

POSTERIOR VITREOUS DETACHMENT WITH DISPASE

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Purpose: To induce a posterior vitreous detachment (PVD) in porcine and human cadaver eyes *in vitro* with Dispase (Gibco, Grand Island, NY).

Methods: Dispase (0.5 mL) was injected into the vitreous cavity of enucleated porcine (0.05–25 U/mL) and human (5 U/mL) eyes. After incubation at 37°C for 15–120 minutes, the globes were hemisected and the extent of PVD was graded as complete, partial, or absent. The structural integrity of the retina was estimated by measuring the elastic constant and maximal stretching before fracture. Retinal cell viability was determined by an intracellular esterase assay. Light, transmission, and scanning electron microscopy were performed to examine the ultrastructure of the vitreoretinal interface.

Results: After 15 minutes, a partial or total PVD was present in 7/10, 8/10, or 9/10 enucleated porcine eyes incubated with 1, 5, or 10 U/mL Dispase, respectively, versus 3/10 control eyes ($P < 0.05$). A partial or complete PVD was present in 3/5, 4/5, 5/5, or 14/15 porcine eyes after 15, 30, 60, or 120 minutes of treatment with 0.1 U/mL Dispase, respectively. Similarly, 19/20 human cadaver eyes developed a complete and 1/20 an incomplete PVD after incubation with 5 U/mL Dispase for 15 minutes. Microscopy demonstrated that Dispase cleaved the attachment of the posterior hyaloid to the internal limiting membrane with minimal damage to the inner retina. Retinal cell viability and the mechanical properties of the retina were similar for Dispase-treated and control eyes.

Conclusion: Dispase disrupts the attachment of the posterior hyaloid to the inner limiting membrane with minor morphologic changes in the inner retina. The enzyme may be useful in removing cortical vitreous during vitreous surgery.

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The posterior hyaloid surface of the vitreous gel is adherent to the inner retinal surface in the normal human eye, most prominently at the vitreous

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base, optic disc, and along the major retinal vessels.¹ The development of a posterior vitreous detachment (PVD) will sometimes leave small islands of cortical vitreous attached to the inner retinal surface.¹ Separation of the posterior hyaloid from the retinal inner limiting membrane (ILM) has an impact on the natural course of many vitreoretinal diseases, including diabetic retinopathy,^{3–5} vitreomacular traction syndrome,⁶ idiopathic macular holes,⁷ and retinal detachment.

Removing the posterior hyaloid during pars plana vitrectomy is often necessary, especially in cases of macular hole, penetrating ocular trauma, and proliferative diabetic retinopathy.^{8,9} Mechanical removal

of the cortical vitreous, however, may cause retinal breaks or bleeding from a retinal vessel, whereas remnants of residual posterior hyaloid may provide a surface for subsequent cell proliferation and fibrocellular contraction. For this reason, several workers have attempted to induce a PVD prior to or during vitreous surgery by using intravitreal injection of different enzymes,^{10–13} homologous blood,¹⁴ an expansile gas,¹⁵ or intraocular diathermy.¹⁶ None of these techniques has led to specific cleavage of the binding sites between the ILM and the posterior hyaloid surface.

Dispase (Gibco, Grand Island, New York) is a neutral 35.9 kD protease obtained from *Bacillus polymyxa*,¹⁷ which cleaves the basal membrane in various tissues including skin,¹⁸ testis,¹⁹ and retinal pigment epithelium (RPE).²⁰ Dispase acts on type IV collagen and fibronectin, whereas other components of the extracellular matrix such as laminin and type V and VII collagens are resistant to the enzyme.²¹ Based on the molecular^{22,23} and structural²⁴ resemblance of the ILM to other basal lamina, we hypothesized that Dispase might selectively cleave the attachment of the posterior hyaloid to the ILM. We tested this hypothesis using enucleated porcine and human eyes, and determined the effects of Dispase treatment on the morphology, cell viability, and mechanical properties of the retina.

Materials and Methods

Preparation of Dispase Solution

Stock solutions of Dispase at a concentration of 25 U/mL (388 milliosmole, pH = 7.4) were prepared in calcium- and magnesium-free phosphate-buffered saline (PBS), filtered through a 0.2- μ m pore size filter, aliquoted, and stored at -20°C until further use. Stock solutions were thawed and filtered again as needed and diluted to a final concentration of 0.05–10 U/mL with sterile calcium- and magnesium-free PBS.

