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## SYNTHETIC HYDROGEL AS AN ARTIFICIAL VITREOUS BODY. A ONE-YEAR ANIMAL STUDY OF ITS EFFECTS ON THE RETINA

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### Abstract

A hydrogel with a high water content was assessed *in vitro* and *in vivo* as a possible vitreous substitute. From a large series of polymers produced by the aqueous polymerization of methyl acrylamidoglycolate methyl ether (MAGME), a gel synthesized in 80% water was selected for an animal study. The gel was injected intravitreally into rabbit eyes and followed clinically by ophthalmoscopy, tonometry, and fundus photography. The gel was clinically well tolerated, but after 6 months ophthalmoscopy revealed progressive pallor of the optic nerve head. The eyes were enucleated one year after injection of polymer. Histopathological examination by light microscopy of retinal and vitreal sections revealed significant retinal disorganization, degeneration of the optic nerve and retinal neural elements, retinal detachment, and inflammatory changes. Analysis of immunohistochemically labeled retinal sections revealed loss of ganglion cells and extensive pathological reaction of the Müller cells and astrocytes. All these findings were consistent with a toxic effect of the polymer itself or some residual contaminants. The cytotoxicity of the hydrogel was assessed *in vitro* using cultured mouse (Balb/c-3T3) fibroblasts. The bioassay showed both cytostatic and cytotoxic effects of the polymer. Our results indicate that hydrogels produced from MAGME monomer cannot function as vitreous substitutes because of severe toxic reaction elicited to the posterior segment of the eye.

**Key words:** Hydrogels, methyl acrylamidoglycolate methyl ether, vitreous substitute, histology, immunohistochemistry, glial fibrillary acidic protein, cytotoxicity.

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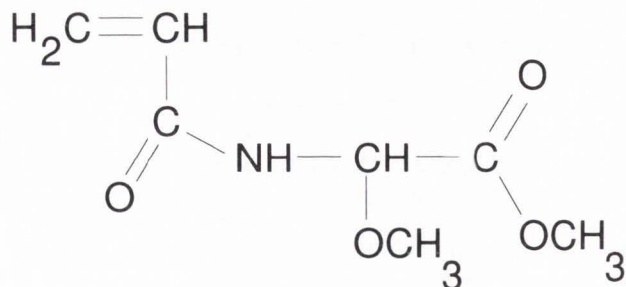
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### Introduction

After a century of recorded attempts to replace the vitreous body of the eye with a foreign material in the surgical treatment of various vitreoretinal pathologies, there is still no ideal substitute available to function permanently as a vitreous. Many materials have been proposed or tested as vitreous substitutes (Ohm, 1911; Cutler, 1946; Gartner and Priestley, 1947; Katzin and Blum, 1947; Scuderi, 1954; Castren, 1961; Kishimoto *et al.*, 1964; Balazs and Hultsch, 1976; Chan *et al.*, 1984), and some are successful as temporary substitutes. The synthetic and semisynthetic polymers have been episodically considered as candidates for vitreous substitution over the last four decades. Their use and performance, and the knowledge gained from their experimentation were recently reviewed in depth (Chirila *et al.*, 1994). It appears that some water-swallowable synthetic gels (hydrogels) may be promising as permanent vitreous substitutes if they fulfil at least two prerequisites, i.e., they do not undergo extensive fragmentation during surgical manipulation, and they do not induce toxic reaction to tissues in the eye. However, there are many other requirements for an ideal vitreous substitute (Refojo, 1975; Balazs and Hultsch, 1976). Realistically, a hydrogel to fulfill all these requirements is yet to be found.

We investigated certain hydrogels with high and very high water contents. They were prepared by polymerization of methyl 2-methoxy-2-(1-oxopropenylamino)acetate (see formula in Fig. 1). This substance is better known as methyl acrylamidoglycolate methyl ether and its acronym, MAGME. Apart from the homopolymer, poly(MAGME), copolymers of MAGME with 2-hydroxyethyl methacrylate (HEMA) and other comonomers were also synthesized, by polymerization in large amounts of water. From more than 100 polymers of MAGME produced, one was eventually selected for experiments in animals as a potential temporary or permanent vitreous substitute. We now report the manufacture and characterization of this synthetic hydrogel, the results after its injection into the vitreous cavity of the rabbit eye, and effects on cells in an *in vitro* bioassay.



**Figure 1.** Formula of MAGME. This monomer is an N-substituted derivative of acrylamide.

## Materials and Methods

### Preparation of a poly(MAGME) hydrogels

MAGME monomer was supplied by American Cyanamid Company (Wayne, NJ) as MAGME<sup>®</sup>100, with a purity of 90%. According to the supplier, the residual content of acrylamide is 1%. The purity was checked qualitatively in a Hewlett-Packard 5890A gas chromatograph, using an Ultra 2 capillary column (12 m x 0.2 mm x 0.33  $\mu\text{m}$ ) and a temperature program from 40°C to 220°C at a rate of 10 degrees/minute. The presence of acrylamide was confirmed, and also N,N'-methylene-bis-acrylamide was detected (traces). Since the latter compound can perform the crosslinking, its presence explains in part the formation of water-insoluble gels following polymerization, even without adding any crosslinking agent. However, most of the crosslinking of the macromolecular chains was probably caused by the self-condensation of the N-methoxymethyl amide side groups, which can be easily induced by an acidic medium, as documented in the technical information provided by the supplier. The fact that the pH of aqueous solutions of MAGME was acid (e.g., pH 2.6 for a solution 20% wt MAGME) supports this hypothesis. Prior to polymerization, MAGME was purified by recrystallization from toluene. After recrystallization the color of MAGME became lighter, however the gas chromatographic analysis indicated that the levels of various contaminants, most of them unidentified, were reduced to a small extent.

A large number of polymers of MAGME were synthesized by polymerization in solution. A homopolymer of MAGME (designated as V180 in our code-naming system) was eventually selected for the present experimental study. This polymer was prepared as follows. Recrystallized MAGME was dissolved in pure water (MAGME/water ratio was 20/80 by weight) in an ultrasonic bath. By "pure" water, we designate deionized

water which was subsequently distilled twice in all-glass equipment. The components of a redox initiating couple (each 0.5% wt of the monomer amount) consisting of aqueous solutions (10% wt concentration) of ammonium persulfate and sodium metabisulfite, were added in succession to a monomer solution. The clear, homogeneous liquid was then equally distributed (approximately 2.5 ml portions) among polypropylene molds fitted into the cavities of a bottom plate of a molding unit designed by us. The unit was closed and sealed, and vacuum was produced by pump-evacuating the air for 5 minutes. High purity nitrogen was then admitted through an inlet valve until an overpressure (100-150 mbar) was created in the molding unit. The unit was placed in a water bath in which the temperature was controlled by a digital program controller with a built-in microprocessor. A temperature program was run in the bath, consisting of three 10-hour steps at 30, 40 and 50°C, respectively. On completion of the cycle, the polymer buttons were removed from the molds and placed in containers with pure water, where they were stored for 2 weeks, with daily water exchanges. At the end of this period, the water was replaced with phosphate-buffered saline (PBS) and stored for a further 2 to 3 weeks, when pH of the medium reached about 7.2. The measurements of pH were performed every two days using a pH-meter, and PBS was changed after each measurement. The neutral gels were then placed in glass containers with balanced salt solution (BSS) and sterilized in an autoclave. Throughout this processing and until surgery, the samples were stored at 4°C to minimize the opportunity for bacterial growth and consequent toxin production.

