PSEUDOEXFOLIATIVE MATERIAL CONTAINS AN ELASTIC MICROFIBRILLAR-ASSOCIATED GLYCOPROTEIN*

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INTRODUCTION

IN THE PSEUDOEXFOLIATIVE (PSX) SYNDROME, A FIBRILLAR PROTEIN ACCUMULATES on and within many of the tissues bathed by the aqueous[1-5] and some of the adnexal tissues.[6-8] It is still unclear whether this material is of purely local origin and of what it is composed. The possibility that PSX material bears some relation to the zonules arises from shared histochemical reactions, including some unusual ones, such as positivity with Gomori's chrome alum hematoxylin[9,10] and oxytalan staining.[11,12] PSX material and zonular fibers have a similar lectin-binding profile[13] which together with the histochemistry suggest a complex glycoprotein-proteoglycan nature. Morphologically, the resemblance of PSX fibers to zonules is not striking, although there are similarities. The PSX fiber is much larger in diameter, but has a similar macroperiodicity of 25 to 50 nm, and contains some microfibrils of 6 to 8 nm with a 10 to 12 nm periodicity,[5] which is closer to zonular fibril morphology.

To determine whether there is a close structural similarity between the two fibrillar proteins, we have compared them immunologically. As described in a preliminary report,[14] there was strong binding of a polyclonal zonular antiserum to PSX material. Since zonular fibers appear to be part of a wide-spread elastic-microfibrillar system,[15] we looked for evidence of an affinity between PSX material and other members of this system. In the present study, we compare the results of staining PSX material with bovine zonular antisera,[15] microfibrillar protein (MFP) antisera derived

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from bovine ligamentum nuchae,[16] and a recently described monoclonal antibody to elastin-associated microfibrils.[17]

MATERIALS AND METHODS

Ten human lenses, with the clinical diagnosis of PSX disease, were obtained in the operating room immediately after extraction and frozen in liquid nitrogen within 20 minutes. Cataract lenses without PSX disease were processed in the same way, as controls. For antibody staining, the lenses were thawed and sectors of the anterior and equatorial capsule were immediately cut from the lens and immersed in phosphate-buffered saline. Remaining cortex and epithelium usually separated cleanly from the capsule during washing before immunostaining, giving an almost pure capsular preparation. After immunostaining, the tissues were fixed in 2.5% glutaraldehyde, embedded in Mollenhauer's medium, and processed for electron microscopy. Portions of three capsules were taken for whole mounts, without further staining. Grids for transmission electron microscopy were examined both unstained and double-stained with uranyl acetate and lead citrate. Controls for PSX and non-PSX disease lenses had omission of immune serum in the staining sequence or substitution of normal rabbit serum.

Antibodies to fresh bovine zonules were raised in rabbits, as described previously.[15] Antibodies to elastic MFP, extracted from bovine ligamentum nuchae, were similarly raised, as detailed before.[16] Monoclonal antibody to an elastic microfibrillar-associated protein[17] (presently called fibrillin) was kindly supplied by Dr Lynn Sakai. All antisera and the monoclonal antibody were used in full strength or 1:10 dilution. For immunohistologic staining, the indirect peroxidase-labeled staphylococcal protein A method was used for the polyclonal antibodies,[15,16] and an indirect peroxidase-labeled second antibody technique for the monoclonal antibody. Whole avian serum 1:10 or 4% bovine serum albumin (BSA) were used to block nonspecific staining, before application of primary antibody.

RESULTS

In PSX disease, there are three characteristic zones of different morphology in the lens capsule. The most familiar clinical accumulations are the small bush-like aggregates on the anterior capsule in its midperiphery, but they also occur over the whole zonular insertion region in advanced disease. They are composed of thick (20 to 55 nm) fibers banded at 25 to

50 nm and a small number of thin (6 to 8 nm) fibrils (Fig 1A). On the undersurface of the capsule, more laterally, is the fibrogranular zone,[1] where the multilaminar capsule is expanded in round patch-like areas, by infiltration with exuberant vertically-oriented bundles of 7 to 12 nm fibrils. These fibrils splay out horizontally and are associated with granular material (Fig 1B). The third zone consists of fibrogranular inclusions, lying horizontally within the lens capsule almost throughout its width, appearing similar to common aging inclusions,[9] but larger and more profuse (Fig 1C). The latter two zones lie, for the most part, over the germinative region of the lens epithelium. A less well defined zone is a central disc of superficial capsular thickening, which will not be considered here.

After reacting the lens capsule with zonular antisera, without counterstain, PSX vegetations stood out as black nodules, in light microscopy of the plastic thick sections (Fig 2). The larger vegetations were most heavily stained in their outer portions. The fibrogranular deep patches were sharply demarcated with their multiple columns of vertical fibrils. Inclusions within the capsule were visible but paler, and the capsule itself was negative. Staining was always densest in the outer portions of the stained areas, which was probably related to the limited ability of antibodies to penetrate tissue. In paraffin sections where a full thickness of capsule was exposed to the antibody, the distribution of stain was the same. No staining was visible in the controls, except on any adherent zonules and occasional small aging inclusions.

