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Etiology of the developing eye in myelencephalic blebs *(my)* mice

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Summary. The etiology of the eye defects in myelencephalic blebs *(my)* mutant mice has been poorly understood for almost seventy years. Embryos from 9 to 14 ½ days of gestation were subjected to Alcian blue 8GX staining for acidic glycosaminoglycan deposition in basement membrane structures of the developing eye in *my* stock and control specimens. In addition 12 day embryos were subjected to avidin-biotin-peroxidase labelling for laminin. At 9 - 9 ½ days of gestation more Alcian blue positive extracellular matrix was found in the region between the optic vesicle and the overlying putative lens ectoderm in the *my* stock embryos. By 12 days, there was an irregular and lesser amount of deposition of glycosaminoglycans in the len's capsule and in the «inner limiting membrane» of the presumptive neural retina; however, the deposition of laminin appeared to be greater in the inner limiting membrane of the *my* eye. By 14 days, the damage to the eye in the *my* embryos can be quite extensive, and the deposition of glycosaminoglycans was very meager in this situation. It appears that irregular deposition of glycosaminoglycans in the extracellular matrix and possible increase in the amount of laminin in basement structures in *my* embryos indicate disruption of the normal histochemistry involved in the development of the eye. Altered histochemistry may in turn indicate changes in permeability between cells of the developing tissues which result in the blebbing.

Key words: Eye, Blebs, Basement membrane, Glycosaminoglycans

Introduction

Although the anomalies were first described almost seventy years ago, (as cited by Grüneberg, 1952; Carter, 1959), the etiology of the eye defects in myelencephalic blebs *(my)* mutant mice has remained poorly understood. Therefore, we undertook a systematic investigation of successive stages in the expression of the *my* anomaly during the development of the eye in these abnormal embryos. Since it is obvious that the cornea and lens are markedly affected in mutant *my* embryos (Grüneberg, 1952), and since basement membranes «form the *in vivo* substratum for epithelial and endothelial cells» (Paulsson et al., 1986), it seemed appropriate to examine the eyes of *my* embryos with a special concern for abnormalities of the basement membrane and other extracellular matrix components. In addition, since a number of investigations (Ekblom et al., 1980; Timpl et al., 1983; Kleinman et al., 1985) have emphasized the role of laminin in the basement membrane and in embryonic development, it was also determined that preliminary immunochemistry procedures which are specific for laminin would be utilized in our investigations of the *my* eye anomalies. Eyes of C57BL/6 and in a few cases, of Patch *(Ph)* stock embryos (Center et al., 1988) were utilized as control specimens. It has been suggested recently that the Fraser syndrome in humans might be homologous to one of the «bleb» mutants of the mouse (Winter, 1990). This possibility heightens interest in the development of the *my* mutant which may serve as a model for the above syndrome in humans. The blebs were established as a likely cause of the eye anomalies in *my* mutant mice a number of years ago (Grüneberg, 1952), but the histological etiology of the myelencephalic blebs abnormalities was not determined.

Materials and methods

Twenty-one mouse embryos ranging from 9 - 14 ½ days of gestation were included in our investigation of the histochemical changes in the basement membrane

