Domain and Basement Membrane Specificity of a Monoclonal Antibody against Chicken Type IV Collagen

JOHN M. FITCH, EILEEN GIBNEY, RALPH D. SANDERSON, RICHARD MAYNE, and THOMAS F. LINSENMAYER
Developmental Biology Laboratory, Massachusetts General Hospital, Boston, Massachusetts 02114; Departments of Medicine and Anatomy, Harvard Medical School, Boston, Massachusetts 02114; and Department of Anatomy, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294

ABSTRACT   A monoclonal antibody, IV-IA8, generated against chicken type IV collagen has been characterized and shown to bind specifically to a conformational-dependent site within a major, triple helical domain of the type IV molecule. Immunohistochemical localization of the antigenic determinant with IV-IA8 revealed that the basement membranes of a variety of chick tissues were stained but that the basement membrane of the corneal epithelium showed little, if any, staining. Thus, basement membranes may differ in their content of type IV collagen, or in the way in which it is assembled. The specificity of the antibody was determined by inhibition ELISA using purified collagen types I-V and three purified molecular domains of chick type IV collagen ([F1]$_2$F2, F3, and 7S) as inhibitors. Only unfractionated type IV collagen and the (F1)$_2$F2 domain bound the antibody. Antibody binding was destroyed by thermal denaturation of the collagen, the loss occurring at a temperature similar to that at which previous optical rotatory dispersion studies had shown melting of the triple helical structure of (F1)$_2$F2. Such domain-specific monoclonal antibodies should prove to be useful probes in studies involving immunological dissection of the type IV collagen molecule, its assembly within basement membranes, and changes in its distribution during normal development and in disease.

Basement membranes, a class of extracellular matrices which separate epithelia from connective tissues and surround some types of cells such as smooth and skeletal muscle, have been implicated in mediating cell and tissue interactions during embryonic development, tissue repair, and regeneration (for reviews, see references 1 and 2). Evaluation of the proposed roles for these structures requires specific and sensitive probes for identification, localization, and manipulation of their various molecular components. One such probe is the monoclonal antibody (3–5), which can be selected not only for specificity to an individual molecular component, but for specificity to an individual domain within a molecule.

Type IV collagen is a major component of many basement membranes. Recent work on the mouse EHS-sarcoma (6), chicken gizzard (7), human placenta (8), and bovine lens capsule (9) have provided some general information about the structure of the molecule (reviewed in references 10 and 11). The native type IV collagen molecule is composed of three chains (each ~180,000 mol wt) representing at least two differ-

ent gene products. These are assembled so that each molecule contains one or more triple-helical regions (collagenous domains) and at least two regions in which the chains are not folded into the triple-helical conformation (noncollagenous domains). A model has been proposed to explain how type IV molecules may be arranged as supramolecular aggregates within basement membranes (12, 13). Its salient features have been inferred from specific enzymatic digestions coupled with electron microscopic observations of rotary shadowed preparations. The model proposes that four molecules are arranged so that a specific collagenous domain at or near one end of each of them overlaps with the same domain of the other three. The overlap region (termed 7S in reference to its sedimentation coefficient when isolated) is stabilized by multiple interchain disulfide bonds. It is highly resistant to enzymatic digestion and has a very high melting temperature (14), both of which are substantially decreased by disulfide reduction. Extending from the 7S domain are long triple-helical domains which are probably interrupted repeatedly by noncollagenous sequences

(15, 16). Rotary shadowing observations of acid-extracted type IV collagen from a mouse tumor (13) suggest that each collagenous chain terminates distally in a noncollagenous globular domain (termed NC-1) which may associate with the same domain on other molecules to form an interlocking collagen lattice.

From type IV collagen extracted from chicken gizzard and kidney by pepsinization, Mayne and co-workers (7, 17) have isolated and identified two other domains in addition to 7S. The generation of these domains results from the presence of an additional pepsin-sensitive site that appears to be absent from mammalian type IV collagen. One of these domains, termed F3, is largely triple-helical, contains interchain disulfide bonds, and may be close to 7S. The other, termed $(F1)_2F2$, is also largely triple-helical but contains no interchain disulfide bonds. This latter region constitutes >90% of type IV collagen preparations extracted from chicken gizzard by limited pepsin digestion.

Many questions are still unanswered. Do all basement membranes contain the same type IV collagen, or does type IV constitute a class of separate gene products with tissue specific distributions? Is type IV collagen universally distributed in basement membranes or do some of them lack this molecule? How are the molecules assembled? Do the different structural domains of type IV interact independently with other molecular components of basement membranes, such as the recently characterized basement membrane proteoglycans (18) and glycoproteins (19, 20)? Do any of the domains of type IV serve as "informational macromolecules" capable of influencing cell and tissue behavior during development or tissue repair?

We think that many of these questions can be approached most directly through the use of monoclonal antibodies specific for the different regions of type IV collagen. In this initial study we describe a monoclonal antibody which specifically binds to a conformational-dependent determinant located within the $(F1)_2F2$ domain of type IV collagen extracted from chick gizzard. In addition, we demonstrate by immunofluorescence histochemistry that most basement membranes react strongly with the antibody, demonstrating the presence of the antigenic determinant; several others, however, stained very lightly, if at all.