On electron microscopy, the appearance of all PSX areas was similar (Fig 3). In the superficial aggregates, antizonular reaction product covered the whole fiber, both thick and thin types, with occasional evidence of increased stain at intervals of 45 to 55 nm (Fig 4). In cross-section, the thicker fibers showed rims of darker stain. The deep fibrogranular zone had extensive stain associated with the vertical fibrillar bundles and their branches (Fig 5). The inner capsular surface was free of stain or had a few small nodular clumps, like those of vertical fibrils, occasionally confluent with the next adjacent fibrillar bundle. The capsule in some specimens also had a faint granularity in the fibrogranular zone, not seen in the rest of the capsule. Clumping of reaction product on the vertical fibrils obscured their details, but in the exposed fibrillar bundle "tails," when spread out, a diffuse coating was visible as in the surface PSX, darker around the fibril itself with rare macroperiodicity visible. Capsular inclusions which were sufficiently close to either surface and sometimes even those in midcapsule, were moderately stained by the zonular antisera (Fig 6).

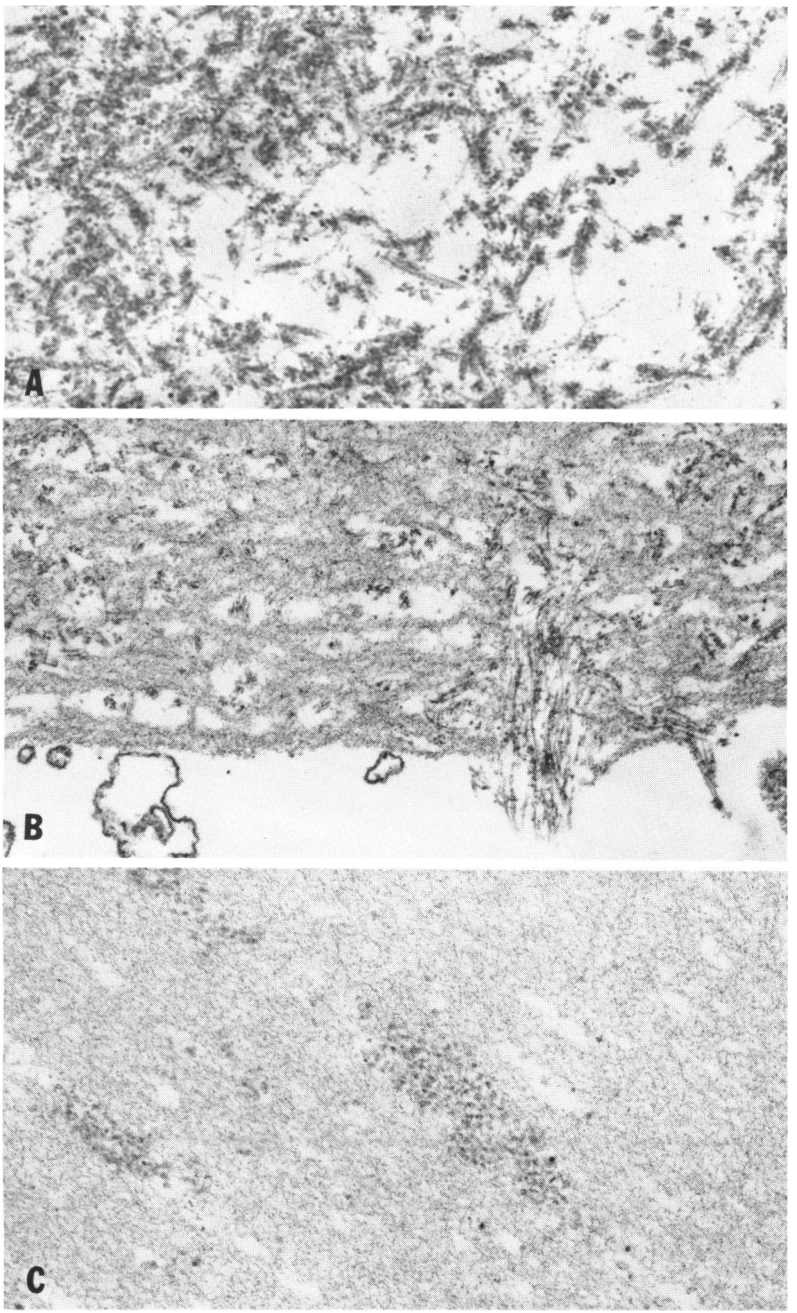
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FIGURE 2
Lens capsule from PSX disease reacted with zonular antisera, showing binding to patches of deep fibrogranular zone (*arrows*) on one side and to nodular PSX aggregates on the other. Linear capsular inclusions also visible but rest of capsule is unstained. APO method, no counter-stain (magnification, × 420).

FIGURE 1
A: Nodule of PSX material on anterior lens capsule. Note both thick fibers with macroperiodicity, and thin fibrils (magnification, × 30,000). B: Fibrogranular zone showing bundles of thin fibrils with some associated granular material, entering capsule and splaying out between lamellae of BM (magnification, × 43,700). C: A fibrogranular inclusion within midzone of lens capsule (magnification, × 56,000).

