Monoclonal antibody X18A4 identifies an oncofetal fibronectin epitope distinct from the FDC-6 binding site

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OBJECTIVE: Oncofetal fibronectin reactive with antibody FDC-6 has been associated with trophoblastic implantation and chorion structural stability. Abnormal release of this fibronectin into cervical and vaginal secretions has identified patients at risk for preterm labor and delivery. The aim of this study was to determine whether trophoblast-derived oncofetal fibronectin contains other novel epitopes distinct from the FDC-6 binding site.

STUDY DESIGN: Antitrophoblast fibronectin hybridomas were generated and screened by comparative immunoassays. One specific monoclonal antibody, X18A4, was identified and compared with antibody FDC-6 by immunocytochemical and immunoblot analyses. Both antibodies were also evaluated in "sandwich"-type double monoclonal immunosorbent assays.

RESULTS: X18A4 and FDC-6 bind avidly and noncompetitively to distinct epitopes within oncofetal fibronectin. They exhibit similar immunohistochemical staining of the extracellular matrix within placental tissue, ovarian epithelial tumors, and cultured trophoblasts. However, in contrast to FDC-6, X18A4 has no detectable binding activity to human plasma fibronectin, and its binding to oncofetal fibronectin was unaffected by enzymatic deglycosylation. Immunoblot analyses of oncofetal fibronectin proteolytic digests suggest that X18A4 binds near or within the alternatively spliced type III connecting segment domain.

CONCLUSIONS: X18A4 identifies and binds with high affinity to a new epitope within oncofetal fibronectin, distinct from the FDC-6 binding site. Because X18A4 displays no detectable binding to plasma fibronectin, it could be used as an important adjunctive antibody for enhancing the specificity of clinically based oncofetal fibronectin diagnostic assays. (Am J Obstet Gynecol 1995;172:1526-36.)

Key words: Oncofetal fibronectin, monoclonal antibody, preterm labor detection.

Oncofetal fibronectin, a distinct class of adhesive extracellular matrix molecules, has recently been associated with the biologic processes of trophoblastic implantation, placentation, and chorionic membrane stability.[1-3] During pregnancy oncofetal fibronectin is normally found within the trophoblast-associated extracellular matrix of the uteroplacental junction and chorion[1-3] and in soluble form in amniotic fluid.[4] As such, detection of oncofetal fibronectin that is abnormally released into the cervix and vagina before 37 weeks of gestation has helped identify patients at high risk for preterm labor and delivery.[5-8]

The original identification of oncofetal fibronectins in pregnancy and tumor tissues was based on the isolation of a novel monoclonal antibody, FDC-6. Elegant studies by Matsuura and Hakomori[4] and Matsuura et al.[9, 10] have demonstrated that FDC-6 binds to a specific *O*-linked N-acetylgalactosaminylated hexapeptide epitope within the fibronectin type III connecting segment. This binding site, which requires both the peptide backbone and the carbohydrate moiety to generate the epitope, is not found in high abundance in normal adult fibronectins. Thus FDC-6 has been incorporated into a sensitive enzyme-linked immunosorbent assay for the clinical detection of cervicovaginal oncofetal fibronectin.[5] Recent evidence indicates that FDC-6 binds specifically to 1% to 4% of circulating plasma fibronectin from pregnant and nonpregnant women and males.[11] Therefore significant potential exists for obtaining false-positive readings in patients screened for cervicovaginal oncofetal fibronectin with FDC-6 alone.

Trophoblasts are likely to be a major cellular source of pregnancy-associated oncofetal fibronectin, both in vivo and in vitro. As such, we have hypothesized that a specific isoform of oncofetal fibronectin—trophouter-

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onectin—could function as a trophoblast-uterine "glue," or connecting protein, for implantation of the early conceptus and subsequent anchorage of the developing placenta to the uterine wall.[1-3] Moreover, oncofetal fibronectin production by cultured cytotrophoblasts is significantly stimulated in response to transforming growth factor-β.[12] Glucocorticoids inhibit oncofetal fibronectin production by trophoblasts, leading Guller et al.[13] to propose this observation as a significant factor contributing to the initiation of parturition.

Because oncofetal fibronectin appears to be a regulated, trophoblast-adhesive molecule throughout pregnancy, we wondered whether other distinct oncodevelopmental epitopes within fibronectin could be identified. To approach this question, we used trophoblast oncofetal fibronectin as an immunogen for generating murine-based hybridoma clones. By comparative immunoassay analyses, X18A4 was identified as one monoclonal antibody that, like FDC-6, binds avidly to oncofetal fibronectins. However, because X18A4 exhibits certain important binding properties distinct from FDC-6, we hypothesized that X18A4 could be used to enhance the specificity of clinical-based oncofetal fibronectin assays.

Methods

Cytotrophoblast culture and oncofetal fibronectin isolation. Immediately after delivery human cytotrophoblasts were purified from the placentas of uncomplicated term pregnancies by serial trypsin-deoxyribonuclease digestions and Percoll gradient centrifugation, as previously described by Kliman et al.[14] Yields of viable cytotrophoblasts ranged from 60 to 100 × 10^6 cells per 30 gm of starting placental tissue. The cytotrophoblasts were cultured in Dulbecco's modified Eagle's medium containing 25 mmol/L glucose and 25 mmol/L HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), supplemented with 4 mmol/L glutamine and 50 μg/ml gentamicin. The Dulbecco's modified Eagle's medium also contained 10% newborn human cord sera as a maximal stimulator of oncofetal fibronectin synthesis in vitro.[2] After 36 to 40 hours in serum-containing media the cells were washed thoroughly with serum-free Dulbecco's modified Eagle's medium and cultured for an additional 36 to 50 hours in serum-free Dulbecco's modified Eagle's medium. In spite of the lack of serum at this stage of the culture, the cells continued to synthesize and secrete abundant FDC-6–reactive fibronectin, with media concentrations of 10 to 15 μg/ml. Serum-free conditioned media from human HepG2 hepatoma cells and residual amniotic fluid after therapeutic amniocentesis for hydramnios were also used as a sources of oncofetal fibronectin. Fibronectin from conditioned media or amniotic fluid was purified by gelatin-Sepharose 4B chromatography (Pharmacia, Piscataway, N.J.).[15]

