue for purification of this protein,<sup>1,4</sup> an antibody specific for human tissue factor is a highly desirable reagent. Using small quantities of human tissue factor purified by affinity chromatography on immobilized human factor VII, we successfully produced a monoclonal antibody that recognizes both brain and placental tissue factor.

### MATERIALS AND METHODS

Coagulation factors VII and X were purified and prepared for use in these experiments as previously described.<sup>5-7</sup> Bovine brain tissue factor was purified using monoclonal antibody TF1-F7,<sup>4</sup> whereas human tissue factor was isolated using factor VII-Affi Gel.<sup>2,3</sup> Tissue factor was reconstituted with phosphatidylserine and phosphatidylcholine (30/70) (Supelco, Bellefonte, PA) using octylglucoside followed by dialysis<sup>8</sup> or with mixed brain lipids using the deoxycholate-CdCl<sub>2</sub> protocol.<sup>9,10</sup> Tissue factor activity was measured with a two-stage clotting assay<sup>8,11</sup> or a continuous chromogenic rate assay,<sup>12,13</sup> using bovine factors VIIa and X.

Plasmacytoma cells P3-NSI-1-Ag4-1 (NS-1) were cultured as

previously described,<sup>14</sup> and NCTC 109 was included at 5% in the HY media. The following immunization protocol provided hyperimmune spleen cells from a single mouse, which were used to obtain hybridomas.<sup>15</sup> Human brain tissue factor (HBTF), 20 µg, was combined with 1 µg of cholera toxin (from Dr Alex Kurosky, University of Texas Medical Branch at Galveston), 210 µL of 0.9% NaCl and 200 µL of Freund's complete adjuvant (GIBCO, Grand Island, NY). The mouse received the entire inoculum intraperitoneally (IP) on day 1. Three weeks later, the animal received an IP booster containing 13 µg of HBTF in 200 µL of saline and 200 µL of Freund's incomplete adjuvant (GIBCO). On day 39, 7 µg of HBTF was administered intravenously (IV) followed by another IV boost of 7 µg on day 57. On day 60, the mouse was killed, and cells harvested from the spleen were fused with NS-1 cells using polyethylene glycol (PEG) 1540 (American Type Culture Collection).<sup>14</sup> Cells were plated and grown in selective medium as previously described.<sup>14</sup>

Culture supernatants were screened for antibodies recognizing TF using a multistep enzyme-linked immunosorbent assay (ELISA) similar to the one developed for use with bovine TF.<sup>4</sup> The wells of an Immulon II microtiter plate were sequentially incubated (interspersed with washes) with rabbit antibodies against mouse immunoglobulins (DAKO, Santa Barbara, CA), spent culture media from the supernatants to be tested, Tris-saline (0.05 mol/L of Tris, 0.1 mol/L of NaCl, 0.01% NaN<sub>3</sub>, pH 7.6) containing 10 mg/mL of bovine serum albumin (BSA), biotinyl-HBTF, and avidin-horseradish peroxidase conjugate. Bound peroxidase activity was detected using phenylenediamine.<sup>16</sup> The biotinyl-HBTF used was HBTF, oxidized with sodium metaperiodate, reacted with biotin-hydrazide (Sigma, St Louis), and reduced with sodium cyanoborohydride.<sup>17,18</sup> Avidin was conjugated to horseradish peroxidase (Sigma, type VI) using the two-step glutaraldehyde method.<sup>16</sup> Cultures that produced positive ELISA results (visible peroxidase product in the well) were expanded, cloned, and frozen.<sup>4,14</sup>

Tissue factor on nitrocellulose (Schleicher and Schuell, BA85) either as "dot blots" or Western blots<sup>19</sup> was detected with HTF1-7B8. After TF was bound, the nitrocellulose was blocked with 3% gelatin in Tris-saline. The blot was incubated overnight at room temperature with 1% gelatin, 0.05% Tween 20, and HTF1-7B8 (0.4 µg/mL) in Tris-saline. Following washes with 0.05% Tween 20 in Tris-saline, the blot was incubated with horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin (Bio-Rad, Richmond, CA, 1/2,000) in 1% gelatin, 0.05% Tween 20, Tris-saline for 2 hours. After further washing, the bound antibodies were developed using 4-chloro-1-naphthol.<sup>20</sup> No staining was observed when HTF1-7B8 was omitted from the sequence.