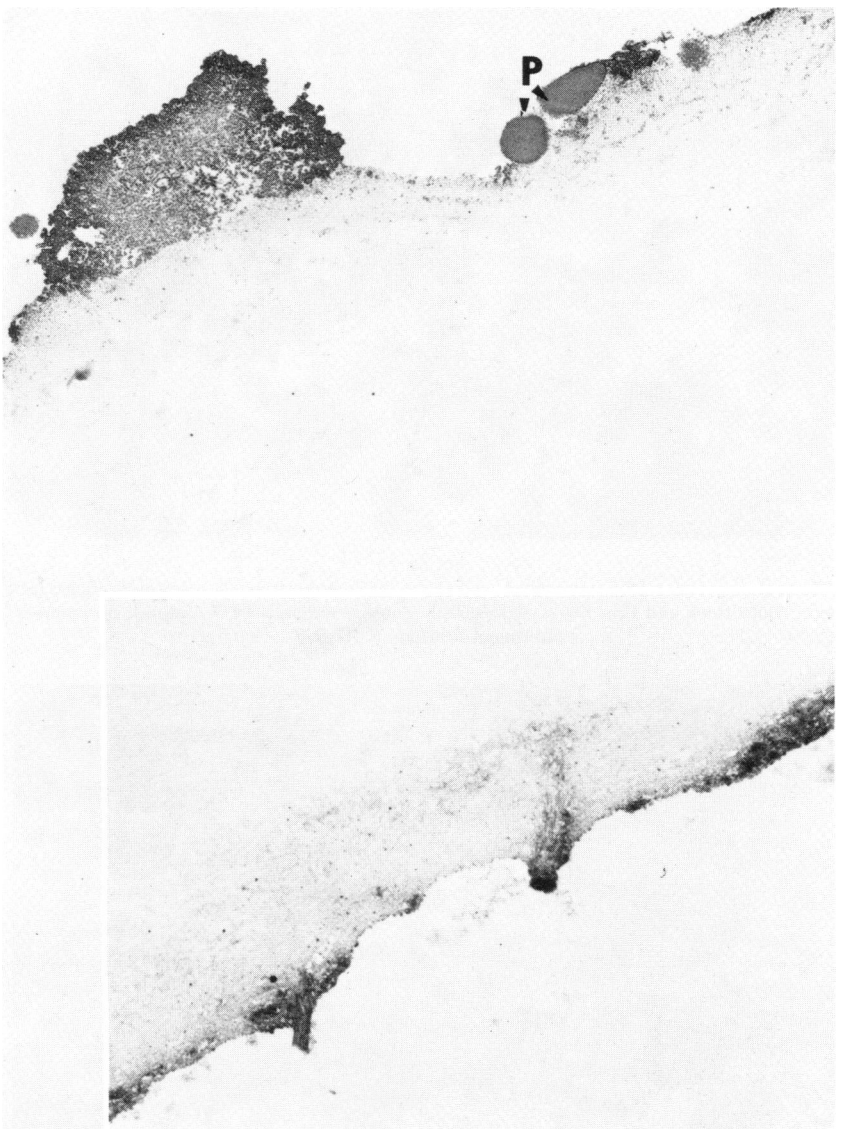


FIGURE 3

Zonular antisera binding to PSX nodules on superficial lens capsule. Note iris pigment granules (P). Inset shows patches of deep fibrogranular zone under this area with deeper stained "tails." Penetration of this thick fibrogranular zone is incomplete. APO method, no counter-stain (magnification, × 9200).

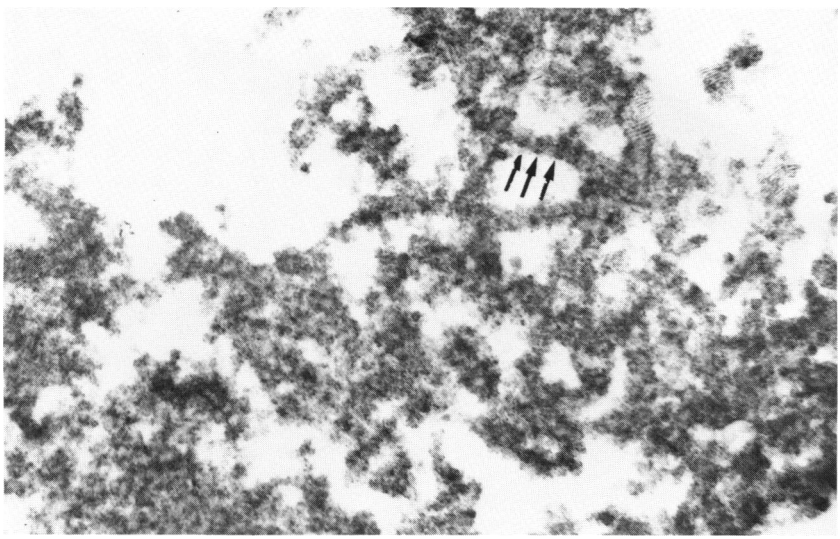


FIGURE 4
Diffuse staining of a PSX superficial aggregate, with suggestion of macroperiodicity (*arrows*). Both thick and thin fibers are stained. Zonular antisera-APO method, no counter-stain (magnification, × 47,500).

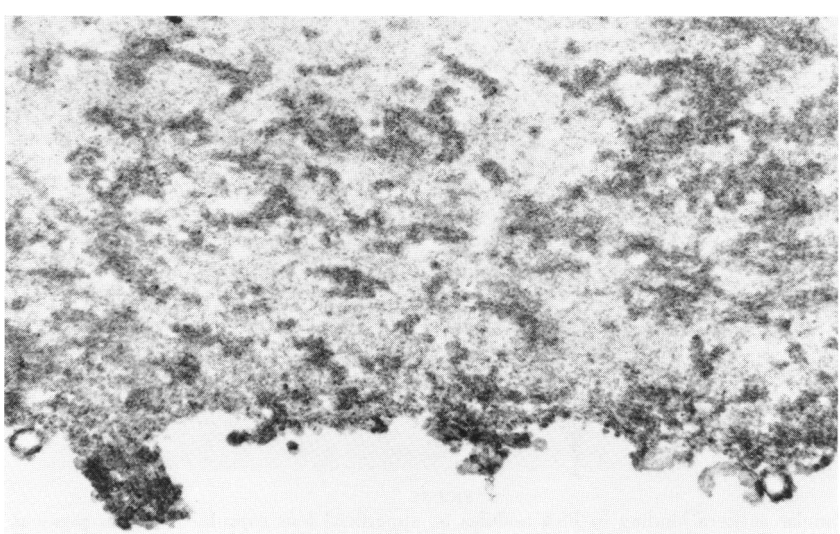


FIGURE 5
Dense staining of a fibrogranular "tail" and branches in lens capsule. Some similar clumps at inner capsular edge. Capsule itself has a fine granularity. Zonular antisera-APO method, no counter-stain (magnification, × 36,200).