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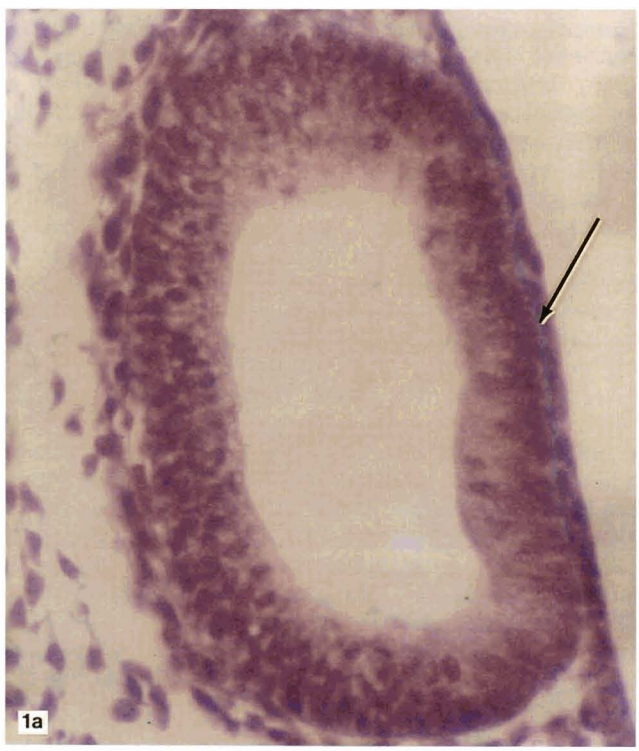
and other components of the extracellular matrix associated with various structures in the developing eye utilizing Alcian blue 8GX staining. Two main groups of embryos were studied: these were the myelencephalic blebs *(my)* mice and C57BL/6 normal control mice. Five embryos from a Patch *(Ph)* stock were included as controls since the embryos from the Patch stock showed no eye abnormalities and were classed as normal. Pregnancies were timed using the vaginal plug method (plug = day zero) and correlation with Grüneberg's morphological staging (Grüneberg, 1943).

Pregnant female mice were sacrificed by cervical dislocation and embryos were removed and placed in 0.9% NaCl. The extraembryonic membranes and placentae were removed and the embryos were fixed in 10% neutral buffered formalin at room temperature for two to three days. After this time, the embryos were transferred to 70% ethanol and stored at room temperature. The embryos were embedded in paraplast at 56° C to 59° C and the head of each embryo was serially cross sectioned at 10 μm, albumin was used as an adhesive in order to adhere the sections to the slides. Staining was done by use of a modification of the method developed by Steedman (Bancroft, 1975) for staining at a pH of 2.5 for the presence of acidic glycosaminoglycans. In order to provide contrast, hematoxylin (Harris, modified without acetic acid) was used as a counterstain.

Five embryos of 12 days gestation (C57BL/6 and *my*) were utilized for study of the differences in the distribution of laminin in the basement membrane structures of the developing eye. The timed embryos were removed from the pregnant females as stated above, but they were placed in cold 0.9% NaCl and fixed in cold 10% neutral buffered formalin for two days and stored at 4° C; they were then transferred to cold 70% ethanol for storage at 4° C. These embryos were embedded in parawax at 54° C and the head of each embryo was cross-sectioned at 8 μm under a constant flow of CO_2 vapor. To avoid tryptic digestion of the adhesive during the staining procedure, the slides were treated with Vectabond Reagent (Vector Laboratories, Burlingame, CA) prior to section adhesion. A number of alternative techniques e.g., poly-l-lysine, proved to be unsuccessful. Sections were cleared and dehydrated in xylene and 100% ethanol after fixation to the slides.

Staining for laminin was done utilizing a modified version of avidin-biotin-peroxidase complex labelling (D'Errico et al., 1986). Endogenous peroxidase activity was blocked using a 0.3% H_2O_2 solution in PBS, pH 7.2. The tissues were then incubated for five minutes at 37° C in Tris buffer and then for 20 minutes at 37° C in a 0.1% trypsin/0.1% $CaCl_2$ in Tris buffer solution to digest any inhibitors introduced by the formalin. Non-specific staining was blocked using 10%



