Production and Characterization of a Monoclonal Antibody to Human Type IV Collagen

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We have produced a monoclonal antibody to human basement membrane Type IV collagen. The antibody reacts with the pepsin-resistant, collagenase-sensitive domain of Type IV collagen isolated from placental membranes, but not with human collagens of Types I, II, III, V, 1α, 2α, and 3α. The antibody precipitates biosynthetically labeled human Type IV procollagen, and the precipitate contains both the α1(IV) and α2(IV) chains, suggesting the occurrence of both of these chains within the same triple-helical molecule. When used in indirect immunofluorescence, the antibody gives brilliant staining of basement membranes from a variety of human tissues but does not stain tissues of bovine, canine, rabbit, rat, or mouse origin. It is suggested that this antibody will be of value in research on the structure of human basement membrane collagen, on the distribution of this collagen in various basement membranes, and particularly for the study of basement membranes in normal human development and pathologic processes. (Am J Pathol 1982, 108:310–318)

THE TERM COLLAGEN presently encompasses at least seven genetically distinct, but similar molecules, and there is good evidence for the existence of at least four additional different molecular varieties of collagen.[1-4] Each collagen molecule is composed of three polypeptide subunits called alpha chains, and each type of collagen molecule is constructed from a unique combination of structurally distinct alpha chains. It is generally believed that primary structural differences between these collagens specify the manner in which collagen molecules of a single type interact with each other, with other collagen types, and with noncollagen macromolecules. The large variety of biomechanical properties demonstrated by the various connective tissues is probably a direct consequence of such interactions.

Basement membranes are an example of a highly specialized connective tissue. In addition to their role as supporting structures, basement membranes also function as semipermeable filters, serve as a boundary between different cell types or between cell layers and subjacent interstitial connective tissues, provide scaffolds for tissue repair and remodeling, and may play critical roles in tissue morphogenesis and differentiation. In a number of human diseases, ultrastructural and functional alterations of basement membrane(s) have been observed, notably various glomerulonephritides, disseminated lupus erythematosus, diabetes mellitus, and the Alport and Goodpasture syndromes.[5-7]

Type IV collagen is the main collagenous component of basement membrane.[8] It is composed of at least two different alpha chain subunits termed α1(IV) and α2(IV).* These may be components of the

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* Nomenclature: By present convention,[1] the individual collagen types are indicated by roman numerals I, II, etc.,

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same or different molecules.[1,11-13] Based on recent studies, a model for the structure of Type IV collagen is proposed.[14] Three distinguishable domains of the molecule are proposed: a large central triple-helical region flanked by a nonhelical globular region at one end, and by a triple-helical "7S" domain[15,16] at the other end. The "7S" is thought to be the major site of intermolecular crosslinking to other Type IV molecules.[14-16]

It is presently unclear whether collagen types other than Type IV, such as Type V, are also present in basement membranes.[17] The noncollagen components of basement membranes include fibronectin, laminin, and proteoglycans.[18,19] The modes of interactions between these various components are the subject of substantial current investigations.

Ultrastructural localization of the individual basement membrane components is a first approach to understanding the complex sets of interactions responsible for basement membrane structure, and analysis of the disordered distributions of these components in disease offers an approach to the pathogenesis and pathophysiology of disorders affecting basement membranes. The production of specific immunologic probes have proven effective for localization of the collagen components of several systems.[20-26] However, due to weak immunogenicity, as well as to structural similarities between the different collagen types, large amounts of antiserums with the desired monospecificity have been difficult to produce by conventional immunologic techniques.[22,26] For this reason, it is highly desirable to construct hybridomas producing monoclonal antibodies to different collagen types. Hybridoma technology has been successfully applied to several collagen types.[28-31]

In this communication, we describe the properties of an antibody specific for the triple-helical domain of human Type IV collagen. The antibody reacts also with Type IV procollagen isolated from *in vitro* cell culture but does not react with the triple-helical portions of any of the six other types of collagen tested. The antibody specifically stains basement membranes of human tissues by indirect immunofluorescence, but not those of bovine, canine, rabbit, rat, or mouse.

We anticipate this antibody to be useful for microlocalization studies of Type IV collagen in both normal and abnormal basement membranes, for studies of the distribution and interrelationships of basement membrane components, and for structural studies of Type IV collagen.