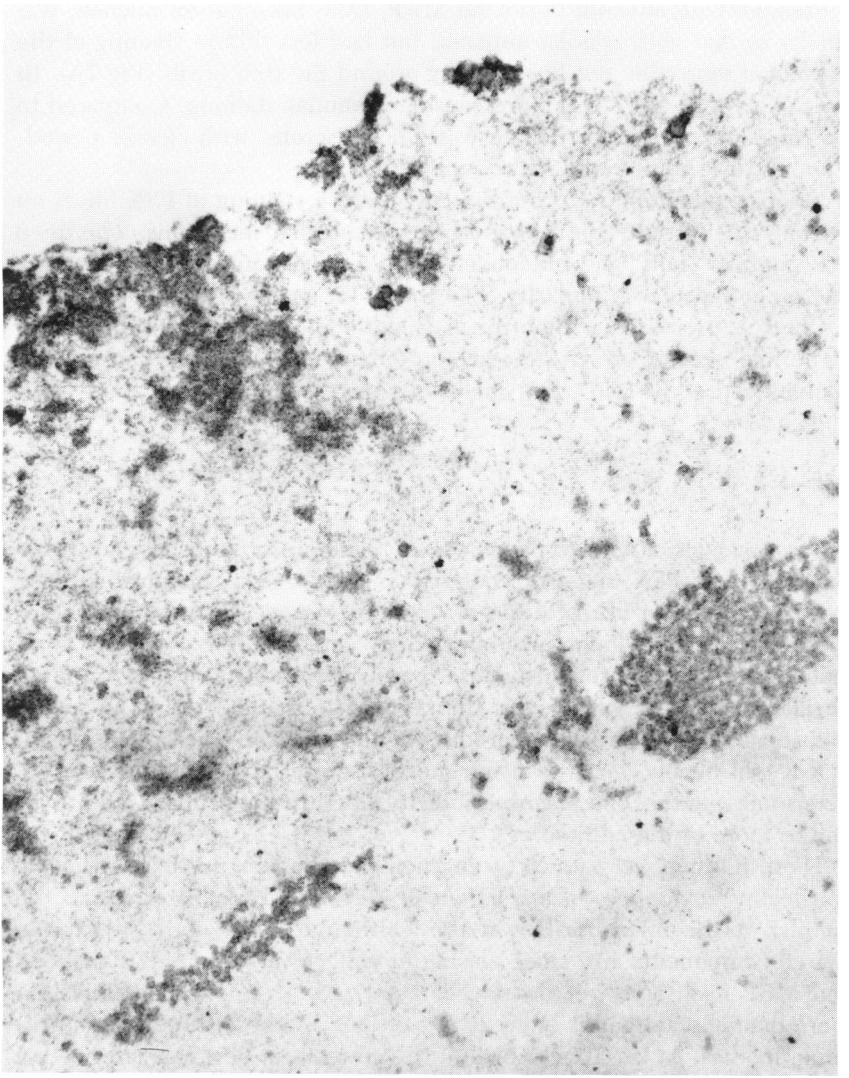


FIGURE 6
Surface of capsule with infiltration of PSX fibers. Large horizontal capsular inclusions also stained. Zonular antisera-APO method, no counter-stain (magnification, × 40,000).

Staining with antisera to bovine MFP, from ligamentum nuchae, was similar to that with zonular antisera, but had less diffuse staining of the fibers and more fine dot-like staining around the thin fibrils (Fig 7A). In the deep zone, there was more capsular granular staining. Compared to zonular antisera, the fibril stain was more discrete, with clearer periodicity, and had penetrated less deeply (Fig 7B).

Monoclonal antibody to fibrillin gave diffuse staining of PSX fibers on the anterior capsule (Fig 8A) and of most capsular inclusions. The deep fibrogranular "tails" and branches were also well stained (Fig 8B) with a fine inner capsular granularity, like that from most of the other stains.

Controls processed without primary antibody or with the substitution of normal sera, were unstained. In the non-PSX disease lenses, the zonules and occasional aging inclusions were stained by all three antibodies.