### Physical evaluation

Hydration was performed in pure water and PBS and the equilibrium water content (W), as weight percentage, was calculated using the equation  $W = 100(w_1 - w_2)/w_1$ , where  $w_1$  and  $w_2$  are the weights of a fully hydrated specimen, and of a dried specimen, respectively.

The injectability was evaluated subjectively by the force necessary to push the gel out of the syringe through 21-gauge or 23-gauge needles, and the degree of gel fragmentation when it comes out from the needle. Cohesiveness was also appreciated subjectively, by visual examination.

The transparency of polymer V180 was evaluated using the method proposed by Refojo and Zauberman (1973).

### *In vivo* experiments

Six Dutch belted rabbits were used in this study. The animals were anaesthetized by halothane/nitrous oxide/oxygen mixture delivered continuously through an oxyflow machine and a mask in a semi-closed system.

In the first stage, in one eye of each rabbit, a gas-mediated vitrectomy was performed according to the technique described by Thresher *et al.* (1984). After 4 weeks, when the gas had been completely absorbed leaving a quiet eye with a fluid vitreous, the second stage was undertaken. The gel V180 was injected into five of the six gas-compressed eyes, as follows. A 4-mm, 20-gauge infusion cannula, connected to a compressed filtered air line, was inserted into the vitreous cavity, 3 mm posterior to the limbus at the inferior temporal quadrant. A 20-gauge flute needle was then inserted at the superior nasal quadrant and an air-fluid exchange was performed. The gel was placed in a 20-ml sterile syringe and injected through a blunt 20-gauge needle into the air-filled vitreous cavity. The sclerostomies were sutured with 6-0 Vicryl sutures.

The eyes were examined daily for the first week, and thereafter weekly until sacrifice, using biomicroscopy and indirect ophthalmoscopy to evaluate the cornea, anterior chamber, lens, optic nerve, and retina. A portable fundus camera was used to photograph the fundus, and the intraocular pressure was measured with a Schiotz tonometer. The operated animals were followed for 12 months.

### Histology

Three animals were sacrificed by intravenous injection of Lethobarb and the eyes were immediately enucleated, and a slit was made just below the pars plana. The eyes were fixed in Karnovsky's fixative for 1 hour. The corneas were then removed, and the eye cups returned to the fixative solution where they were stored overnight. The eyes were then stored in 10% sucrose for 2 days. Pieces (2 x 2 mm) of full thickness retina and choroid from the periphery, center and the disc area, as well as transverse slices (1 mm) from the optic nerve were excised. Pieces of scar tissue from the vitreous cavity were also removed. All specimens were fixed in osmium tetroxide, dehydrated in graded ethanols, and infiltrated and embedded in an epoxy resin (Durcupan<sup>®</sup> ACM, Fluka AG, Switzerland) for light microscopy. Semithin (2 µm) sections were cut using an ultramicrotome (LKB 2088 Ultratome V, LKB-Produkt AB, Sweden), and stained with toluidine blue.

### Immunohistochemistry

The retinæ of two eyes containing polymer V180 and of one eye which had only undergone gas-mediated vitrectomy without insertion of polymer, all enucleated 12 months after surgery, were examined by wholemount immunohistochemistry for neurofilaments (NF) and glial fibrillary acidic protein (GFAP). A control eye, in which no surgery was performed, was also enucleated and examined. The wholemount technique gives an overview of the whole retina allowing confident assess-

ment of which regions are most damaged. The NF antibody (clone 2F11, supplied by Monosan, The Netherlands) recognizes phosphorylated forms of the heavy and medium weight neural filaments which normally only exist in the axons, therefore both the density of axons and the health of the ganglion cells could be assessed (cell soma labeling indicates pathology). GFAP is normally expressed only by astrocytes, but under pathological circumstances Müller cells also express GFAP.

A procedure developed in our laboratory was used (Humphrey *et al.*, 1993). After enucleation, the anterior chamber and lens were removed by a circular cut through the sclera about 2 mm posterior to the limbus. The eye cup was then fixed by immersion in periodate-lysine-paraformaldehyde fixative (McClellan and Nakane, 1974) for 2 hours. The polymer was then gently removed, the eye cup bisected, and the half-retinæ separated from the other layers. The retinal halves were then washed 3 times in phosphate buffer (PB), and incubated in 10% normal goat serum (NGS) and 0.5% Triton X-100 in PB for 2 hours. One half of each retina was placed into a solution of monoclonal mouse anti-GFAP (clone G-A-5, supplied by Sigma-Aldrich, USA) diluted 1:200 in 5% NGS and 0.5% Triton X-100 in PB, and kept for 3 days. The other half was similarly incubated in a mouse monoclonal antibody to NF (clone 2F11) in the same solution at a dilution of 1:50. All retinæ were then washed in 5 changes of PB over a day and placed in 1:200 biotinylated goat anti-mouse IgG (Caltag Laboratories, USA) in the same diluent for 2 days. Following other 5 washes in PB, the retinæ were incubated overnight in avidin-biotin complex (Vectastain<sup>®</sup>Elite, Vector Laboratories, USA) with 0.5% Triton X-100 in PB. Following 5 washes in PB, the retinæ were incubated in 0.05% diaminobenzidine and 0.05% hydrogen peroxide in PB for 30 minutes. They were then washed twice in PB and mounted in 9:1 glycerol/PB.

### Evaluation of cytotoxicity in cell culture

Cultured mouse (Balb/c-3T3) fibroblasts were used to determine the cytotoxicity of hydrogel V180. The following 4-day protocol was used. On day 1, the confluent fibroblasts were trypsinized and plated (5000 cells in 400 µl/well) in RPMI 1640 medium with L-glutamine, 5% foetal calf serum (FCS) and 1% penicillin/streptomycin, on a 8-well chamber slide (Nunc, #177402, Denmark) and then incubated at 37°C in a humidified 5% CO<sub>2</sub>/air atmosphere. At 24 hours, the medium was replaced in the wells by serum-free medium. At 48 hours, 40 µl PBS were added into the first well (negative control), 40 µl FCS into the second well (positive control), 2 drops of gel V180 from a microsyringe into the third well, and 2 drops of gel together with

**Table 1.** Equilibrium Water Uptake of Poly(MAGME) Gels in Aqueous Media\*

Sample (Code name)	Water Uptake (% wt)†		
	In pure water(pH < 7)	In PBS (pH 7.4)	In Water/PBS‡
V180§	99.64	98.33	97.75
V180/02**	99.45	97.30	97.37
V180/08††	99.49	97.28	97.40
VH180/02-10‡‡	99.11	97.07	96.53
VH180/08-10§§	99.26	96.87	96.70

\* All gels were prepared with 80% concentration of water in the initial monomer mixture. The gels were hydrated for 8 days at 4°C.

† Averaged values of three measurements.

‡ Following 8-day hydration in pure water, these gels were then transferred in PBS for an additional 8-day period.

§ Homopolymer of MAGME, no crosslinking agent.

\*\* As above, 0.2% crosslinking agent in the initial monomer mixture. In all compositions, the crosslinking agent was N,N'-methylene-bis-acrylamide.

† As above, 0.8% crosslinking agent.

‡ Copolymer 90% MAGME/10% HEMA, 0.2% crosslinking agent.

§§ As above, 0.8% crosslinking agent.

40  $\mu$ l FCS into the fourth well. At 72 hours, the cellular effects were assessed by monitoring cell proliferation and cell death. Proliferating cells were identified immunocytochemically by the inclusion of 5-bromo-2'-deoxyuridine (BrdU) for 1 hour (i.e., hour 73), followed by an additional incubation for 1 hour with anti-BrdU. The cells were fixed with acid ethanol, and the BrdU-positive cells (dividing cells) were detected by using the Amersham RPN 20 cell proliferation kit. The slides were counter-stained lightly with hematoxylin.