The capacity of HTF1-7B8 IgG to inhibit TF activity was ascertained using both coagulation and chromogenic assays. Increasing amounts of HTF1-7B8 IgG or a control IgG (RIg9C<sup>14</sup>) were added to a constant amount of human brain homogenate used as a TF "standard."<sup>11</sup> Activity was then quantitated in the presence of

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factor VIIa and factor X, using concentrations as described in the Results section. Curves were fit and equation parameters were estimated using the direct-plot method.<sup>21</sup>

Tissue factor was immunoaffinity purified using 22 mg of HTF1-7B8 IgG coupled to 50 mL of Affi-Gel 10 (Bio-Rad). Acetone powders of human placenta and brain were prepared and extracted with Tris-saline, 0.1% Triton X-100, and finally with 2% Triton X-100 essentially as reported,<sup>1,11,22</sup> using 10 mL of solution per gram of powder. Approximately 150 mL of extract was applied to the IgG-Affi-Gel, which was then washed with 5 column vol of 0.1% Triton X-100 in Tris-saline followed by 0.1% Triton X-100 in Tris-saline with 0.9 mol/L of NaCl. The TF was eluted with 0.1 mol/L of glycine, 0.1 mol/L of NaCl, 0.1% Triton X-100, pH 2.<sup>1,4</sup> Gel filtration on Ultragel AcA 44 (LKB) in Tris-saline with 0.1% Triton X-100 removed minor high-mol-wt proteins, providing TF that was homogeneous on polyacrylamide gels. The immunoaffinity column has been used repeatedly over a period of 6 months with no apparent loss of effectiveness. The amino acid compositions of the TF preparations were obtained by the Yale Protein Chemistry Facility following 16-hour hydrolysis in 6 N HCl containing 0.2% phenol at 115°C.

## RESULTS

Of the cultures that produced antibodies against HBTF as indicated by the ELISA, the 7B8 culture was most consistently positive, rapidly growing, and stable after cloning. Immunodiffusion against chain type-specific antibodies indicated that this hybridoma produced an IgG with  $\gamma_1$  and  $\kappa$  chains. This hybridoma, designated HTF1-7B8, produced ample ascites when grown in pristane-primed mice, and the IgG was easily purified by ammonium sulfate precipitation, chromatography on diethylaminoethanyl (DEAE) Sephacel and, as required, gel filtration on Sephacryl S-300 to remove transferrin.<sup>23,24</sup>

Having shown that HTF1-7B8 bound biotinyl-TF in the ELISA, we tested the purified IgG for effects on the activity of TF reconstituted with phospholipids. This antibody suppressed TF activity in both clotting and continuously monitored chromogenic assays. Kinetic effects of HTF1-7B8 IgG were ascertained using the chromogenic assay, with a 1/12,000 dilution of human brain homogenate used as "standard" tissue factor,<sup>11</sup> and varying concentrations of both the antibody and factor VIIa. As shown in Fig 1, TF activity was inhibited by added HTF1-7B8 IgG, and inhibition approached 100% as antibody exceeded 30 nmol/L. As shown in the inset in Fig 1, the maximum attainable inhibition was not affected by the concentration of factor VIIa, but the concentration of HTF1-7B8 IgG required to achieve half-maximal inhibition decreased as the factor VIIa concentration was reduced to <0.5 nmol/L.

HTF1-7B8 also recognized TF antigen on nitrocellulose. HBTF, purified by affinity chromatography on factor VII-Affi-Gel,<sup>3</sup> was subjected to electrophoresis in a 10% polyacrylamide gel after reduction and denaturation in sodium dodecyl sulfate (SDS).<sup>25</sup> The TF, electrophoretically transferred onto nitrocellulose, was immunochemically detected with HTF1-7B8 IgG (Fig 2). As in previous reports,<sup>2,4</sup> an antigen with electrophoretic mobility somewhat greater than that of the phosphorylase b mol-wt standard was also demonstrable. In "dot" blots, <1 ng of human TF was clearly

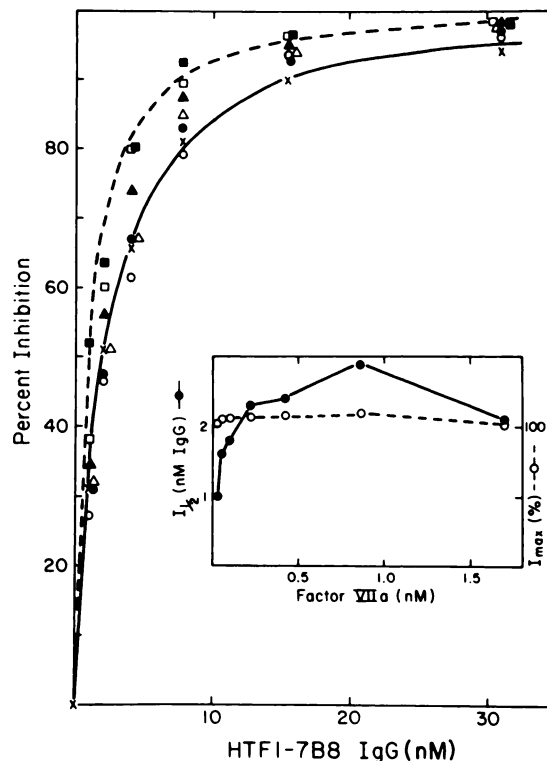
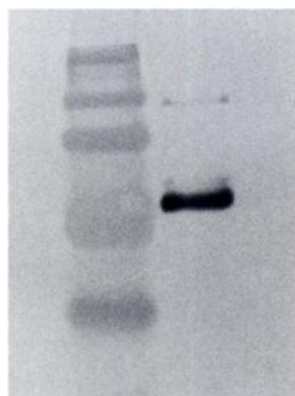