Ability of Dispase to Induce PVD

Adult domestic pig eyes were used because porcine eyes have a thick posterior hyaloid that strongly adheres to the ILM.²⁵ Adult domestic pig eyes were obtained within 3 hours after death from a local slaughterhouse and transported to the laboratory on ice. Eyes were cleaned of extraneous tissue, and 0.5 mL of Dispase (1, 2, 5, 10, or 25 U/mL) was injected into the midvitreal cavity of 10 eyes; 0.5 mL of

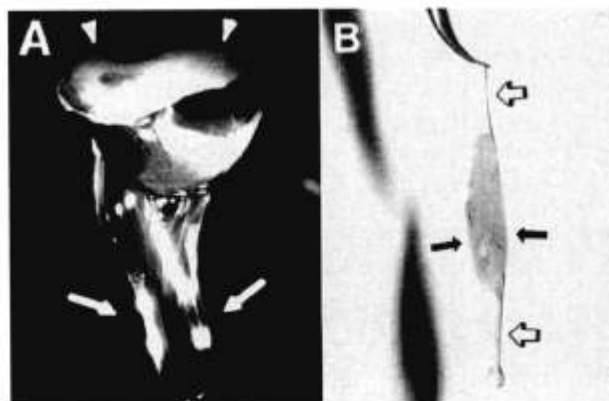


Fig. 1. A, Removal of the anterior segment (arrowheads) of a porcine eye after Dispase treatment frequently results in the removal of the whole vitreous (arrows) as an intact gelatinous mass adherent to the anterior segment. This eye would be graded as a complete posterior vitreous detachment (1.4 $\times$). B, In control eyes, the vitreous (open arrows) remains adherent to the sensory retina (closed arrows). This eye would be graded as no posterior vitreous detachment (0.7 $\times$).

calcium- and magnesium-free PBS was injected into another 10 eyes as a control.

Two different incubation conditions were chosen to mimic the way that Dispase might be used clinically: (1) In some clinical situations, intravitreal injection of Dispase might be performed immediately before pars plana surgery. To mimic this clinical use, eyes were incubated at 37°C in 5% CO_2 /95% air for 15 minutes. At the end of the incubation period, a circumferential incision was made 4 mm posterior to ora serrata, and the anterior segment was removed. The vitreous was frequently adherent to the anterior segment; if not, the vitreous was removed by tilting the eye cup and pouring vitreous out of the eye cup. The extent of a PVD was graded as complete (if there was no adhesion between the vitreous and the neurosensory retina, Figure 1A), partial (if the vitreous remained adherent to the retina in some locations), or absent (if the entire retina remained adherent to the excised vitreous, Figure 1B). (2) In other clinical situations, a single intravitreal injection of Dispase might be used to induce a PVD without performing vitreous surgery. To mimic this situation, we determined the lowest effective concentration of Dispase that would induce a PVD within 120 minutes after injection. We limited the observation period to 120 minutes because the results at longer incubation periods might be confounded by postmortem release of proteinases.²⁶ Adult domestic porcine eyes were injected with 0.5 mL of Dispase (0.05, 0.1, 0.25, 0.5, 1, 2, or 4 U/mL), and the same amount of PBS was injected into