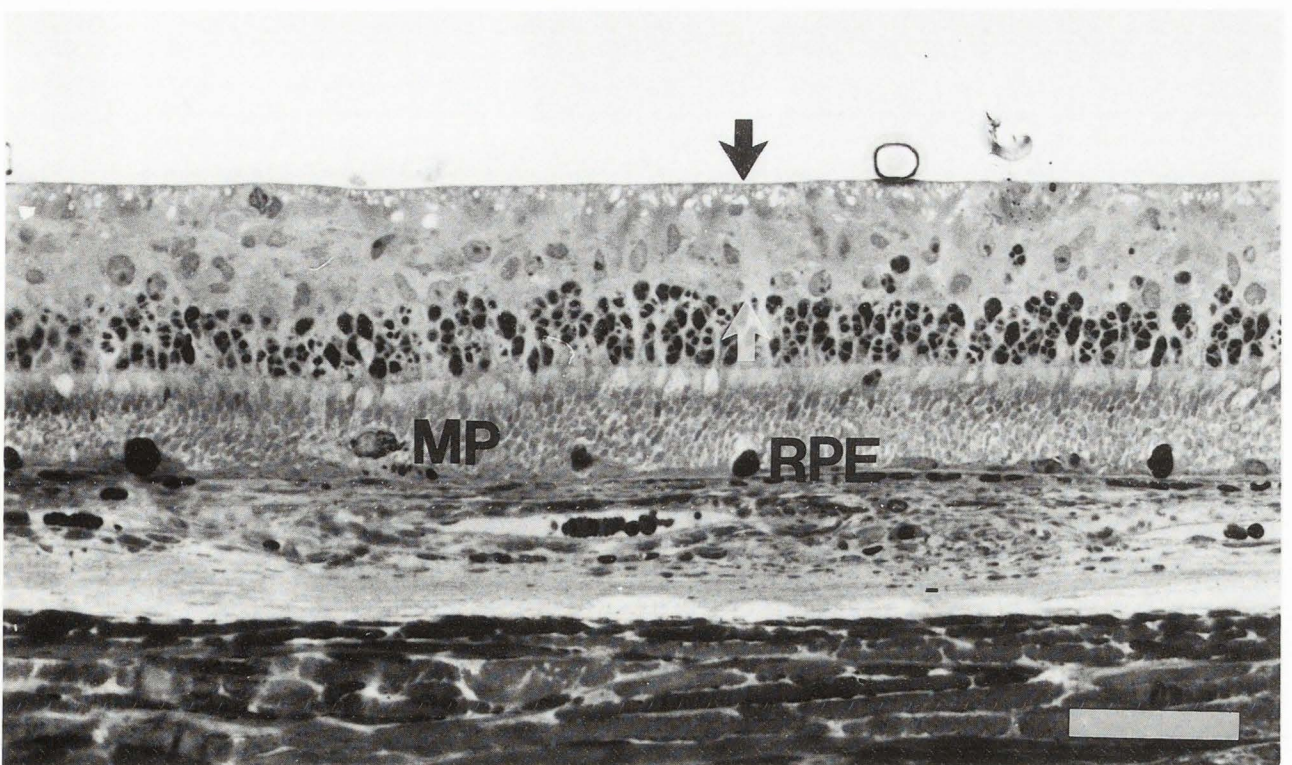
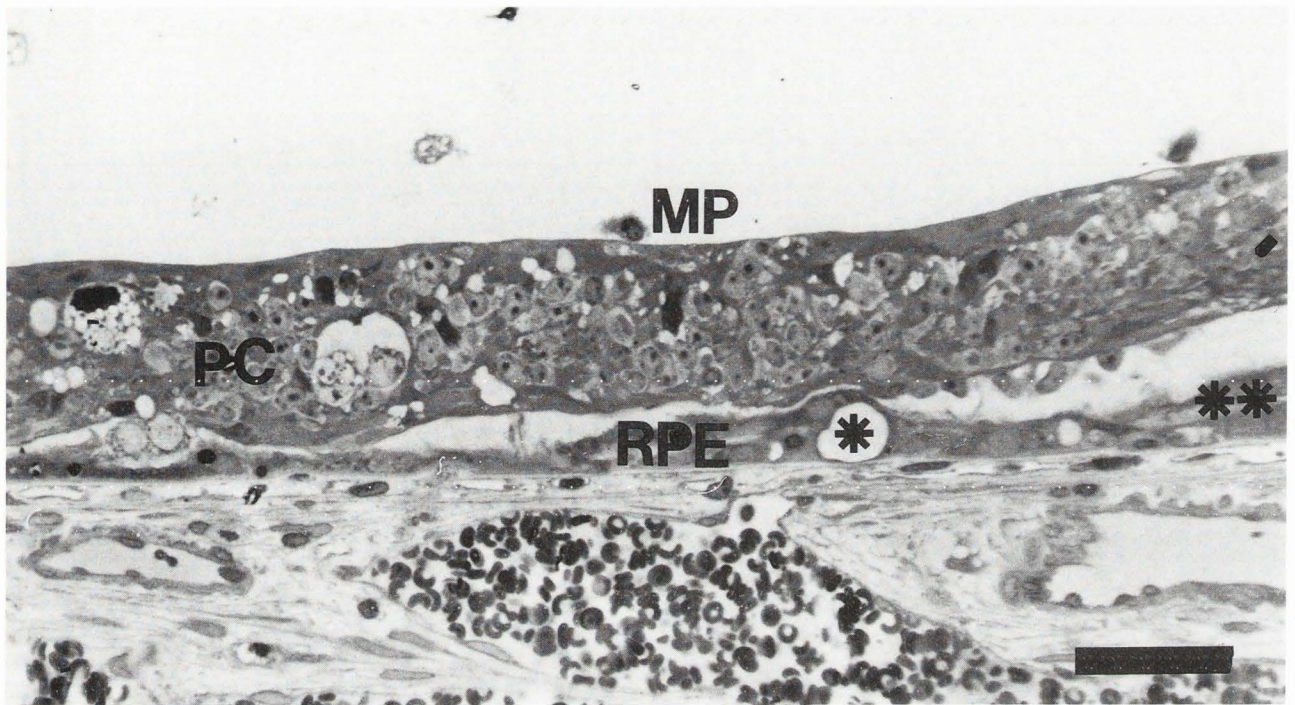
This assay was repeated 5 times. The BrdU-positive cells (brown nuclei), pyknotic cells (dark blue nuclei), and BrdU-negative cells (blue nuclei) were counted from 15 fields of view at 40x from each of the four samples, for each assay. A cytostatic or cytotoxic effect of polymer was assigned when there was a statistically significant ( $P < 0.05$ ) difference between the appropriate test and control. Comparisons were made by analysis of variance using the general linear models procedure of the SAS® (Version 6) statistical package (SAS Institute Inc., USA). The following comparisons were made with respect to BrdU-positive cells and pyknotic cells. The proportion of proliferating cells (i.e., the number of BrdU-positive cells/total number of cells/field in 15 fields) in wells with polymer and without polymer were compared in each of the serum-free and 5% FCS conditions. A significant reduction of proliferating cells in the presence of polymer was assigned as a cytostatic effect. The proportion of pyknotic cells (i.e., the number of pyknotic cells/total number of cells/field in 15 fields) in wells with polymer and without polymer were com-

pared in each of the serum-free and 5% FCS conditions. A significant increase of pyknotic cells in the presence of polymer was assigned as a cytotoxic effect. In every assay run, the positive control for proliferation (i.e., 5% FCS without polymer) showed greater than 2.5 times increase on the negative control (i.e., serum-free without polymer). There was no difference in the number of pyknotic cells present in serum-free or 5% serum conditions.