MATERIALS AND METHODS

Preparation of Collagens

Chick collagen types III, IV, and V were obtained by limited pepsin extraction of adult chicken gizzard or kidney and separated by fractional salt precipitations as previously described (7, 21). Some preparations were further purified by ion-exchange chromatography on carboxymethyl-cellulose (CM-cellulose) or diethylaminoethyl-cellulose (DEAE cellulose) columns run under nondenaturing conditions (7, 21). Type I collagen was an acid extract of lathyritic chick skin, and type II was a pepsin extract of adult chicken sterna purified as previously described (5). The antigen consisted of type IV collagen that had been passed over CM-cellulose in native conditions (7). Characterization of the antigen was performed by SDS PAGE, agarose gel filtration (A-5m), and rotary shadowing. The preparation consisted of >90% $(F1)_2F2$ together with 7S domains from which one or more arms of average length 215 nm were observed (17).

Highly purified $(F1)_2F2$ was isolated first by CM-cellulose chromatography in native conditions followed by molecular sieving at 4°C on a 1.5 × 150-cm column of Agarose A-15, equilibrated with 1 M $CaCl_2$. F3 and 7S + arms were isolated by agarose gel filtration and, in the case of F3, by CM-cellulose chromatography in denaturing conditions (7). The relative abundance of these domains within each of these fractions was confirmed by rotary shadowing.

Preparation and Isolation of Hybridomas

Female SJL/J mice (Jackson Laboratories, Bar Harbor, ME) were immunized with type IV collagen isolated from gizzard. The first injection (200 μg in complete Freund's) was administered subcutaneously at several sites along the flank. Two booster shots (~200 μg in incomplete Freund's) were administered intraperitoneally at 2-wk intervals. The mice were rested for 6 mo. On the four consecutive days before being used for a hybridoma fusion, they received daily intraperitoneal injections of 20 μg of immunogen in incomplete Freund's.

The procedure used for the production of hybridomas is described in detail elsewhere (22) and is briefly summarized here. All cultures of hybridomas and the NS-1 myeloma cells used for hybridoma production were grown in Dulbecco's Modified Eagle's Medium (4.5 g/l glucose) (Gibco, Grand Island Biological Co., Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Sterile Systems, Logan, UT) and gentamycin (50 μg/ml). Splenocytes (9 × 10^7) were obtained from the immunized mice and fused with myeloma cells (2 × 10^7) in 35% polyethylene glycol. The cells were suspended in complete medium and plated into 96-well cultures plates at a density of 5 × 10^4 myeloma cells/well. The next day, HAT medium was added to select for hybridomas.

For reasons to be described later, the initial selection for those hybridomas producing the desired antibodies was made by immunofluorescence histochemistry. Frozen sections (8 μm) of the anterior portion of eyes from 17- to 18-d-old chicken embryos were mounted on 12-spot glass slides (Shandon Scientific, Shandon Southern Instruments, Inc., Sewickley, PA); a drop of supernatant from a hybridoma culture well was added. The slides were incubated overnight at 4°C, rinsed in phosphate-buffered saline (PBS), incubated with rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) for 1 h at room temperature, rinsed again in PBS and mounted in glycerol. Sections were examined by fluorescence optics with a Zeiss photomicroscope equipped with an epiilluminator. Selected hybridomas were cloned by limiting dilution, and positive cultures were expanded. The culture supernatants were harvested, and aliquots of cells were frozen and stored in liquid nitrogen.

Immunofluorescence Observations

Fluorescence histochemistry was performed with 8 μm frozen sections of organs from 18-d-old chick embryos. Slides were stained with antibody as described above. Some slides were pretreated with testicular hyaluronidase (4,000 U/ml in PBS; 37°C, ½ h) or pepsin (10 μg/ml in 0.5 M acetic acid (HOAc); 20 min, 24°C). In all experiments, staining was done with antibody-containing medium from cultures of cloned hybridomas. Observations were usually done blind. Photographs were taken with Kodak Tri-X film rated at ASA 1,600. Control sections were treated and photographed in the same way as the experimental ones, except that the primary antibody was substituted with one of several different high-titre IgG antibodies against determinants not related to type IV collagen and not available in the tissues examined.

Immunological Characterization

Antibodies were characterized by both passive hemagglutination as previously described (4, 5) and an enzyme-linked immunosorbent assay (ELISA). ELISAs (23, 24) were performed using a Hybridoma Screening Kit (Bethesda Research Laboratories, Rockville, MD) and Immulon microtiter plates (Dynatech Corp., Alexandria, VA). Wells were coated for at least 3 d with type IV collagen in 20 mM sodium carbonate buffer, pH 8.6. For most assays, each well was coated with 2 μg of collagen, but 0.2 μg worked equally well. The hybridoma antibody to be examined was added (100 μl/well), and the plates were incubated overnight at 4°C. Subsequent steps were performed according to the Bethesda Research Laboratory directions. The wells were washed and then incubated with the enzyme-linked secondary antibody (β-galactosidase-conjugated sheep anti-mouse IgG) for 1 h at room temperature. The wells were washed, and the p-nitrophenyl-β-D-galactoside substrate was added and allowed to react for 1 h at room temperature. The reaction was stopped by the addition of 0.5 N Na_2CO_3, and the wells were diluted with H_2O to a final volume of 0.8 ml and read at 414 nm in a Gilford spectrophotometer.