Materials and Methods

Preparation of Human Collagens

Type IV collagen was isolated by salt precipitation from pepsin digests of human chorioamniotic membranes.[2] Following neutralization of the pepsin digest with a saturated solution of TRIS† base to pH 8.6, this suspension was clarified by centrifugation, NaCl solution was added to 1.2 M, and the solution was acidified with CH_3COOH to pH 3.5. The resulting precipitate was harvested by centrifugation, dissolved in 1 M NaCl, 50 mM TRIS-HCl, pH 7.5, at 4 C, and dialyzed against 2.7 M NaCl, 50 mM TRIS-HCl, pH 7.5, at 4 C. The resulting precipitate, containing collagen Types I, III, and IV was redissolved in TRIS-buffered 1 M NaCl and dialyzed against 0.01 M Na_2HPO_4. The precipitate formed was removed by centrifugation, and the Type IV collagen in the supernatant solution was precipitated by dialysis against distilled water. The Type IV collagen precipitate was redissolved in 0.5 M CH_3COOH and lyophilized. This preparation contained collagenous peptides with relative molecular weights of 180 K, 160 K, 140 K, and 70 K, as well as very high molecular weight aggregates by SDS-polyacrylamide gel electrophoresis after disulfide reduction with 2-mercaptoethanol (Figure 1). Total amino acid compositions were consistent with basement membrane-like collagen (data not shown). Human collagens Types I, II, III, V, 1α, 2α, and 3α were prepared as previously reported.[2,3]

Preparation of Hybrid Cell Lines and Hybridoma Antibodies

Hybridomas were produced according to the method of Kohler and Milstein[32] with the modifications described.[33,34] SJL/J mice (Jackson Laboratory, Bar Harbor, Maine) were primed with an intraperitoneal injection of 50 μg of native Type IV collagen in PBS

and their subunit α chains are indicated by Arabic numerals. For example, Type V collagen contains three α chains, previously designated A, B, and C, and now termed α1(V), α2(V), and α3(V), respectively. The terms 1α, 2α, and 3α describe three collagen chains found in cartilage[3,9,10] which have not yet been assigned a type designation.

† TRIS, tris (hydroxymethyl)-aminomethane; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIA, radioimmunoassay; Tween 20, polyoxyethylene (20) sorbitan-monolaurate (trademark of the J. T. Baker Chemical Company); PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PVC, polyvinylchloride.

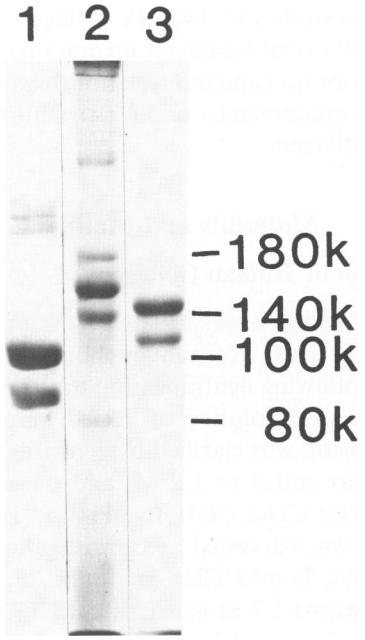

Figure 1—SDS-polyacrylamide gel electrophoretogram of human Type IV collagen, the antigen (lane 2), human bone Type I collagen (lane 1), and human amniotic Type V collagen (lane 3). Samples were disulfide-reduced by incubation with 2-mercaptoethanol prior to electrophoresis. Much of the antigen is covalently associated, even following disulfide reduction. The major peptides demonstrate mobilities consistent with relative molecular weights of approximately 180,000, 160,000, 140,000, and 80,000.