## Results

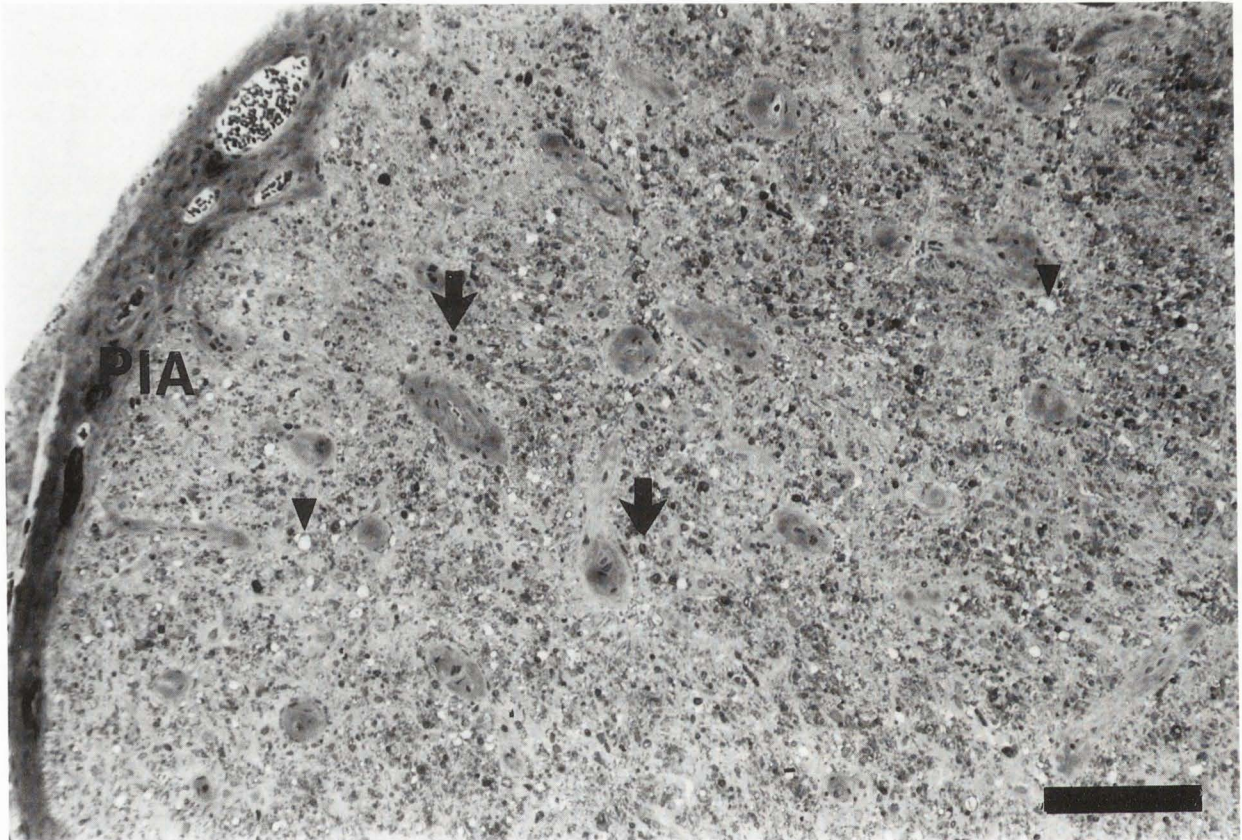
### Physical properties of poly(MAGME) hydrogels

Upon hydration in pure water, most of the MAGME-based polymers swelled to a very high equilibrium water content. This is shown in Table 1 for a homopolymer (V180) and some copolymers of MAGME. By rising only slightly the pH of the hydrating medium, as when immersed in PBS, all polymers underwent a volume contraction due to the decrease of the equilibrium water content. As seen in Table 1, the water uptake was reduced to approximately the same value both in the gels swollen in PBS directly after their synthesis and in those firstly hydrated in pure water and subsequently stored in PBS. Obviously, the MAGME-based hydrogels are pH-sensitive. (Incidentally, the natural vitreous is also pH-sensitive (Balazs, 1968), but no conjecture can be surmised at this stage on the need for artificial substitutes to have the same property.) This feature, like the very high water uptake, is also probably due to the presence of residual acrylic acid.



**Figure 2 (top).** Photomicrograph of the central retina showing a vacuolated (\*) and hypertrophic (\*\*\*) pigment epithelium (RPE). A large infiltration of plasma cells (PC) are seen occupying most of the retina in which a large number of cells had been lost. Macrophages (MP) are also seen in the vitreous cavity. Bar = 40  $\mu\text{m}$ .

**Figure 3 (bottom).** Photomicrograph of the peripheral retina in which the retinal pigment epithelial (RPE) cells appear flattened. A macrophage (MP) is seen in the subretinal space. Pyknotic nuclei (arrow) are seen in the outer nuclear layer. The inner layers of the retina (between black and white arrows) are disorganized and show a loss of retinal elements. Bar = 50  $\mu\text{m}$ .



**Figure 4.** Photomicrograph of the optic nerve showing degeneration of most of the nerve components, within the pia mater (PIA). Axons are shrunken and darkly stained (arrow). Small vacuoles (arrow head) are seen which may represent degenerated axons. Bar = 100  $\mu\text{m}$ .

The hydrogel V180 maintained a good transparency after its passage through 20-gauge or 23-gauge needles. Although it was not easy to inject the hydrogel, its fragmentation was minor and the resulting material displayed a certain cohesiveness. The fragments of gel mixed together quickly in such a manner that almost no individual particles were distinguishable. However, in visual acuity tests, when the occurrence of the "nude in the shower" phenomenon (Miller, 1972; Refojo and Zauberman, 1973) was precluded, the results were poor which rather suggested the use of this hydrogel as a temporary substitute only.

