Monoclonal Antibodies against Chicken Type V Collagen: Production, Specificity, and Use for Immunocytochemical Localization in Embryonic Cornea and Other Organs

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ABSTRACT Two monoclonal antibodies have been produced against chick type V collagen and shown to be highly specific for separate, conformational dependent determinants within this molecule. When used for immunocytochemical tissue localization, these antibodies show that a major site for the *in situ* deposition of type V is within the extracellular matrices of many dense connective tissues. In these, however, it is largely in a form unavailable to the antibodies, thus requiring a specific "unmasking" treatment to obtain successful immunocytochemical staining. The specificity of these two IgG antibodies was determined by inhibition ELISA, in which only type V and no other known collagen shows inhibition. In ELISA, mixtures of the two antibodies give an additive binding reaction to the collagen, suggesting that each is against a different antigenic determinant. That both antigenic determinants are conformational dependent, being either in, or closely associated with, the collagen helix is demonstrated by the loss of antibody binding to molecules that have been thermally denatured. The temperature at which this occurs, as assayed by inhibition ELISA, is very similar to that at which the collagen helix melts, as determined by optical rotation. This gives strong additional evidence that the antibodies are directed against the collagen. The antibodies were used for indirect immunofluorescence analyses of cryostat sections of corneas and other organs from 17 to 18-day-old chick embryos. Of all tissues examined, only Bowman's membrane gave a strong staining reaction with cryostat sections of unfixed material. Staining in other areas of the cornea and in other tissues was very light or nonexistent. When, however, sections were pretreated with pepsin dissolved in dilute HAc or, surprisingly, with the dilute HAc itself dramatic new staining by the antibodies was observed in most tissues examined. The staining, which was specific for the anti-type V collagen antibodies, was largely confined to extracellular matrices of dense connective tissues. Experiments using protease inhibitors suggested that the "unmasking" did not involve proteolysis. We do not yet know the mechanism of this unmasking; however, one possibility is that the dilute acid causes swelling or conformational changes in a type-V collagen-containing supramolecular structure. Further studies should allow us to determine whether this is the case.

Within both developing embryos and adult organisms, most cells and tissues are intimately associated with some form of collagenous extracellular matrix. Such matrices fall into two general categories: (*a*) the stromas, which generally become populated with mesenchymal cells and consist largely of extracellular materials having structural and supportive roles within tissues and organs, and (*b*) the basement membranes, which are acellular sheets found at all epithelial-mesenchymal interfaces and surrounding some types of individual cells. The collagenous components of these matrices constitute a hetero-

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geneous group of at least seven, and probably more, distinct molecules composed of chains whose synthesis is directed by at least ten separate genes (for review see reference 5). Numerous biochemical, biosynthetic, and immunocytochemical studies have been performed to determine the cell and tissue distribution of the collagens. The results obtained for collagen types I-IV have generally been in agreement that different collagens occur preferentially in either stromal matrices or basement membranes, but not both. In adult organisms, the interstitial collagens, types I, II, and III, are thought to occur chiefly in stromas, and type IV exclusively in basement membranes. In early avian embryos, however, some interstitial collagens may also be found transiently in the specialized, basement-membrane-like matrices of the cornea (21, 23, 31, 60) and the notochord (30, 36).

In the case of type V collagen, however, seemingly conflicting results have been the rule rather than exception, with respect to both the structure of the molecule(s) and their tissue distribution and *in situ* location. Part of the problem may result from molecular heterogeneity, since type V collagen may in reality constitute a class of distinct but related molecules. As originally isolated from a pepsin digest of human placenta (8, 10), type V contained two different α-chain sized polypeptides termed αA and αB, now designated α2(V) and α1(V), respectively (see reference 5). Since these were found in a ratio of 1:2 it was suggested that these occur as a single, triple-chain heteropolymer, $[\alpha 1(V)]_2\alpha 2(V)$. Numerous subsequent studies have suggested that this indeed may be the predominant molecular species of type V collagen found in most tissues (3, 24, 28, 41, 53, 54). Results have also been obtained suggesting, at least in some cases, that a portion of the α1(V) chains may be assembled in a triple-chained homopolymer $[\alpha 1(V)]_3$ (12, 29, 38, 45, 49); in one cell line this is the only molecule assembled (14, 20). Some tissues also contain small amounts of a molecule(s) containing a third chain, the αC or α3(V) chain (6, 7), and it has been suggested that molecules of composition $\alpha 1(V)$ $\alpha 2(V)$ $\alpha 3(V)$ may also occur (51).

Additional sources of molecular heterogeneity have recently been suggested by in vitro biosynthetic experiments. Several different studies have shown that the chains of the smallest type V molecule produced are considerably larger than their counterparts produced by limited pepsin digestion (28, 35). This suggests that the type V molecules which are normally incorporated into tissues may be in forms resembling "partially processed" procollagen molecules containing noncollagenous extensions (29). Possibly even other peptides may be covalently bound to the molecules (14).

Since the initial isolation of type V collagen was from the basement-membrane-rich placenta, and since the α1(V) and α2(V) chains have certain compositional similarities to those of type IV collagen, it was suggested that basement membranes could be the normal *in situ* location. Subsequent biochemical analyses showed the corneal stroma to have a relatively high concentration of this collagen (11, 24, 46, 58, 61, 62). Since this structure is devoid of basement membranes, an exclusive basement-membrane location of the molecule is unlikely.

Immunocytochemical studies with conventional antibodies against $[\alpha 1(V)]_2\alpha 2(V)$ molecules have also seemingly given inconsistent results and have raised further questions as to the *in situ* localization of type V collagen(s). Stenn et al. (55) reported that antibodies produced against $[\alpha 1(V)]_2\alpha 2(V)$ molecules stained the epidermal basement membrane of mouse skin, and Bailey et al. (1) reported similar results for skeletal muscle in which their antibodies located this molecule in the endomysium (see also 4 and 39). Conversely, Pöschl and von der Mark (46) have reported that their antibodies against $[\alpha 1(V)]_2\alpha 2(V)$ collagen (59) give a reaction pattern similar to that of type I collagen, staining, for example, the entire cornea. Antibodies produced against native type V collagens and subsequently affinity purified against isolated α1(V) chains give yet other staining patterns. With these, staining appears pericellularly (17, 18, 40), as well as in association with striated collagen bundles (40). This has led to the suggestion that some type V may function as a bridge between basement membranes and connective tissues.