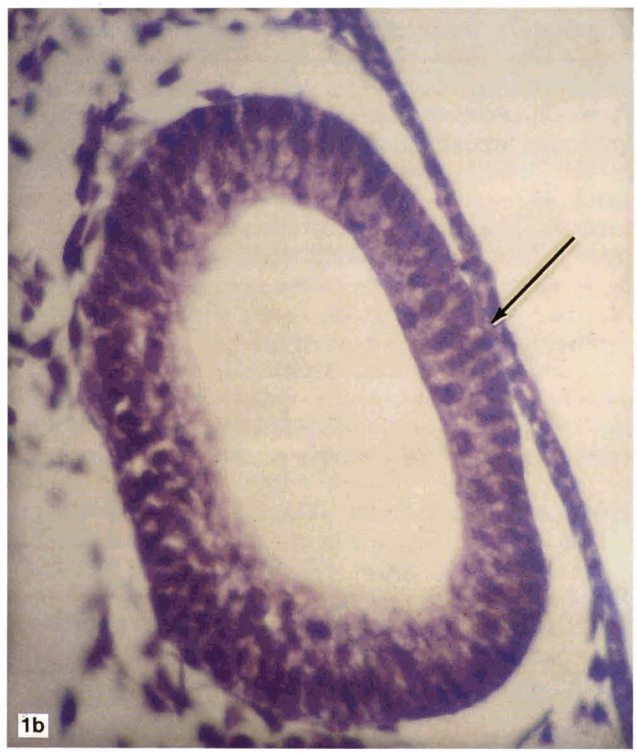
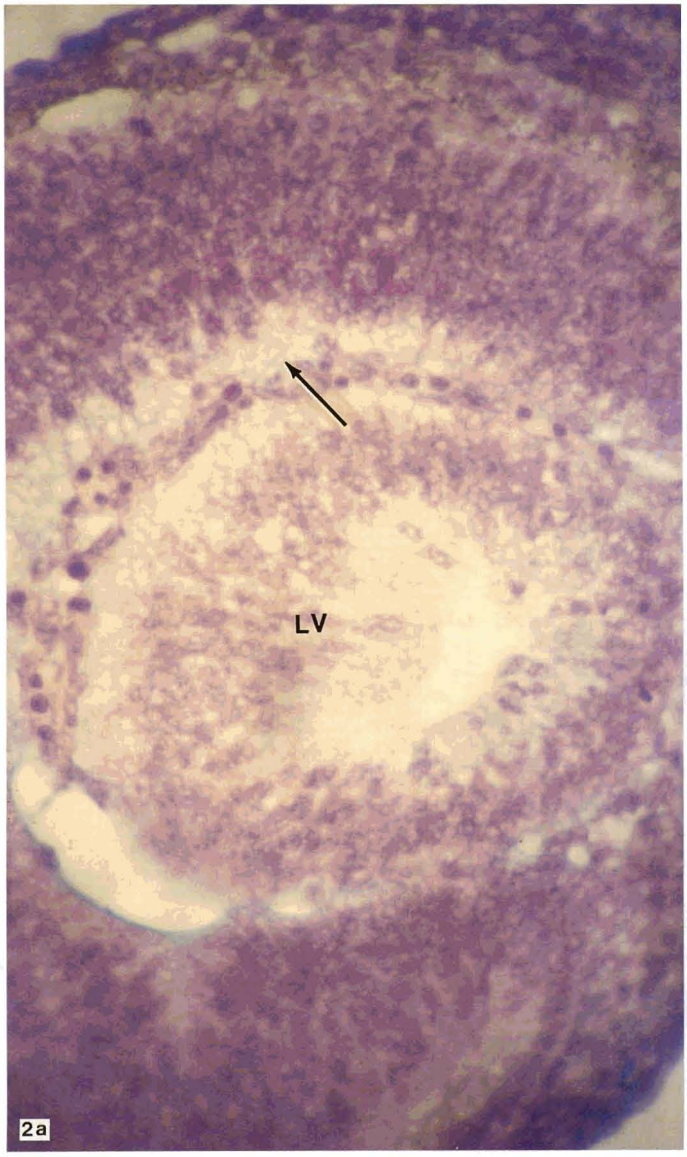


Fig. 1a. Section of a developing eye in a 9 ½ day *my* stock embryo showing the optic vesicle and overlying presumptive lens ectoderm. Arrow indicates area of deposition of glycosaminoglycans in the extracellular matrix between the optic vesicle and overlying ectoderm × 480. **Fig. 1b.** Section of a normal developing eye in a 9 ½ day Patch stock embryo showing the optic vesicle and overlying ectoderm. There is almost no extracellular matrix (arrow) between the optic vesicle and overlying ectoderm. × 525. (Fig. 1 Alcian blue and hematoxyling staining).

normal goat serum (Vector Laboratories) in PBS for 20 minutes at room temperature in a moist chamber, as were the following incubations. The slides were drained and incubated in laminin antiserum (rabbit anti-mouse laminin, Gibco Laboratories, Grand Island, NY) for 60 minutes. Subsequently, the slides were incubated in biotinylated anti-rabbit IgG (Vector Laboratories) for 30 minutes, and then were incubated in the avidin-biotin complex (Vector Laboratories) for 30 minutes. Afterwards, the slides were stained with Hanker-Yates Reagent (Polysciences, Inc, Warrington, PA) and counterstained with hematoxylin. All slides in the investigations reported here were examined by means of light microscopy.