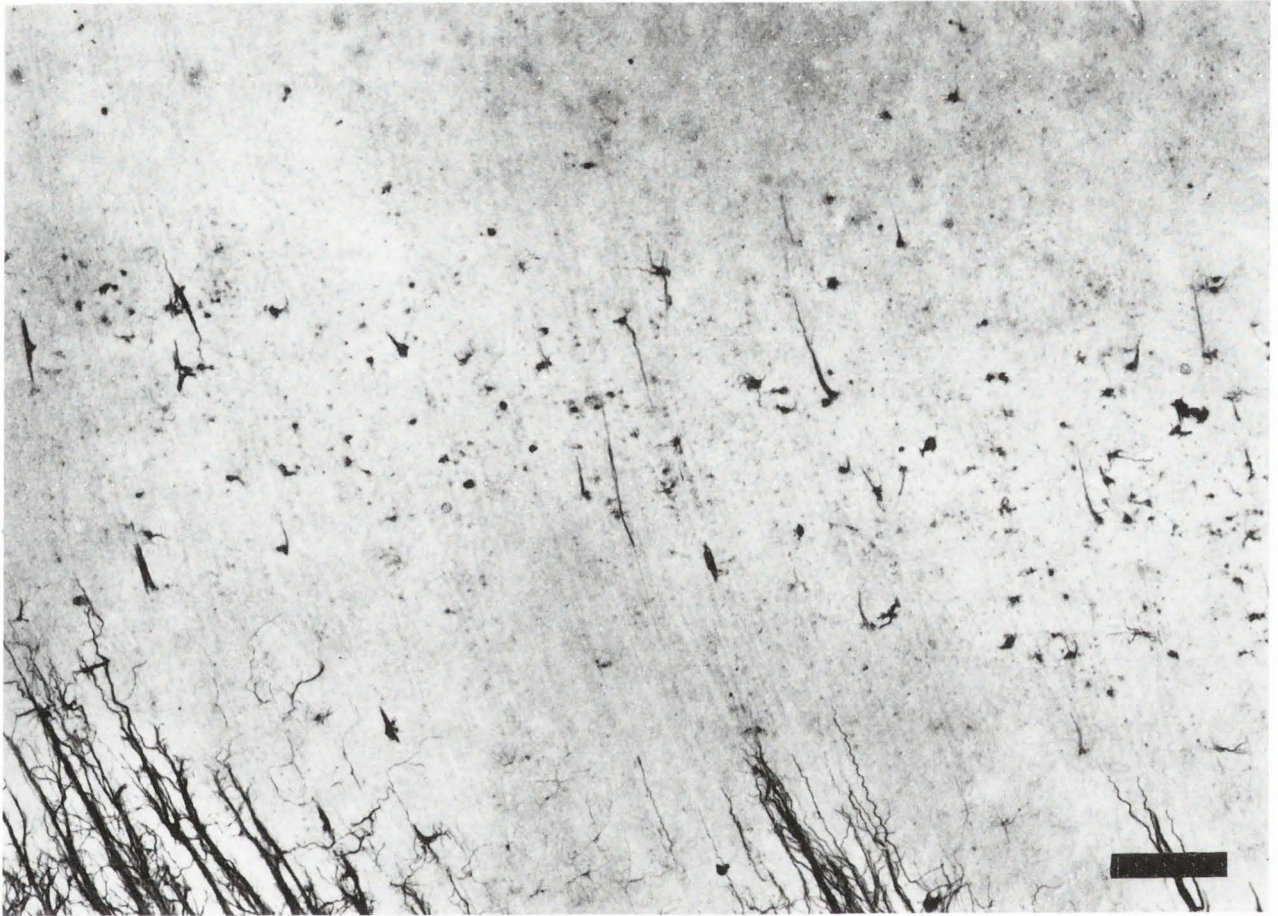
#### Clinical observations

The gel V180 was readily injected into the air-filled vitreous cavity, completely displacing the air. Clarity was less than perfect, but acceptable. No significant inflammation was observed. Applanation tonometry showed no rise in intraocular pressure. However, after 6 months definite optic dish pallor was seen in all eyes.

#### Histopathologic observations

Histological examination by light microscopy of the central retina revealed after 12 months severe pathological changes extending from the retinal pigment epithelium to the inner layers of the retina (Fig. 2). The retinal pigment epithelium was vacuolated, hypertrophic in some areas and denuded in other areas. The retina had detached and there was a loss of rod outer segments in most areas and a rarefaction of nuclei of the inner segments. Extensive disorganization of the inner layers of the retina, with loss of nerve fibers, loss of the parallel architecture of the glial columns, proliferation of glial cells, and infiltration of a large number of plasma cells and macrophages were also seen. The peripheral retina showed vacuolated and flattened retinal pigment epithelial cells. Macrophages were seen in the sub-retinal space and the rod outer segments were distorted in some areas. There appeared to be a reduction in the nuclei of the inner segments and some of them had undergone





**Figure 5.** Photomicrograph of a retinal section in an eye subjected to gas-mediated vitrectomy. Twelve months following vitrectomy the retina appears normal, except for the presence of GFAP-positive glial cells along the visual streak. Bar = 200  $\mu$ m.

pyknosis. The inner layers were also affected by loss of neural cells, proliferation of glial cells and infiltration of inflammatory cells (Fig. 3). However, the damage to the periphery was less than that to the central retina.

Light microscopy of the optic nerve showed extreme damage with the degeneration of most of the nerve components (Fig. 4). The axons appeared shrunken and myelin sheaths irregular. Small vacuoles were seen which may represent degenerated axons. The neat fascicular arrangement of the nerve had been lost. Extravasation of red blood cells and macrophages were also seen.

Light microscopic examination of the pieces from the vitreous cavity revealed polymer interlaced with pigment-laden macrophages, multinucleate giant cells and fibrous tissue.

### Immunohistochemistry

In the eye subjected only to gas-mediated vitrectomy the NF labeled ganglion cell axons were normal in appearance, with no beading or swelling, and their density was in the normal range. No ganglion cell somas were labeled, which indicates that the neurofilaments were only being phosphorylated in the axon and the cells were therefore relatively healthy. GFAP labeling was confined to the astrocytes in the medullary rays and there was no labeling of peripheral Müller cells. The visual streak region, however, contained much higher than normal numbers of GFAP-positive cells (Fig. 5). Some of these were confined to the superficial nerve fiber layer, whilst others had processes extending into the retina. These are most probably activated Müller cells which



**Figure 6.** Photomicrographs of retinal sections following GFAP labeling in eyes containing polymer. (a, above) Even at 12 months after injection the Müller cells were GFAP-immunoreactive indicating an injury response. As shown here, most of this is probably secondary to retinal detachment due to glial strands (elongated cells) forming in the vitreal space and exerting traction on the retina (arrow). (b, on the facing page) In some regions, particularly over the medullary rays, there was a massive outgrowth of GFAP-positive glial cells into the polymer. Often the polymer was separated into clumps surrounded by cells (asterisks). Bar = 100  $\mu$ m.

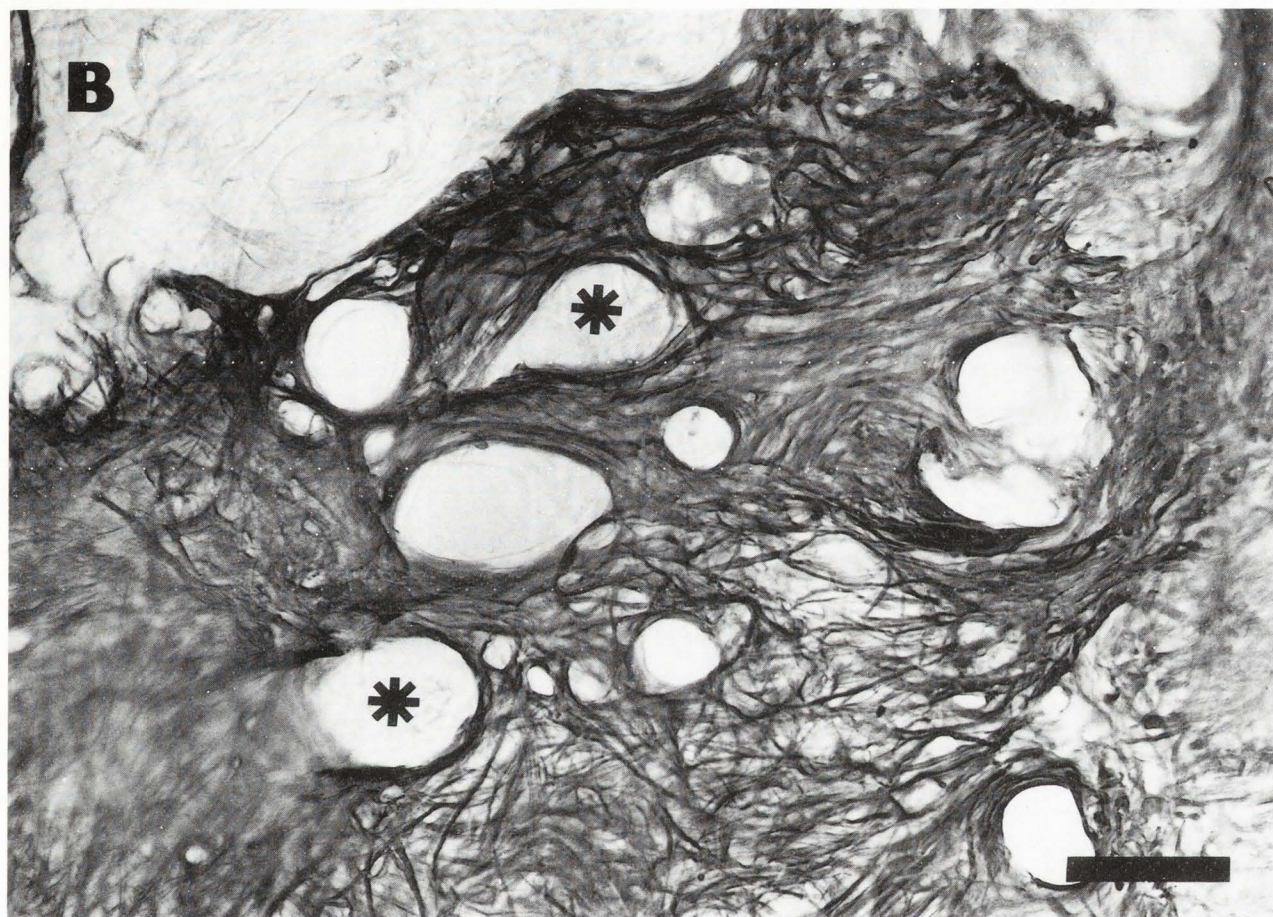
have left their normal position between the inner and outer limiting membranes, and migrated partly or completely onto the inner retinal surface. This finding indicates that gas-mediated vitrectomy alone can cause minor but permanent changes in the glial cells.