We felt that at least some of the apparently inconsistent results might be resolved by an immunocytochemical study with anti-collagen monoclonal antibodies (32, 34) against type V. In this present report we describe the production, isolation, and characterization of two monoclonal antibodies specific for different antigenic determinants in native type V collagen isolated from chicken gizzard. In addition, we present immunocytochemical evidence that a major *in situ* location of type V molecules containing these determinants is in dense connective tissue stromas. However, the determinants are normally inaccessible to antibodies unless the tissues are subjected to an "unmasking" procedure to be described.

MATERIALS AND METHODS

Preparation of Collagens: Chick collagen types III, IV, and V were obtained by limited pepsin extraction of adult chicken gizzard and separated by fractional salt precipitations (42, 43). Some preparations of type IV were further purified by ion exchange column chromatography on CM-cellulose (42, 43), and some of type V on DEAE-cellulose (3), both run under non-denaturing conditions. Highly purified type V was also prepared by dialysis of fractional-salt-separated type V against 2 M urea, 0.02 M NaCl, 0.05 M Tris HCl, pH 8.6 (44, 50). In this procedure, type V collagen is precipitated whereas minor amounts of other collagens, if present, remain in solution. Type I collagen was an acid extract of lathyritic chick skin, and type II a pepsin extract of adult chicken sterna purified as previously described (32, 34).

Characterization of the type V preparations was performed by SDS PAGE, CM-cellulose chromatography and amino acid analysis (see Results; Fig. 1; and reference 48).

Hybridoma Production: Female SJL/J mice (Jackson Laboratories, Bar Harbor, ME) were initially immunized with 200 μg of type V emulsified in complete Freund's adjuvant and injected subcutaneously. Booster injections (200 μg) were given intraperitoneally at two weeks (in incomplete adjuvant) and three months (without adjuvant) after the first injection. The mice were rested for 10 wk, and then were given one final injection (100 μg, without adjuvant) four days before using their splenocytes for the hybridoma fusion.

The procedure used for the production of hybridomas (27) is described in detail elsewhere (33) and is briefly summarized here. All cultures of hybridomas and the NS1 myeloma cells used for hybridoma production were grown in Dulbecco-Modified Eagle's Medium (4.5 g/l glucose) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Sterile Systems), and gentamycin (50 μg/ml). Splenocytes (2×10^8) were obtained from the immunized mice and fused with myeloma cells (6×10^7) in 35% polyethylene glycol. The cells were suspended in complete medium and plated into 96-well culture plates at a density of 6×10^4 myeloma cells/well. The next day, HAT medium was added to select for hybridomas. Hybridomas producing desired antibodies (see below) were cloned twice by limiting dilution.

Immunological Characterization: Antibodies were characterized both by passive hemagglutination as previously described (2) and by ELISA (19, 49).

For passive hemagglutination, type V collagen (0.3% solution in 0.1 M calcium acetate) was coupled to human type 0-positive erythrocytes (10% solution) with glutaraldehyde. 100 μl aliquots of the derivatized erythrocytes (diluted 1:20) were then added to 100-μl portions of spent medium from hybridoma cultures in conical-bottom microtiter wells. The plates were allowed to develop overnight at 4°C before being scored.

ELISAs were performed using a Hybridoma Screening Kit (Bethesda Research Laboratories, Bethesda, MD) and Immulon microtiter plates (Dynatech Labo-

ratories, Alexandria, VA). Wells were coated for at least 3 d with type V collagen in 20 mM sodium carbonate buffer pH 8.6. For most assays, each well was coated with 2 μg of collagen. The hydridoma antibody to be examined was added (100 μl/well), and the plates were incubated overnight at 4°C or for 1 h at room temperature. Both gave equivalent results. 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 2 h at room temperature. The wells were washed again, 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 M Na_2CO_3, and the wells diluted with H_2O to a final volume of 0.8 ml and read at 414 nm in a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH).

Inhibition ELISAs were performed using nonequilibrium conditions (49). Aliquots of the collagens in phosphate-buffered saline (PBS) (pH 7.1), were added to the culture medium antibody, diluted as specified in the results, 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.

Antibody Binding to Thermally Denatured Collagen: In some ELISA experiments, the inhibitory collagens in PBS (pH 7.1), were first heated at the desired temperature for 0.5 h, and then were rapidly cooled to 0°C to prevent renaturation. These samples were used in inhibition ELISAs as described above.

Measurement of the Thermal Denaturation Temperature of the Type V Collagen Helix: The helical structure of the type V collagen was monitored at a wavelength of 221 nm in a Cary Model 61 spectrapolarimeter equipped with a 1-cm water-jacketed flow cell and an internal thermocouple. The temperature of the collagen sample (250 μg/ml in 0.4 M NaCl, 20 mM Na phosphate buffer, pH 7.4) was increased at a rate of 30°C/h. The total time of UV exposure at 221 nm was kept to <15 min. to minimize damage by ultraviolet irradiation (22).

Immunofluorescence Histochemical Observations: Fluorescence histochemistry was performed on frozen sections of organs from 18-d chick embryos. The freshly removed organs were dropped into freon cooled to its freezing point with liquid N_2. The tissues were mounted in OTC Compound (Tissue Tek), 8 μm sections were cut on a cryostat and placed on 12-spot glass slides (Shandon Scientific, Sewicklay, PA). Each section was covered with a drop of culture supernatant antibody, and the slides were incubated overnight at 4°C. The slides were rinsed in PBS (pH 7.4), incubated with rhodamine-conjugated goat anti-mouse IgG diluted 1:50 (Cappel Laboratories, Cochranville, PA) for 1 h at room temperature, rinsed again in PBS and mounted in glycerol-PBS. As controls, duplicate slides were stained with several different monoclonal antibodies specific for different domains of type IV collagen (15), or others specific for lens fiber cells (16).