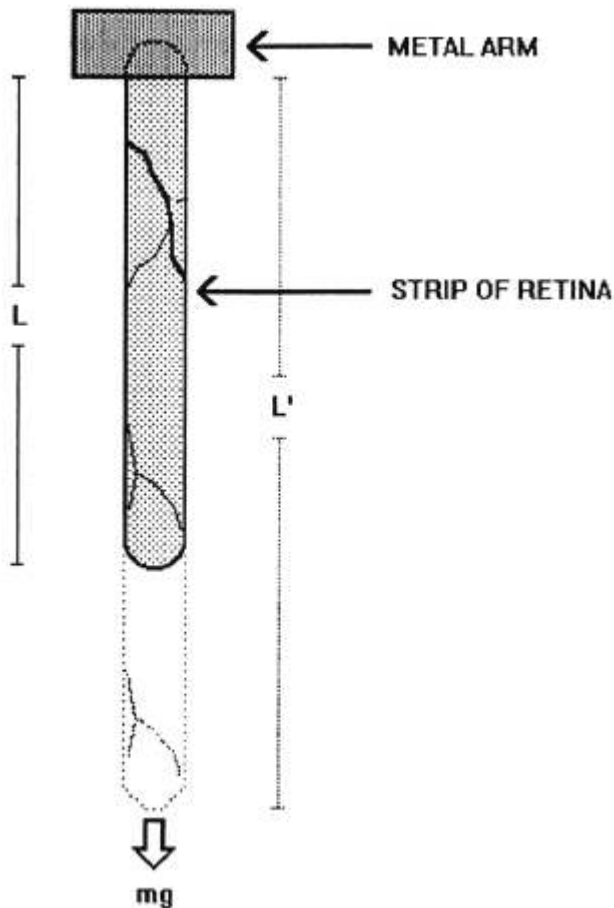


Fig. 2. A strip of human sensory retina (20×5 mm) was cut and clamped to a metal arm. Different weights (mg) were applied to the lower end of the strip, and the change in length of the stretched retina ($L' - L = \Delta L$) was measured at each weight. The ratio of the longest distance the strip stretched before fracture to the original length was defined as maximal retinal stretching before fracture.

other eyes as a control. Eyes were incubated at 37°C in 5% $\text{CO}_2/95\%$ air for 120 minutes; the extent of PVD was graded as described previously.

The ability of Dispace to induce a PVD was also tested using the posterior pole of 20 human cadaver eyes obtained from the Mid-America Eye Bank (St. Louis, MO). Eyes from young donors with a low chance of spontaneous PVD were included in this study.^{2,27} The death-to-experiment time was restricted to <72 hours (mean, 23.21 ± 12.6 hours; range, 7.1 ± 54.1 hours); there is minimal effect of death on the strong adhesion between the retina and the vitreous during this period.²⁸ The average age of the donors was 39.1 ± 13.2 years (range, 18 ± 61 years). Two of the eyes were from female donors and 18 were from male donors. Eight (40%) died following motor vehicle accidents, five (25%) from

myocardial infarction, three (15%) from cerebrovascular accident, three (15%) from respiratory failure, and one (5%) from cancer. None of the eyes had signs of ocular trauma. The suprachoroidal space of the posterior poles was sealed by adhering the iris root to the sclera using cyanoacrylate glue as previously described.²⁰ Dispace (0.5 mL at 5 U/mL) was injected into the midvitreal cavity of one eye; the fellow eye received the same amount of calcium and magnesium-free PBS as a control.²⁹ Eyes were incubated for 15 minutes at 37°C in 5% $\text{CO}_2/95\%$ air and then hemisected and graded for the presence and extent of a PVD.

Histologic Examination

Dispace solution (0.5 mL at 0.1 U/mL or 5 U/mL) was injected into the vitreous of three enucleated domestic pig eyes, and the eyes were incubated at 37°C in 5% $\text{CO}_2/95\%$ air for 120 or 15 minutes, respectively. Intravitreal injections of the same amount of calcium and magnesium-free PBS were performed in control eyes. After the incubation, the globe was punctured 3 mm posterior to the limbus at four points and fixed in an isotonic solution of 1% formaldehyde and 1.25% glutaraldehyde in phosphate-buffered saline at 4°C for 24 hours.³⁰ The anterior segment and vitreous were then removed and discarded, and the retina was removed and post-

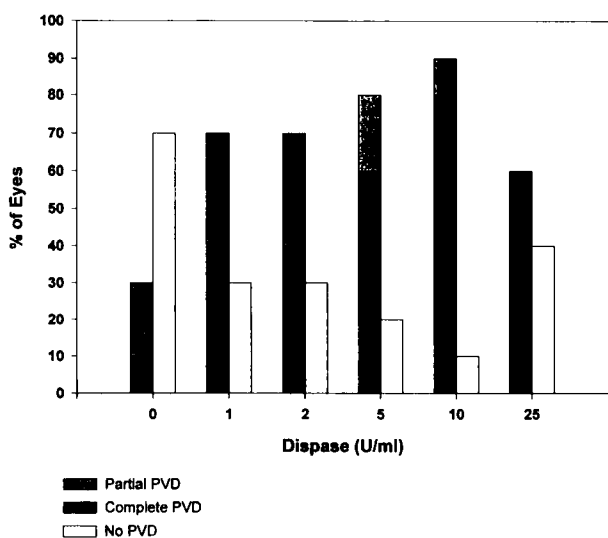


Fig. 3. The extent of a posterior vitreous detachment was graded as complete, partial, or no posterior vitreous detachment 15 minutes after injection of Dispace into the vitreous of enucleated porcine eyes ($n = 10$ at each concentration); 5 U/mL is the lowest concentration of Dispace that induces a statistically higher rate of posterior vitreous detachment compared with phosphate-buffered saline controls ($P = 0.03$).