The changes were much more dramatic in the retinae of the eyes which had received the polymer gel as a vitreous substitute (Fig. 6). In all retinae, there was an extensive Müller cell reaction which varied from the expression of GFAP with normal structure through to the formation of scars of flattened fibroblast-like GFAP-positive cells. In many cases, strands of cells entered the vitreous body and wrapped around particles of polymer. The majority of cells which had migrated into the vitreous were also GFAP-positive. The most extensive glial scarring was generally associated with sever

retinal thinning, and loss or severe abnormalities of the neurons. The NF labeling showed that there were surviving ganglion cells but the axonal density was very low and in some regions the somas were labeled due to defective axonal transport. Amacrine and horizontal cells were relatively unaffected except in regions of severe scarring where they became hypertrophic (Fig. 7).

#### Cytostatic/cytocidal effects of hydrogel

Photomicrographs of representative fields from control and test samples are shown in Figure 8. The negative control (serum-free without polymer) (Fig. 8a) shows few BrdU-positive cells (dark nuclei) whereas the positive control (5% FCS without polymer) (Fig. 8b) shows many BrdU-positive nuclei. The test wells, i.e., serum-free with polymer (Fig. 8c) and 5% FCS with



polymer (Fig. 8d) show both cytostatic effect (reduced BrdU-positive cells), and a cytotoxic effect (increase in pyknotic cells).

Statistical results from the comparisons of a representative assay are presented as a graph in Figure 9.

### Discussion

Physical investigation of a poly(MAGME) hydrogel indicated a set of acceptable properties (transparency, autoclavability, stability, cohesiveness and injectability) which initially encouraged us to use it as a vitreous substitute, at least as a temporary one. After injection of gel into the vitreous cavity, the eyes were quiet and clear upon routine ophthalmoscopic examination, with the exception of a mild cloudiness of the gel which subsided within 2 to 4 weeks in all eyes. However, further histopathological and immunohistochemical investigations of the enucleated eyes, as well as bioassay of the gel in cell culture revealed a different situation.

The findings from histological examination by light microscopy indicated a strong toxic effect of the polymer to the retina and optic nerve, which both were severely

damaged. The damage to the peripheral retina was less than that to the central retina in which most of the neural elements were severely affected. A chronic inflammatory reaction persisted in the retina and vitreous, with the infiltration of a large number of inflammatory cells for as long as one year after insertion of polymer into the vitreous cavity.

The wholemount immunohistochemistry revealed a partial loss of retinal ganglion cells and an extensive pathological reaction of the Müller glia and astrocytes. The loss of ganglion cells is indicative of the presence of a neurotoxic agent. The occurrence of regions with very few ganglion cells but a normal complement of neurofilament-labeled far-field amacrine cells and horizontal cells suggests that the ganglion cells are selectively vulnerable to the toxic effects of the polymer itself or of low-molecular-weight residual contaminants, such as acrylamide or other derivatives. Acrylamide is indeed a known neurotoxic agent and specific damage to neural elements of the visual system has been proven experimentally (Souyri *et al.*, 1981; Chretien *et al.*, 1981; Vidyasagar, 1981; Merigan *et al.*, 1982).

The regions with more severe neuronal changes are



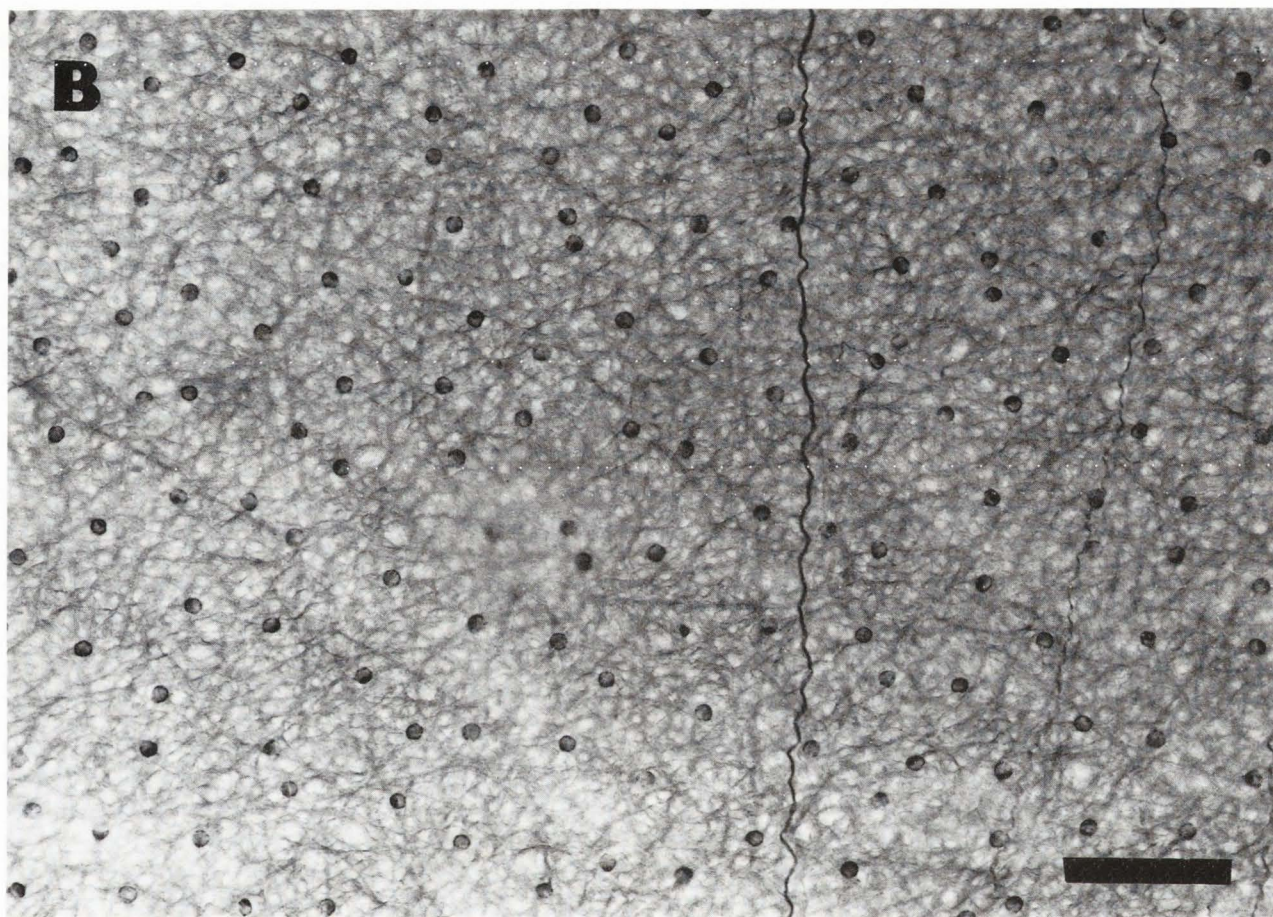
**Figure 7.** Photomicrographs of retinal sections following neurofilament labeling in control eyes and in eyes containing polymer. **(a, above)** In control retinae the 2F11 antibody labels the axons of larger retinal ganglion cells, but not their somas or dendrites, and also a population of far-field amacrine cells. **(b, on the facing page)** Following polymer injection, many regions of retina had a normal distribution of far-field amacrine cells but very few surviving ganglion cell axons. These axons were not associated with labeled cell somas and were therefore normal in terms of axonal transport. Bar = 100  $\mu$ m.