Results

Alcian blue staining indicated that in the 9 - 9 ½ day *my* stock embryo there was more acidic glycosaminoglycans in the extracellular matrix between the overlying ectoderm and the optic vesicle than was evident in the normal eye of a 9 ½ day Patch stock embryo (Fig. 1). In the 9 day C57/BL/6 embryo in the same region noted above, there was also a lesser amount of glycosaminoglycans than in the *my* stock embryo. At this stage there was no evidence of the conditions that would lead to the formation of blebs and the obstruction of normal eye development in the *my* embryos. The optic vesicle and overlying ectodermal



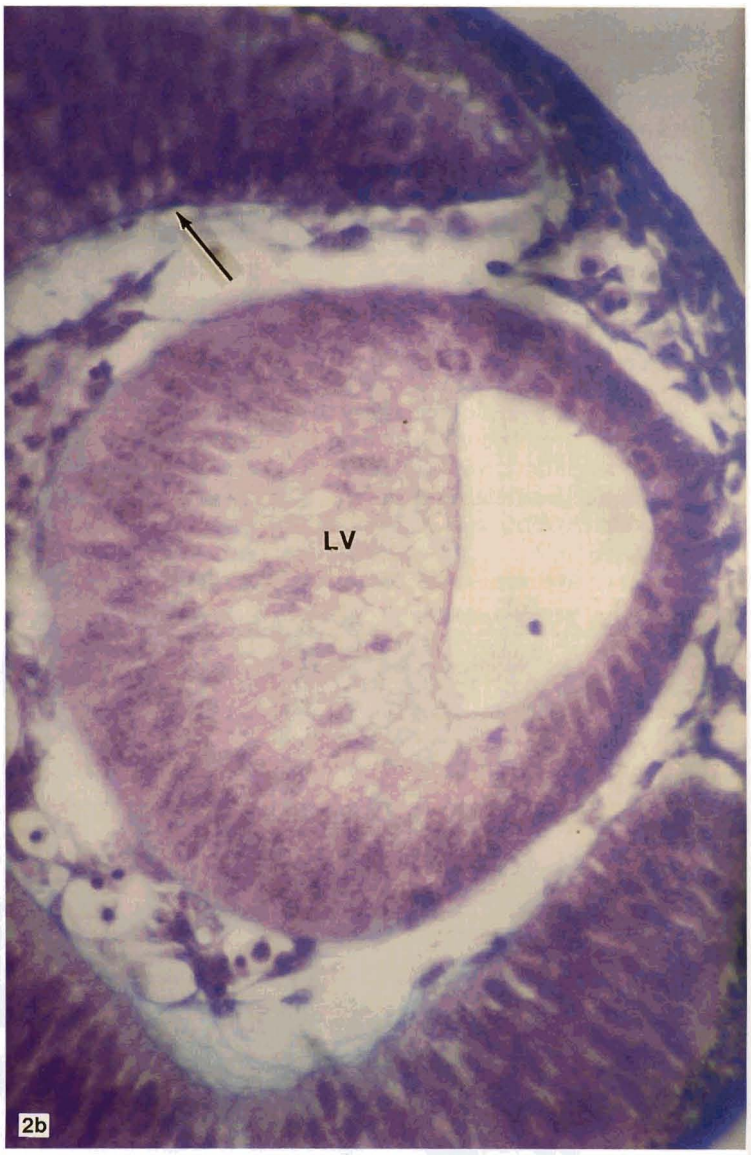


Fig. 2a. Developing lens vesicle (LV) in a 12 day *my* embryo. Lens capsule is disrupted and the inner limiting membrane (arrow) of the putative neural retina is irregular in deposition. Both tissues show less deposition of glycosaminoglycans. × 560. **Fig. 2b.** Developing lens vesicle (LV) of a normal 12 day C57BL/6 embryo. Cells of the developing lens are well ordered. The lens capsule and inner limiting membrane (arrow) show regular deposition of glycosaminoglycans. × 560. (Fig. 2 - Alcian blue and hematoxylin staining).

cells appeared to be normal. No differences in the morphology of these structures in the developing eye were noted between the eyes of the *my* stock embryos and the controls.

