Key words: open-sky vitrectomy, rabbit, ions, osmotic pressure, blood circulation

REFERENCES


Antibody to bovine zonules raised in rabbits gave diffuse staining of zonular fibrils with prominent accumulations at 35 to 45 nm sites of overbanding by means of the indirect immunoperoxidase technique. Antibody binding occurred in both fresh and paraffin-embedded material and was not species specific. The same pattern of heavy antibody binding and 35 to 45 nm periodicity was found on the microfibrils of elastic tissue in human and bovine ciliary body, calf ligamentum nuchae, and chick aorta. This cross reaction is the first direct evidence of a structural similarity or identity between components of the zonules and the microfibrils of elastic tissue, correlating with similarities in their morphology and amino acid profiles. Interstitial collagen fibers appear to have a minimal quantity of antigenically similar material at crossbanding sites. These relationships may hold important clues to the site of systemic involvement in connective tissue diseases associated with lens dislocation. Zonular antibody should prove useful in investigating abnormalities of the zonular-elastic microfibrillar system and cross-banding sites.

The ocular zonules are unique fibers whose relationship to other structural fibrous tissues is still uncertain. Although originally thought to be collagenous like the adjacent vitreous, the zonules contain no hydroxyproline and are insensitive to collagenase. Morphologically, zonular fibrils most closely resemble the microfibrils of elastic tissue, which are fine fibrils surrounding the amorphous elastin-containing core of the elastic fiber. The amino acid profile of the zonules, including an unusually high cysteine content, also resembles that of elastic tissue microfibrils. To determine whether a structural relationship may exist between these two types of fibrils, they were compared immunohistochemically.

Materials and methods. Zonules were cut from fresh bovine eyes with the use of microinstruments under a dissecting microscope, carefully avoiding contamination with lens capsule and vitreous. Zonular antibodies were raised in 2.5 kg male New Zealand white rabbits by injecting a 1:1 mixture of zonules sonicated in 0.01M phosphate-buffered saline (PBS, pH 7.3) and complete Freund's adjuvant. The rabbits received 1 ml of the mixture divided among five sites subcutaneously at 2 week intervals for 8 weeks. Sera were then tested for the presence of antibody in Ouchterlony plates. Because zonules are insoluble without treatment, antigen for the reaction was prepared by reducing fresh zonules with 4M urea, 1% sodium dodecyl sulphate (SDS), and 0.04M dithiothreitol (DTT). The reduced zonular solution was then dialyzed against 0.04M DTT before immunodiffusion.

Tissues were examined for antibody binding by both light and electron microscopy, with peroxidase-labeled staphylococcal protein A as an immunoreagent. The protein A was conjugated by the peroxidase method. For light microscopy,
bovine and human eyes were fixed in 10% neutral buffered formalin, and the tissue was embedded in paraffin. Sections mounted on coverslips were deparaffinized and washed in Tris-buffered saline (TBS) 0.05M, pH 7.6. To block nonspecific immunologic reactions the sections were treated with a 1:10 solution of whole chicken serum in a moist chamber for 10 min. Chicken serum was chosen because protein A does not bind to chicken IgG. After sections were washed with TBS, the indirect immunoperoxidase technique was followed, with the antizonular antiserum at 1:10 to 1:100 dilution and the peroxidase-labeled staphylococcal protein A (APO) at 1:100 dilution. Reagents were applied for 10 min each, followed by washing with TBS and fixation in cold 2.5% glutaraldehyde PBS 0.01M, pH 7.4, for 30 min. Sections were then washed in PBS and reacted with Karnovsky’s diaminobenzidine (DAB) reagent in a 0.05M citric acid phosphate buffer (pH 5) for 10 min in the dark. After washing with PBS, sections were dehydrated and mounted in permount.

For transmission electron microscopy (TEM), a slight modification of these methods was used. Tissues examined were small pieces of fresh bovine and human ciliary body with adherent zonule, lens capsule with zonule, calf ligamentum nuchae, and 17 day chick aorta. Times for rinsing and for reactions with whole chicken serum and whole or 1:10 zonular antiserum were increased to 30 min. Incubation time in 1:100 peroxidase-labeled staphylococcal protein A was increased to 2 hr, as was fixation time in 2.5% glutaraldehyde. The tissues were rinsed in PBS overnight and reacted with Karnovsky’s DAB for 30 min. Post-fixation in 2% osmium tetroxide was carried out, and the tissues were embedded in Mollenhauer’s medium. Sections were examined both unstained and stained with uranyl acetate and lead citrate.

Controls for light and electron microscopy included sections with no added serum, normal serum, and blanks containing only DAB. Zonular antisera were also absorbed with whole vitreous at 37° C for 2 hr before use on tissues as a further test of antibody specificity.

Results. Immunodiffusion of zonular antisera against zonules solubilized by reducing agents and detergents gave one precipitin line close to the antibody wells. No reaction was obtained between zonular antisera and normal calf serum or human or rat fibronectin. Staining of tissue was not affected by absorption of antisera with whole bovine vitreous.

By light microscopy, staining of the bovine zonule with zonular antibody in paraffin sections of the anterior segment gave a sharp demarcation of zonular bundles (Fig. 1). Lens capsule and cortex, ciliary body, cornea, sclera, and vitreous were all unstained. The reaction was identical with three
different zonular antisera. The human zonule is much more delicate than the bovine but also reacted well in paraffin sections.