homogenized with an equal volume of complete Freund's adjuvant. Two weeks later, on 3 consecutive days, injections of 200, 400, and 400 μg of Type IV collagen in PBS (without adjuvant) were administered, and the spleens were removed on the fourth day.[35] Single cell suspensions of approximately 10^8 splenic lymphocytes were mixed with 5×10^7 NS-1 (P3-NS1/1-AG4-1) myeloma cells, a nonsecreting variant of the P3-X63-AG8 mouse myeloma line[36] (a gift from Dr. R. Dulbecco with permission of Dr. C. Milstein). The cells were fused in 1 ml of 50% polyethylene glycol 1500 (BDH Chemicals, Ltd., Poole, England) in serum-free Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) and gradually diluted with 15 volumes of serum-free medium over 8 minutes. Hybrid cells were selected in HAT medium (DMEM supplemented with 10% fetal calf serum and 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) in 96-well tissue culture plates for 2 weeks. Culture supernates were assayed for anti-Type IV collagen antibody production with the use of a solid-phase RIA. Cultures of interest were fed with HT medium (HAT without added aminopterin), and then were grown and cloned by limiting dilution in complete Dulbecco's medium. For large-scale antibody production, approximately 10^7 hybridoma cells were injected intraperitoneally into athymic nude mice (a gift from Dr. William Benedict); and after approximately 3 weeks, the antibody-rich ascites fluid was collected. Alternatively, antibody was precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation to the spent culture media.

Assays

Solid-Phase Radioimmunoassays

Purified triple-helical collagens were dissolved in 20 mM Na_2CO_3 (pH 9–10), and 100 μl of a 10 μg/ml solution was dispensed into PVC microtiter wells (Cooke 1-22-24N, Cooke Laboratory Products, Alexandria, Va) and incubated overnight at 4 C. After extensive washing with PBS containing 0.05% Tween 20 for removal of unadsorbed collagen, the plates were used for assay as follows: individual culture supernates were added in 100-μl aliquots to duplicate wells and incubated at room temperature for 2 hours and then washed three times with PBS–Tween 20 buffer. For most experiments, a 1/500 dilution of rabbit anti-mouse IgG (Miles Laboratories, Elkhart, Ind) was added to each well and incubated for 1 hour, followed by three additional washes. Subsequently, 100 μl aliquots of ^{125}I-labeled Protein A were added to each well and incubated for 60 minutes at room temperature. After six washes to remove unbound Protein A, the wells were punched out and their content of ^{125}I was measured. Later assays were conducted similarly, but with the omission of the rabbit anti-mouse IgG step. In all assays, both positive (mouse antiserums to the relevant antigen) and negative (buffer only) controls were included.

For demonstration that antibody was directed against collagen, coated wells were digested with 28 units of Clostridial collagenase (Form III, 2800 units/ml, Advance Biofactures, Lynbrook, NY) in 50 mM TRIS-HCl, pH 7.4, containing 10^{-3} M N-ethylmaleimide and 10^{-3} M $CaCl_2$ for 30 minutes at 30 C. This enzyme has previously been shown to be devoid of nonspecific proteolytic activity.[2] After extensive washing, antibody binding to these wells was tested as described above. Antibodies were also tested in plates coated with heat-denatured collagen as described above.

The ability of various collagens to inhibit the binding of the hybridoma antibody to Type IV collagen was tested. A $1:5 \times 10^5$ dilution of antibody was preincubated with 0 to 10 μg of Type IV collagen, or 10 μg collagen Types I, II, III, V, and the minor cartilage collagens, for 18 hours before testing for binding to Type IV coated plates as described above. The re-

sults are expressed as [1-(cpm bound at "X" ng inhibitor/cpm bound without inhibitor)] × 100.

Antibody Isotyping

Ouchterlony double diffusion gels were prepared with 0.7% agarose, 4.0% polyethylene glycol 1500 in 0.1 M Na_2HPO_4 on glass microscope slides. Antiserums specific for mouse IgG_1, IgG_{2A}, IgG_{2B}, IgG_3, and IgM and for kappa and lambda light chains were used (Miles Laboratories, Elkhart, Ind) to test concentrated spent culture medium.

Immunoisolation of Type IV Collagen

Immunoprecipitation of human Type IV procollagen was performed by Dr. Lisa Fessler. ^{3}H-Leucine containing Type IV procollagen was isolated after metabolic labeling of SV40 DNA-transformed human umbilical cord endothelial cells in culture as previously described.[37] The radiolabeled Type IV procollagen was purified from the culture medium by 45% $(NH_4)_2SO_4$ precipitation, and the collagen fraction was isolated from the precipitate by sucrose gradient velocity sedimentation. Forty microliters of this radiolabeled Type IV collagen was incubated either with 1 ml of anti-Type IV antibody-containing culture medium, with 0.3 ml of this medium plus 0.7 ml of PBS, or with 1 ml of PBS alone as control. The mixtures were incubated for 18 hours at 7 C. Following the incubation, 150 μl of Pansorbin (Calbiochem-Behring Corp., La Jolla, Calif) were added and incubated for 30 minutes at room temperature. The supernatant solution was removed by centrifugation. The pellet was suspended in and washed with 50 mM TRIS-HCl, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100. Bound proteins were extracted from the Pansorbin with 50 μl of 2% SDS, 2 M urea, buffered to pH 7.5 with TRIS-HCl and containing 10% glycerol and 1 mM dithiothreitol, and analyzed by 4.5% polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE).[38] Radiolabeled proteins were subsequently visualized by fluorography[39] and scanned with the use of an Ortec densitometer.