By 12 days of gestation, the situation appeared reversed with regard to the amount of glycosaminoglycans in the embryonic eyes. There was an irregular and lesser amount of deposition of glycosaminoglycans in the developing «inner limiting membrane», which is a basal lamina of the presumptive neural retina and in the capsule of the lens vesicle of the *my* eye. The lens capsule appeared to be disrupted and the inner limiting membrane of the putative neural retina was irregular in deposition. In addition, the cells of the lens vesicle appeared less well ordered in the *my* eye. There was an obvious overall decrease in size of the lens vesicle (Fig. 2). While it is out of the field shown in figure 2, the cornea was also affected, showing some collapse and closer proximity to the lens vesicle. The blebbing was grossly visible at this stage and the development of the eye appeared to be directly affected. In the embryos which were utilized for laminin investigation, the developing inner limiting membrane of the presumptive neural retina appeared to be thicker and contained more laminin in the *my* embryo than was seen in the eye of a control C57BL/6 embryo of a comparable stage of development (Fig. 3).

By 14 - 14½ days, the abnormalities of the eyes were of varying degrees of severity in the *my* embryos. In one case, the *my* eye was malformed and smaller with slightly more glycosamynoglycans deposited in the inner limiting membrane of the neural retina than in the normal eye (Figs. 4a, b). In another case, extreme malformation of the eye was evident (Figs. 4c, d). The eye had almost completely degenerated and it appeared that red blood cells had invaded the eye (Fig. 4d). While the neural retina was still discernible, the cornea and other structures of the eye were not easily identifiable (Fig. 4c). The lens capsule was very disrupted with only a hint of glycosaminoglycans remaining; there was also a possible remnant of the inner limiting membrane (Fig. 4d).

Discussion

It appears that irregular deposition of glycosaminoglycans in the extracellular matrix of certain structures of the eye plays a role in the development of abnormal eyes in *my* mutants. This mutation which is located on Chromosome 3 (Davisson et al., 1976) has been studied by a number of investigators, (cited in Grüneberg, 1952; Carter, 1959; Center, 1977; Eicher and Lane, 1980). The abnormal deposition of acidic glycosaminoglycans and the increase in laminin indicate disruption of the normal histochemistry involved in the development of the eye. Beginning on day nine in the *my* stock embryos, there is an altered pattern of glycosaminoglycan deposition. These irregular changes in the chemistry of development may indeed be the cause instead of the result of the blebbing. Histochemical changes in the extracellular matrix, particularly in the sulfated glycosaminoglycans, were found associated with the eye defects found in aphakia mutant mice (Zwaan and Webster, 1984) and in anopthalmic mice (Webster et al., 1984). In working with the anopthalmic strain, Webster et al. noted that normally there is strong staining for sulfated glycosaminoglycans between the optic cup and the lens rudiment when these tissues are in close proximity. As these rudiments separate, the staining becomes less intense. If the amount of glycosaminoglycans is

