Müller's cells and the "middle limiting membrane" of the human retina

An electron microscopic study

Ben S. Fine* and Lorenz E. Zimmerman

Electron microscopy of 3 normal human retinas has provided evidence which generally supports the conventional ideas that have been based on light microscopy of Müller's cells. This study has revealed even greater cytoplasmic differences between the inner and outer halves of Müller's cells than has been appreciated in the past: the inner has properties of fibrous astrocytes whereas the outer resembles oligodendrocytes. Certain structural characteristics of the inner part of the outer plexiform layer give it both the appearance and properties of a limiting membrane. This structure tends to restrain the passage of hemorrhages and exudates out of the outer plexiform layer; we have proposed the name "middle limiting membrane" for this distinctive zone.

Interest in the structure and physiology of Müller's cells (radial fibers of Müller) has been reawakened by the recent histochemical studies of Cogan and Kuwabara,1-4 Pearse,5 and Berkow and Patz.6,7 Long considered merely a coarse glial skeleton which constitutes the main framework of the retina, these cells are now known to possess great lactic dehydrogenase activity as well as an ability to synthesize and store glycogen. Both the lactic-diphosphopyridine nucleotide (DPN) dehydrogenase activity and the storage of glycogen may be increased in pathologic states associated with gliosis. In view of these new observations and in light of their potential importance in pathologic conditions of the retina, it seemed essential to determine by electron microscopy the cytologic characteristics of the normal Müller cell.

The conventional ideas about the structure of Müller's cells as determined by light microscopy are sufficiently well described in standard textbooks5-10 that they need not be reviewed here. To date we are not aware of any detailed electron microscopic observations of these cells in the normal human retina. Sjöstrand, in his study of the retinal rod synapses of the guinea pig eye11 and in his subsequent study of the retinal receptors of the verte-
brate eye, published some observations of the outer (scleral) portion of Müller's cells. Ladman briefly described the inner part of these cells in the cat retina. Cohen included the inner part of the cells in his description of the nerve fiber layer of the retina of the rhesus monkey.

One purpose of this paper, therefore, is to describe and illustrate the varying characteristics of Müller's cells in the several layers of the human retina as determined by electron microscopy. In the course of this study it became apparent that besides the four well-known "limiting membranes" of the retina, there is still another which might be called the "middle limiting membrane" because it lies in a plane that is approximately midway between the internal and external limiting membranes of the sensory retina. Thus, a second purpose of this presentation is to describe the "middle limiting membrane" and to indicate the role played in certain pathologic states.

Materials and methods
All material for the electron microscopic studies was obtained and fixed promptly at the time of surgical enucleation. Three eyes were used: one from a 57-year-old white woman who had a melanoma of the ciliary body, another from a 40-year-old white man who had a melanoma of the upper temporal choroid, and a third from a 61-year-old white man who had a melanoma of the upper nasal choroid. In each case the retina overlying the tumor was only minimally affected and the remainder of the retina was uninvolved. The eyes, except for their uveal tumors, were otherwise considered normal by clinical and histopathologic examination. Suitable portions of nasal and macular retina, as distant as possible from the tumor areas, were used for this study. The macular area was further subdivided into nasal macula, between the fovea and the optic disc, and the temporal macula, just temporal to the fovea centralis. Immediately after enucleation and within the operating room, the globes were opened through the equator and portions of retina, together with choroid and sclera, were cut out and placed in Dalton's chrome-osmium fixative for 30 minutes at room temperature. The tissue was then washed thoroughly with 10 per cent ethanol, dehydrated through ascending concentrations of alcohol, and finally embedded in Epon.

Sections of the embedded tissue were cut in a Porter-Blum ultramicrotome with a glass knife and some of the mounted sections were then treated for 10 minutes with 1 per cent uranyl acetate in 50 per cent ethanol, washed with distilled water, drained, and allowed to dry before examination. Other sections were examined without treatment with uranyl acetate. The sections were examined in an RCA-EMU 3D or 3F electron microscope. The scale markers on the micrographs represent 1 μ unless otherwise indicated.