Before application of the antibodies, some slides were pretreated. The treatments included: (*a*) pepsin (0.1 μg/ml) in 0.5 M HAc, 0.5 h, 22°C; (*b*) HAc (0.5 M, 0.1 M, and 0.01 M), 0.5 h, 22°C; (*c*) 0.1 M HAc containing pepstatin (20 μg/ml), EDTA (25 mM), phenylmethylsulfonylfluoride (PMSF: 100 μg/ml), and paramercuribenzoate (100 μg/ml).

Observations were usually done blind. Photographs were taken with Kodak Tri-X film rated at ASA 1,600 and developed in Diafine developer.

RESULTS

Characterization of the Type V Collagen

The type V collagen preparations used as an immunogen, and for routine screening and assay of antibodies by ELISA, were pepsin extracts of adult chicken gizzard in which the different collagen types had been separated by fractional salt precipitations (42, 43). For some ELISA experiments, type V collagen preparations were further purified by DEAE-cellulose chromatography performed under nondenaturing conditions (3), and precipitation from 2 M urea at alkaline pH as described in Materials and Methods and (50).

When analyzed by SDS PAGE and CM-cellulose chromatography, all preparations of type V collagen were identical. As can be seen in Fig. 1 *inset*, SDS PAGE analyses of the preparations showed prominent bands of α1(V) and α2(V) chains, two slower migrating bands corresponding to β-chain dimers, and a very small amount of diffuse material migrating slower than the β-chains. Preparations of denatured material chromatographed on CM-cellulose columns (Fig. 1) showed major peaks of the α2(V) and α1(V) chains, and a small peak eluting later than α1(V), (labeled *post α1(V)*). The identity of all peaks was verified by amino acid analysis, and by SDS PAGE of both the intact chains and the CNBr peptides derived from them (data not shown). By these criteria, the major peaks were α1(V) and α2(V). The material in the *post α1(V)* peak was indistinguishable from α1(V), and probably represents a modified form of the α1(V) chain (Mayne and Zettergren, unpublished results). From the areas of the CM-cellulose chromatographic peaks, the ratio of α1(V) [including *post α1(V)*] to α2(V) is 2:1 (range 1.97–2.28, six preparations). Thus, most probably the predominant, if not exclusive, form of the type V collagen used in this study had the chain composition $[\alpha1(V)]_2\alpha2(V)$.

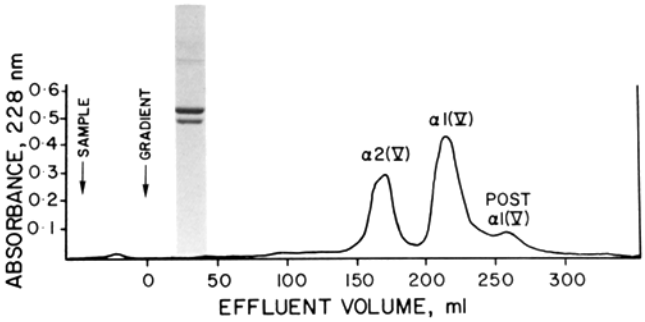

FIGURE 1 CM-cellulose chromatography and SDS PAGE of one of the type V collagen preparations used in the present investigation. The CM-cellulose chromatogram shows major peaks corresponding to α1(V) and α2(V) chains and a small "post α1(V)" chain peak. The material in the "post α1(V)" peak is, by all other criteria, indistinguishable from an α1(V) chain (see text). The ratio of α1(V) to α2(V) chain is about 2:1. Note the negligible size of the forepeak in which most noncollagenous materials, if present, would elute. The SDS PAGE gel (*inset*) shows predominant bands corresponding to α1(V) and α2(V) chains, two slower bands of β-chain dimers, and a very small amount of diffuse material migrating slower than the β-chains.

Hybridoma Production and Selection

The hybridoma cultures were produced by fusion of NS1 myeloma cells with splenocytes from a type V-immunized mouse, and antibody-positive clones were selected by passive hemagglutination with type V collagen-coated erythrocytes. Of 960 culture wells tested, 19 were positive. Two were selected for their high hemagglutination titers, their specificity in ELISA (described below), and their stable antibody production upon cloning. They were designated V-DH2 and V-AB12. Both were cloned by limiting dilution; spent culture medium from the resulting clones was used as a source of antibody in all subsequent studies.

Immunoglobulin Analysis

The immunoglobulin class to which each of the hybridoma antibodies belonged was determined by double immunodiffusion against heavy-chain-specific, anti-mouse immunoglobulin antibodies. Both V-AB12 and V-DH2 are IgGs. Clones of V-AB12 belong to the IgG1 subclass; those of V-DH2 belong to IgG2b.

Antibody Characterization and Specificity

Characterization of the antibodies was continued by ELISA and inhibition ELISA performed under nonequilibrium conditions according to Rennard et al. (49). Wells coated with 2 μg of the collagen produced a strong colorimetric reaction (Fig. 2), yielding highly reproducible results. Digestion of the collagen used to coat the wells, or of coated wells themselves, with highly purified bacterial collagenase in the presence of the protease inhibitors *N*-ethylmaleimide (2 mM) and PMSF (1 mM) (46) abolished antibody binding (data not shown).

For the inhibition ELISA, we wanted to use a concentration of antibody sufficient to produce a maximum colorimetric reaction without having a large antibody excess. This was determined by examining the reaction produced by twofold serial dilutions of each hybridoma culture supernatant (Fig. 2). The reaction with V-AB12 began to decrease between the second and third serial dilution, and no significant reaction was obtained after the seventh one (an absolute antibody dilution of 1/128). The maximum reaction with V-DH2 began to decrease between the third and fourth dilutions and approached a minimum at the eighth. From these data, we chose to perform all the inhibition ELISAs using antibody V-AB12 at a dilution of 1:5 and V-DH2 at 1:10.