Immunization scheme and immunoassays. The identification of specific antioncofetal fibronectin antibodies used an approach incorporating in vivo suppression of the immune response to plasma fibronectin, and an in vitro boost of splenic lymphocytes with purified trophoblast oncofetal fibronectin. Six-week-old female Balb/c mice were immunized intraperitoneally with 20 μg of human plasma fibronectin; cyclophosphamide (100 mg/kg of body weight) was administered 2 days later[16] to suppress the immune response to plasma fibronectin. An alternating series of intraperitoneal and subcutaneous injections with trophoblast oncofetal fibronectin was initiated 1 week after cyclophosphamide immunosuppression. After four injections of oncofetal fibronectin (15 μg each, 2 weeks apart), one mouse was killed, the spleen was dissected, and splenocytes were perfused out. Erythrocytes were lysed by suspension in 0.14 mol/L ammonium chloride for 8 minutes at 4° C, and the splenocytes were incubated for 2 days in 0.1 μg/ml oncofetal fibronectin in hybridoma medium (72% Dulbecco's modified Eagle's medium, 4.5 gm/L glucose, 8% NCTC135, 20% fetal calf serum, with 4 mmol/L L-glutamine, 0.15 mg/ml oxaloacetate, 0.05 mg/ml pyruvate, 0.2 IU/ml insulin, 100 U/ml penicillin, and 100 U/ml streptomycin) supplemented with 20% Origen growth supplement (IGen, Rockville, Md.) and 20 U/ml interleukin-2.

The in vitro stimulated lymphocytes were fused with Sp2/0Ag14 myeloma cells according to the procedure of Lane et al.[17] Hybridomas were cultured in hybridoma medium supplemented with 10% P388 supernatant, 30 μg/ml carboxyethyl GABA (γ-amino-n-butyric acid), 0.1 mmol/L hypoxanthine, and 6 μmol/L azaserine. Hybridomas were fed at 1 week after fusion, and yellow supernatants from wells containing hybridomas were harvested and tested for reactivity to plasma and oncofetal fibronectin. Hybridomas from positive wells were cloned by limited dilution in hybridoma medium supplemented with 10% Origen or P388 supernatant, and positive subclones were identified, cultured, and frozen.

The initial comparative immunoassay screen with hybridoma supernatants used microtiter plates precoated with amniotic fluid oncofetal fibronectin, trophoblast oncofetal fibronectin, or human plasma fibronectin. Immunodetection of antibody binding was performed with a biotinylated antimouse secondary antibody, avidin, and biotinylated horseradish peroxidase, according to the manufacturer's instructions (ABC Vectastain, Vector Labs, Burlingame, Calif.). Hybridoma supernatants that demonstrated selective binding to oncofetal fibronectins were further analyzed by comparative Western immunoblots.

For immunoblot analyses samples were electrophoresed in 6% sodium dodecyl sulfate–polyacrylamide gels under reducing or nonreducing conditions. Gels

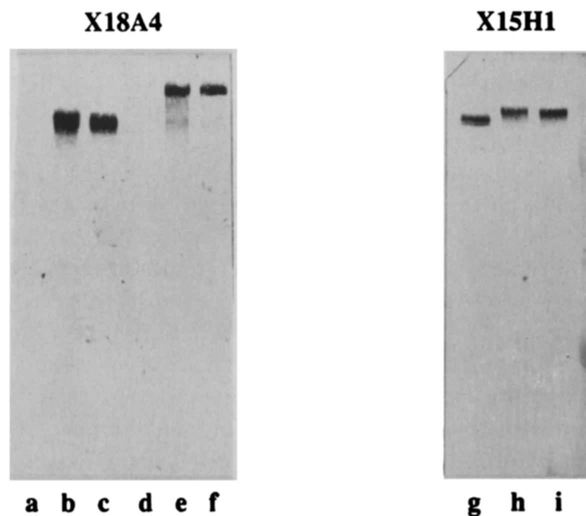

Fig. 1. Monoclonal antibody X18A4 does not bind to purified human plasma fibronectin (*lanes a* and *d*) but reacted strongly with 500 ng of purified amniotic fluid fibronectin (*lanes b* and *e*) and 500 ng of purified trophouteronectin (*lanes c* and *f*). Specificity of binding is apparent whether samples are electrophoresed in reducing (*lane a* through *c*) or nonreducing (*lanes d* through *f*) buffers. Monoclonal antibody X15H1, typical of the nonspecific antitrophoblast fibronectin antibodies, binds equally well to plasma fibronectin, amniotic fluid fibronectin, and trophouteronectin (*lanes g* through *i*). X184A also binds to single moiety when whole amniotic fluid is analyzed by Western blot (not shown).

were electrotransferred to nitrocellulose (Schleicher and Schuell, Keene, N.H.) overnight. Blots were incubated with FDC-6 or X18A4 hybridoma supernatants at concentrations of 1 to 3 μg/ml. Immunodetection was carried out with ABC Vectastain kits (Vector Labs) and 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis) as the chromogen.

Double monoclonal FDC-6 and X18A4 immunoassay. Enzyme-linked immunosorbent sandwich assays were carried out with 96-well plates precoated with an immunoglobulin G fraction of murine ascites containing FDC-6, kindly provided by Adeza Biomedical, Sunnyvale, California. Known standards of oncofetal fibronectin were used as the antigen. Immunodetection was performed with X18A4 and a biotinylated secondary antibody specific for the murine immunoglobulin μ chain, using the Vectastain protocol. Graphic analysis was carried out with CricketGraph 1.3.2 for the Macintosh (Computer Associates, Garden City, N.Y.), with R^2 values >0.98.