fixed in 1% osmium tetroxide in 0.16 M cacodylate buffer (pH 7.4) for 1 hour, stained in 1% uranyl acetate in 0.1 M sodium acetate buffer, dehydrated in a graded series of ethyl alcohol, and embedded in EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA). One-micrometer sections were cut and stained for 1 minute with Richardson's stain. Ultrathin sections were cut with an ultramicrotome (Ultramicrotome MT-2, Sorvall, Norwalk, CT) and examined with a transmission electron microscope (Model 100B, JEOL Ltd., Tokyo, Japan) using an acceleration voltage of 80 kV. For scanning electron microscopy, the retina was then embedded in EMbed 812 without a catalyst and placed in gelatin capsules. Sections were frozen at -80°C for 20 minutes and then cracked in half. The resin was removed with propylene oxide, and the specimens were desiccated for 5 minutes in hexamethylsilazane followed by air drying overnight. The specimens were then coated with a 30-nm gold-palladium coating and examined in a Hitachi S-450 scanning electron microscope (Tokyo, Japan) using an acceleration voltage of 15 kV.

Cell Viability Analysis

Dispace (0.5 mL at 0.1 U/mL or 5 U/mL) was injected into the midvitreous cavity of five adult domestic pig eyes. The eyes were incubated at 37°C in 5% CO_2 /95% air for 120 or 15 minutes, respectively. Human cadaver eyes injected with 0.5 mL of Dispace at 0.1 U/mL (five eyes) or 5 U/mL (15 eyes) were incubated for 120 minutes or 15 minutes, respectively. Fellow eyes receiving the same amount of intravitreal calcium and magnesium-free PBS were used as controls. At the end of the incubation period, the eyes were hemisected, and the anterior segment and vitreous were discarded. The retina was dissected free of its attachment to the optic nerve and transferred to a petri dish containing minimum essential medium. Pieces of retina 5×5 mm in size were cut and transferred onto a glass slide. Cell viability was assessed using Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR).³¹ This kit contains calcein and ethidium probes and relies on intracellular esterase activity within living cells to cleave the calcein to form an intracellular green fluorescent product.³¹ Ethidium passes through the compromised membranes in dead cells and attaches to the DNA, yielding red fluorescence. The number of viable and nonviable cells was counted in at least three different areas, and the retinal cell viability was expressed as the average

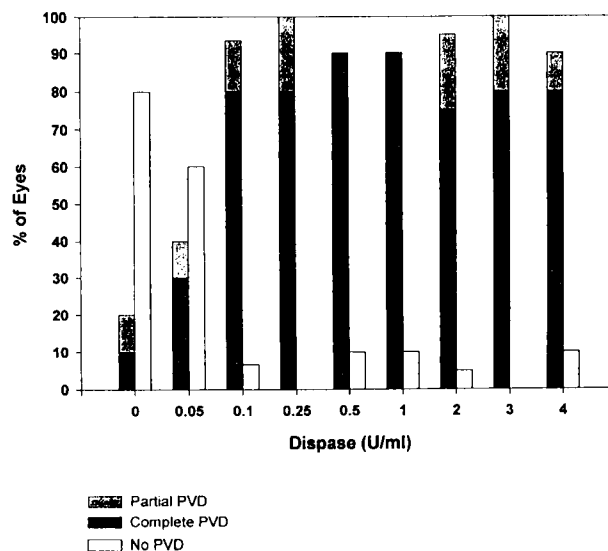


Fig. 4. The extent of a posterior vitreous detachment was graded as complete, partial, or no posterior vitreous detachment 120 minutes after injection of Dispace into the vitreous of enucleated porcine eyes ($n = 10$ at each concentration); 0.1 U/mL is the lowest concentration of Dispace that induced a statistically higher rate of posterior vitreous detachment compared with phosphate-buffered saline controls ($P = 0.0003$).

ratio of live cells to the total number of cells in these three areas.