Observations
Our principal observations are described in detailed legends which accompany each illustration (Figs. 1 to 15). The electron micrographs are generally arranged in such a sequence that the Müller cell is traced from the inner to the outer limiting membrane. We will not repeat all details here, but will merely summarize.

The inner ends of Müller's cells form the inner surface of the sensory retina to which a basement membrane (the internal limiting membrane of the retina) is intimately attached. The outer ends of Müller's cells and the adjacent receptor cells contain apposing cytoplasmic and plasma membrane densities interpreted as terminal bars which account for the structure designated external limiting membrane by light microscopists. Villous projections of the Müller cells pass outward beyond the external limiting membrane. In all retinal layers between the internal and external limiting membranes, cytoplasmic extensions of Müller's cells surround and fill in the "spaces" between nerve cells, dendrites, and axons. Other interstitial cells (glia) are comparatively few and limited to the vascular layers of the retina. No interstitial connective tissue or ground substance is identified between the cellular elements of the retina, except in that portion outside the external limiting membrane where there is both histochemical and electron microscopic evidence of a mucoid interstitial substance between receptor cells and about the villous terminations of the Müller cells.15
For legends for electron micrographs see pages 321 to 323.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 7.
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Fig. 10.
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Fig. 13.
Fig. 15.
Fig. 1. Human retina (nasal), celloidin-embedded and cut at 12 to 14 μ for orientation. The inner ends (IM) of Müller's "fibers" are observed passing through the inner plexiform, ganglion cell, and nerve fiber layers to terminate at the internal limiting membrane (ILM). The outer portions (OM) of Müller's cells are not visible as "fibers" but appear as clear spaces between the axons (RA) of receptor cells. (Hematoxylin and eosin stain. ×440.)

Fig. 2. The inner boundary of the Müller cell ends as an irregularly infolded plasma membrane (PL) to which is intimately applied a thick basement membrane (BM) measuring here 0.4 to 1.2 μ. This membrane is smooth on its innermost surface from which the filaments of the vitreous framework are presumed to have separated in these preparations. Double membranes representing the plasma membranes of adjacent Müller cells can be seen clearly at PM. Neurites (N) cut obliquely are seen in the nerve fiber layer at the bottom of the micrograph. The cytoplasm of the Müller cells contains elongated, moderately electron-dense mitochondria (M) and large aggregates of radially oriented intracellular filaments (F). Among these filaments there are electron-dense particles (P). Larger vesicles (V) of lesser electron density are found throughout the cytoplasm. (Nasal macula, uranyl acetate treated. ×12,000.)

Fig. 3. Within the nerve fiber layer of the retina the Müller cells (upper right portion of field) contain large aggregates of radially oriented, delicate (100 to 120 Å) intracellular filaments (F) among which are frequently found elongated mitochondria (M) that are also oriented radially. Cytoplasmic extensions (E) of the Müller cells partially enclose small groups of neurites (N) and so serve as an intercellular matrix within the nerve fiber bundles. Vesicles of moderate electron density (V) are present in many areas of the cytoplasm. (Nasal retina, untreated. ×17,000.)

Fig. 4. Although the nuclei of Müller's cells may be found at all levels of the inner nuclear layer, in this micrograph the nucleus of a Müller cell (NUC) is seen at the junction of the inner plexiform and inner nuclear layers. Interweaving neurites of the inner plexiform layer (IPFL) are present in the upper right corner of the micrograph. The cytoplasm of the Müller cell here is less filamentous but more densely packed with linearly oriented granules and vesicles of moderate electron density measuring about 400 to 500 Å. In the outermost part (lower left corner) of the Müller cells in this micrograph, clusters of small (150 Å) dense granules interpreted as probable ribonucleoprotein particles (RNP) are seen. Several artifactitious linear densities (A) are present. The irregularly lucent area in the cytoplasm at D is interpreted as early degeneration due to extreme sensitivity of the Müller cell to the manipulations involved in obtaining and preparing the tissue. (Nasal retina, untreated. ×17,000.)