Inhibition ELISAs were performed under nonequilibrium conditions (24). Aliquots of the collagens to be tested were dissolved in PBS (pH 7.1), added to the culture medium antibody (diluted 5:1), and reacted overnight at 4°C. 100-μl portions of the mixture were added to the collagen-coated wells and incubated for 1 h at room temperature to allow free antibody to bind. Succeeding steps in the assay were performed as described above.

Thermal Denaturation

Before some ELISA experiments, the inhibitory collagens, in either 0.1 M HOAc (pH 2.5) or PBS (pH 7.1), were thermally denatured at the desired temperature for 0.5 h and then were rapidly cooled to 0°C to prevent renaturation (see Discussion). These samples were then used in inhibition ELISAs as described above. Those denatured in PBS were reacted directly with antibody; those denatured in acid were first dialyzed against PBS.

RESULTS

Antibody Selection and Characterization

The method of initial screening by immunofluorescence histochemistry with cryostat sections of anterior eye tissues proved efficient and offers two advantages over other methods (25). It excludes any antibodies directed against antigenic determinants that are masked or otherwise inaccessible in tissues *in situ*, thus ensuring that the antibodies obtained will be useful for experiments involving immunohistochemical analyses of intact tissues. Furthermore, it allows the initial detection of antibodies against each of the components and molecular domains of a complex antigen or an antigenic mixture, regardless of their relative quantities.

Of 359 hybridoma wells tested, 60% showed positive immunofluorescent staining of one or more of the basement membranes within the anterior eye (see, for example, Fig. 4A–C). No antibody stained a structure other than a basement membrane, indicating that the original immunogen preparation consisted largely of type IV collagen components; antigenic contaminants, if any, could only have been derived from other basement membrane components. About fifty positive wells were selected. The cells in some wells were immediately cloned by limiting dilution; in others, the uncloned cell populations were expanded and frozen. Antibody-containing culture supernatants from all cloned and uncloned cultures were collected and stored at 4°C for further use. All experiments were performed using spent culture media as the sources of antibodies.

The culture supernatants were examined for their reactivity in passive hemagglutination using type IV collagen-coated erythrocytes and by ELISA using type IV collagen-coated microtiter wells. Only two antibodies gave a positive response in the passive hemagglutination assay. One, designated IV-IA8, was strong, and the other was weak. In the more sensitive ELISA, all but one of eight antibodies tested gave a positive response in wells coated with 20 ng of collagen (data not shown). The results of the ELISA indicated that most of the antibodies were probably directed against domains present in type IV collagen. The results of the hemagglutination assay suggested that the two positive antibodies were produced in response to quantitatively major components of the type IV collagen preparation. IV-IA8 has been characterized, and the tissue distribution of its antigenic determinant has been documented in 18-d-old embryos (see below).

The immunoglobulin subclass to which IV-IA8 belongs was determined by double immunodiffusion against anti-mouse-immunoglobulin heavy-chain-specific antibodies. It is an IgG1.

The determination of antibody specificity by inhibition ELISA indicated that IV-IA8 did not react with highly purified native collagen types I, II, III, or V but could be completely inhibited by preincubation with type IV (Fig. 1). The preparation of type III used to generate the data in Fig. 1 had been chromatographically purified by DEAE-cellulose chromatography run under nondenaturing conditions; the type V had been purified by precipitation in 2 M urea, 0.05 M Tris HCl pH 8.6 (26). They were free of any type IV collagen components as determined by SDS PAGE. In less pure preparations of types III and V that had been separated from type IV only by conventional fractional salt precipitation, the inhibition ELISA detected 1–3% cross-reactive material (data not shown).

The type IV collagen preparations that had been used as the immunogen, and for coating the wells for the ELISA, were analyzed by SDS PAGE. They consisted largely of the $(F1)_2F2$ component, with smaller amounts of the 7S and F3 domains.

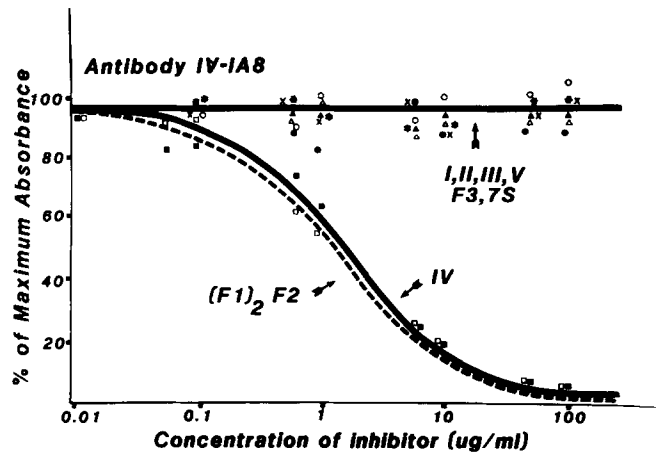FIGURE 1   Inhibition ELISA using antibody IV-IA8, and various concentrations of native collagens (abcissa) as inhibitors. The ordinate reports the percentage of the maximum absorbance measured at 414 nm. The upper solid line shows the absence of inhibition by collagen types I (open triangles), II (filled triangles), III (open circles), and V (filled circles), and by the F3 (asterisks) and 7S (X's) domains of type IV collagen. Unfractionated type IV (filled squares; lower solid line) and the isolated $(F1)_2F2$ domain (open squares; dashed line) gave nearly identical patterns of strong inhibition.
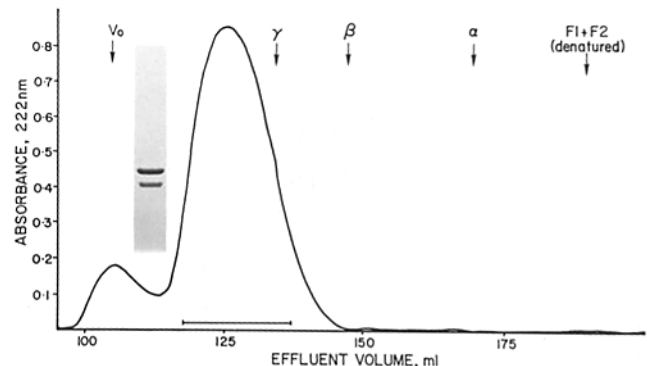