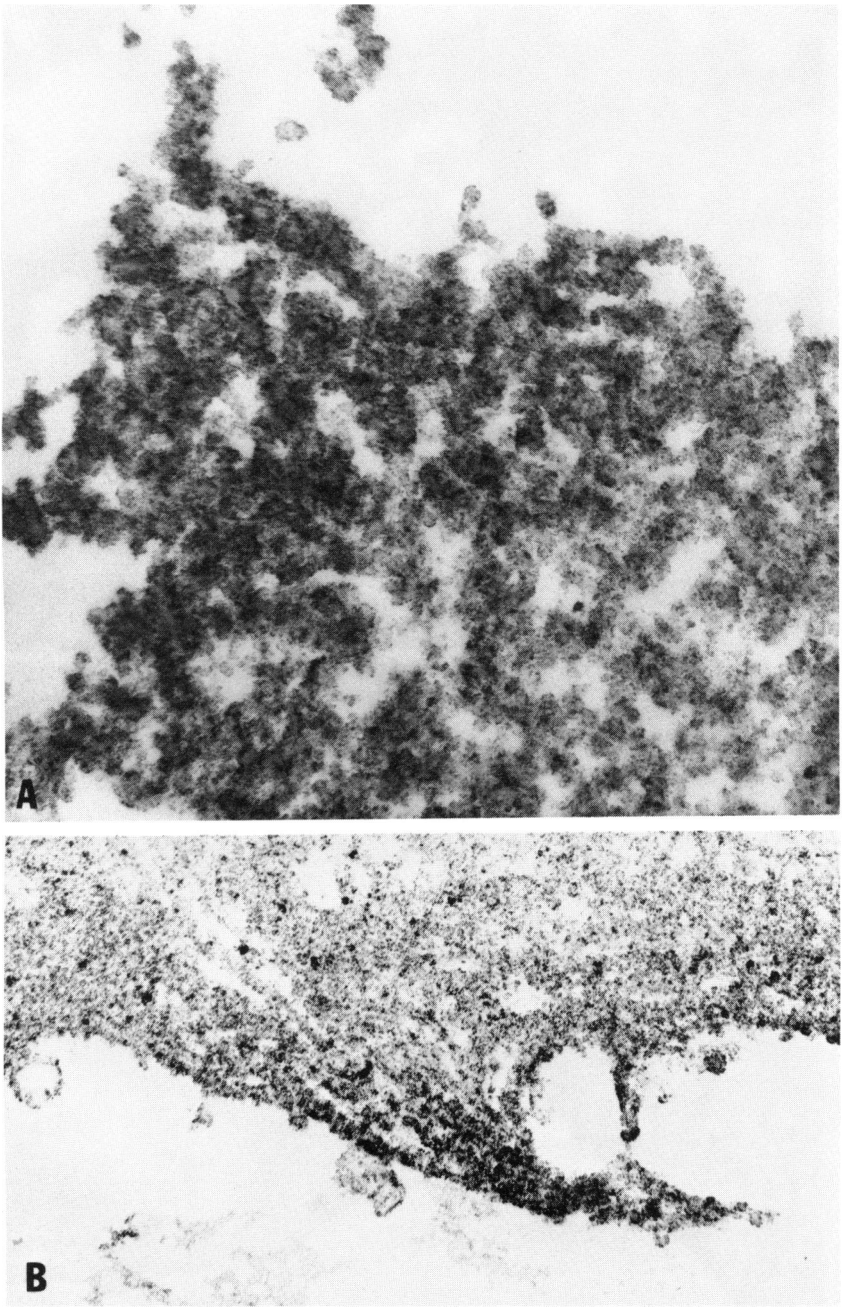
DISCUSSION

There was considerable similarity in the staining produced by all three antibodies on PSX material. Zonular antibody gave the most intense stain, especially in fibrils of the fibrogranular zone. All three antibodies accentuated a 35 to 45 nm macroperiodicity on PSX fibers, and on accompanying zonules, as previously described for zonular and ligament microfibrillar antisera.[15,16] However, the peroxidase method results in such a diffuse aggregate of reaction product at the electron microscopic level, that it will be necessary to use a more discrete label, such as gold, to determine whether the macroperiodicity sites are the primary affinity for one or more of the antibodies.

These findings very much strengthen the previous histochemical[11,12] and lectin[13] evidence, of a relationship between PSX material and the zonular-elastic microfibrillar system, although not defining what the shared components are, since elastic microfibrils are complex structures and MFP itself is as yet poorly characterized. It is quite possible that more than one antigen is being demonstrated by these three microfibrillar antibodies. At least one, fibrillin, the newest but best characterized of the three microfibrillar antigens, is a glycoprotein.[17]