Immunocytochemistry. Sections of frozen placental or metastatic ovarian carcinoma tissue 5 μm thick were placed on glass slides (Probe-On, Fisher Scientific), fixed with cold acetone for 15 minutes, and stored in a −20° C freezer until use. SPA-26 cells, a first-trimester immortalized trophoblast cell line (gift of Dr. Janice Chou, National Institutes of Health)[18] were cultured on glass coverslips and fixed with 2% to 3% formalin for 15 minutes. Primary antibodies FDC-6 (American Type Culture Collection, Bethesda, Md.) and X18A4 were diluted from hybridoma supernatants and were used at antibody concentrations of 1 to 3 μg/ml. Control slides, which yielded negative staining, were incubated with undiluted ATCC P3X63Ag8 mouse myeloma cell line supernatant. Immunodetection with Vectastain ABC and 3,3-diaminobenzidine, followed by counterstaining with hematoxylin, were carried out as recommended by the manufacturer (Vector Labs).

Protease and deglycosylation reactions. Purified hepG2 hepatoma oncofetal fibronectin was digested with 1.25 U/ml cathepsin D (Sigma) or 2.5 U/ml trypsin (Sigma) in a buffer containing 50 mmol/L Tris–hydrochloric acid, pH 9.0, 2.0 mol/L urea, 20 μmol/L dithiothreitol. Hepatoma oncofetal fibronectin was also treated with 1 to 10 U/ml glycopeptidase F (Sigma), which released N-linked carbohydrate moieties, in a buffer containing 0.25 mol/L sodium phosphate, pH 8.6. De-*O*-glycosylation was carried out by preincubation with 1.0 U/ml sialidase for 1 hour, followed by addition of 30 mU/ml of endo-α-N-acetylgalactosaminidase (O-Glycanase, Genzyme, Boston) in a buffer containing 50 mmol/L sodium acetate and 1 mmol/L calcium chloride, pH 6.0, and 37° C overnight. Control samples for all enzymatic reactions were incubated in the appropriate buffers in the absence of enzyme. Digestion products were analyzed by Western immunoblots.

Results

X18A4 isolation and binding specificity. Of the 1337 hybridoma clones screened, 13 (1%) demonstrated some specificity of binding to oncofetal fibronectin and were subcloned. Of these, three clonal supernatants—X18A4, X20C4, and X8E3—exhibited reproducible and specific binding to amniotic fluid and trophoblast oncofetal fibronectin, but not plasma fibronectin, on enzyme-linked immunosorbent assays. Fig. 1 demonstrates the specific binding activity of X18A4 to oncofetal fibronectin by Western immunoblot under both reducing and nonreducing conditions. Clone X15H1 is an example of the many nonspecific antifibronectin clones identified in the screening that bind equally to plasma and oncofetal fibronectin. Of the three specific clones identified, X18A4 exhibited stronger binding for oncofetal fibronectin at much lower antibody concentrations than did X20C4 or X8E3. For immunoassays X18A4 maintained high sensitivity at antibody concentrations of 0.5 to 2 μg/ml, whereas X20C4 and X8E3 binding typically required ≥20 μg/ml of antibody. Antibody X18A4 is an immunoglobulin M molecule, whereas X20C4 and X8E3 are immunoglobulin G antibodies, as determined by isotyping immunoassays using immunoglobulin class-specific

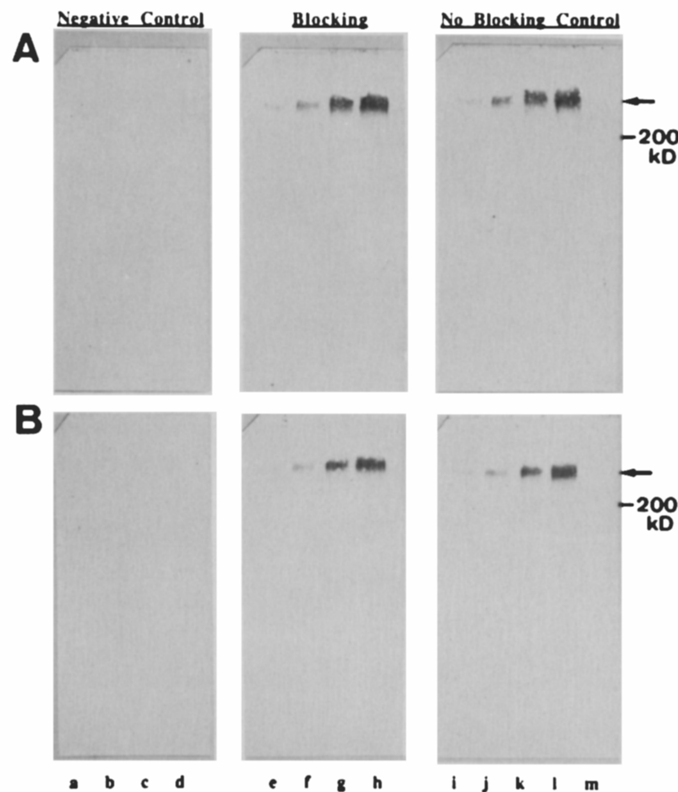

Fig. 2. Monoclonal antibody X18A4 does not compete with FDC-6 for binding to oncofetal fibronectin. **A,** Blots were preincubated with X18A4 (*lanes a* through *h*) or control diluent (*lanes i* through *m*) in parallel, then reacted with diluent (*lanes a* through *d*) or FDC-6 (*lanes e* through *m*). Blots in **A** were then developed with biotinylated anti–γ-chain–specific antibody to detect FDC-6 but not X18A4 binding. Preincubation with X18A4 had no demonstrable blocking effect on FDC-6 binding. **B,** Blots were preincubated with FDC-6 (*lanes a* through *h*) or diluent (*lanes i* through *m*), then reacted with diluent (*lanes a* through *d*) or X18A4 (*lanes e* through *m*). Blots in **B** were then developed with a biotinylated anti–μ-chain–specific antibody to detect X18A4 but not FDC-6. Preincubation with FDC-6 does not block or diminish X18A4 binding to oncofetal fibronectin. Samples loaded: oncofetal fibronectin 50 ng (*lanes a, e,* and *i*), 100 ng (*lanes b, f,* and *j*), 250 ng (*lanes c, g,* and *k*), 500 ng (*lanes d, h,* and *l*); plasma fibronectin 500 ng (*lane m*).

antimouse antibodies (Boehringer-Mannheim, Vector Labs).