FIGURE 2   Elution profile of pepsin-extracted type IV collagen chromatographed under nondenaturing conditions (1 M $CaCl_2$, 4°C) on a column of agarose A-15M; the material in the fractions of the major peak (shown by bar) were pooled and analyzed by SDS PAGE on a 5–10% gradient gel (*insert*). It is exclusively $(F1)_2F2$. α, β, and γ show the elution patterns of monomeric α-chains, β-chain dimers, and γ-chain trimers. The elution position of the denatured F1 and F2 chains is also shown.

Since IV-IA8 seemed to be directed against a quantitatively major component, we thought that its antigenic determinant might be within $(F1)_2F2$. To test this, $(F1)_2F2$ was separated from the other type IV collagen domains by gel filtration on an Agarose A-15M column run under nondenaturing conditions. As can be seen in Fig. 2, native $(F1)_2F2$ elutes slightly after the void volume and thus can be obtained free from any other detectable component, as determined by SDS PAGE. When this highly purified $(F1)_2F2$ fraction was tested by inhibition ELISA (Fig. 1, dashed line), it inhibited at least as well as the unfractionated type IV collagen preparation. Purified 7S and F3 domains gave no inhibition (Fig. 1). Unfractionated type IV collagen purified from kidney showed inhibition equal to that from gizzard (data not shown).

The melting temperature of the triple helix of $(F1)_2F2$ has recently been found to be 33°–34°C in 0.1 M HOAc (pH 2.5), as measured by optical rotation (17). Since most anti-collagen
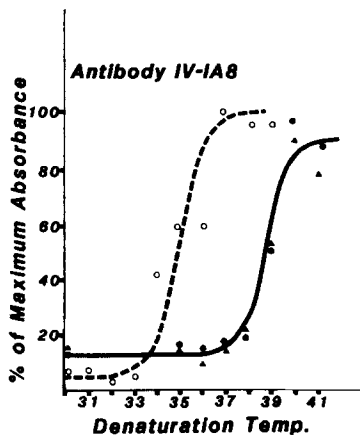
FIGURE 3 Thermal denaturation of the antigenic determinant of antibody IV-IA8 determined by inhibition ELISA. Wells coated with 100 μl type IV collagen (20 μg/ml.). Abcissa = temperature to which the inhibitor type IV collagen had been raised for 30 min. The ordinate reports the percentage of the maximum absorbance measured at 414 nm. The dashed line shows inhibition by samples that had been heated in 0.1 M HOAc and then dialyzed to neutrality at 4°C (open circles); the solid line by samples that had been heated in PiBS in the presence (closed triangles) or absence (closed circles) of 5 mM DTT.

antibodies produced in mice are conformational-dependent and do not bind to denatured collagen (22, 27), we asked whether the antigenic site within (F1)$_2$F2 was also conformational-dependent, and, if so, how this relates to the melting temperature of (F1)$_2$F2. Samples of type IV collagen dissolved in 0.1 M HOAc (pH 2.5) were raised to progressively higher temperatures, rapidly cooled, brought to neutrality with PBS, and antibody binding was tested by inhibition ELISA. A failure to bind antibody was observed in samples heated to temperatures >34°C (Fig. 3, dashed line). When thermal denaturation was performed under more physiological conditions of pH and ionic strength (see Discussion), inhibition of ELISA with IV-IA8 revealed a shift of the denaturation temperature to 38°–39°C (Fig. 3, solid line). The 38°–39°C melting temperature was unaffected by the addition of reducing agents, a result also consistent with the determinant being within (F1)$_2$F2 which lacks cysteine.