FIGURE 7

A: Heavy staining of PSX aggregates on lens capsule reacted with bovine MFP antisera. APO method, no counter-stain (magnification, × 54,400). B: Fibrogranular "tail" with same antisera. More granular staining of capsule, discrete clumps on vertical fibrils, and less penetration around fibril into capsule compared to zonular AB. APO method, no counter-stain (magnification, × 48,700).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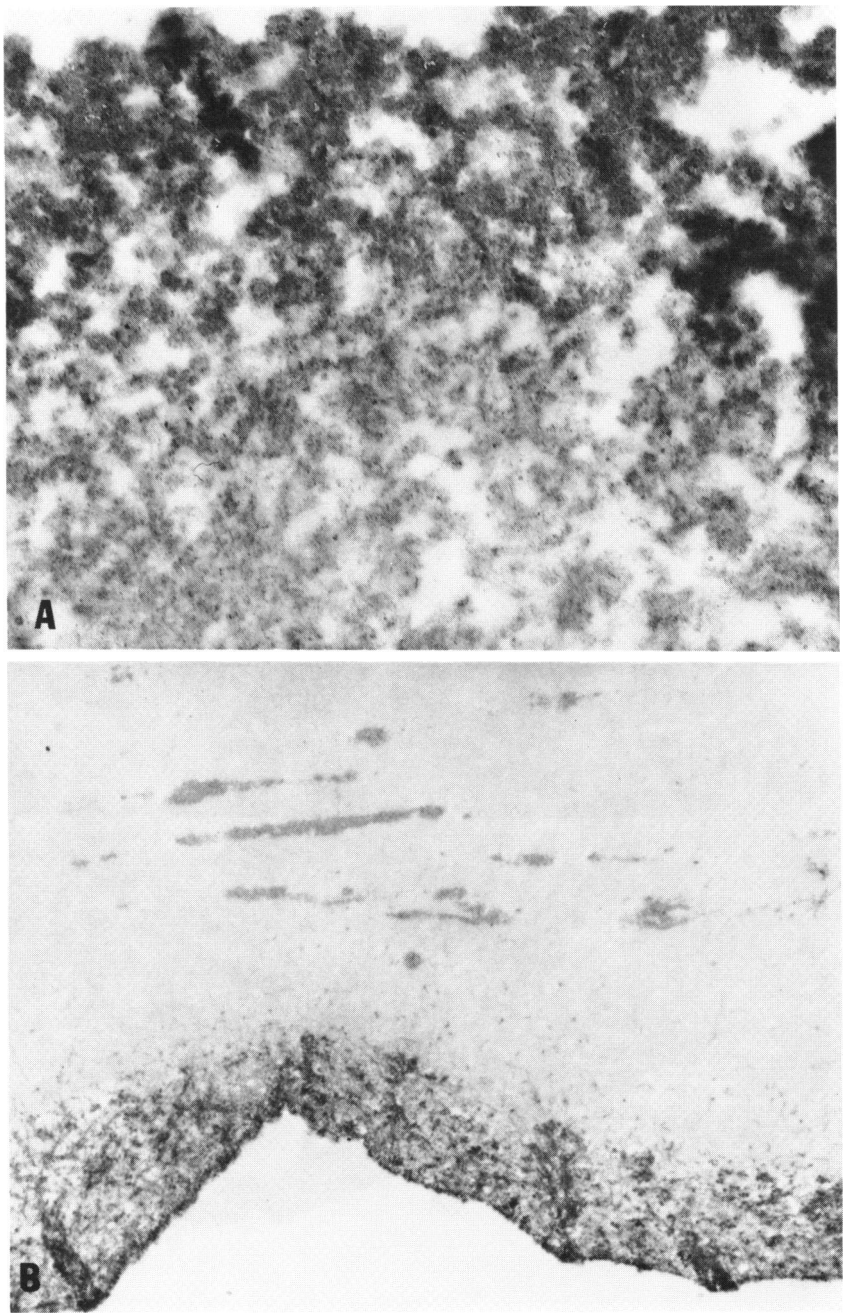
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Several suggestions about the nature of PSX material have been advanced in recent years. Eagle et al[8] believe that PSX material is abnormal banded basement membrane (BM), and that the disease should be called the BM exfoliation syndrome. This theory is based on the morphology of the fibers and the fact that they occur in and around several of the BM of the anterior segment and adnexa, which are often themselves unhealthy. Such proximity appears to implicate the respective BM-producing cells, but it does not necessarily mean that PSX material is composed of BM in any usual sense. BM is composed primarily of type IV collagen, which there is no evidence of, in this study, in any of the antigens used to raise the antibodies. Of course, BM has a number of other components besides collagen which might be involved. We have found no cross-reactivity (unpublished work) between PSX material and three of these components: type IV collagen, laminin, and BM proteoglycan (predominantly heparan sulfate). Harnish et al,[18] using an earlier antibody for the latter, did find positivity in several PSX capsular sites. In digestion studies of PSX material,[19] we found evidence of a small amount of heparan sulfate and chondroitin sulfate, but concluded that proteoglycan did not constitute a major component of PSX fibers. In a series of studies using histochemical techniques, Davanger[20] also inferred the presence of proteoglycans in PSX material, but came to the same conclusion that they did not constitute the central fiber itself.

The other frequent suggestion about PSX composition, first made by Ringvold and Husby,[21] was that it was an amyloid-like substance. PSX material does indeed show some features of amyloid, such as fluorescence with thioflavin T,[10] but it does not show the polarization dichroism with Congo red, presently required for the histological diagnosis. Morphologically, there are also significant differences in the fibers, although the designation "amyloid-like" is not unreasonable.

The possibility, that a variety of ocular region BM-producing cells may be secreting material which polymerizes as microfibrils, with components antigenically like elastic microfibrils and zonules, has some support from the behavior of many such cells in tissue culture. A number of BM-producing mesenchymal cells, besides fibroblasts, are known to produce

FIGURE 8

A: PSX aggregates on anterior capsule with fibrillin monoclonal antibody showing a diffuse stain on fibers, with rare evidence of macroperiodicity. Indirect peroxidase method, no counter-stain (magnification, × 41,600). B: Deep capsule with complete staining of fibrogranular zone fibrils, and capsular horizontal inclusions. Indirect peroxidase method, no counter-stain (magnification, × 16,200).

large quantities of tubular microfibrils of this type, in culture. Microfibrils, secreted by epithelial cells in culture, are less definitely of this type, but tubular fibrils have been described.[23] It might be expected that cells in other than the ocular region, would be able to produce PSX-like abnormal microfibrils; a more extensive search for these should be made. An intriguing question is, what stimulus turns on this secretory product? Is it a local change in environment or a systemic one? Are there hereditary factors, or is PSX material possibly an abnormal gene product from the outset? What relation does PSX material have to the main disease associated, namely glaucoma? It is hoped that further studies of PSX composition will begin to answer these questions.