The collagen-type specificity of the two different antibodies was determined by inhibition ELISA, using purified collagen types I-V as inhibitors (Fig. 3). Strong inhibition of both antibodies was obtained with type V collagen as well as with the alkaline-urea purified preparation (Fig. 3, solid line). Neither antibody showed detectable inhibition by collagen types I-IV (Fig. 3, dashed line). Nor were they inhibited by any of the minor "type V-like" collagens from cartilage (Mayne, Sanderson and Linsenmayer, unpublished observations, see Discussion).

As can be seen in Fig. 2, we consistently observed that the maximum colorimetric reaction produced by antibody V-DH2 was considerably greater than that produced by V-AB12. Two possibilities could explain this observation. One was that both antibodies are against the same antigenic determinant, but V-DH2 binds with a greater affinity. The other was that, in addition to affinity differences, each was also against a different antigenic site(s) within the molecule.

To distinguish between these, we used the ELISA to compare binding of mixtures of the two antibodies to that of each individually, to see if they would be additive. If both were against the same antigenic site, the amount of antibody bound from the mixture should be no greater than that of the individual antibody with the higher affinity. If, however, each was directed against a different determinant, the amount of antibody bound from the mixture should be expected to be additive. Fig. 4 shows the ELISA results obtained from a series of twofold dilutions of V-AB12 and V-DH2 compared to a series of dilutions made from a mixture of the two. The data clearly show, at each antibody concentration, that the mixture is greater than either individual. At higher concentrations they are essentially additive, suggesting that each is directed against a different determinant.

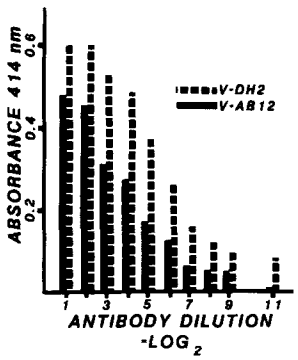

FIGURE 2 ELISA absorbance values produced by twofold serial dilutions ($-\log_2$) of monoclonal antibody-containing culture supernatants by hybridomas V-AB12 and V-DH2. ELISA performed as described in Materials and Methods and read at 414 nm.

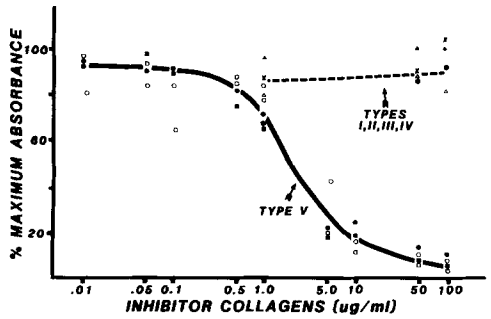

FIGURE 3 Inhibition ELISA of monoclonal antibodies V-AB12 and V-DH2 using different types of collagens. Inhibitor collagens at the concentrations shown were preincubated overnight at 4°C with the monoclonal antibodies. Then, to assay for free antibody, each inhibitor-antibody mixture was added to a microtiter well previously coated with 2 μg of type V collagen and incubated at room temperature for 1 h. The amount of antibody that bound to the type V collagen-coated well was assayed by an enzyme-linked, β-galactosidase-antibody reaction, performed as described in Materials and Methods and read at 414 nm. Solid line, inhibition produced on the two different antibodies using type V collagen as an inhibitor: open circles and open squares are with V-AB12, and closed circles and closed squares are with V-DH2; squares are with conventionally purified type V collagen and circles are with alkaline-urea-purified type V. Dashed line, inhibition produced by collagen types I-IV on antibody V-DH2: X's, type I; asterisks, type II; open triangles, type III; and closed triangles, type IV. A similar complete lack of inhibition was observed when these collagens (I-IV) were used as inhibitors with antibody V-AB12 (not shown).

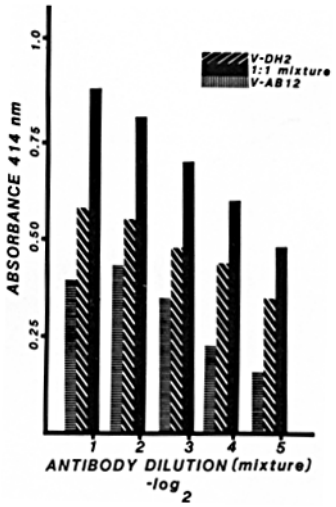

FIGURE 4 ELISA absorbance values produced by twofold serial dilutions ($-\log_2$) of monoclonal antibody-containing culture supernatants by hybridomas V-AB12 and V-DH2, and equivalent 1:1 mixtures of the two antibodies. ELISA performed as described in Materials and Methods and read at 414 nm.

Further characterization of the antigenic sites was obtained by examining their conformational dependency. In collagens, conformational-dependent determinants are generally thought to occur within the body of the molecule, and to require an intact triple helix for their generation (for reviews see references 30 and 56). Upon denaturation of the collagen, such a determinant would cease to exist. In addition, the helical conformation within a collagen generally melts at a temperature

considerably lower than that at which most other proteins become denatured. Thus, a positive correlation between the melting temperature of the collagen helix and a loss of antibody binding would provide strong additional evidence that the antibodies were indeed directed against collagen.

The conformational dependence of the antigenic determinants was examined by inhibition ELISA, using as inhibitors type V collagen samples that had been heated to progressively higher temperatures. The data were then compared to a thermal denaturation curve of the same type V collagen obtained by optical rotation measurements at 221 nm. As can be seen in Fig. 5, the melting curve of helicity obtained by optical rotation (solid line) was very similar to that of the temperature-dependent loss of binding of the two antibodies as measured by competition ELISA (dashed line and slashed line). The major changes in optical rotation of the collagen occurred between 39° and 43°C with a midpoint of about 41°C, similar to that reported for both chick type V collagen (57) and type I collagen (22) at neutral pH. The curve generated by the temperature-dependent loss of antibody binding of V-AB12, over most of its length was almost congruent with that of helicity; the one produced by antibody V-DH2 was similar but ~1.5°C higher. Both antibodies appeared to show some slight, but still detectable changes in binding at higher temperatures, a result which was more noticeable with V-DH2 than V-AB12. At present we