Immunofluorescence Histochemistry

The tissue distribution of the antigenic determinant recognized by IV-IA8 was surveyed in a variety of tissues from 18-d-old chicken embryos. Representative immunofluorescence micrographs are shown in Figs. 4 and 5. In sections of anterior eye (Fig. 4), antibody IV-IA8 outlined the profiles of the ducts and/or blood vessels of the limbal region (Fig. 4 B, arrowheads), and the basement membrane of the overlying conjunctival epithelium (Fig. 4 B, arrow). Bright fluorescence was also associated with the anterior lens capsule (but not the posterior lens capsule) and the basement membranes of the anterior epithelium of the iris and the iris muscle cells (Fig. 4 A). The basement membranes of the ciliary body were consistently stained (Fig. 4 C). We were surprised to find greatly diminished fluorescence, if any, associated with the basement membrane of the corneal epithelium (Fig. 4 A, arrow). Staining of the corneal endothelial basement membrane (Descemet's membrane; Fig. 4 A, open arrow) with this antibody was also markedly reduced compared to that of most other basement membranes and to the staining of Descemet's elicited by a variety of antibodies we have produced against noncollagenous basement membrane components (25). Nevertheless, faint fluorescence was generally visible in this structure.

Staining of the basement membranes of other organs was extensive. In the kidney (Fig. 5 A), bright fluorescence was
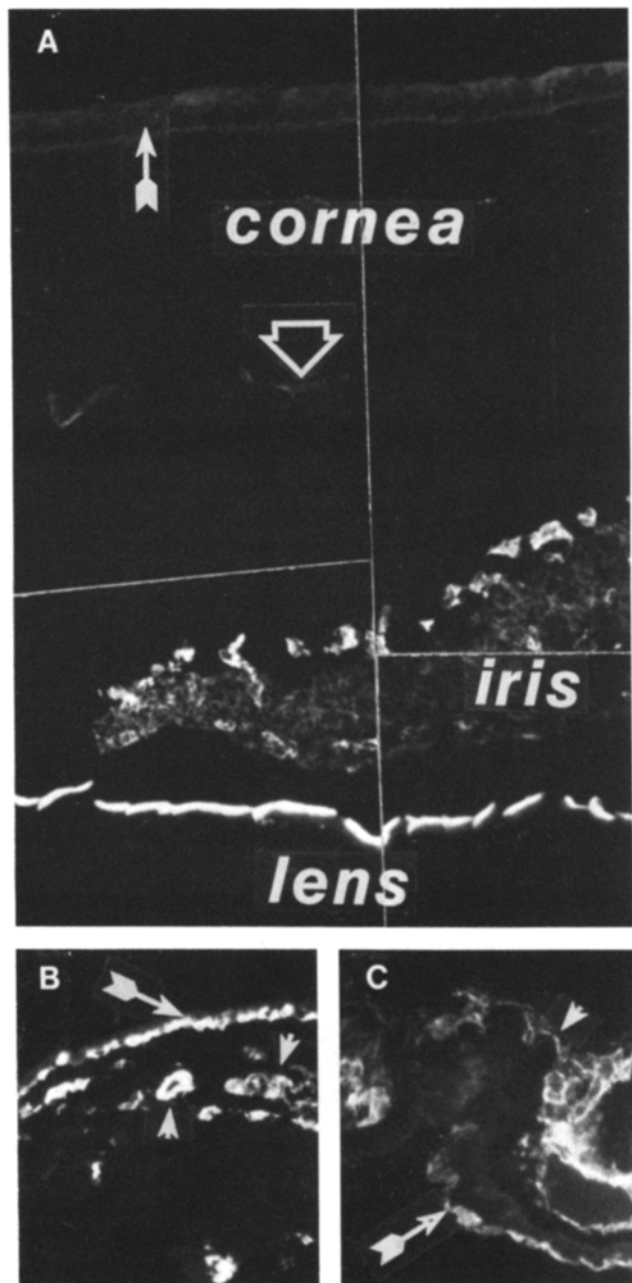
FIGURES 4 and 5 Photomicrographs of frozen sections of tissue from 18-d-old chicken embryos. All sections were treated with antibody IV-IA8, followed by rhodamine-conjugated goat antimouse IgG secondary antibody, and viewed by fluorescence microscopy. Control and experimental sections were treated identically, except the former received one of several high-titer monoclonal antibodies (all IgG) against antigenic determinants not detectable in the tissue instead of IV-IA8. Fig. 4 (above) shows anterior eye including cornea, iris, and lens (A), sclera (B), and ciliary body (C). Controls (not shown) were completely negative and comparable to those shown in Fig. 5. X 113.