Fig 1. Inhibition of human brain tissue factor procoagulant activity by HTF1-7B8 IgG in the continuous chromogenic rate assay. Factor X was present in all assays at 100 nmol/L. Factor VIIa concentrations ranged from 26.7 pmol/L (■), to 1.71 nmol/L (X), with direct-plot fitted curves as indicated (---■--- and ---X---), respectively. Inset illustrates the effect of factor VIIa concentration on the maximum attainable inhibition (---○---) and on the concentration of HTF1-7B8 IgG required to achieve half-maximal inhibition (---●---) of factor X activation.

detectable by the immunochemical staining (Fig 3). This experiment also revealed immunologic reactivity of HTF1-7B8 IgG with bovine brain TF with an empirically estimated fourfold decrease in sensitivity relative to the reactivity with HBTF (Fig 3).

HTF1-7B8 IgG, coupled to Affi-Gel 10, was used to isolate TF factor from 2% Triton X-100 extracts of human brain and placenta. The immunoaffinity matrix routinely removed all detectable TF activity from the extracts, and homogeneous TF protein was recovered from the affinity matrix. From 150 mL of 2% Triton X-100 extract, 140 to 150  $\mu$ g of tissue factor was routinely obtained, representing an estimated recovery of 75%. The 10% polyacrylamide gel analysis of the brain and placental TF isolates confirmed their electrophoretic similarities (Fig 4).<sup>26</sup> This comparison also established that the immunoaffinity and factor VII affinity-purified tissue factors were electrophoretically indistinguishable. When reconstituted into lipid vesicles, the brain and placental tissue factor isolates exhibited similar specific activities in the clotting assay. One picogram of bovine brain TF, reconstituted into PS/PC (30/70) vesicles was defined as one unit.<sup>1,8</sup> This purified and reconstituted TF provided a log-log calibration curve of units v clot time for



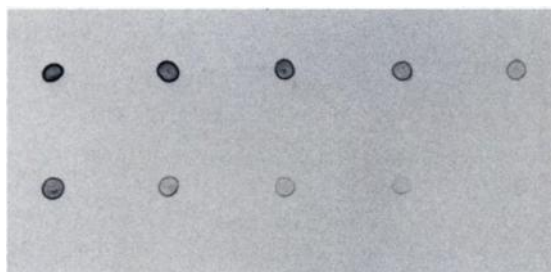
**Fig 2.** Western blot of factor VII affinity-purified human brain tissue factor using HTF1-7B8 as the immunochemical probe. The bands in the lane farthest left are the prestained electrophoretic standards (BRL), from top to bottom, myosin H chain (mol wt 200,000), phosphorylase b (mol wt 97,400), bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,000),  $\alpha$ -chymotrypsinogen (mol wt 25,700),  $\beta$ -lactoglobulin (mol wt 18,400), and lysozyme (mol wt 14,300).

the two-stage coagulation assay which was linear from 1,000 to 125 U/mL. When reconstituted into mixed brain lipids, both human brain and placental TF had specific activities of 0.8 U/pg. Both brain and placental TF reconstituted into PS/PC (30/70) using octylglucoside had specific activities of 1.0 U/pg.

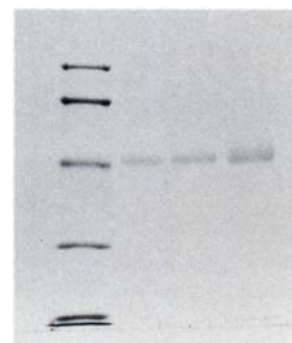
In further investigation of their similarities, the TF apoproteins from brain and placenta were subjected to acid hydrolysis and amino acid analysis. As shown in Table 1, brain and placental TF have indistinguishable amino acid compositions.