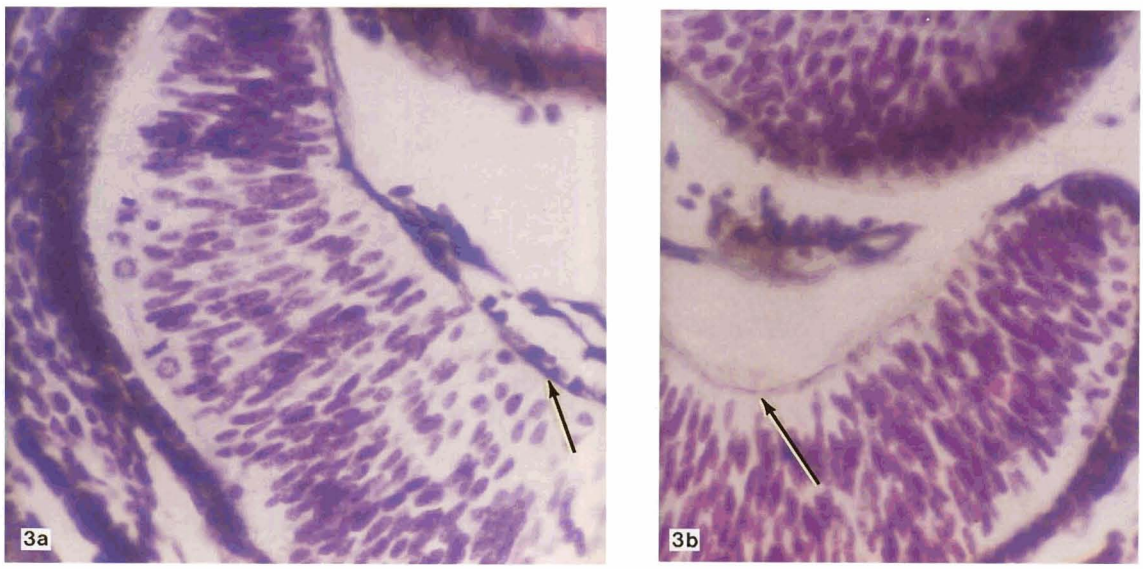


Fig. 3a. Section of a developing eye of a 12 ½ day my *embryo*, arrow indicates an area of greater laminin deposition in the inner limiting membrane of the retina than is seen in Fig. 3b. × 640. **Fig. 3b.** Section of a normal developing eye of a 12 day C57BL/6 embryo, arrow indicates laminin deposition in the inner limiting membrane of the retina. × 640. (Figure 3 - Avidin-biotin-peroxidase labeling of laminin).