observed in all tubules, ducts, and glomeruli. Staining within skeletal muscle (Fig. 5 B) included the basement membrane surrounding each muscle fiber plus intervening capillaries. In the connective tissue surrounding each bundle of muscle fibers, the walls of arteries and veins were brightly stained; light staining was also found within bundles of nerve (not shown). The staining within the walls of the arteries and veins appeared to be associated with smooth muscle cells. In larger blood
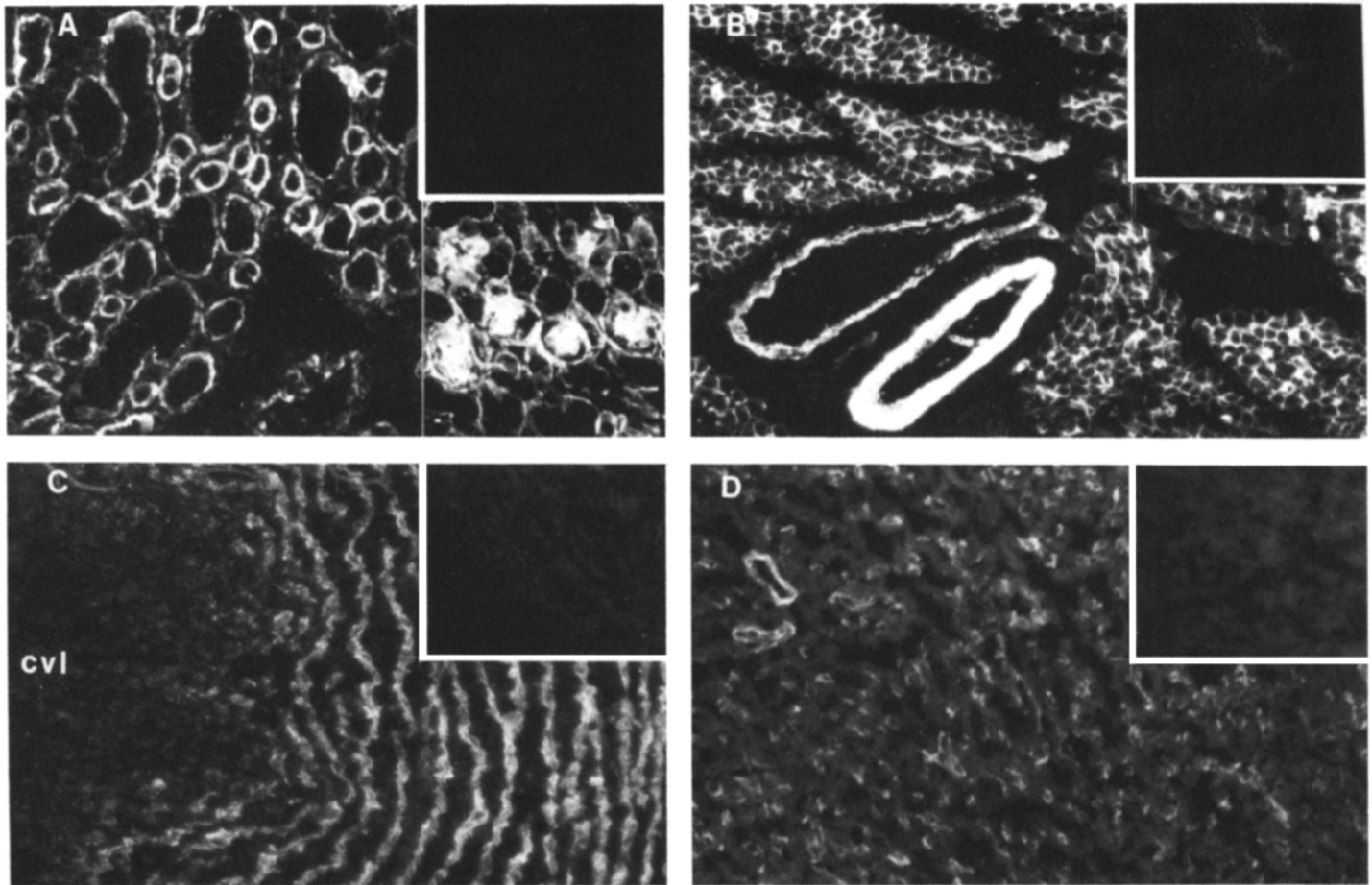
FIGURE 5 (*A*) kidney, (*B*) skeletal muscle, and (*C*) large blood vessel. *cvl* overlies the region of the collapsed vascular lumen, which is unstained. (*D*) Cardiac ventricle. Control sections at the same magnification and exposure times are included as inserts in the upper right-hand corner of each picture. × 113.

vessels (Fig. 5 *C*), we observed concentric rings of fluorescence that seemed to outline the layers of smooth muscle, but no staining was found in the region of the lumen (*cvl*, Fig. 5 *C*). In contrast, similar sections stained with antibodies to native lens capsule (25) clearly stain the basement membrane of the vascular endothelium in a stellate pattern that seems to parallel the profile of the collapsed arterial lumen (in preparation). That the basement membranes of at least some forms of vascular endothelium are positive, however, is evident by bright staining of capillaries. This is clearly visible in the myocardium of the heart ventricle, in which the capillary network is the predominant structure stained (Fig. 5 *D*).

In all of these experiments, the controls (inserts in the upper corner of each figure) were completely negative. Attempts to alter the staining patterns by pretreating the slides to remove potential masking substances, such as testicular hyaluronidase-sensitive proteoglycans or pepsin-sensitive proteins, were unsuccessful.

DISCUSSION

By immunohistochemical staining, we have identified hybridomas producing antibodies against components of a highly purified type IV collagen preparation. A number of criteria indicate that one of these, antibody IV-IA8, is directed against the $(F1)_2F2$ component. Preliminary characterization of several other antibodies by similar procedures suggests that the antigenic determinant for some of them may be within the F3 domain, and for others within 7S. If so, these will provide

comparisons for antibody IV-IA8 and should allow us to evaluate: (*a*) the current model of type IV collagen and its assembly within basement membranes, (*b*) the variety of antigenic determinants that exists within type IV collagen, and (*c*) the interaction of different molecular domains of type IV collagen with other basement membrane components.