Immunofluorescence

Deep-frozen tissues were cut in 5-μm sections, air-dried on glass slides, and fixed in cold acetone. Hybridoma culture fluids were buffered by the addition of 1/10 volume of 0.15 M phosphate buffer, pH 7.2. Samples were incubated on the fixed tissue sections for 2 hours. Tissue-bound antibody was detected with the use of fluoroscein-labeled goat anti-mouse IgG (1 : 40 dilution, Cappel Laboratories, Cochranville, Pa). The slides were mounted in buffered glycerol and examined in an Olympus inverted microscope with fluorescent attachment.

Table 1 — Bacterial Collagenase Inhibition of Binding (CPM)*

Antibody source	(−) Collagenase	(+) Collagenase
Hybridoma supernate #117-3	6234	751
Hybridoma supernate #124-20	840	850
Culture medium alone	872	708
Immune mouse serum (1:10)	44,688	52,342
Nonimmune mouse serum	6757	6242

* Type IV collagen-coated plates were incubated with bacterial collagenase (Methods) and then used to assay antibody binding. For this experiment, culture supernates from actively growing hybridomas were used without concentration: 117-3 producing antihuman Type IV antibody; 124-20 producing IgG antibody not directed against Type IV, and culture media including fetal calf serum but not exposed to hybridoma cells.

Results and Dicussion

Production of Monoclonal Antibody

The fusion mixture from one spleen was plated into a total of 500 microtiter wells. Hybrid growth was observed in 14 wells, suggesting that these hybrids might be monoclonal from the initial growth dilution. RIA indicated that supernates from two of these wells were strongly positive for antibody against the immunizing antigen. Only one of these positive wells (designated 117-3) was sensitive to specific collagense digestion or heat-denaturation (see Table 1). This culture was cloned by limiting dilution into 300 microtiter wells. Growth was observed in 44 of these wells, and the medium from all 44 was positive for reactivity with the immunizing antigen, indicating the monoclonality of the original culture. A vigorously growing subculture from the cloning experiment producing a high titer of antibody was selected and, after expansion, was injected intraperitoneally into athymic nude mice for ascites production. The antibody was determined to be IgG_{2B} (kappa light chain) by Ouchterlony double diffusion from ammonium sulfate-concentrated media and exhibited a titer of $1:5 \times 10^5$ against Type IV collagen in solid-phase RIA (Figure 2).

Specificity of Hybridoma Antibody

Preincubation of Type IV collagen-coated plates with Clostridial collagenase abolished the binding of