X18A4 and FDC-6 do not compete for binding to oncofetal fibronectin. Two previously published attempts to detect specific moieties associated with oncofetal fibronectin, but not plasma fibronectin, have led to identification of only the FDC-6 epitope.[4, 19] Therefore we wished to determine whether X18A4 and FDC-6 bind to the same or different epitopes within oncofetal fibronectin. Competitive binding studies with different dilutions of X18A4 and FDC-6 hybridoma supernatants were carried out with Western immunoblots containing 50 to 500 ng of purified oncofetal fibronectin. As shown in Fig. 2, *A,* preincubation of the immunoblots with a tenfold higher concentration of X18A4 (*A,* lanes e through h) did not compete with or inhibit FDC-6 binding compared with the control blot not preincubated with X18A4 (*A,* lanes i through l). The negative control (*A,* lanes a through d) was also incubated with X18A4, and no signal is present because the biotinylated secondary antibody used in *A* (anti–immunoglobulin G) is directed specifically against FDC-6. We assayed up to 35-fold higher X18A4 antibody concentrations and found no blocking of FDC-6 binding.

Conversely, FDC-6 preincubation had no demonstrable effect on X18A4 binding (Fig, 2, *B,* lanes e through h) compared with the control blot analyzed in parallel (lanes i through l). The negative control blot in *B* was also incubated with FDC-6, but no signal is seen because the secondary anti–immunoglobulin M antibody reacts only with X18A4. We assayed up to 100-fold higher FDC-6 antibody concentrations and found an absence of blocking activity for X18A4 binding. Neither FDC-6 nor X18A4 demonstrate detectable binding to 500 ng of human plasma fibronectin (*A* and *B,* lane m).

The lack of competitive inhibition in this analysis suggests that X18A4 and FDC-6 bind to distinct epitopes within the oncofetal fibronectin molecule. Fur-

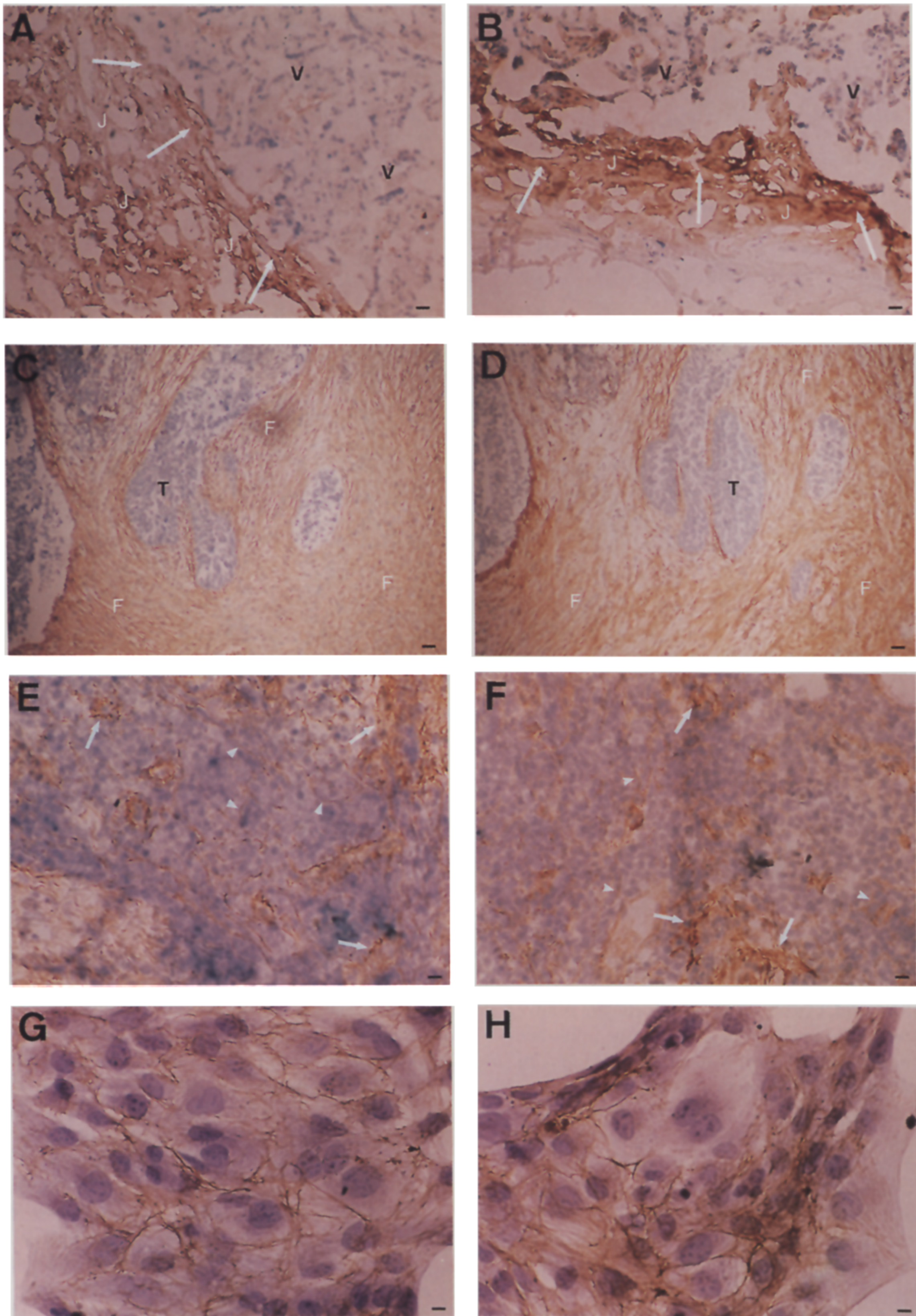

Fig. 3. For legend see opposite page.

ther evidence for independent binding to different epitopes is based on our ability to use X18A4 and FDC-6 in double monoclonal "sandwich"-type immunosorbent assays (see Fig. 8). Interestingly, X18A4 preincubation with immunoblots containing oncofetal fibronectin blocked both X20C4 and X8E3 antibody binding (not shown), suggesting that these three antibodies recognize a closely associated or identical epitope.