On the basis of the inhibition ELISA analysis, the antigenic determinant recognized by IV-IA8 appears to be located within, or closely associated with, the $(F1)_2F2$ domain. Additional evidence comes from inhibition ELISA with collagen thermally denatured in 0.1 M HOAc, which demonstrated that the loss of antibody binding occurs at a temperature similar to that at which $(F1)_2F2$ loses its helical structure, as determined by optical rotation (17). While further implicating the $(F1)_2F2$ region as the source of the conformational-dependent IV-IA8 binding site, this information on thermal stability also raised a question. How could a conformational-dependent antibody against an immunogen that denatures at 33°–34°C be generated in a mouse whose body temperature is considerably higher? One possible answer, that the thermal stability of the $(F1)_2F2$ helix is greater in more physiological conditions, was indicated by inhibition ELISA with type IV collagen denatured in neutral phosphate buffer. This revealed a shift in the temperature at which antibody binding was lost to 38°–39°C.[1]

---

[1] Although some renaturation may have occurred after cooling, Mayne and Bruckner (17) have observed that the helicity of $(F1)_2F2$ as measured by optical rotation in 0.1 M HOAc does not readily return under these conditions. Furthermore, the failure to detect binding to

Another possibility is that some of the type IV may be in aggregate form in neutral conditions. If so, or if such aggregates rapidly form upon injection, the melting temperature is likely to be substantially elevated, as is the case for type I collagen (28).

The precise biochemical nature of the determinant remains to be defined. The overall amino acid composition of $(F1)_2F2$ shows it to be largely collagenous; analysis by optical rotatory dispersion and its appearance in rotary shadowed electron microscopic preparations (17) are consistent with a collagenlike triple helical structure. The amino acid sequence has not been determined, but partial sequence analysis of homologous regions of type IV collagen from other species indicates that helical domains may be interrupted by noncollagenous sequences (15, 16). To establish the type of domain to which IV-IA8 binds will require isolation and characterization of a fragment of $(F1)_2F2$ containing the binding site. Preliminary experiments suggest that it may be possible to generate such a fragment by limited digestion with bacterial collagenase.

The immunofluorescence histochemical experiments revealed that most basement membranes were stained by IV-IA8, but those of the corneal epithelium and the endothelium of large blood vessels reacted very slightly, if at all, and that of the corneal endothelium showed a weak but positive reaction. This difference could be due to: (a) masking of the antigenic determinant in certain basement membranes; (b) the existence of a heterogeneous family of immunologically distinct type IV collagens that have tissue-specific distributions; or (c) a greatly reduced content, or possibly an absence, in some basement membranes of type IV collagen.

We have attempted to remove potential masking substances from the corneal basement membranes by pretreating sections with testicular hyaluronidase and pepsin. Neither enzyme produced observable changes, suggesting the absence of type IV collagen in some tissues. This is supported by preliminary immunofluorescence histochemical experiments with other monoclonal antibodies to the F3 and 7S regions of type IV, which behave like IV-IA8 (unpublished observations). Nevertheless, this negative result cannot eliminate the possibility that type IV collagen is present but strongly and completely masked in these tissues but might be exposed by other procedures. We have found type V collagen to be masked in a variety of tissues stained with monoclonal antibodies (manuscript submitted for publication), but the procedures for unmasking this collagen do not appear to affect the staining pattern of IV-IA8 or other monoclonal antibodies to type IV collagen.

The possibility that tissue-specific type IV collagens exist cannot be eliminated and indeed remains an intriguing question for future investigation.

Finally, the possibility that certain basement membranes, at least in some species, contain little or no type IV collagen must be considered. In the human cornea both epithelial and endothelial basement membranes have been reported to stain with a conventional, affinity-purified antibody against type IV collagen from the EHS-mouse tumor (29; Fujikawa, Foster, and Colvin, personal communication; submitted for publication). On the other hand, Fujikawa, Foster, and Colvin (personal communication; submitted for publication), using the same

type IV collagen denatured under both acid and neutral conditions indicates that the extent of specific reformation of the binding site was negligible. This is probably due in part to the absence of disulfide bonding in $(F1)_2F2$.

anti-type IV collagen antibodies, have been unable to stain the corneal epithelial basement membrane in rabbits or guinea pigs, although the basement membranes of other tissues from these animals react strongly. These differences could therefore be species-related. In addition, there may exist age-related differences, since we have observed temporal changes in other basement membrane antigens during embryonic development (25). Future studies with monoclonal antibodies against different domains of the type IV molecule and other basement membrane components should make it possible to distinguish between these alternatives. In the least, the differences in staining with the anti-type IV collagen antibody reported here may reflect a significant heterogeneity in the quantity or assembly of this molecule within different basement membranes.