Effect of Dispace on the Elastic Properties of the Retina

The structural integrity of the treated retina was determined by measuring the elastic constant (k) and maximal amount of retinal stretching before fracture. For this purpose, 0.5 mL of Dispace (0.1 U/mL or 5 U/mL) was injected into the midvitreous cavity of five human cadaver eyes, with calcium and magnesium-free PBS injections serving as the controls. The eyes were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 120 or 15 minutes, respectively. The anterior segment was then removed, and the sensory retina was dissected and transferred to a petri dish containing CO_2 -free medium (Gibco). We then cut 5×20 -millimeter strips of retina extending from the periphery to the posterior pole, avoiding the large vessels and the thicker peripapillary retina. Strips with visible tears were discarded. The strips were transferred onto the surface of a Teflon (Cope Plastics, St. Louis, MO) plate, and excess fluid was removed with a filter paper. A thin plastic thread was glued to each end of the retinal strips, and one end of the strip was clipped onto a metal arm (Figure 2). A

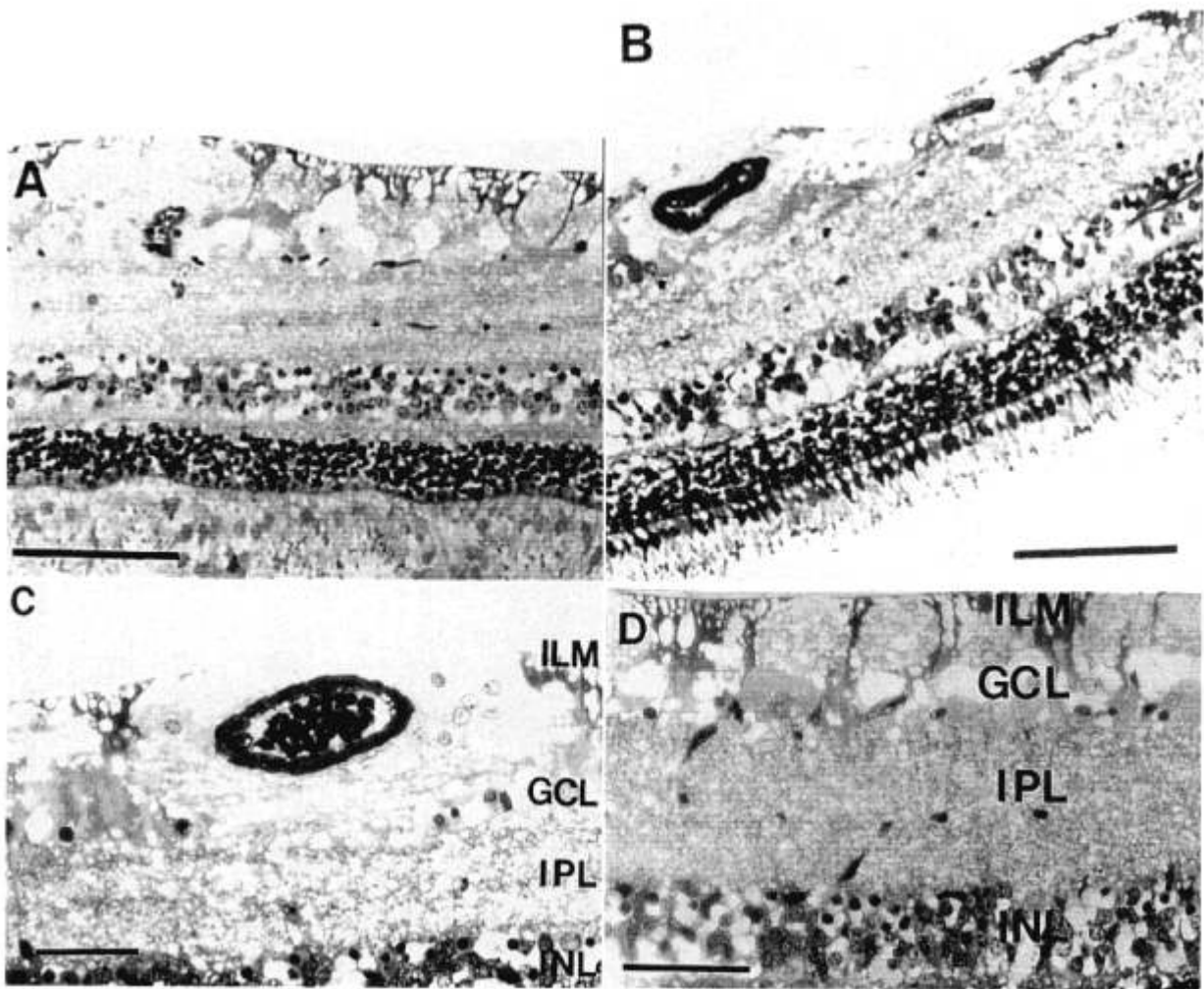


Fig. 5. **A**, Control pig retina. Vacuoles are seen in the inner limiting membrane (ILM) similar to changes noted in Dispace-treated eyes (Richardson's stain, bar = 50 micrometers; GCL, ganglion cell layer, IPL, inner plexiform layer, INL, inner nuclear layer). **B**, Dispace treatment at 5 U/mL for 15 minutes did not alter the morphology of the pig retina on light microscopy (Richardson's stain, bar = 50 micrometers). **C**, Note the retinal vessel wall is intact after Dispace treatment at 5 U/mL for 15 minutes (Richardson's stain, bar = 25 micrometers). **D**, Similar results were observed after treatment with Dispace at 0.1 U/mL for 120 minutes (Richardson's stain, bar = 25 micrometers).