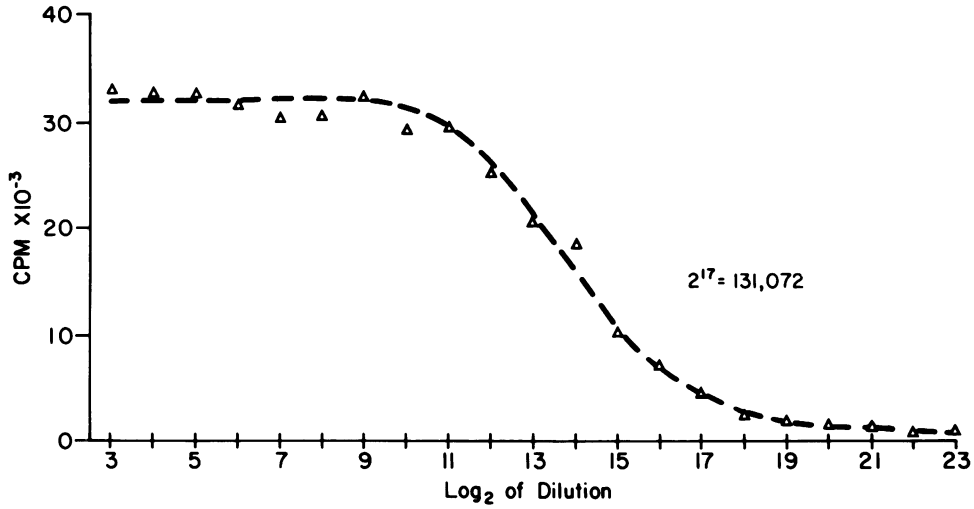

Figure 2—Titration curve of serial twofold dilutions of hybridoma supernatant IgG binding to native human Type IV collagen by solid-phase radioimmunoassay. For this experiment, the cell medium from hybridomas producing anti-IV monoclonal antibody was concentrated tenfold by precipitation with ammonium sulfate. The data have not been corrected for a background of 750 cpm.

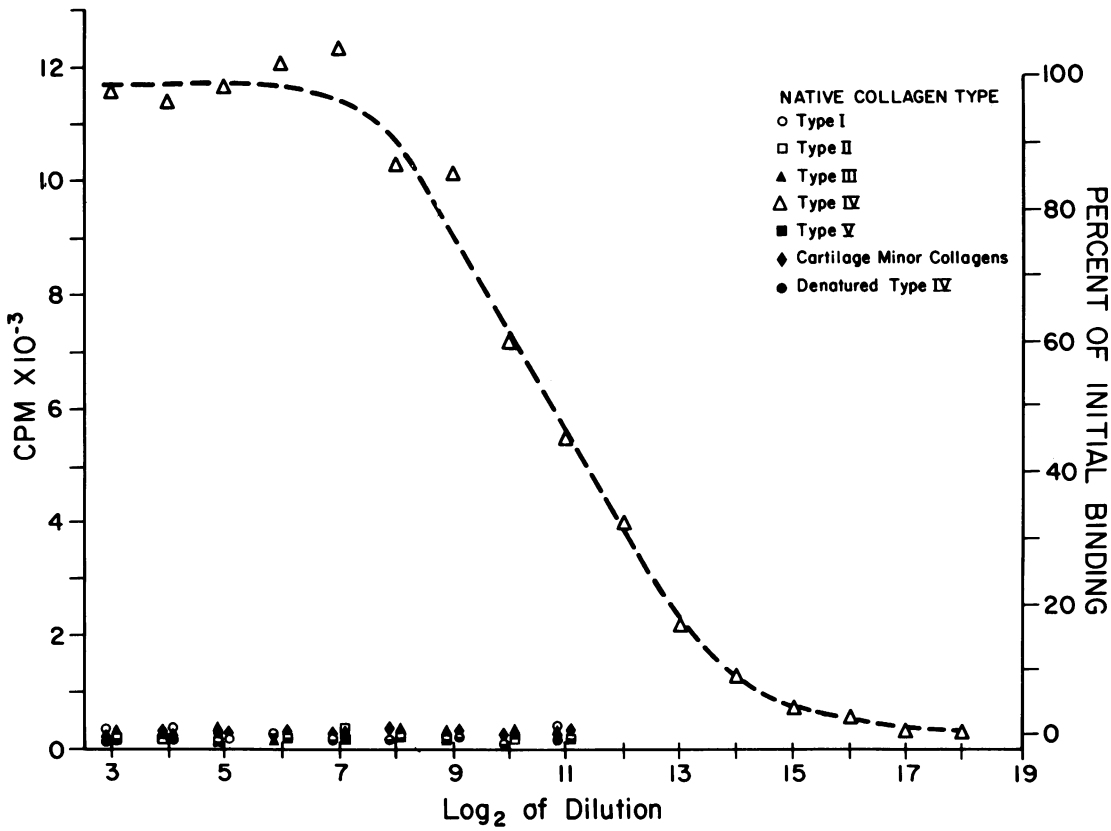

Figure 3—Solid-phase radioimmunoassay for cross-reactivity of antihuman Type IV monoclonal antibody against other native human collagens. Concentrated hybridoma supernate was used as the source of the monoclonal antibody. For this experiment, antibody binding was amplified by incubation of the antibody-antigen complex with rabbit antimouse IgG (1:500 dilution) for 1 hour prior to incubation with ^{125}I-labeled Protein A. O, Type I; □, Type II; ▲, Type III; △, Type IV; ■, Type V; ◆, 1α, 2α, and 3α cartilage collagens; ●, heat-denatured Type IV. Mouse antiserums to each of these collagens were used as positive controls. The data have not been corrected for a background of 200 cpm. The different level of plateau binding in this figure, as compared with Figure 2, is due solely to the use of ^{125}I-labeled Protein A of a different specific activity.