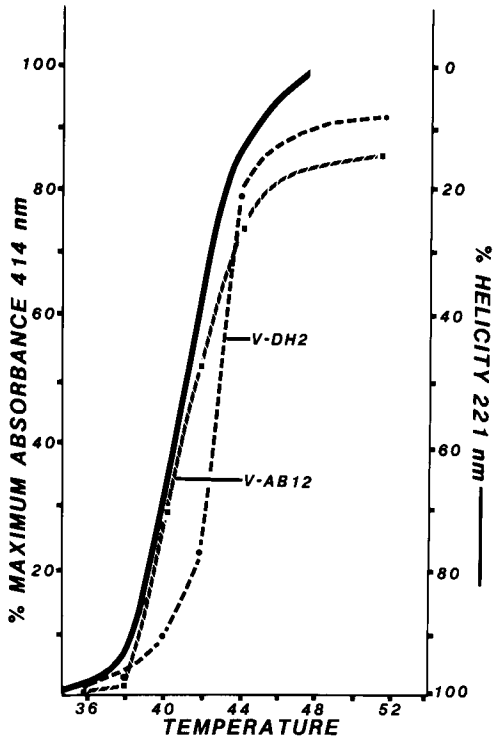

FIGURE 5 Comparison of the thermal melting curve of the helical structure of type V collagen with the thermal destruction of the antibody-binding sites against which the type V antibodies are directed. The melting curve of the collagen helix was obtained by optical rotation measured at 221 nm (solid line, % helicity). The thermal destruction of the antigenic determinants was determined by inhibition ELISA, using collagen samples which had been heated to progressively higher temperatures as inhibitors. The ELISA values are recorded as the percent of the maximum absorbance recorded at 414 nm compared to wells with no inhibitor collagen added. Slashed line, ELISA values obtained with antibody V-AB12. Dashed line, ELISA values obtained with antibody V-DH2.

do not know whether (*a*) this reflects the normal statistical distribution of native and denatured antigenic determinants generated at the different temperatures, (*b*) a greater than average thermal stability of some portions of the molecule as also suggested by the enzyme digestion experiments of Sage et al. (54), or (*c*) renaturation of a small portion of the determinants during the subsequent incubation period with antibody.

Fluorescence Immunohistochemistry

Antibodies V-AB12 and V-DH2 were used as the primary antibodies for immunofluorescent staining of cryostat sections of unfixed corneas and other tissues from 18-d-old chick embryos. The results obtained with these were largely negative. In fact, in the organs examined the only structure exhibiting strong fluorescence was Bowman's membrane (Fig. 6*A*). Organs which showed no staining at all were skeletal muscle (Fig. 7*A*), large blood vessels (Fig. 7*D*) and heart ventricle (not shown). Light staining was observed in the corneal stroma, probably extending into Descemet's membrane (Fig. 6*A*). It was also discernible in the connective tissue layers of the gizzard (Fig. 6*D*, arrowheads).

There were at least two possible reasons for this paucity of staining. The antibodies could have been directed against largely artifactual determinants proteolytically generated in the type V collagen either by the pepsin used in its isolation or by endogenous tissue proteases. Alternatively, the lack of antibody staining might be the result of *in situ* unavailability of the antigenic determinants, due either to masking by juxtaposed, noncollagenous molecules or steric hindrances created during assembly of the type V molecules into a supramolecular structure.

To try to obtain more positive staining, we pretreated tissue sections either with dilute pepsin dissolved in 0.5 M HAc, and, as a control, with the HAc solution itself. After washing with PBS, the tissues were reacted with antibodies. The initial experiments were performed on sections of cornea with attached scleral tissue, and subsequently on sections of the other tissues.

Both pretreatments resulted in dramatic new staining in the cornea, very similar to that shown in Fig. 6*C* which had been pretreated with a lower concentration of HAc (0.1 M). The 0.1 M HAc pretreatment (but not 0.01 M) resulted in staining at least as bright and extensive as did the 0.5 M HAc or the pepsin, and in addition maintained better tissue structure. In the pretreated corneal sections, the stroma and Descemet's membrane, which had been only lightly stained in sections without pretreatment, now stained brightly. Bowman's membrane, which had been positive in the untreated sections, remained so in the acid pretreated ones. The bright staining of all three corneal structures became noticeably diminished at the corneal-scleral border.

The staining produced by both the pepsin and HAc treatments was specific for the anti-type V collagen antibodies, and was not due to a general increase of IgG binding. As a control we always processed identically pretreated sections, substituting for the primary anti-type V antibody a high titer monoclonal against type IV (basement membrane) collagen (15). As can be seen in Fig. 6*B*, with this latter antibody neither the acid nor the pepsin (not shown) pretreatments produced any corneal staining, not even of the epithelial basement membrane.[1]