Comparative immunochemistry. Immunohistochemical analysis of the placental-decidual junction from a normal term pregnancy (Fig. 3, *A* and *B*) revealed prominent extracellular staining with both antibodies. As previously described for FDC-6,[1, 2] X18A4 did not stain fibronectin within the villous mesenchymal core or in fetal blood vessel walls. The two antibodies yielded an identical staining pattern within parallel sections from a metastatic implant of ovarian adenocarcinoma, demonstrating strong staining around some epithelial tumor nests (*C* and *D*) and fine fibrillar staining within others (*E* and *F*). SPA-26 cells, a first-trimester trophoblast line immortalized by SV40 transfection,[18] also produced abundant FDC-6 and X18A4 reactive extracellular fibronectin fibrils (*G* and *H*).

Enzyme digest analysis. To further compare the X18A4 and FDC-6 binding sites,[4] oncofetal fibronectin was digested with cathepsin D or trypsin and assayed by Western immunoblot. As shown in Fig. 4, X18A4 reacted with a broad doublet of approximately 90 and 110 kd generated by cathepsin D digestion, similar to FDC-6 reactive fragments analyzed in parallel. These fragments correspond to the major carboxy terminus regions of the fibronectin monomer and include the type III connecting segment domain.[4] Similarly, trypsin digestion yielded several discrete bands in the 55 to 65 kd range, reactive with both FDC-6 and X18A4 (Fig. 5). As described by Matsuura and Hakomori,[4] the size diversity of these bands corresponds closely to the different lengths of alternatively spliced type III connecting segment peptide backbone represented in the tryptic fragments.

Interestingly, two smaller trypsin-generated subunits with molecular weights of 47 and 40 kd displayed binding to X18A4 but not to FDC-6. These bands, which appeared after more prolonged proteolysis, correspond closely in size to tryptic fragments containing the carboxy terminus fibrin binding domain and small portions of type III connecting segment, as described by Sekiguchi et al.[20] and Humphries et al.[21] FDC-6 would not be expected to bind to these fragments, because the FDC-6 epitope is located toward the amino terminus side of the type III connecting segment, approximately 60 to 90 amino acids away from the fibrin-binding domain. Thus tryptic digest mapping provides evidence that X18A4 binds to an epitope near or within the carboxy terminus portion of the type III connecting segment peptide region.

To determine whether the X18A4 epitope is associated with *O*-glycosylation, oncofetal fibronectin was treated enzymatically with neuraminidase and endo-α-N-acetylgalactosaminidase (*O*-glycanase). Although FDC-6 binding was sensitive to enzymatic de-*O*-glycosylation (Fig. 6, *B*), X18A4 binding was not affected (Fig. 6, *A*). Enzymatic deglycosylation of N-linked carbohydrate residues also had no effect on X18A4 binding (Fig. 6, *C*). Therefore the X18A4 epitope appears to differ significantly from the FDC-6 binding site by its insensitivity to sequential neuraminidase/O-Glycanase deglycosylation.

Comparative binding to human plasma fibronectin. We have recently found that FDC-6 binds 1% to 4% of circulating plasma fibronectin from normal males and from pregnant or nonpregnant females,[11] suggesting that low but clinically significant levels of FDC-6–reactive fibronectin may be produced by normal adult tissues. Interestingly, X18A4 differs significantly from FDC-6 by exhibiting no detectable binding to plasma fibronectin on Western immunoblot (Fig. 7) or enzyme-linked immunosorbent assay (not shown). Thus, for the clinical determination of oncofetal fibronectin in settings where blood or plasma may be present, X18A4 potentially provides greater detection specificity.

Combined FDC-6 and X18A4 immunoassay. As one potential modality to improve the specificity of oncofetal fibronectin assays we wished to determine whether FDC-6 and X18A4 could be used in a combination

Fig. 3. Comparative immunocytochemical analysis with antibodies X18A4 (**A, C, E,** and **G**) and FDC-6 (**B, D, F,** and **H**). **A** and **B**, Immunostaining of placental-decidual junction *(white arrows)* demonstrated prominent extracellular staining surrounding junctional anchoring trophoblasts *(J)*, with negative placental villous *(V)* staining. **C** and **D**, Prominent fibrillar staining *(F)* surrounding tumor nests *(T)* within parallel sections of metastatic peritoneal implant of ovarian adenocarcinoma. **E** and **F**, Delicate fibrillar staining within certain tumor nests revealed very thin fibronectin fibrils *(arrowheads)*, along with more developed, thicker areas of fibronectin *(arrows)*. **G** and **H**, SPA-26 cells, a first-trimester nonmalignant trophoblast cell line, produce and deposit abundant X18A4– and FDC-6–reactive exracellular fibronectin fibrils. *Bars* represent 20 μm (**A** and **B**), 40 μm (**C** and **D**), or 10 μm (**E** through **H**).