most probably secondary to the formation of scar tissue strings in the vitreous with subsequent traction, because they were always associated with extensive glial scarring. The vitreal strands were composed largely of GFAP-positive cells suggesting that Müller cells and astrocytes had no difficulty in growing around the polymer. Outside the regions of glial scarring the surviving ganglion cells did not have labeled somas with the 2F11 antibody, therefore they are probably healthy. These last two observations suggest that the polymer or its contaminants may only be transiently toxic. The observation that gas-mediated vitrectomy can alone produce long-term alterations in the Müller glia along the visual streak agrees with recent section studies which show that lensectomy-vitrectomy induces Müller cell GFAP expression (Yoshida *et al.*, 1993).

Following the disturbing results revealed by the histopathological and immunohistochemical analysis, we assessed bioactivity of the polymer *in vitro*. The assay indicated a cytotoxic effect of the synthetic polymeric gel. The addition of polymer to cells induced a significant inhibition of cell proliferation in both serum-free and 5% serum conditions. There was a significant increase in the number of pyknotic cells in all the cultures containing the polymer. Although the presence of serum did lessen the toxicity of polymer, the latter was still significantly cytotoxic in 5% FCS.

### Conclusion

A representative polymer of the class of hydrogels produced from methyl acrylamidoglycolate methyl ether



(MAGME) showed physical characteristics suitable for a vitreous substitute. However, it has failed to meet most of the biocompatibility requirements for a long-term vitreous substitute. Extensive retinal disorganization and degeneration of the optic nerve and neural elements of the retina preclude the use of these hydrogels in the vitreous cavity.

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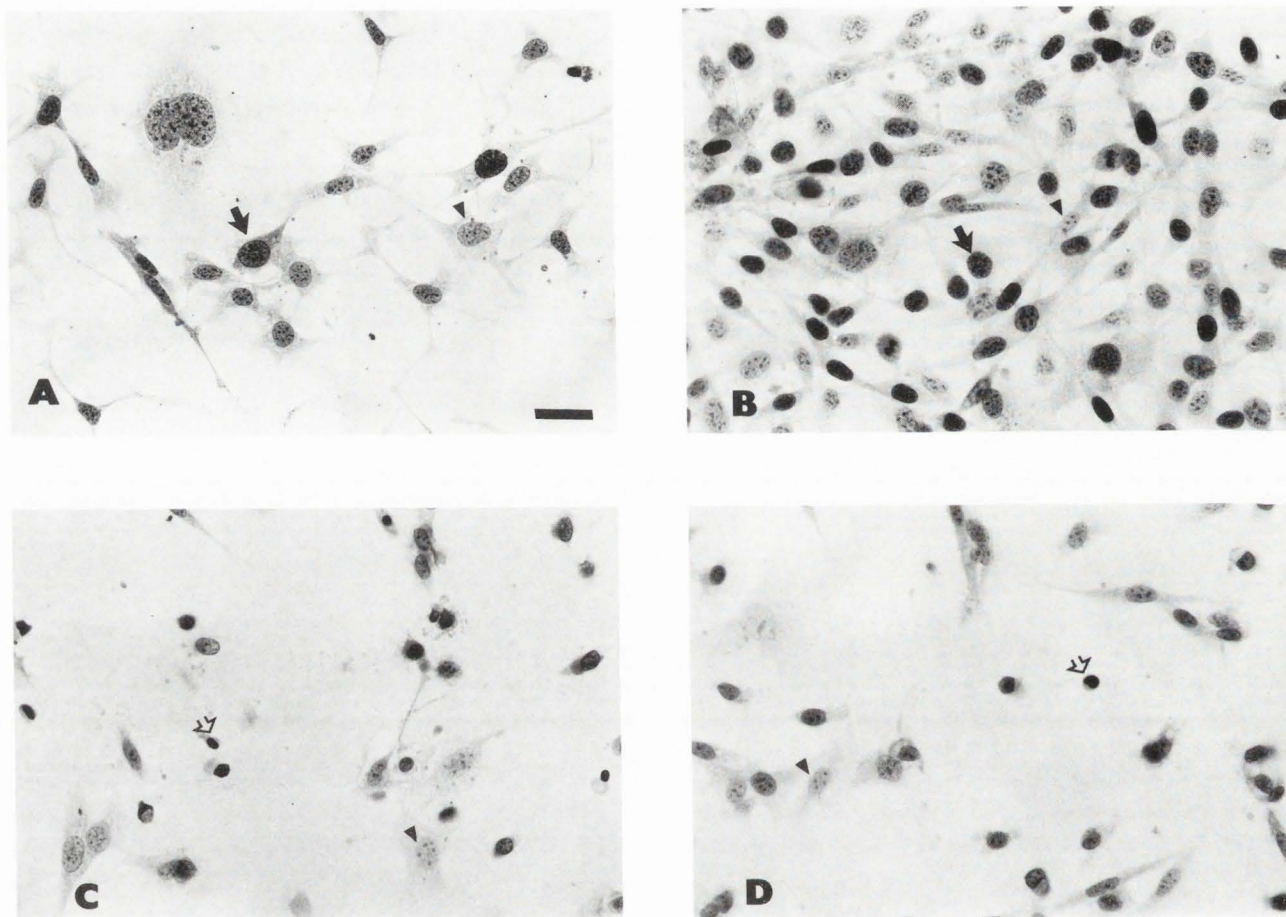
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**Figure 8.** Photomicrographs of the cell culture samples: (a) with PBS showing proliferating cells (BrdU-positive, arrow) and non-proliferating cells (BrdU-negative, arrow head); (b) with FCS: large number of proliferating cells (arrow) are seen among non-proliferating cells (arrow head); (c) with polymer showing cells with pyknotic nuclei (open arrow) and non-proliferating cells (arrowhead); (d) with FCS and polymer: pyknotic cells (open arrow), proliferating cells (arrow) and non-proliferating cells (arrow) are seen. **The four figures are at identical magnification; bars = 20  $\mu$ m.**