small weight (25, 50, 100, 200, 400, or 600 mg) was attached to the lower end of the strips, and we determined the length of the retina at each weight. The elastic constant (k) was defined as $mg/\Delta L$, where m = attached mass, g = gravitational constant, and ΔL = change in length of the retinal strip (Figure 2).³² The amount of retinal stretching was plotted as a function of the force applied, and the elastic constant (k) was determined from the slope of the linear regression line using a personal computer supplied with Sigmaplot 3.01 software (Jandel Corp., San Rafael, CA). Three measurements were obtained and averaged. The ratio of the longest distance the strip stretched before fracture to the original length was defined as maximal retinal stretching before fracture.

Statistical Analysis

The rate of PVD induction for Dispace-treated eyes was compared with control eyes undergoing injection of calcium and magnesium-free PBS using Fischer's exact test.³³ The cellular viability, elastic constant (k), and maximal retinal stretching before fracture of Dispace-treated and control eyes were compared with the Mann-Whitney-U test.³⁴ A confidence level of $P < 0.05$ was considered statistically significant.

Results

Ability of Dispace to Induce a PVD in vitro

The ability of different concentrations of Dispace to induce a PVD in enucleated porcine eyes within

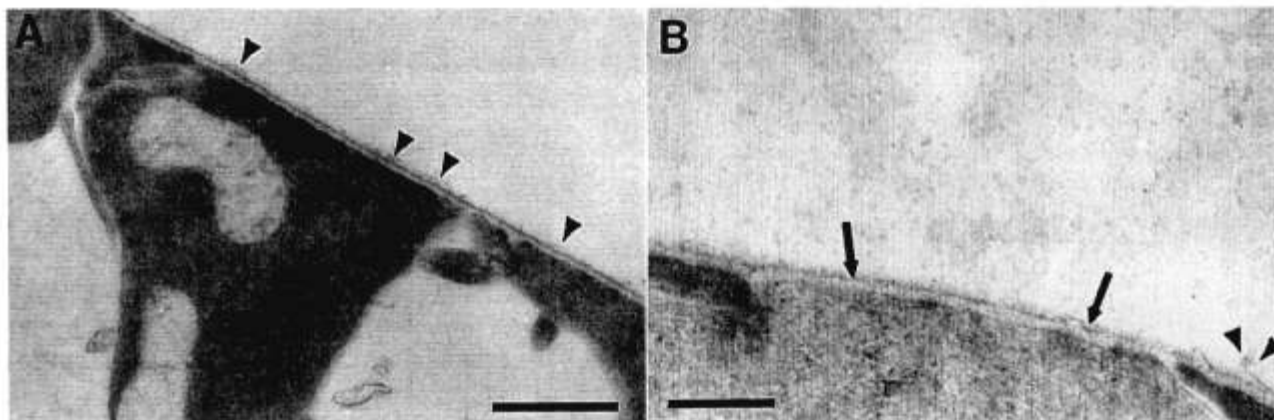


Fig. 6. **A**, Transmission electron microscopy of the vitreoretinal junction in a control pig eye. Dense collagen fibrils of the posterior hyaloid (arrowheads) are oriented parallel to the inner limiting membrane (bar = 1 micrometer). **B**, Transmission electron microscopy of the vitreoretinal junction in a Dispace-treated porcine eye (5 U/mL for 15 minutes). Note that the collagen fibrils of the posterior hyaloid and the lamina rara externa of the internal limiting membrane are not present. A small remnant of the cleaved posterior hyaloid is visible (arrowheads). The lamina densa is amorphous, and its borders (arrow) are less distinct (bar = 0.5 micrometers).