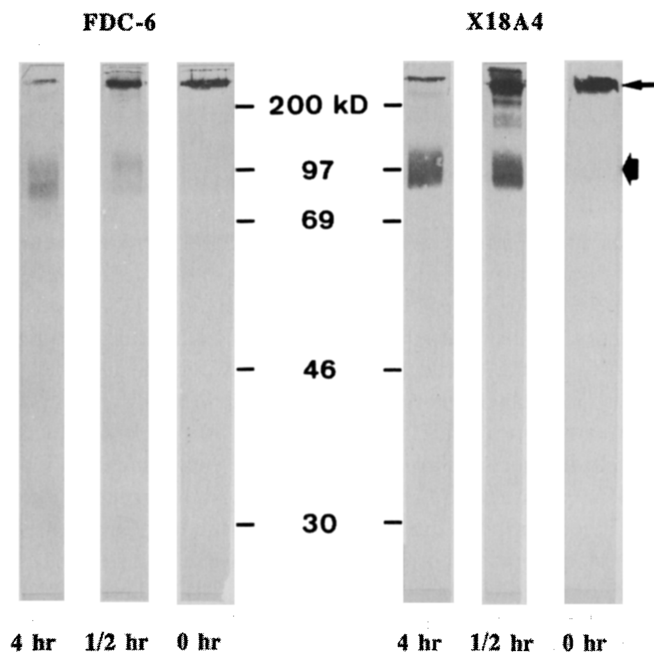

Fig. 4. Cathepsin D digestion of purified hepG2 cell oncofetal fibronectin (5 μg) demonstrated similar pattern of digestion fragments with FDC-6 or X18A4. After 4 hours of digestion both antibodies reacted with a doublet of 90 and 100 kd *(arrow)*.

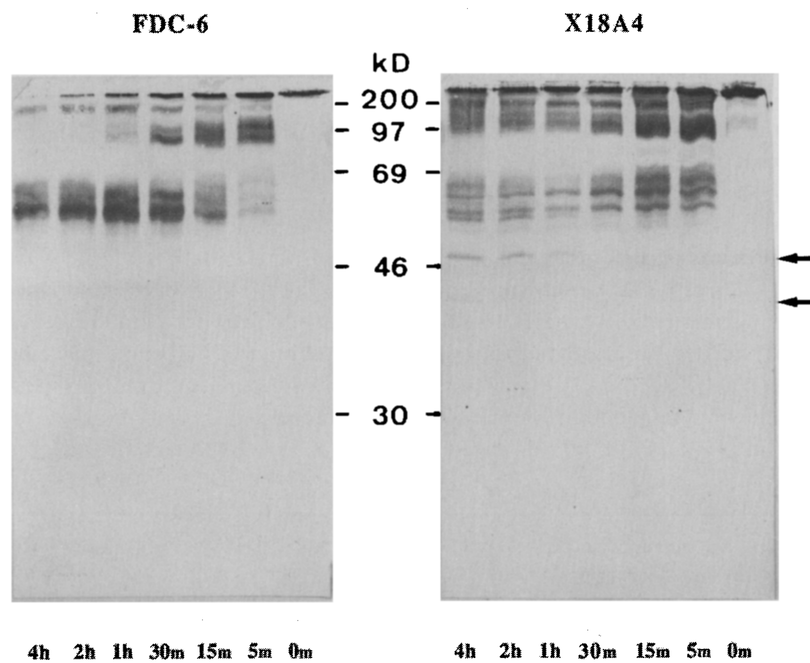

Fig. 5. Trypsin digestion of hep G2 cell oncofetal fibronectin (10 μg) over 4 hours yielded multiple bands in 55 to 65 kd range reactive with both FDC-6 and X18A4. As previously described, size diversity of these bands corresponds closely to different lengths of type 3 connecting segment peptide backbone represented in tryptic fragments.[4] Two smaller 47 and 40 kd fragments *(arrows)* bind to X18A4 but not to FDC-6.