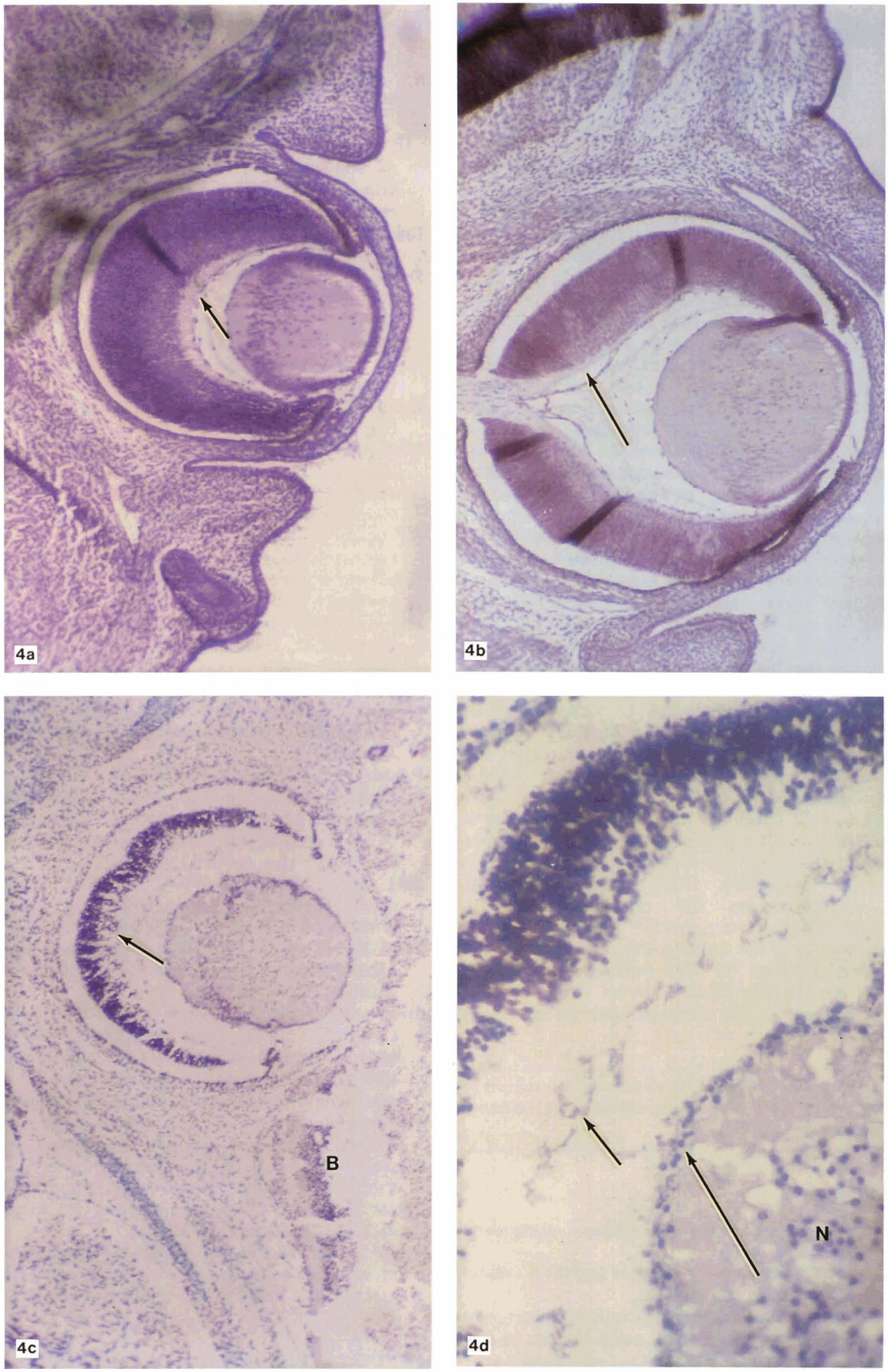


Fig. 4a. Section of the eye of a 14 ½ *my* embryo with a somewhat normal appearing eye. However, the eye is smaller than the normal eye found in a comparable 14 ½ day C57BL/6 embryo (Fig. 4b). Arrow indicates glycosaminoglycans in the inner limiting membrane of the retina. × 100. **Fig. 4b.** Section of a normal eye of a 14 ½ day C57BL/6 embryo. Arrow indicates the inner limiting membrane of the retina. x 100. **Fig. 4c.** Section of a severely affected eye of a 14 ½ day *my* embryo with a large bleb (B) over the eye. Arrow indicates the degenerating neural retina. × 105. **Fig. 4d.** Higher magnification of Fig. 4c. showing a remnant of the inner limiting membrane (short arrow) of the neural retina and a lens capsule (long arrow) which shows degeneration and only a slight amount of glycosaminoglycans. N indicates the nuclei of red blood cells that have invaded the lens. x 445. (Fig. 4 - Alcian blue hematoxylin staining).

excessive, it is possible that the optic vesicle and presumptive lens tissues remain in contact for too long a period of time and abnormal development results.

Carter (1959) cited excess «mesenchymal intercellular fluid» as the «most probable source of the bleb fluid» in the *my* mice. It is of interest to note that Pino et al., (1982) suggested that some disorders affecting the function of the eye may involve changes in the chemistry of the basal laminae which result in changes in permeability. The functioning of basement membranes as «filtration barriers» has been recognized for some period of time (Mohan and Spiro, 1986; Turksen et al., 1985). In addition, basement membranes have been implicated in a number of disease conditions including autoimmune diseases and neoplastic conditions (Timpl et al., 1983). Timpl et al. suggest that excessive development of the basement membrane may interfere with the transfer of substances into and out of tissues. It is relevant in this regard, that more deposition of laminin was noted in the putative inner limiting membrane of the developing neural retina in the twelve-day old *my* embryo. Thus, it seems reasonable to propose that alterations of the histochemistry of the extracellular matrix and in particular, of the basement membranes components are the basic cause of the accumulation of fluid which causes blebbing. The blebbing in turn results in defective eyes found in the *my* mutants. We are extending our investigation to the study of the postnatal glomerular basement membrane and Bowman's capsule basement membrane in the kidneys of juvenile and adult *my* mice since *my* mutants are known to have kidney anomalies in addition to eye abnormalities (Grüneberg, 1952). Preliminary results indicate alterations in the deposition of the glycosaminoglycan components of basement membrane tissues in the kidneys from *my* mice. Thus, a widespread change in the semipermeability of basement membranes may prove to be the fundamental cause of the abnormalities found in the *my* mutants.

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