[1] The absence of detectable staining of the corneal epithelial basement

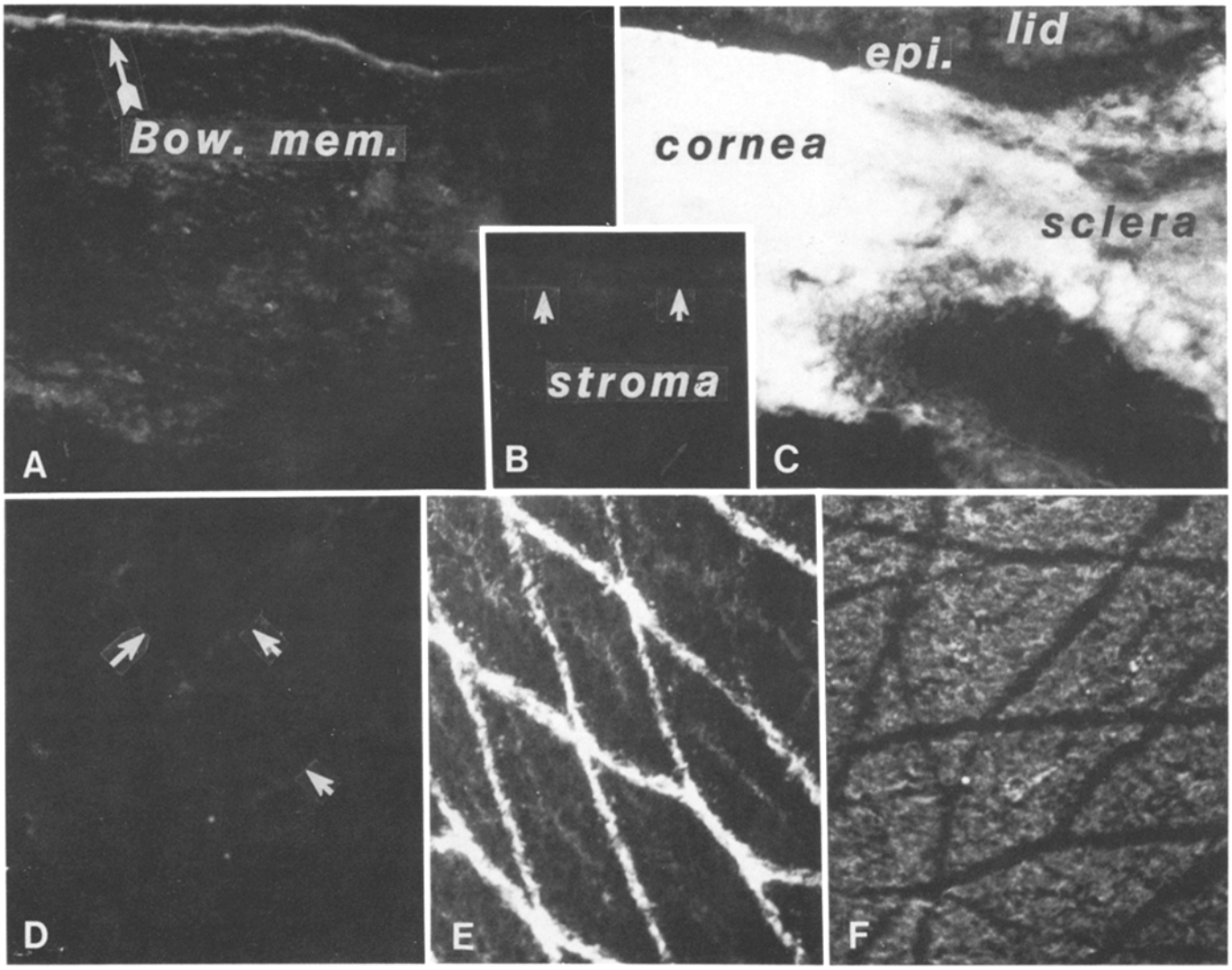

FIGURE 6 Fluorescence micrographs obtained using the anti-type V monoclonal antibodies for indirect immunofluorescence staining of sections of cornea plus sclera (*A* and *C*) and gizzard (*D* and *E*) from 17-d-old embryonic chicks. *A* and *D* were obtained by staining fresh, untreated tissue sections with the anti-type V collagen antibodies. Note in the cornea the relatively strong staining of Bowman's membrane (*A*, *Bow. mem.*) and the light staining of the stroma. In the gizzard (*D*), the connective tissue layers (arrowheads) show very light staining. The results in *C* and *E* were obtained with sections that had first been treated with 0.1 M HAc before staining with antibody. In the treated cornea (*C*) extremely bright staining was observed extending from directly beneath the epidermal cell layer (*epi.*) to the endothelial cell layer. Thus staining is obtained in Descemet's and Bowman's membrane layers as well as the stroma proper. The staining decreases at the corneal-scleral junction, being noticeably less intense in the sclera. Staining is also observed in the connective tissue of the eyelid (*lid*). In the treated gizzard (*E*), staining is localized in the dense connective tissue bands that run between the layers of smooth muscle. *B* and *F* are acid-pretreated sections stained, for purposes of comparison, with a high titer, IgG monoclonal antibody specific of chick type IV (basement membrane) collagen (15). *B* shows the anterior portion of the cornea, and *F* shows the gizzard. In the cornea there is a complete absence of staining, even of the epithelial basement membrane which is one basement membrane we have found to be negative with all of our anti-type IV monoclonal antibodies (see reference 15).[1] The gizzard shows anti-type IV staining to be associated with the smooth muscle layers, but none in the intervening connective tissue layers which stain with the anti type V antibodies.

Since acid pretreatment alone produced increased staining, the possibility of artifactual determinants produced by pepsin was eliminated; other results suggest that endogenous tissue proteases are also not involved. When sections were pretreated with 0.1 M HAc containing a mixture of protease inhibitors (see Materials and Methods) increased staining by the antibodies was obtained just as in the absence of these inhibitors.

The remaining possibility, which at present we consider most likely, is that the acid treatment facilitates antibody access to the antigenic determinant by producing swelling, either of the entire tissue or of a type V collagen supramolecular structure within the extracellular matrix.

Whatever the mechanism of HAc treatment, it is not re-

membrane with the anti-type IV collagen monoclonal antibody is indeed correct. This antibody, which is against a specific helical domain of the type IV molecule (15), produces little if any staining of this basement membrane. It does, however, stain the basement membranes of most other tissues (see reference 15, and Figs. 6 *F*, and 7 *C* and *F*). Using monoclonal antibodies we have produced against other domains of type IV, we have observed a similar lack of staining of the corneal epithelial basement membrane but extensive staining of most other basement membranes (unpublished observations).