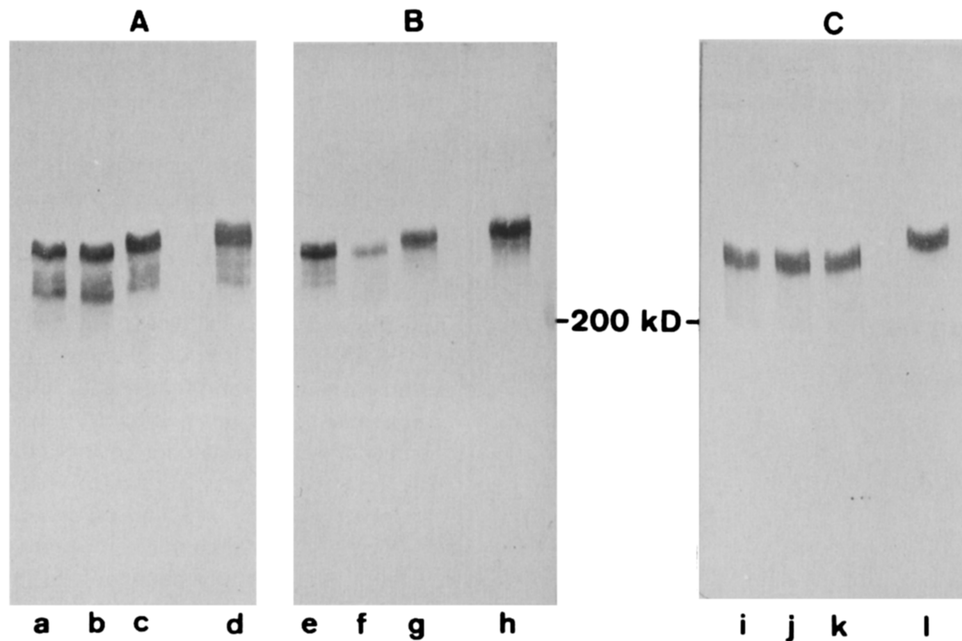

Fig. 6. Glycosidase digestion of oncofetal fibronectin. **A** and **B**, Oncofetal fibronectin (10 μg) was treated enzymatically with neuraminidase alone (*lanes a* and *e*), serial digestions with neuraminidase and endo-α-N-acetylgalactosamindase (*O*-Glycanase) (*lanes b* and *f*), *O*-glycanase alone (*lanes c* and *g*), or no enzyme control (*lanes d* and *h*). In **A** X18A4 binding to de-*O*-glycosylated oncofetal fibronectin (*lane b*) was similar to control (*lane d*), whereas in **B** FDC-6 binding to de-*O*-glycosylated oncofetal fibronectin (*lane f*) was significantly reduced compared with control (*lane h*). **C,** X18A4 binding to hep G2 oncofetal fibronectin (3.0 μg) was not affected by deglycosylation with N-glycanase (*lane i,* 1.0 mU/ml; *lane j,* 5.0 mU/ml; *lane k,* 10 mU/ml) compared with no enzyme control (*lane l*). FDC-6 yielded identical binding pattern (not shown). Slight shifts in electrophoretic mobility on immunoblot indicate effectiveness of deglycosylation reactions.

"sandwich" immunoassay. X18A4 was substituted for the polyclonal antifibronectin antibody in commercially obtained 96-well plates precoated with FDC-6. As shown in Fig. 8, a standard curve with increasing quantities of oncofetal fibronectin was generated, whereas plasma fibronectin had no activity in this assay. As a control, the immunoassay was carried out in parallel with the polyclonal "reporter" antibody, yielding a curve identical to the manufacturer's product insert. Both FDC-6 and X18A4 could also be used together when the position of the antibodies was reversed (i.e., when X18A4 was used as the "capture" antibody on the bottom of the sandwich and FDC-6 was used as the "reporter" antibody on top) (not shown). The ability to use X18A4 and FDC-6 together in this type of sandwich immunoassay provides further evidence that the two monoclonal antibodies bind to distinct epitopes within oncofetal fibronectin.

Comment

Molecular characterization of oncofetal fibronectin has ramifications for the understanding of normal and aberrant implantation processes, the pathophysiolgic mechanisms of preterm labor, and perhaps oncogenesis. To date, much work has focused on monoclonal antibody FDC-6, which originally identified and defined the oncofetal class of fibronectins as distinct from normal adult tissue and plasma fibronectins.[4, 10, 11] In pregnancy tissue oncofetal fibronectin is found primarily within the trophoblast-associated extracellular matrix of the chorion and uteroplacental junction and in amniotic fluid.[1-4] Concomitant with these findings, a clinical assay of FDC-6–reactive cervicovaginal fibronectin has been used as a novel modality for identifying patients at potential risk for preterm labor.[5-8]

We hypothesized that trophoblast oncofetal fibronectin, in addition to its FDC-6 binding site, might contain other interesting molecular regions not previously identified. To determine whether this was so, we speculated that new antibodies could be raised as probes to localize novel regions within this fibronectin class. Therefore oncofetal fibronectin purified from trophoblast-conditioned media was used as an immunogen to generate a series of antifibronectin monoclonal antibodies. Inter-

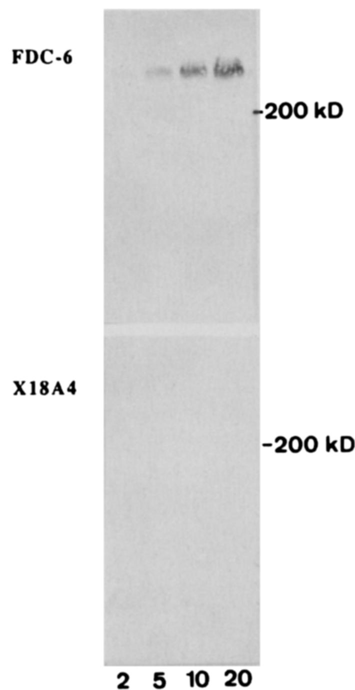

Fig. 7. FDC-6 binds quantitatively to increasing amounts (2 to 20 μg) of purified human plasma fibronectin. In parallel immunoblot X18A4 has no detectable binding to plasma fibronectin.

estingly, the comparative antibody screen we used identified very few specific antibodies, suggesting that the number of novel immunogeneic motifs within trophoblast oncofetal fibronectin was restricted. Although three specific antibodies were identified by the comparative screen, X18A4 exhibited much higher binding activity than did X20C4 or X8E3. Furthermore, X18A4 blocked the binding of X20C4 or X8E3 to oncofetal fibronectin, suggesting that these three antibodies recognize a similar or identical epitope.

Several lines of evidence suggest that X18A4 and FDC-6 recognize distinct epitopes. In Western immunoblots designed to assay competitive binding activity neither antibody was capable of blocking the other. In double monoclonal sandwich-type immunoassays both antibodies were used concomitantly to detect and quantitate oncofetal fibronectin. Immunoblot analyses with protease digests of oncofetal fibronectin suggest that the X18A4 epitope may be located near or within the alternatively spliced type III connecting segment region. In contrast to FDC-6, the X18A4 epitope is not sensitive to deglycosylation by neuraminidase and *O*-glycanase. However, this finding does not eliminate the possibility that glycosylation or other posttranslational modifications contribute to the X18A4 binding site. The requirement for a novel structural motif in this epitope seems necessary, in view of the fact that plasma fibronectin contains the type III connecting segment peptide backbone[22] yet is nonreactive with X18A4.

In spite of binding independently to distinct fibronectin epitopes, the two antibodies behaved very similarly on immunohistochemical analysis. Both antibodies specifically stained the fibronectin matrix of the placental-decidual junction but not the fibronectin of the villous mesenchymal core or fetal vessel walls. The dense fibronectin matrix of a metastatic implant derived from an epithelial ovarian malignancy was strikingly positive with both antibodies, as were the less prominent and delicate fibronectin fibrils interspersed within some tumor nests. Interestingly, the pattern of oncofetal fibronectin staining we have observed in ovarian carcinomas is very similar to that described by Mandel et al.[19] in oral squamous cell carcinomas. SPA-26 cells, a first-trimester nonmalignant trophoblast cell line,[18] also produce abundant X18A4 and FDC-6 immunoreactive fibronectin fibrils.