Fig. 5. Müller cell cytoplasm near the nucleus (NUC) from a section of nasal retina cut obliquely. Mitochondria (M) are present but they do not have the narrow, elongated appearance of those in the innermost part of the cell. Intracellular filaments (F) are found in little aggregates, poorly oriented within the more electron-lucent cytoplasm. Very short segments of granular endoplasmic reticulum (ER) and some clusters of free ribonucleoprotein particles (RNP) are present. Tubular (T) and vesicular (V) profiles are observed in the cytoplasm. (Nasal retina, uranyl acetate treated. ×24,600.)

Fig. 6. Cytoplasm of Müller cell passing outward through the inner plexiform layer. Small numbers of radially oriented filaments (F) are present as well as elongated mitochondria (M) and moderately electron-dense granules, vesicles, or short segments of tubules (T). The Müller cell cytoplasm (MC) extends laterally to occupy all "space" and to serve as an interstitial matrix between the neurites. The large numbers of vesicles (V) within the neurites of the inner plexiform layer suggest the presence of synapses nearby. (Nasal macula, uranyl acetate treated. ×14,400.)

Fig. 7. A Müller cell can be seen extending from its nucleus (NUC) within the inner nuclear layer through the innermost portion (IP) of the outer plexiform layer to reach past the synaptic layer into the "spaces" between the receptor cell axons of the "fiber" portions of the outer plexiform layer (lower right corner of field). The cytoplasm of the Müller cell here differs from that near the internal limiting membrane in being less fibrous and less compact in its arrangement. NI, nuclei of neurons in the inner nuclear layer. (Nasal macula, uranyl acetate treated. ×9,900.)
The cytoplasm of a neuron containing a grouping of granular endoplasmic reticulum and clusters of free ribonucleoprotein granules in a pattern similar to Nissl substance is seen at Fig. 9. The cytoplasm of a Müller cell in the innermost portion of the outer plexiform layer. The peculiarly electron-lucent cytoplasm of the field. Small numbers of disoriented intracellular filaments (F) are seen within the Müller cell. The Müller cell cytoplasm extends laterally (MC) to occupy all "space" between the neural cells. (Nasal macula, uranyl acetate treated. ×15,500.)

Fig. 10. Outer plexiform layer of the retina cut obliquely to show more of Müller cell cytoplasm (MC) among the neurites (N) and about the synaptic terminals of the receptor cells (ST). A few densities (free arrows) of adjacent neurite plasma membranes and cytoplasm are seen in this plexiform layer (for more detailed demonstrations, see Figs. 11 and 12). The cytoplasm of Müller's cell contains filaments, dense particles, fragments of granular endoplasmic reticulum, small and large vesicles of low electron density, and mitochondria similar to those found elsewhere in the cell, but, here, these elements appear to be less concentrated and less well oriented. (Nasal retina, uranyl acetate treated. ×34,250.)

Fig. 11. Innermost portion of outer plexiform layer showing some dendrites (D) of a single cell leading from synaptic connection with a multisynaptic cone foot (CF). The neurites of this layer are interwoven in a complex manner but short densities (desmosomes, Ds) can be seen between a number of these neurites. Similar segmental plasma membrane densities (SD) are clearly seen in the synaptic areas. DT, is a dendrite leading away from a synaptic connection with the cone foot. (Nasal retina, untreated. ×18,600.)