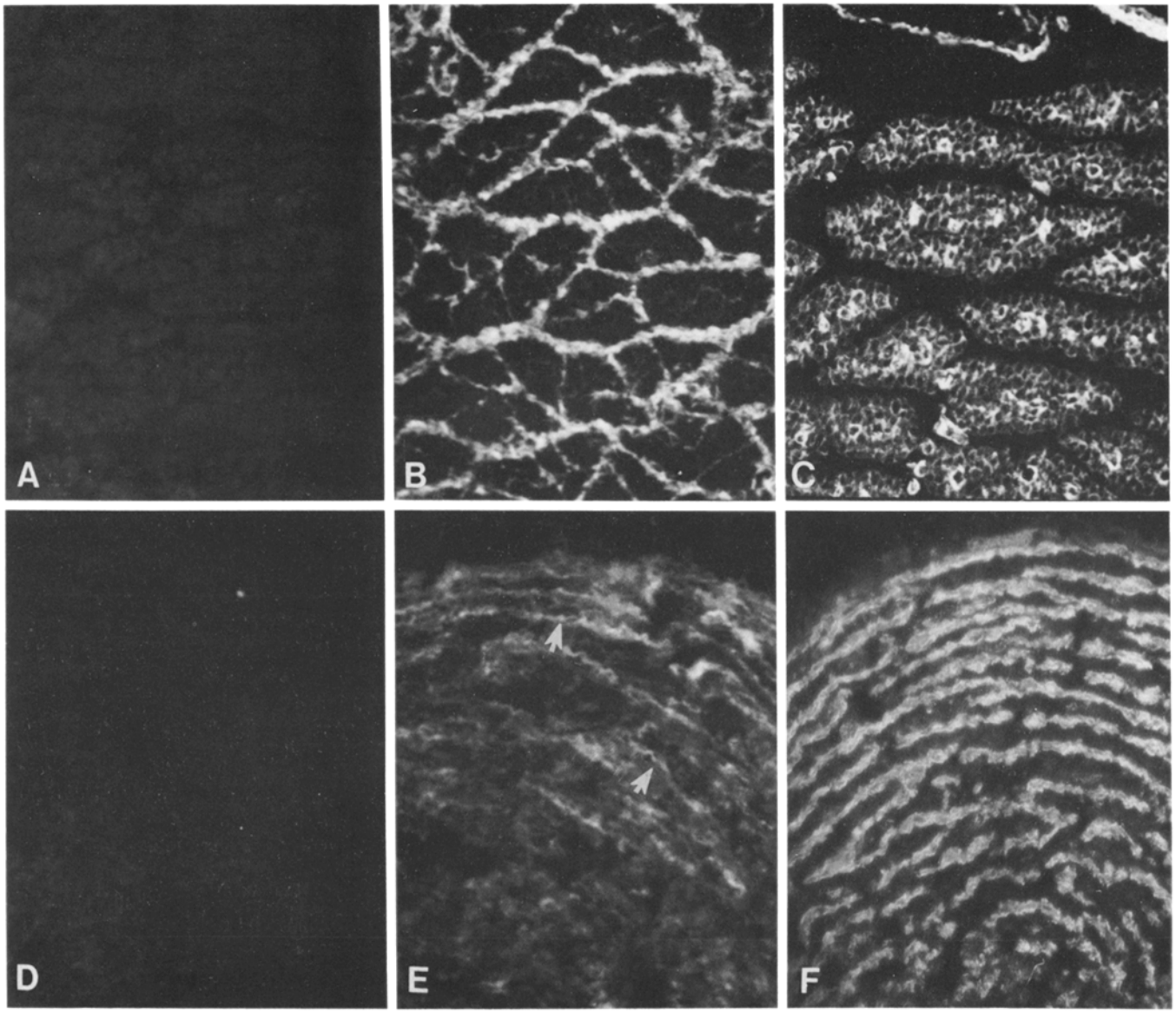

FIGURE 7 Fluorescence micrographs obtained using the anti-type V monoclonal antibodies for indirect immunofluorescence staining of sections of skeletal muscle (*A* and *B*) and large blood vessel wall (*D* and *E*) from 17-d embryonic chicks. *A* and *D* were fresh, untreated tissue sections. Note the complete absence of staining. Sections that had been first treated with 0.1 M HOAc before staining with antibody are shown in *B* and *E*. In the pretreated skeletal muscle (*B*), bright staining was observed in the connective tissue layers surrounding bundles of muscle fibers. In the pretreated large blood vessels (*E*), the major staining was observed in fibrous strands, although more diffuse staining was also observed. For purposes of comparison the same monoclonal antibody against chick type IV (basement membrane) collagen used in Fig 6 *B* and *F* was used to stain acid-pretreated sections of skeletal muscle (*C*) and blood vessel (*F*). These figures again show that acid pretreatment does not produce its effect by causing nonspecific binding of IgG to the acid-treated matrices, since the structures stained with the anti-type IV antibodies are entirely different from those stained with the anti-type V. The only effect of acid treatment on the staining with the anti-type IV collagen antibodies is a slight decrease in the staining of certain basement membranes (such as those surrounding the skeletal muscle fibers) when compared to that of nonpretreated sections stained with this antibody (not shown, see reference 15).

stricted to corneal matrices. We have observed greatly enhanced staining of specific extracellular matrices in most organs examined. For example, in sections of pretreated gizzard (Fig. 6 *E*), the anti-type V collagen antibodies brightly stained the fibrous connective tissue layers, whereas, before treatment the same bands showed only very light staining (compare Fig. 6 *D* and *E*). In the acid-pretreated gizzard stained with anti-type IV (basement membrane) collagen antibody as a control (Fig. 6 *F*), all staining was associated with the layers of smooth muscle cells which alternate with the fibrous ones. Thus, the antibodies against the two different types of collagens produce essentially reciprocal staining.

In acid-pretreated skeletal muscle, the anti-type V staining (Fig. 7 *B*) was located in the connective tissue layers surrounding bundles of muscle fibers. No detectable staining was present in the basement membranes surrounding the individual muscle fibers or in those associated with intervening capillaries. In acid-pretreated sections reacted with the anti-type IV (basement membrane) collagen antibody (Fig. 7 *C*), the basement membranes of these latter two structures stained brightly.[2] In

[2] In most tissues, the basement membrane staining produced by the

large blood vessels, acid pretreatment uncovers anti-type V antibody staining in fibrous strands (Fig. 7 *E*, arrowheads); similar strands are found in pretreated sections of heart ventricle (not shown). When stained with the anti-type IV collagen antibody, pretreated sections of large blood vessels show bright staining associated with the bands of smooth muscle (Fig. 7 *F*), a pattern identical to that produced by this antibody in non-pretreated sections (not shown).