The presence of novel epitopes such as the FDC-6 and X18A4 binding sites within trophoblast-derived fibronectin may help explain the unique function of this adhesive glycoprotein during prengancy-related processes. For example, the FDC-6 epitope is located just nine amino acids from the tripeptide Leu-Asp-Val (L-D-V), a major α_4 integrin (VLA-4)–mediated cell-binding site within the fibronectin type III connecting segment region.[22] Glycosylation and alternatively spliced moieties confer conformational alterations in the fibronectin molecule,[23] thus potentially influencing integrin-mediated cellular interactions and fibronectin matrix assembly. Oncofetal fibronectin, reactive with both FDC-6 and X18A4, has recently been identified immunocytochemically within the blastomeres and zona pellucida of early human embryos.[24] Interaction of embryos with fibronectin appears to enhance their in vitro development and the expression of metalloproteinases implicated in implantation.[24] The presence of fibronectin receptor proteins such as the α_4 integrin within the endometrium during the purported secretory phase "implantation window"[25] provides appealing evidence for the significance of embryonic oncofetal fibronectin during the adhesive phases of human implantation.

One striking contrast between FDC-6 and X18A4 is the binding activity they manifest for normal adult plasma fibronectin. Although FDC-6 binds to 1% to 4% of circulating plasma fibronectin,[11] X18A4 has no detectable binding. Thus X18A4 may be a more specific antibody probe than FDC-6 for identifying trophoblast-derived and other oncofetal fibronectins. This difference may have important clinical ramifications. Diagnostic assays of cervicovaginal FDC-6 reactive fibronectin have a significant rate of false-positive results,[5, 7, 8] perhaps related, in part, to overt or occult bleeding and

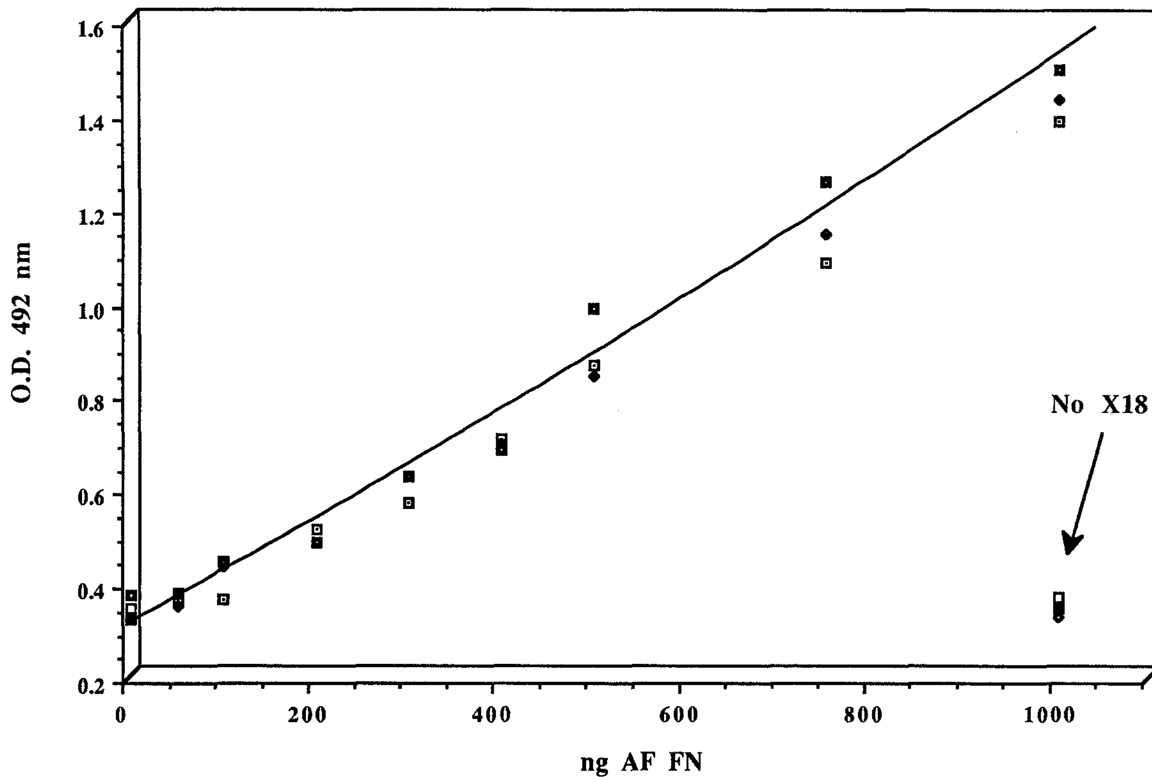

Fig. 8. In this double monoclonal "sandwich"-type immunosorbent assay X18A4 reacted quantitatively with increasing amounts of purified amniotic fluid *(AF)* oncofetal fibronectin *(FN)* but had no binding activity to plasma fibronectin. Each *symbol* represents average of triplicate determinations. For this assay commercially available microtiter plates precoated with FDC-6 were used. *O.D.*, Optical density.

the presence of plasma fibronectin. Because X18A4 exhibits equivalent sensitivity to FDC-6 on Western immunoblots and enzyme-linked immunosorbent assays, studies are underway to compare the specificity of these antibodies for cervicovaginal fibronectin detection and subsequent risk of preterm labor.

In summary, our findings indicate that oncofetal fibronectins contain at least one additional novel epitope—defined by antibody X18A4—which is distinct from the FDC-6 binding site. Furthermore, our results suggest that the number of unique epitopes within trophoblast oncofetal fibronectin are relatively restricted. Future studies will attempt to precisely characterize the molecular structure and function of the X18A4 epitope and to explore the antibody's utility for detecting trophoblast and tumor-associated oncofetal fibronectin.

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