REFERENCES

1. Kefalides, N. A., editor. 1978. Biology and Chemistry of Basement Membranes. Academic Press, New York/London.
2. Grant, M. E., J. G. Heathcote, and R. W. Orkin. 1981. Current concepts of basement-membrane structure and function. *Biosci. Rep.* 1:819–842.
3. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.).* 256:495–497.
4. Linsenmayer, T. F., C. D. Little, and M. J. C. Hendrix. 1979. Production and characterization of a monoclonal antibody to chicken type I collagen. *Proc. Natl. Acad. Sci. U. S. A.* 76:3703–3707.
5. Linsenmayer, T. F., and M. J. C. Hendrix. 1980. Monoclonal antibodies to connective tissue macromolecules: type II collagen. *Biochem. Biophys. Res. Commun.* 92:440–446.
6. Timpl, R., G. R. Martin, and P. Bruckner. 1978. Structure of basement membrane collagen obtained from a mouse tumor. *Front. Matrix Biol.* 7:130–141.
7. Mayne, R., and J. G. Zettergren. 1980. Type IV collagen from chicken muscular tissues. Isolation and characterization of the pepsin-resistant fragments. *Biochemistry.* 19:4065–4072.
8. Glanville, R. W., A. Rauter, and P. P. Fietzek. 1979. Isolation and characterization of a native placental basement-membrane collagen and its component α-chains. *Eur. J. Biochem.* 95:383–389.
9. Schwartz, D., T. Chin-Quee, and A. Veis. 1980. Characterization of bovine anterior-lens-capsule basement-membrane collagen. *Eur. J. Biochem.* 103:21–27.
10. Bornstein, P., and H. Sage. 1980. Structurally distinct collagen types. *Annu. Rev. Biochem.* 49:957–1003.
11. Heathcote, J. G., and M. E. Grant. 1981. The molecular organization of basement membranes. *Int. Rev. Connect. Tissue Res.* 9:191–264.
12. Kuhn, K., H. Wiedemann, R. Timpl, J. Risteli, H. Dieringer, T. Ross, and R. W. Glanville. 1981. Macromolecular structure of basement membrane collagens. Identification of 7S collagen as a cross-linking domain of type IV collagen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 125:123–128.
13. Timpl, R., H. Weidemann, V. VanDelden, H. Furthmayr, and K. Kuhn. 1981. A network model for the organization of type IV collagen molecules in basement membranes. *Eur. J. Biochem.* 120:203–211.
14. Risteli, J., H. P. Bachinger, J. Engel, H. Furthmayr, and R. Timpl. 1980. 7-S collagen: characterisation of an unusual basement membrane structure. *Eur. J. Biochem.* 180:239–250.
15. Schuppan, D., R. Timpl, and R. W. Glanville. 1980. Discontinuities in the triple helical sequence gly-X-Y of basement membrane (Type IV) collagen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 115:297–300.
16. Glanville, R. W., and A. Rauter. 1981. Pepsin fragments of human placental basement-membrane collagens showing interrupted triple-helical amino acid sequences. *Hoppe-Seyler's Z. Physiol. Chem.* 362:943–951.
17. Mayne, R., H. Wiedemann, W. Dessau, K. von der Mark, and P. Bruckner. Structural and immunological characterization of type IV collagen isolated from chicken tissues. *Eur. J. Biochem.* In press.
18. Hassell, J., P. G. Robey, J. J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin. 1980. Isolation of a heparan sulfate-containing proteoglycan from basement membrane. *Proc. Natl. Acad. Sci. U. S. A.* 77:4494–4498.
19. Timpl, R., H. Rhode, P. G. Robey, S. I. Rennard, J. M. Foidart, and G. R. Martin. 1979. Laminin-a glycoprotein for basement membranes. *J. Biol. Chem.* 254:9933–9937.
20. Carlin, B., R. Jaffe, B. Bender, and A. E. Chung, 1981. Entactin, a novel lamina-associated sulfated glycoprotein. *J. Biol. Chem.* 256:5209–5214.
21. Mayne, R., J. G. Zettergren, P. M. Mayne, and N. W. Bedwell. 1980. Isolation and partial characterization of basement membrane-like collagens from bovine thoracic aorta. *Artery.* 7:262–280.
22. Linsenmayer, T. F., and M. J. C. Hendrix. 1982. Production of monoclonal antibodies to

collagens and their immunofluorescence localization in embryonic cornea. *In* Immunochemistry of Extracellular Matrix. H. Furthmayer, editor. Academic Press, NY. 179–198.

23. Gosslan, B., and H. Barrach. 1979. Enzyme-linked immunosorbent microassay for quantification of specific antibodies to collagen type I, II, and III. *J. Immunol. Methods.* 29:71–77.
24. Rennard, S. I., R. Berg, G. R. Martin, J. M. Foidart, and P.G. Robey. 1980. Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Biochem.* 104:205–214.
25. Fitch, J., and T. Linsenmayer. Monoclonal antibody analysis of ocular basement membranes during development. *Dev. Biol.* In press.
26. Rhodes, R. K., and E. J. Miller. 1978. Physicochemical characterization and molecular organization of the collagen A and B chains. *Biochemistry.* 17:3442–3448.
27. Linsenmayer, T. F. 1982. Immunology of purified collagens and their use in localization of collagen types in tissues. *In* Collagen in Health and Disease, M. I. V. and Jayson Veiss J. B., editors. Churchill Livingston, Edinburgh, England. 244–268.
28. Gross, J. 1964. Thermal denaturation of collagen in the dispersed and solid state. *Science* (*Wash. D. C.*). 143:960–961.
29. Newsome, D. A., J. M. Foidart, J. R., Hassell, J. H., Krachmer, M. M. Rodriques, and S. I. Katz. 1981. Detection of specific collagen types in normal and keratoconus corneas. *Invest. Ophthalmol. Vis. Sci.* 20:738–750.