In general, then, the staining with these antibodies is predominantly localized in what would be considered to be fibrous, stromal matrices.

DISCUSSION

We have isolated two monoclonal antibodies against different antigenic sites within a type V collagen. Several different lines of evidence suggest that the antibodies are completely specific for conformational dependent antigenic determinants in, or closely associated with, the triple helical structure of the molecule, and are present in no other known type of collagen molecule.

By passive hemagglutination with type V collagen-coated erythrocytes and by ELISA using type V-coated wells, both antibodies gave strong reactions. In competition ELISA they were both strongly inhibited by native type V collagen, including molecules which had been purified by DEAE-cellulose chromatography and by alkali-urea fractionation. The antibodies were not inhibited by any other collagen types, including native molecules containing the 1α, 2α, and 3α chains from cartilage (Mayne et al., unpublished observations) the first two of which resemble type V collagen chains (9, 48). These antibodies then seem to be uniquely specific for type V collagen.

Treatment of the type V collagen with highly purified bacterial collagenase abolishes the reaction in ELISA, so that the antigenic determinants are probably localized in, or at least closely associated with, a helical region of the molecule. Additional evidence for such a location is provided by the thermal denaturation experiments. In these, the curves generated by the loss of antibody binding activity, over most of their length were similar to loss of helicity, as determined by optical rotation. This strongly suggests that the antigenic determinants are within the collagen molecule itself. An alternative possibility which we consider somewhat unlikely, but have not yet been able to eliminate, is that the determinants are not within the collagen helix, but instead are in a component(s) so intimately associated with the helical structure that any conformational change in the helix also destroys or changes the structure of the hypothetical, bound component(s). Even if this were the case, the bound component itself would still seem to be highly specific for type V and no other collagen. In the present study, the collagen types III and IV were derived from the same preparations of gizzard as was the type V, and these did not bind antibody.

The immunocytochemical data we obtained using these antibodies on acid-pretreated sections from a variety of tissues suggests that a major *in situ* location for the molecule is dense connective tissue, a conclusion also drawn by Pöschl and von der Mark (47). Little if any staining was found in basement membranes as evidenced by comparative sections stained with monoclonal antibodies against type IV basement membrane collagen (15). The only exception we have noted thus far is Descemet's membrane, one of the specialized basement-membrane-like structures of the cornea. As shown here, this structure gives a strong reaction when stained with the anti-type V collagen antibodies; when stained with antibodies against type IV collagen, it exhibits a weak, yet generally detectable reaction (15).[1] Descemet's membrane, however, is probably not a typical basement membrane. At the ultrastructural level it is morphologically complex (25, 26), and by biochemical and immunocytochemical analyses, it has been shown to contain several different interstitial collagens, as well as type IV (21, 23, 58, 60). Our general conclusion, then, would be that the type V collagen against which our antibodies are directed has a tissue distribution pattern similar to that of an interstitial collagen such as type I or type III.

Our results should not, however, be interpreted as eliminating the possibility that other type V collagen molecule(s) may have tissue distributions different from the ones observed here. For example, Gay and co-workers (17, 18) have demonstrated a type V collagen which occurs in a pericellular localization, although they also find the molecule closely associated with type I collagen fibrils (40). This latter distribution is consistent with our results reported here. Others have reported type V to be associated with basement membranes (1, 52, 55). Due to the potential heterogeneity of type V with respect to chain composition and molecular assembly (12, 14, 20, 29, 45, 49) and the presence of accessory peptides (14), different antibodies may indeed detect antigenic determinants within different type V collagen molecules. In addition, the high degree of *in situ* masking we have observed leaves the possibility that different unmasking procedures might uncover additional locations for the molecule(s). Thus, only positive staining can be interpreted with any degree of confidence, the absence of staining is very much a negative result.

The staining we have obtained with the anti-type V monoclonal antibodies is consistent with known biochemical data. In nonpretreated sections, some structures which have previously been shown to contain type V (i.e. large blood vessels) (13, 43), were completely negative when stained with the antibodies. After acid pretreatment, however, staining of connective tissue elements in these previously negative structures became clear-cut, and the staining of other structures that had reacted only lightly in untreated tissues became intense. The correlation between biochemical analyses and immunocytochemical staining is especially evident in cornea. In acid-pretreated corneas, equally intense fluorescence is observed in the stroma proper and both Bowman's and Descemet's membranes. Biochemical analyses from several different laboratories (11, 26, 46, 58, 61, 62) have shown corneas to be rich in type V collagen, with each of the connective tissue layers containing it in approximately the same percentage (58).

We do not know for certain the mechanism of unmasking by the acid pretreatment, but since it works in the presence of protease inhibitors, proteolytic changes are probably not involved. Our current working hypothesis is that the unmasking is due to swelling, either of the entire tissue or more probably of a supramolecular structure in which the type V collagen molecules may be arranged. It is not known in what fibrillar or other supramolecular form(s) type V collagen molecules are arranged *in situ*. However, if they are conformationally arranged such that the antigenic determinants are unavailable to the antibody, simple swelling of the structure by the acid may

anti-type IV collagen antibody on acid pretreated sections is exactly the same pattern as is produced by it with non-pretreated sections (15). The only detectable difference is that the staining of some basement membranes, such as those surrounding skeletal muscle cells, is somewhat less intense in the acid pretreated sections.

be sufficient for unmasking, by allowing antibody penetration. The same mechanism of unmasking could work, for example, if type V collagen molecules were arranged within, or complexed with, fibrils composed largely of another collagen such as type I, a possibility that has been suggested previously (40, 47). In this case, the absence of complete masking in structures such as Bowman's membrane and the cornea stroma could be due to a different supramolecular arrangement of a portion of the molecules or to the presence of newly synthesized molecules that have not yet completely assembled. Alternatively, the low pH treatment could work by inducing conformational changes in masking substances, such as glycoproteins (37), or even within the structure of the type V collagen molecule itself. We are currently trying to distinguish among these possibilities.

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