By TEM the normal zonular fibril was slender, straight, and 10 to 11 nm in diameter, with a periodicity of 12 to 14 nm (Fig. 2, inset A). An over-banding at 35 to 45 nm was seen occasionally where fibrils were tightly aggregated. In cross section the fibrils had an osmiophilic tubular profile. After staining with zonular antibody there was diffuse globular staining of the whole zonular fibril (Fig. 2), doubling its width and obscuring the
microperiodicity. In addition, there was a regularly occurring increase in stain at 35 to 45 nm intervals on both individual and aggregated fibrils (Fig. 2, inset B). The clumps of peroxidase reaction product producing this wider banding were 20 to 30 nm aggregates of the apparent 6 to 8 nm smallest complexes. Often the clumps were offset from the vertical giving a spiraled appearance.

Elastic tissue microfibrils were seen by TEM as 10 to 12 nm tubular fibrils sparsely surrounding the central amorphous core of elastin, with a few fibrils penetrating the core (Fig. 3). Their periodicity varied from 12 to 14 nm. After staining with zonular antibody the microfibrils were heavily covered by globular reaction product similar to that on the zonules (Fig. 4). A prominent wider periodicity of 35 to 45 nm was visible where fibrils were cut longitudinally (Fig. 4, inset B). The elastin core remained unstained. Staining was identical around elastic fibers in the ciliary body stroma, calf ligamentum nuchae, or 17-day chick aorta. A faint dotlike stain was seen irregularly on interstitial collagen fibers, but not on vitreous fibers, frequently at 35 to 62 nm intervals (Fig. 4). The 5 to 10 nm dots often coincided with collagen banding sites, which have a 45 to 62 nm periodicity in the ciliary body and calf ligamentum nuchae. No such dots were observed with nonimmune sera.

**Discussion.** The marked binding of zonular antisera to zonular fibrils and not to lens capsule or vitreous fibrils supports the purity of the dissected
Fig. 4. Microfibrils of elastic fibers reacted with antizonular antibody. The elastin cores (EL) are unstained. A fine dotlike stain is visible on collagen fibers (C). (×67,500.) Inset A, Dense stain around individual elastic fibers (EL). (×13,700.) Inset B, Macroperiodicity on longitudinally sectioned microfibrils. (×67,500.) (All from calf ligamentum nuchae; APO method; unstained.)

zonular material indicated by previous amino acid analyses. We have found no evidence of collagen contamination in protein analyses on six different bovine zonular samples. The prominence of antibody binding at 35 to 45 nm intervals on the fibrils was unexpected because of the infrequency of this overbanding with routine staining. A periodicity that was not measured but appeared to be of the same order was reported on elastic microfibrils after staining with antibody to microfibrillar protein. It is not possible to say whether the antigenic determinants of the zonule reside in its protein core or in associated carbohydrate moieties of this glycoprotein. The prominence at overbanding sites might suggest some relation to the latter.

The heavy staining of elastic tissue microfibrils with zonular antibody in a pattern identical to that of the zonules is strong evidence that these two fibrils have structural similarities in one or more components. Morphologically the fibrils are similar but not identical, since elastic microfibrils have a slightly wider range of diameters than that of zonules, and their periodicity is often more difficult to see. They also may have a lower content of cysteine, the most distinct feature of the zonules. However these discrepancies may be more related to differences in tissue site and in preparation methods, since microfibrillar protein must be extracted from whole tissue.

The small quantities of elastic microfibrils in these tissues were not visualized by light micros-
copy with the present antibody. A somewhat stronger antibody obtained recently after repeated booster injections has shown a faint stain around the large elastic fibers and in the walls of some blood vessels by light microscopy, but for study of the microfibrils TEM is clearly the preferred method.

The presence of a small amount of staining material on interstitial collagen fibers has also been described with the use of monospecific antisera to elastic microfibrils. No binding was obtained to polymeric collagen treated with pepsin to remove glycoprotein. In our study, the dotted stain was often observed to coincide with collagen banding sites. In spite of the minor degree of stain, the reaction appeared to be an immunologic one, since it was only present with immune sera. No evidence of fibronectin was obtained in the antisera by immunodiffusion.

The importance of establishing a close similarity if not identity between components of zonules and microfibrils relates to the occurrence of lens dislocation in systemic connective tissue diseases such as Marfan's syndrome and homocystinuria. To date, the mechanism of these diseases has been sought primarily in abnormalities of collagen or elastin. If the zonule that contains neither collagen nor elastin is deficient in one of these diseases, the defect may reside instead in the zonular-microfibrillar system. Alternatively, it could lie in a component of this system shared with collagen. Antizonular antibody should prove useful in further investigation of the systemic lens dislocating diseases and also of ocular diseases displaying abnormal banded fibrillar material such as the pseudoexfoliative syndrome.

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Corneal re-epithelialization from the conjunctiva. Michael S. Shapiro, Judith Friend, and Richard A. Thoft.

After debridement of the entire corneal epithelium, epithelial cells of conjunctival origin cover the exposed corneal surface. Four to five weeks later, these cells undergo a morphologic transformation to normal-appearing corneal epithelium. To study this transformation the entire corneal epithelium was removed from rabbits with the use of n-heptanol, after which the histologic appearance of and the number of goblet cells in the regenerated epithelium were noted. Five stages of transformation were observed. Immediately after healing, the epithelium consisted of one to two squamous cell layers with no goblet cells apparent at the light microscope level (stage 1). In the following weeks goblet cells appeared at the limbal edge of the cornea (stage 2), reached a uniform distribution across the cornea (stage 3), and subsequently receded.