hybridoma antibody to the plates. Table 1 summarizes one such experiment. These data show that binding of supernate monoclonal antibody to plates coated with Type IV collagen does not take place after treatment of the coated plates with collagenase. In contrast, antibody binding from immune mouse antiserum is essentially unaffected, or is slightly improved, and hybridoma culture supernates from a culture not producing anti-Type IV antibody demonstrated no binding beyond background. The interesting lack of inhibition of binding of immune mouse antiserums following collagenase digestion is susceptible to several interpretations. First, the major serum antibody response to Type IV collagen may be against regions of the molecule not susceptible to collagenase digestion. It is, in fact, now clear that the major antigenic determinant(s) of Type IV collagen in terms of serum antibody response reside in the 7S domain, and this domain is not degraded by collagenase.[40] Alternatively, this observation may suggest that the major antibody response is directed toward noncollagen contaminants in the immunizing and test preparations of Type IV collagen; this possibility is consistent with previous studies of monoclonal antibody directed against human Type II collagen which indicated that a substantial fraction of immune serum antibody binding is insensitive to removal of the triple-helical portion of the antigen and is, therefore, presumably directed toward noncollagen contaminants.[31]

The hybridoma antibody was assayed for cross-reactivity to other native human collagen types with the use of the solid-phase RIA procedure. PVC plates were coated individually with human native collagen Types I, II, III, IV, and V, respectively. Native collagens containing the 1α, 2α, and 3α chains (minor cartilage collagens) were coated as a mixture to a single plate, since they cannot presently be separated in the native state. In addition to the native collagens, heat-denatured Type IV was separately adsorbed to PVC plates. Figure 3 depicts the results of one cross-reactivity assay. No cross-reactivity was observed between the Type IV antibody and any native collagen other than Type IV, or with denatured Type IV collagen. This observation indicates that the monoclonal antibody is conformationally specific for one or more sites within the triple-helical domain of Type IV collagen and is monospecific for Type IV collagen. In concert with the previous collagenase susceptibility data, it is also clear that the antibody is directed toward a collagenase-sensitive triple-helical portion of the Type IV molecule.

Table 2—Human Type IV Collagen Inhibition of Binding

Type IV concentration (ng)	% Inhibition
10,000.0	100
1000.0	100
500.0	100
100.0	92.9
50.0	85.7
10.0	64.3
5.0	50.0
1.0	14.3
0.1	0.0
0	0.0

A 1:5 × 10^5 dilution of hybridoma antibody was preincubated with the indicated amount of Type IV collagen. The percentage of inhibition of binding in the solid-phase radioimmune assay is expressed as [1-(cpm bound at "X" ng Type IV)/cpm bound without Type IV collagen)] × 100. One hundred percent binding represents 2300 cpm; a background of 90 cpm was subtracted.