Fig. 12. A portion of a cone foot synaptic area (CS) (lower portion of field) filled with a large number of synaptic vesicles. A typical dense cone synaptic lamella characteristically surrounded by a grouping of synaptic vesicles is seen at SL. The upper part of the micrograph shows the interweaving neurites (N) of the innermost portion of the outer plexiform layer. Shorter densities (D) are observed periodically along adjacent plasma membranes associated with an increase in density of the adjacent neurite cytoplasm. These are interpreted as attachment plates or desmosomes. Similar short dense segments of adjacent plasma membranes are seen along the complex synaptic membranes, synaptic densities (SD). (Nasal macula, untreated. ×60,000.)

Fig. 13. Nuclei (NUC) and axons (A) of the visual cells within the outer nuclear layer occupy most of this field but the proximal part of the inner segments of the rods (R) and cones (C) are present at the bottom of the field. Within the outer nuclear layer the space between adjacent nerve cells is filled by the lucent disrupted cytoplasm of Müller's cells (MC). A series of terminal bar densities (TB) make up the external limiting membrane (see Fine,15 for more detailed description of this structure). The Müller cells terminate in a large number of delicate villous processes which project outward beyond the external limiting membrane between the inner segments of the rods and cones (Fig. 15). (Nasal macula, untreated. ×6,420.)

Fig. 14. A cross section through the outer nuclear layer just internal to the external limiting membrane. Nuclei (NUC) of two receptor cells are present. The Golgi apparatus (G) can be seen in the cytoplasm of one receptor cell. The cytoplasm of low density which forms the outer parts of the Müller cells (MC) occupies the spaces between receptor cells. Several cross sections of rod receptor cell axons (RA) are shown. A mitochondrion (MM) and filaments and granules of varying density are seen in the relatively electronlucent cytoplasm of Müller's cells. (Nasal retina, uranyl acetate treated. ×24,600.)
The cytoplasmic constituents of Müller's cells vary in concentration and in degree of orientation in the several retinal layers. In the inner layers there is the greatest concentration and radial orientation of delicate filaments similar to those that have been observed in fibrous astroglia. Closely associated with these filaments are minute particles. Large, less dense granules and vesicles are also present in considerable numbers. Scattered, elongated, radially oriented mitochondria are present throughout. The concentration and orientation of these cytoplasmic organelles remain much the same throughout the inner portion of the cell, except in the macular area where these structures appear less compacted in the inner plexiform layer. In the vicinity of the nucleus the cytoplasmic constituents seem less concentrated and the filaments are less well oriented. The mitochondria appear less elongated. The decrease in concentration and the lack of orientation of organelles become greater from the nuclear region toward the outer limiting membrane. As a result, the cytoplasm of the Müller cells appears less dense in the outer layers as compared with its appearance in the inner layers.

Throughout the course of this study, it was apparent that the cytoplasm of Müller's cells was more difficult to preserve and therefore interpreted as being more sensitive to artifactitious disruption during preparation as compared with the neural elements of the retina. Similarly, the outer half of the Müller cell appeared to be much more sensitive than the inner half.

In the inner portion of the outer plexiform layer numerous desmosomes were observed between adjacent neurites and similar densities were found along the synaptic membranes at the synaptic endings of the receptor cells.

Discussion

From our electron microscopic observations it would appear that the Müller cell is a peculiar glial cell which has some of the characteristics of epithelial cells, astrocytes, and oligodendrocytes. In common with many epithelial cells it has a somewhat tall columnar outline, a broad base to which there is applied an adherent basement membrane (the internal limiting membrane of the retina), and an apical end containing terminal bars and villous projections into a lumen (the mucoid-filled spaces between the inner segments of receptor cells). The basal half of the cell between the nucleus and the internal limiting membrane contains a large number of radially oriented filaments similar to those of fibrous astrocytes and it also contains many other organelles including mitochondria, segments of smooth-surfaced endoplasmic reticulum, and a variety of granules and vesicles. The outer (apical) half of the cell, by contrast, seems to have properties in common with other glia that have been interpreted as oligodendrocytes, not the least of which is the extreme sensitivity of the cell which permits it to undergo artifactitious alterations so promptly.