SUMMARY

Because of histochemical similarities, there is reason for thinking that some component or components of PSX material are also present in zonular fibers. Since the zonule is a member of the elastic microfibrillar system, PSX material on the lens capsule was tested for immunologic affinity to elastic MFP from three widely divergent sources, using an indirect immunoperoxidase electron microscopic method. Positive staining was obtained with all three antibodies on all components of lens PSX material, including the superficial aggregates, deep fibrogranular zone, and capsular inclusions. The results support our hypothesis that PSX material derives from abnormal polymerization of glycoprotein associated with the zonular-elastic microfibrillar system. Similar staining of the abnormal material within the lens capsule indicates that the lens epithelial cell is involved in processing this protein. It might be suspected that other microfibrillar-secreting cells, even beyond the present range of suspected sources, could produce similar material.

REFERENCES

1. Bertelson TI, Drablos PA, Flood PR: The so-called senile exfoliation (pseudo-exfoliation) of the lens capsule, a product of lens epithelium. *Acta Ophthalmol* 1964; 42:1096-1113.
2. Shakib M, Ashton N, Bloch R: Electron microscope study of pseudo-exfoliation of the lens capsule. II. Iris and ciliary body. *Invest Ophthalmol* 1965; 4:154-161.
3. Ghosh M, Speakman JS: The iris in senile exfoliation of the lens. *Can J Ophthalmol* 1974; 9:289-297.
4. ———: The ciliary body in senile exfoliation of the lens. *Can J Ophthalmol* 1973; 8:394-403.
5. Dark AJ, Streeten BW: Pseudoexfoliation syndrome, in A Garner, GK Klintworth (eds): *Pathobiology of Ocular Disease*. New York, Marcel Dekker, Inc, 1982
6. Ringvold A: On the occurrence of pseudo-exfoliation material in extrabulbar tissue from

patients with pseudo-exfoliation syndrome of the eye. *Acta Ophthalmol* 1973; 51:411-418.
7. Speakman JS, Ghosh M: The conjunctiva in senile lens exfoliation. *Acta Ophthalmol* 1976; 94:1757-1759.
8. Eagle RG, Font RL, Fine BSL: The basement membrane exfoliation syndrome. *Arch Ophthalmol* 1979; 97:510-515.
9. Dark AJ, Streeten BW, Jones D: Accumulation of fibrillar protein in aging lens capsule. *Arch Ophthalmol* 1969; 82:815-821.
10. Dark AJ, Streeten BW, Cornwall CC: Pseudoexfoliative disease of the lens: A study in electron microscopy and histochemistry. *Br J Ophthalmol* 1977; 61:462-472.
11. Streeten BW, Dark AJ, Barnes CW: Pseudoexfoliative material and oxytalan fibers. *Exp Eye Res* 1984; 38:523-531.
12. Garner A, Alexander RA: Pseudoexfoliative disease: histochemical evidence of an affinity with zonular fibers. *Br J Ophthalmol* 1984; 68:574-580.
13. Streeten BW, Gibson SA, Li Z-Y: Lectin binding to pseudoexfoliative material and the ocular zonules. *Invest Ophthalmol Vis Sci* 1986; 27:1516-1521.
14. Streeten BW, Dark AJ, Gibson SA: Cross-reactivity between pseudoexfoliative material and zonular antibody. *Invest Ophthalmol Vis Sci* (Suppl) 1982; 22:4.
15. Streeten BW, Licari PA, Marucci AA, et al: Immunohistochemical comparison of the ocular zonules and the microfibrils of elastic tissue. *Invest Ophthalmol Vis Sci* 1981; 21:130-135.
16. Streeten BW, Licari PA: The zonules and the elastic microfibrillar system in the ciliary body. *Invest Ophthalmol Vis Sci* 1983; 24:667-681.
17. Sakai LY, Keene DR, Clanville RWL: Elastin-associated microfibrils are composed of a unique glycoprotein. *J Cell Biol* 1985; 101:99a.
18. Harnish JP, Barrach HJ, Hassell JR, et al: Identification of a basement membrane proteoglycan in exfoliation material. *Albrecht Von Graves Arch Klin Exp Ophthalmol* 1981; 215:273.
19. Streeten BW, Gibson SA: Digestion studies of pseudoexfoliative material on the lens capsule. *Invest Ophthalmol Vis Sci* (Suppl) 1984; 25:150.
20. Davanger M: Studies on the pseudo-exfoliation material. *Albrecht Von Graves Arch Klin Exp Ophthalmol* 1978; 208:65.
21. Ringvold A, Husby G: Pseudo-exfoliation material—an amyloid-like substance. *Exp Eye Res* 1973; 17:289-299.
22. Cleary EG, Gibson MA: Elastin-associated microfibrils and microfibrillar proteins, in DA Hall, DS Jackson (eds): *International Review of Connective Tissue Research*. New York, Academic Press, 1983, pp 171-178.
23. Inoue S, Leblond CP: The basement membrane-like matrix of the mouse EHS tumor: Ultrastructure. *Am J Anat* 1985; 174:373-380.

DISCUSSION

Dr William H. Spencer. Doctor Streeten and associates are to be congratulated for their painstaking evaluation of the nature of pseudoexfoliative material and its relationship to the lens zonule. With the current emphasis on extracapsular cataract extraction in this country, it must have been difficult to obtain ten lenses removed by the intracapsular technique from patients with pseudoexfoliative disease and from patients without this disease to serve as controls. In her American Ophthalmological Society thesis, Doctor Streeten utilized histochemical, ultrastructural, and biochemical techniques to explore the nature of the normal

human ocular zonule. This established the basis for the current study of abnormal material associated with the zonule; it also provided a basis for future investigations of other conditions involving the zonule, such as the hereditary lens dislocating diseases (Marfan's syndrome, homocystinuria, Weill Marchesani syndrome).