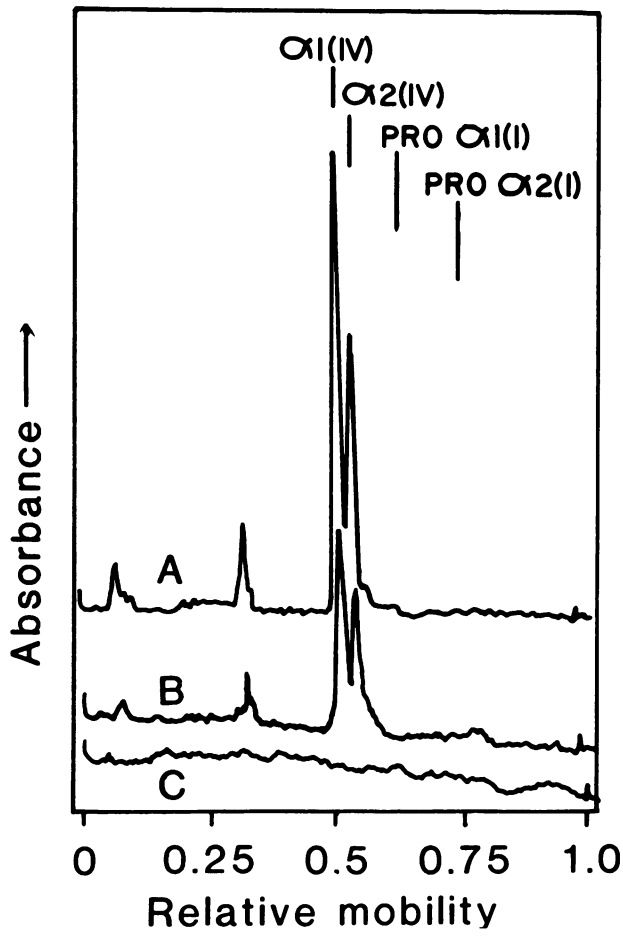

Figure 4—Densitometer tracing of a 4.5% SDS-PAGE fluorogram of ^{3}H-leucine containing human Type IV procollagen precipitated by the addition of (**A**) 1 ml, (**B**) 0.3 ml, or (**C**) 0 ml of concentrated hybridoma supernatant containing anti-Type IV monoclonal antibody and Pansorbin. The electrophoretic positions of pro-α1(I) and pro-α2(I) are indicated. The integrated areas under the peaks are (**A**) α1(IV) 128.7 and α2(IV) 67.6 and (**B**) α1(IV) 50.3 and α2(IV) 24.4

Incubation of the hybridoma antibody with Type IV collagen inhibits the binding of the antibody to Type-IV-coated plates. Preincubation of a 1 : 5 × 10^5