This observation gives us a better understanding of the "edematous" appearance which is so characteristic of the outer plexiform layer in ordinary cellloidin or paraffin sections. The fibers observed in such sec-
tions may represent axons of the receptor cells, whereas the intervening clear spaces represent the disintegrated Müller cells. It is possible, also, that similar cytologic changes occur in pathologic states. For example, in microcystoid degeneration which typically begins in the outer plexiform layer, it is possible that the "cysts" represent a group of disintegrated Müller cells. Since these cysts contain hyaluronic acid, this then would be a mucoid elaboration by the "oligodendroglial portion" of the Müller cell. In microcystoid degeneration and retinoschisis, septa between adjacent cysts have been assumed to be Müller cells matted together by pressure from surrounding cysts. It seems likely, however, that this assumption is incorrect and that the septa actually represent axons of the receptor cells matted together.

At any rate, it has been a common observation in pathologic conditions of the retina that hyaline thickening of Müller's fibers seems to be restricted to the inner half of the retina between the inner nuclear layer and the inner limiting membrane. It is most unusual to observe similar hyaline thickening of Müller's fibers between the inner nuclear layer and the outer limiting membrane. Perhaps the inner half of the cell reacts in the fashion of a fibrous astrocyte whereas the outer half behaves more like an oligodendrocyte. These are, at the moment, speculations, for we have not yet studied retinas with pathologic conditions by electron microscopy.

Although a variety of granules and vesicles are observed in great numbers throughout the inner portion of Müller's cells, we have no way of determining whether any of these represent the glycogen that has been demonstrated in these cells by histochemical techniques. It is of interest to note that the inner portion of the Müller cells which shows the greatest lactic-DPN dehydrogenase activity also contains the greater number of mitochondria.

Our observations of Müller's cells of the human retina are in general agreement with the more limited descriptions of these cells in other animal eyes published by Sjöstrand, Ladman, and Cohen. Sjöstrand, who made brief reference to the outer part of Müller's cells in his study of the retinal rod synapses in the guinea pig eye, called attention to the fact that the cytoplasm of the Müller cell can be easily recognized and distinguished from that of the other components of the retina. Cohen, in his study of the inner end of these cells in the rhesus monkey retina was in agreement that the Müller cell cytoplasm has a distinctive electron microscopic appearance. He also pointed out that near the internal limiting membrane the cells had a fibrous character similar to that of fibrous astrocytes of other parts of the central nervous system. Ladman also described the inner part of Müller's cells as having a distinctly filamentous character. Neither Cohen nor Ladman recorded any observations of the outer part of Müller's cells and the electron microscopic similarity of the outer part of Müller's cells to oligodendrocytes has apparently not yet been reported. A few of the observations made by Cohen and Ladman suggested the possibility of species variation. Ladman, for example, reported that mitochondria were rare in Müller cells of the cat retina. We found them distinctly fewer in Müller cells than in the inner segments of the receptor cells, but, on the other hand, they were certainly not rare in the inner half of the cell in human retinas. They were much less numerous in the outer part of the cell than in the inner. Cohen found that the inner (vitreal) surface of Müller's cells of the rhesus monkey retina was more irregular with more infolding of the plasma and basement membranes than we have noted in the human eye. He observed desmosomes between adjacent glial cells which we have not yet encountered. He reported that the glial cell processes of the nerve fiber layer were not closely packed and even found appreciable extracellular spaces between adjacent cells. Our impression has been that, in the human eye, the Müller
cell processes fill up all potential spaces between bundles of axons in the nerve fiber layer and that there are no appreciable extracellular spaces. Sjöstrand, who has made similar observations, suggested that the glial elements of the retina (the Müller cells), like the neuroglia of the brain, represent the extracellular space. The outer plexiform layer is generally subdivided into three parts: (a) a broad outer band composed of axons of the receptor cells and the outer halves of the Müller cells; (b) a very narrow synaptic band; and (c) another narrow band of interweaving neurites between the synaptic band and the inner nuclear layer. Together, the two narrow bands, (b) and (c), form a sort of "limiting membrane" which tends to restrict the passage of fluids and exudates. In these bands the retinal cells are tightly interwoven, there are innumerable synapses, and desmosomal structures are observed in considerable numbers. All of these structures tend to keep the retinal tissue intact in a plane just external to the inner nuclear layer. We have therefore suggested that this area be considered a "middle limiting membrane." Exudates, hemorrhages, and "cysts" developing in the outer plexiform layer typically are restricted internally by this "membrane" just as they are restricted externally by the outer limiting membrane. It would, therefore, be just as helpful to designate the inner parts of the outer plexiform layer as the "middle limiting membrane" of the retina as it has been useful to designate the series of terminal bars the "outer limiting membrane" although neither is a basement membrane comparable to the inner limiting membrane.