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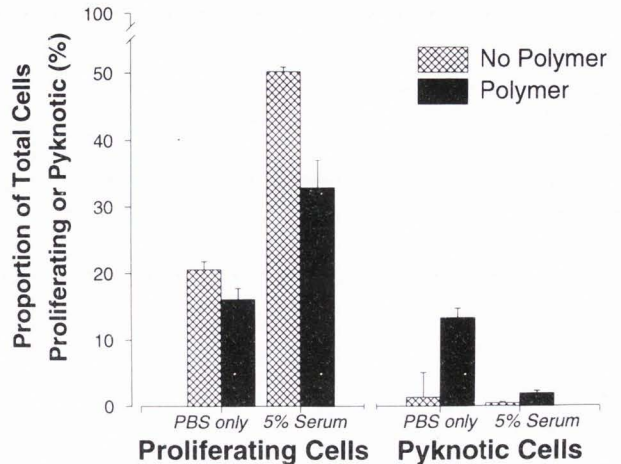
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### Discussion with Reviewers

**Reviewer I:** Considering that the purity of the original monomer was 90%, and that after recrystallization the levels of mostly unidentified contaminants were reduced only to a small extent, and that the stability of the hydrogel under the autoclaving conditions was not ascertained, are the cytotoxicity and *in vivo* intolerance of this hydrogel due to (a) the specific chemical structure of the presumably pure hydrogel, (b) residual impurities in the original hydrogel, or (c) potential products of the hydrolysis of the hydrogel under the sterilization procedure?

**J.M. Schakenraad:** Is the toxic effect observed due to remnants of monomer, pH, or surgical technique (or a combination)?

**Authors:** MAGME monomer is a rather complicated chemical compound. However, it became available on a large scale due to relatively convenient synthetic methods, either from acrylamidoglycolic acid and methyl alcohol, or from glyoxylic acid, acrylamide and methyl alcohol, and to the development of industrial uses such as coatings (enamels, waterborne coatings, automotive re-finish coats etc.), polymer products (graft copolymers, elastomers, functionalized polymers), and in the mineral ores extractive procedures. Obviously, for the mentioned uses a high purity of MAGME is not crucial, and American Cyanamid offers a product with 90% purity (minimum) for the industrial users. On the other hand, its purification is not an easy task by common techniques, as we found during our work. Recrystallization of MAGME from toluene, which appeared to be the only suitable solvent for this operation, was difficult and



**Figure 9.** Graphic statistic comparisons of proliferating and pyknotic cells in various cell cultures showing the cytostatic and cytotoxic effects of polymer V180.

time-consuming (in fact, toluene is not highly recommended as a recrystallization solvent), and reduced the level of contamination rather insignificantly. We expect that during 2 weeks of daily washings in water, and other 2 to 3 weeks in PBS (when also the neutralization took place), all water-extractable contaminants were removed. These contaminants are the only ones which can be released in the ocular environment. Our washing/extraction was actually more extensive than usually applied to the hydrophilic polymers used for the manufacture of ocular devices (contact lenses, intraocular lenses). However, the neurotoxicity manifested *in vivo* may indicate the presence of a neurotoxic compound, probably released as a consequence of the phagocytic activity in the vitreous. At the time of sterilization, the poly(MAGME) hydrogel was already neutral (pH 7.2). It is unlikely that hydrolysis can occur within 20 minutes (i.e., the duration of autoclaving) in a neutral medium. This never happened in our previous experience with other hydrogels subjected to autoclaving. It is unlikely that the toxic response was caused by pH or surgical technique. Gas-mediated vitrectomy is currently used in human patients. Although it has some negative effects (as demonstrated in this paper), the benefits for the visual rehabilitation prevail. As suggested by Reviewer I, probably the specific structure of the poly(MAGME) is responsible for an inherent toxicity of the polymer as such. Whatever the real reason might be, our work clearly shows that this polymeric gel should not be used as a vitreous substitute.

**Reviewer I:** Will the authors comment also on the cohesiveness of the hydrogel after injection. If the fragments of gel coalesced, how is it that the fragments of

gel were separated in clumps surrounded by cells (as in Figure 6)?

**Authors:** The cohesiveness of the gel was tested subjectively, by visual observation. The process of injection subjects any material to enormous shear stress, therefore some fragmentation is unavoidable. Poly(MAGME) gel maintained its transparency after a single injection, which probably indicates that the fragments coalesced enough to maintain the optical properties, but we do not expect the disappearance of the separation interfaces between fragments. The vacuolization of polymer particles observed in the vitreous cavity is very likely caused by phagocytosis. (The high phagocytic activity of the vitreous elements is well known.) The fragmentation caused by injection facilitated the onset of phagocytosis, but did not cause it. As a result of phagocytosis, the polymer was fragmented to smaller particles which were internalized by macrophages. Its molecular weight probably decreased significantly, however within the vacuoles the digestion of polymer cannot actively progress. This behavior does not preclude the use of a polymer as a vitreous substitute, provided that transparency was maintained in the vitreous cavity. Poly(MAGME) fulfilled this prerequisite, but proved to be toxic to the tissues.

**J.M. Schakenraad:** A positive control is defined as material that gives 100% effect in your assay (mostly toxic or adverse). A blank is when no additives are added (background). A negative control is an addition with no effect. You therefore confuse me with FCS as a positive control and PBS as a negative control.

**Authors:** The positive and negative controls are considered here in an immunocytochemical sense, and not in a toxicological sense (when indeed positive means toxic, and negative means nontoxic). This is an immunocytochemical assay which determines cytotoxicity by measuring the proliferation of cells. Here, the positive control refers to the recognition of the antibody to proliferating nuclei; the presence of 5% FCS encourages maximum growth. When FCS is absent, no proliferation is induced, therefore PBS alone (without FCS) represents a negative control.

**J.M. Schakenraad:** Having a relatively unknown polymer gel, why did you test the material *in vivo* first, while your *in vitro* assay demonstrated considerable toxicity?

**Authors:** We were aware of this drawback of our report, and three reviewers mentioned it. However, this was the real sequence of our experiments, whether correct or not: first *in vivo* evaluation, and then- after we examined the results- *in vitro* cytotoxicity assaying to ascertain whether the tissue response was caused by the gel or by the surgical procedure. For the sake of scien-

tific truth, we cannot change this sequence (on paper) or report only half of experiments (either *in vitro*, or *in vivo*). Because MAGME is easily available and it is a monomer which by polymerization leads to gels able to incorporate 99% water (like the natural vitreous body), the idea to use it may occur to other investigators too. These negative results should therefore be made known.

**J.M. Schakenraad:** Have the authors any idea on the actual pH of the in the eye?

**Authors:** We do not expect that pH of the gel (7.2 prior to implantation) will change significantly in the eye, since the pH of the rabbit vitreous is between 7.2 and 7.5.

**T. Matsuura:** What did you do to avoid fragmentation of gels? In our experiments, the change of the crosslinking point of gels led to suitable materials.

**Authors:** We synthesized and tested a large number of copolymers of MAGME. Upon injection, all showed more extensive fragmentation than the gel presented in this paper. We did not use a crosslinking agent in this gel, but in all other crosslinked compositions the variation of crosslinking degree did not diminish the fragmentation.

**T. Matsuura:** You need more precise physical evaluation for gels.

**Authors:** The answer to this question is yes, and we recently improved our instrumentation and techniques for characterization of gels. However, as shown by other investigators (e.g., Chan *et al.*, see **References**), it appears that the tests performed by us are probably sufficient and relevant for a gel intended as a vitreous substitute.

**T. Matsuura:** What is the mild cloudiness of gels? Is this due to fragmentation or bioreaction?

**Authors:** We suspect that the cloudiness of the gel after its injection into the vitreous cavity is due to a fibrinous reaction. It was a transient event, disappearing within 2 to 4 weeks.