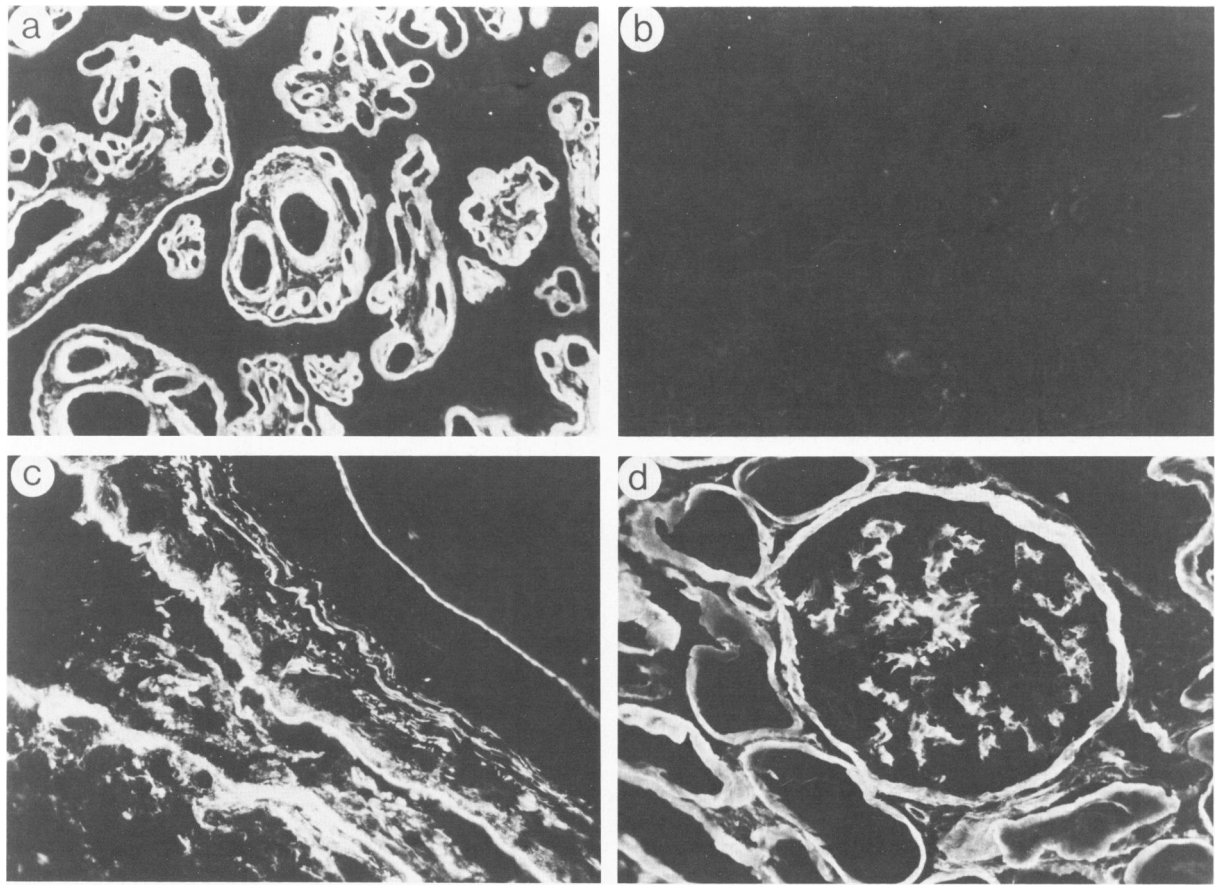

Figure 5—Indirect immunofluorescent staining of sections of placenta (**a** and **b**), chorioamniotic membranes (**c**), and kidney cortex (**d**) with monoclonal antibody to Type IV collagen (**a, c,** and **d**) and an antibody of irrelevant specificity (**b**). (×314)

dilution of hybridoma antibody with 0 to 10 ng native Type IV collagen demonstrates that Type IV collagen can inhibit antibody binding in the solid-phase assay (Table 2). Five nanograms of Type IV collagen inhibits the binding by 50%. One hundred percent inhibition is achieved by preincubation of the antibody with 500 ng of Type IV collagen. Preincubation of the antibody with 10 μg of collagen Types I, II, III, V, or the minor cartilage collagens did not affect the percent binding. These results indicate that the antibody is specific for a site on Type IV collagen, and this site, which might be masked by binding of the various collagens to test plates, is not present in other collagen types.

Type IV collagen can be recovered from hybridoma antibody–antigen complexes. ^{3}H-Leucine-labeled human Type IV procollagen was incubated at two concentrations with anti-Type IV hybridoma supernate and with PBS alone. The resultant immune complexes were absorbed onto Pansorbin, recovered by centrifugation, and resolubilized. The immunoreactive, labeled collagen was visualized by fluorography after SDS-PAGE as shown in Figure 4. No Type IV was precipitated without the addition of hybridoma cell supernate. Both pro-α1(IV) and pro-α2(IV) were recovered from the immune complex. This is the expected observation if both chains occur in a single triple helix with the chain composition [pro-α1(IV)]$_2$ pro-α2(IV), or if two molecules with the chain compositions [pro-α1(IV)]$_3$ and [pro α2(IV)]$_3$ coexist in a single supramolecular aggregate, or if the [pro-α1(IV)]$_3$ and [pro-α2(IV)]$_3$ molecules share an antigenic determinant recognized by the monoclonal antibody. The observed excess of pro-α1(IV) over pro-α2(IV) in the immune precipitate and lack of cross-reactivity with other human collagens suggest that the antigen has the composition [pro-α1(IV)]$_2$ pro-α2(IV), and this molecular species represents a significant portion of the total Type IV molecules.

Indirect Immunofluorescence Using Hybridoma Antibody

Brilliant, crisp staining of basement membranes in human tissues was seen when the monoclonal antibody to Type IV collagen was employed in indirect

immunofluorescence (Figure 5). All basement membranes were specifically stained, including, to date, basement membranes in lung (alveolar and vascular), kidney (glomerulus, tubular, and vascular), skin (epidermal-dermal, adnexal, and vascular), and placenta (vascular). Figure 4 shows the observed patterns of staining of placental vascular basement membranes and of renal glomerular and tubular and vascular basement membranes. Other monoclonal antibodies not directed to basement membrane antigens did not produce basement membrane staining. Notable, in addition to the bright, crisp staining of basement membranes, is the lack of background staining of other structures. These results confirm previous studies using conventional or purified antiserums to type IV collagen which have indicated that all basement membranes share common antigenic determinant(s), presumably due to the common occurrence of Type IV collagen in all studied basement membranes.[17,41-46]

The antibody to human Type IV collagen did not stain tissue sections derived from bovine, canine, rabbit, rat, or mouse tissue. It therefore appears that the antibody, in contrast to conventional or purified antiserums, exhibits considerable species specificity and is specific for human Type IV collagen.

The intensity and sensitivity of the staining reaction seen with this antibody, together with the documented absence of cross-reactivity with other human collagens, suggests that it will be a valuable probe in the study of human basement membranes and their component antigens in health and disease.

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