REFERENCES

functions. However, it seems to be important to emphasize that Müller's fibers must still be considered the main supporting element of the retina; other functions are additional.

The Golgi silver bichromate stain has made it possible for the classical anatomist to obtain a complete demonstration of Müller's fibers. This technique characteristically stains the outlines of only one particular cell with all its processes out of the continuous system of these cells. Cajal, Polyak, and many others showed single radial fibers after Golgi stain interconnecting the outer and inner limiting membranes and with an additional horizontal network of processes and fibrils extending between every neuron and synapse of the retinal layers. Two main difficulties prevented a final understanding of the system of the radial fibers with this technique: (1) it was impossible to stain adjacent elements, and, therefore, a demonstration of the complete system of the radial cells was impossible, and (2) artifacts caused many to doubt the results of the Golgi stain.

With Hortega's silver carbonate technique for the demonstration of glia, it seems to be impossible to obtain a complete demonstration of the Müller cells. Only the astroglia-like part in the inner retinal layers can be well demonstrated. That part which Fine and Zimmerman found to be more oligodendroglia-like does not stain with silver carbonate—just as the true oligodendroglia of the retina, if there is any.

This short review shows clearly how important the present study of Fine and Zimmerman is for the progress in retinal morphology. Fine and Zimmerman are finally able to state that horizontal cytoplasmic extensions from Müller's fibers surpass round and support all nerve cells, dendrites, and axons of the retina.

The findings of Fine and Zimmerman are supported by those of Christopher Pedler of the Institute of Ophthalmology in London, England. Dr. Pedler demonstrated his unpublished electron microscopic findings concerning the structure of Müller's fibers to me this summer. Pedler's views are best summarized by these words from his recent letter, "I now think that the radial fiber represents negative space, that is to say, space where neuronal tissue is not. This may sound a bit strange, but I am sure it is right."

Thus, it becomes clear that the Müller fibers have a supporting function in two dimensions. Their main cell and the astroglia-like fibers support the retina as a whole. Their oligodendroglia-like processes extend between all the delicate neuronal parts of the retina to support and probably also to supply nutrition.

The suggestion of the authors that the inner part of the radial fibers react in the fashion of fibrous astrocytes, whereas the outer half behaves more like oligodendrocytes is a new and very exciting concept. I know that their inner part is very similar to the retinal astroglia and I am ready to believe that the outer part is oligodendroglia-like.

It is good to see that the authors still use the term "inner limiting membrane" after explaining that this is the basement membrane of the retina formed by the inner end of Müller's fibers. This prevents confusion. The structure which Fine and Zimmerman describe as "middle limiting membrane" can be seen with hematoxylin and eosin stain and after silver impregnation. This interrupted layer certainly represents a limit of pathology in many abnormal retinal conditions. Thus, this new term of the authors will help in the understanding and teaching of retinal pathology. The presence of the middle limiting membrane is very obvious in cases of retinoschisis, caused by orbital tumors. This layer as well as the inner limiting membrane is nonelastic and shows most of the folding whereas the outer limiting membrane and the retinal tissues between are more elastic and show less wrinkling.