#### DISCUSSION

An IgG<sub>1</sub> monoclonal antibody has been produced that clearly recognizes human tissue factor. Unlike previous claims,<sup>27,28</sup> this monoclonal antibody binds human TF by multiple criteria. The antibody bound pure biotinyl-TF from solution in the ELISA screening assay. Conversely, pure TF immobilized on nitrocellulose bound HTF1-7B8 IgG from solution in both Western and dot-blot procedures. Significantly, HTF1-7B8 IgG immobilized on Affi-Gel 10 selec-



**Fig 3.** Immunochemical detection of brain tissue factor (TF) on a nitrocellulose dot-blot using HTF1-7B8. The upper row was spotted with human brain TF, and the lower row was spotted with bovine brain TF. From left, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, and 0.05 ng of protein were spotted in each dot in 1  $\mu$ L of Tris-saline with 0.1% Triton X-100.



**Fig 4.** Coomassie R-250-stained polyacrylamide gel (10%) following electrophoresis of purified tissue factor (TF) with sodium dodecyl sulfate (after reduction). The mol-wt standards (left lane) were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase (mol wt 31,000), soybean trypsin inhibitor (mol wt 21,500), and lysozyme. The other three lanes contained (left to right) 3.9  $\mu$ g of factor VII affinity-purified human brain TF, 4.5  $\mu$ g of HTF1-7B8 affinity-purified human brain TF, and 5.6  $\mu$ g of HTF1-7B8-affinity-purified human placental TF.

tively bound TF from among the myriad proteins of the Triton X-100 extract,<sup>26</sup> and enabled immunoaffinity purification of the apoprotein. Furthermore, and of least significance in proving the antibody specificity for TF, HTF1-7B8 IgG inhibits TF activity. In the aggregate, these results present compelling evidence for the specific recognition of TF by this monoclonal antibody.

Using immunoaffinity purification, sufficient quantities of human brain and placental TF have been isolated for partial characterization. Among the first direct results of these experiments, the antibody clearly confirmed the immunologic similarities of human brain and placental TF,<sup>26</sup> and demonstrated partial immunological similarity between human and bovine TF proteins. The electrophoretic mobilities of apoproteins isolated from brain and placenta were the

**Table 1. Amino Acid Composition of Tissue Factor**

Amino Acid	Composition (mol %)		
	Brain (2 Analyses)	Placenta (4 Analyses)	Broze and Colleagues <sup>2</sup>
Asx	10.48	10.93	11.50
Thr	9.00	9.77	9.28
Ser	7.95	7.24	8.28
Glx	11.77	11.93	11.65
Pro	4.99	4.81	4.48
Gly	7.83	6.49	7.75
Ala	5.13	5.09	5.05
½ Cys	ND	ND	0.78
Val	8.30	8.80	8.05
Met	0.79	0.64	0.80
Ile	4.25	4.26	3.95
Leu	7.39	7.56	6.90
Tyr	4.67	4.73	3.98
Phe	4.52	4.68	4.68
His	1.06	0.97	0.95
Lys	7.45	7.74	7.58
Arg	4.44	4.37	4.30
Trp	ND	ND	ND

Abbreviation: ND, not determined.

same on polyacrylamide gels in SDS, consistent with previous conclusions based solely on eluted and reconstituted activity.<sup>26</sup> Furthermore, the immunoaffinity purified and factor VII-affinity purified materials were indistinguishable. On the Western blot, brain TF apoprotein was localized at a position consistent with an estimated mol wt of 47,500, which is larger than bovine TF.<sup>1,3,4</sup> This conclusion is consistent with results of the polyacrylamide gel analysis, where brain and placental TF comigrate. The mol wt, estimated for reduced apoproteins, is less than that previously estimated using activity recovered from polyacrylamide gradient gels without prior reduction.<sup>26</sup> The high-mol-wt band detected near 96,000 mol wt on the Western blot remains to be characterized. A protein of similar apparent mol wt has been observed in preparations of factor VII-affinity purified human TF.<sup>2,3</sup> A protein of 85,000 mol wt has also been

observed in preparations of bovine TF and has been suggested to be a dimeric form of the apoprotein.<sup>1,4</sup> Contrary to one preliminary report,<sup>29</sup> the human brain and placental TF isolated in this study have similar specific activities in the two-stage coagulation assay whether relipidated using octylglucoside<sup>8</sup> or CdCl<sub>2</sub> and deoxycholate<sup>9</sup> reconstitution protocols. Finally, the amino acid compositions of brain and placental TF proteins are indistinguishable from one another, and are not notably different from the previously published amino acid composition of factor VII-purified HBTF.<sup>2</sup> From these data TF apoproteins from human brain and placenta appear to be essentially identical.

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