15 minutes is shown in Figure 3. A partial or complete PVD was observed in 3/10 (30%) control eyes (one complete and two partial), 7/10 (70%) eyes treated with 1 or 2 U/mL of Dispace (four complete, 3/10 partial, $P = 0.08$), 8/10 (80%) eyes treated with 5 U/mL Dispace (six complete, two partial, $P = 0.03$), 9/10 (90%) eyes treated with 10 U/mL Dis-

pase (eight complete, one partial, $P = 0.01$), and 6/10 (60%) eyes treated with 25 U/mL Dispace (five complete, one partial, $P = 0.15$). The lower rate of PVD induction at 25 U/mL was associated with a weakening of the attachment of RPE to Bruch's membrane by the higher concentration of Dispace because the RPE, sensory retina, and vitreous left the eye cup as an intact unit when the eye cup was tilted.

A partial or complete PVD was present in 2/10 (20%) control eyes (one complete, one partial) after 120 minutes. The rate of complete or partial PVD increased to 4/10 (40%; three complete, one partial, $P = 0.24$) with 0.05 U/mL Dispace and to 14/15 (93%; 12 complete, two partial, $P = 0.0003$) with 0.1 U/mL Dispace (Figure 4). Concentrations higher than 0.1 U/mL induced a complete or partial PVD in >90% of eyes. The vitreous did not liquefy at any of these Dispace concentrations.

Of the 20 (95%) human cadaver eyes injected with 0.5 mL of 5 U/mL of Dispace and incubated for 15 minutes, 19 developed a complete PVD, with one eye (5%) developing a partial PVD. None of the eyes that received PBS had a complete PVD, and only one (5%) eye had a partial PVD (data not shown).

Histology of the Dispace-treated Retina

The retinal architecture of the porcine eye was not affected by Dispace treatment at either 0.1 U/mL for 120 minutes or 5 U/mL for 15 minutes on histologic examination (Figure 5, A–D). Transmis-

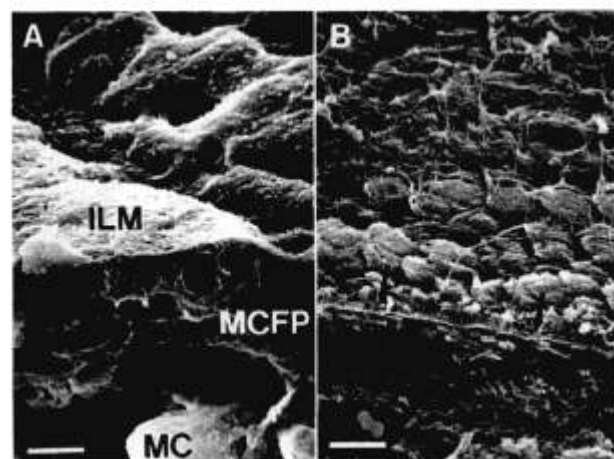


Fig. 7. **A**, Freeze-fracture scanning electron microscopy of the normal vitreoretinal junction in a control pig eye. The internal limiting membrane (ILM) is a homogenous undulating structure that obscures the detail of the cells below. The fan-shaped foot-plates (MCFP) of the Müller cells (MC) terminate in the internal limiting membrane (bar = 5 micrometers). **B**, Freeze-fracture scanning electron microscopy of the sensory retina after treatment with Dispace (at 5 U/mL for 15 minutes). Arrows are pointing to the cracked plane. Dispace cleaves the attachment sites between the ILM and the posterior hyaloid as well as partially digesting the ILM, exposing the mosaic pattern of the inner retinal surface that arises from Müller cell end-foot plates (bar = 5 micrometers).

Table 1. Effect of Dispace Treatment on the Cell Viability and Mechanical Properties of the Retina

Treatment	Retinal Cell Viability (%; mean $\pm$ standard deviation)		Elastic Constant (k) of the Sensory Retina (N.m ⁻¹ ; mean $\pm$ standard deviation)*	Maximal Retinal Stretching Before Tearing (%; mean $\pm$ standard deviation)*
	Pig	Human		
Short-term dispace treatment†	96.2 $\pm$ 2.3	93.8 $\pm$ 5.0	2.3 $\pm$ 1.0	2.5 $\pm$ 0.3
Control	97.3 $\pm$ 1.5	95.0 $\pm$ 3.7	2.0 $\pm$ 0.7	2.2 $\pm$ 0.4
P‡	0.74	0.53	0.46	0.71
Long-term dispace treatment§	94.4 $\pm$ 3.5	92.8 $\pm$ 2.0	2.3 $\pm$ 0.8	2.4 $\pm$ 0.6
Control	92.7 $\pm$ 2.5	95.5 $\pm$ 2.0	2.3 $\pm$ 1.3	2.3 $\pm$ 0.5
P‡	0.63	0.95	0.47	0.38

*Experiments were done in human cadaver eyes.