In the present communication, the authors provide convincing immunologic, histochemical, and ultrastructural evidence to support their hypothesis that pseudoexfoliative material can form as a result of abnormal polymerization of glycoproteins, associated with zonular elastic myofibrils. Doctor Streeten has previously suggested the general name "elastic microfibrillar system" after showing that isolated microfibrillar bundles of the zonule, as well as microfibrils around the elastic fibers, not only closely resemble the microfibrils of elastic tissue morphologically but also share cross-reactivity to anti-bovine zonular antisera, suggesting biochemical structural similarities as well. The concept of a general "elastic microfibrillar system" is strengthened by the demonstration of similar staining of abnormal material in association with the lens epithelial cells, and by the observations of pseudoexfoliative material in proximity to basement membranes in other portions of the anterior segment of the eye and in the conjunctiva. For many years, it has not been known whether the material seen in association with pseudoexfoliation is limited to the eye or is part of a systemic process. I should like to ask Doctor Streeten whether she has attempted to answer this question, by applying these immunologic staining techniques to sections prepared from biopsies taken from other parts of the body, such as the skin or mucosa. I should also like to ask Doctor Streeten whether she believes this process to be one that is developmental or acquired. The exfoliative syndrome seems to occur in older individuals. This suggests that either it is developmental and accumulates throughout life, only becoming clinically apparent later, or that the basement membrane producing cells in the eye undergo an acquired change in adult life, causing them to begin to secrete abnormal microfibrillar material. Doctor Streeten has indicated that mesenchymal cells can produce large quantities of these microfibrils in tissue cultures. Were the cells used for these tissue culture studies from individuals who had the exfoliative syndrome or, from so-called normals?

I very much enjoyed reviewing this interesting manuscript. So many basic discoveries in medicine result from a combination of curiosity and persistence. Doctor Streeten has demonstrated both of these qualities in her studies of the zonule and related conditions, and I look forward to reading her next manuscript.

Dr Clement McCulloch. Doctor Streeten has pursued this subject with great effect; this paper adds to what she has previously described. Doctor John S. Speakman and I found the exfoliation material on the surface of the ciliary body, among the zonular fibers, on the surface of the lens, in the lens capsule, deep to the lens capsule, and among the lens fibers deep to the lens epithelium. There was exfoliation material in the iris and among the conjunctival vessels. The material was, indeed, very widespread. Wondering about the source of the exfoliation material, its presence among the lens fibers deep to the lens epithelium would suggest that one source may have been lens epithelium. Doctor Streeten

may have a comment on the source, or sources, of exfoliation material.

DR ROBERT DREWS. This is an exciting paper. The relationship between zonule and lens capsule, not only anatomically but also microscopically and biochemically is apparently an extremely complex one. I was intrigued by the apparent extension all the way down to the lens epithelium. One wonders if you postulate some relationship between normal zonule and lens epithelium? There appears to be some beginning evidence from extracapsular surgery that there is a difference in attachment of the lens epithelium to lens capsule comparing the equator and zonular area to the central area anteriorly. What you have shown histologically, at least relating to this abnormal material, may have some relationship to the normal lens epithelial anatomy as well. I thought it was a fascinating paper.

DR BARBARA STREETEN. I thank my discussants, Doctors Spencer, McCulloch, and Drews very much for their interesting and kind comments. Doctor Spencer asks whether we have used or plan to use this new method on other tissues. Yes, we are very interested in doing so. We have done quite a bit of electron microscopy on the conjunctiva in a project with Doctor Ritch, investigating further the areas in which pseudoexfoliative material actually accumulates. Unfortunately, we need different methods of immunostaining. On the lens capsule, PSX material is right on the surface, but the antibodies do not penetrate very far into tissue. We are working on a post-fixation method of immunostaining so that we can examine the whole section, which will be necessary when dealing with solid tissues. Doctor Spencer also asked whether we think that this is a developmental corneal disease. I assume that "developmental" would imply some hereditary component and, of course, I don't know the answer to that. Doctor Spencer always asks insightful questions. The possibility of an hereditary component is very interesting because this is often a bilateral disease. But if 11% of people in Scandinavia have pseudoexfoliative disease on their lenses, it seems almost too common for a single gene disease, although there might be multifactorial imput. So I guess we have to say that at present PSX seems most likely to be an acquired disease. I do want to mention that I was not the first to describe the elastic-microfibrillar system. It is a concept which has been put together by many investigators over the years, but we added evidence that the zonule belongs to this system. To date, I don't know of anyone who has cultured cells from patients with the pseudoexfoliative syndrome, which would be a most interesting thing to do to determine what kind of fibrils would be produced. Doctor McCulloch mentions that he and Doctor Speakman saw pseudoexfoliative material deep to the lens capsule. I don't know how deep, but presumably amongst the cortical fibers and superficial cataract. This is fascinating. I haven't seen that, although it would seem possible if the lens epithelium is making this material, as I strongly believe. We have looked at iris and conjunctiva, and confirm all the previous observations. Doctor Drews brings up an interesting point about some relationship between the lens epithelium and zonules. As far as differences in anatomy, we have described zonular attachments on the anterior capsule, equator, and posterior capsule, and there is some difference

in the kinds of attachments. I don't know whether he was leading to this point, but it does involve a most interesting question of whether the lens epithelium normally contributes something biochemically to the zonules, either in their attachment sites, something important for their attachment to the lens capsule, or even in the actual formation of zonular fibers. Again, I don't know the answer to this but it is a very interesting question.