† Injection of 0.5 mL Dispace (5 U/mL) and incubation at 37°C in 5% CO₂/95% air for 15 minutes.

‡ Mann-Whitney-U test.

§ Injection of 0.5 mL Dispace (0.1 U/mL) and incubation at 37°C in 5% CO₂/95% air for 120 minutes.

N.m⁻¹, Newtons/meter.

sion electron microscopy of control eyes with an attached vitreous revealed collagen fibrils in the posterior hyaloid oriented parallel to the surface of the retina and adjacent to the lamina rara externa of the ILM; the lamina densa was observed as a continuous and distinct dark band (Figure 6A). In Dispace-treated eyes, the collagen fibrils of the posterior hyaloid and the lamina rara externa of the ILM were not present. The lamina densa lost its distinct borders and became an amorphous granular layer (Figure 6B). The ILM of control eyes appeared as a homogeneous undulating membrane that obscured the underlying retinal surface structure on scanning electron microscopy (Figure 7A). Müller cell foot plates could be seen as terminal fan-shaped structures. The ILM disappeared after treatment with Dispace at either concentration, revealing a mosaic pattern to the inner surface of the porcine retina, which may be due to the Müller cell foot plates (Figure 7B).

Cell Viability and Mechanical Properties of the Dispace-treated Retina

The overall viability of retinal cells did not change significantly after injection of Dispace into the vitreous of either porcine or human eyes (Table 1). Similarly, Dispace treatment did not alter either the elastic properties or maximal stretching before fracture of the human retina (Table 1).

Conclusion

Our study demonstrates that Dispace, a neutral protease with substrate specificity for fibronectin and type IV collagen,²¹ can be used to cleave the attachment between the posterior hyaloid and the

ILM selectively. Dispace disrupts the collagen fibrils in the lamina rara externa of the internal limiting membrane, with some loss of distinct borders in the lamina densa. The foot plates of the underlying Müller cells, however, are not altered by Dispace treatment.

The concentration of Dispace required to sever the attachment between the posterior hyaloid and ILM depends on the incubation time. With a short incubation time of 15 minutes, 5 U/mL is the lowest concentration of Dispace that can induce a statistically higher rate of posterior vitreous attachment in enucleated porcine and human eyes. With an incubation time of 120 minutes, a lower concentration of Dispace (0.1 U/mL) can induce a posterior vitreous detachment. Dispace can sever the attachment between the posterior hyaloid and ILM without altering the cellular viability or mechanical properties of the adjacent neural retina.

Dispace cleaves the attachment of the posterior hyaloid to the ILM without causing liquefaction of the vitreous gel.^{21,35,36} Presumably this is related to the relative specificity of Dispace for fibronectin and type IV collagen, which is present in basal lamina, rather than type II collagen, which is present in the vitreous gel.^{1,37} Fibronectin and type IV collagen are also found in the epiretinal membranes of macular pucker and proliferative vitreoretinopathy.³⁸ Thus, Dispace may prove to be a useful adjunct during vitreous surgery for these conditions.

Several previous workers have reported promising methods to sever the attachment between the posterior hyaloid and the ILM enzymatically. Non-specific proteases such as trypsin²⁸ and pronase³⁹ can digest the ILM, although these substances have the

potential to alter retinal architecture because of their broad substrate specificity. Hyaluronidase and collagenase partially digest the cortical vitreous collagen, but the posterior hyaloid frequently remains attached to the ILM after treatment with these enzymes.^{10,39} Ultrastructural studies suggest that chondroitinase is ineffective at separating the posterior hyaloid from the internal limiting membrane of humans.³⁹ Plasmin severs the attachment between the posterior hyaloid and the ILM,¹³ and appears to have little cytotoxicity although it is a nonselective protease.⁴⁰ Plasmin, however, is inactivated in the presence of serum,⁴¹ and lacks a direct effect on type IV collagen.¹³ Dispase remains active in the presence of serum, which is particularly important because leakage of serum into the vitreous occurs in many vitreoretinal disorders.

In conclusion, our studies suggest that Dispase can effectively induce a posterior vitreous detachment in porcine and human eyes *in vitro*. Further studies are necessary to determine the possible toxicity of this enzyme *in vivo* and to delineate the utility of this enzyme in managing patients with vitreoretinal disorders.

Key words: Dispase, internal limiting membrane, posterior vitreous detachment, vitreoretinal junction, vitreoretinal surgery.

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