Reports

Modulation of Human Lymphocyte Proliferation by Normal Bovine Vitreous

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A heat stable antiangiogenic protein (Bovine Vitreal Protein or BVP) isolated from normal bovine vitreous and known to inhibit growth of endothelial and smooth muscle cells was studied for its in vitro effect on human lymphocyte proliferation. Unheated BVP inhibited lymphoproliferation in 12 normal, healthy donors in response to tetanus toxoid (TT) and to Concanavalin A (Con A), but had little effect on responses to irradiated allogeneic peripheral blood mononuclear leukocytes (PBML) or Pokeweed mitogen (PWM). In contrast, heated (95°C/10 min) BVP strongly inhibited (more than 50%) proliferative responses to allogeneic cells, TT, and Con A, and strongly augmented responses to PWM. Invest Ophthalmol Vis Sci 28:753–756, 1987

In the normal adult eye, vitreous is both avascular and devoid of leukocytes. During the course of recurrent or chronic posterior uveitis, however, both neovascularization of the retina and infiltration of lymphoid cells into the vitreous may be observed. Whether vascularization precedes inflammation or vice versa, or whether lymphoid and vascular endothelial cells respond concurrently to the same stimulus is not clear. Vascular endothelial cells and lymphocytes have been shown to interact under experimental conditions and to have profound effects upon one another. Previous studies, for example, have demonstrated that vascular endothelial cells can activate lymphocytes by acting as antigen-presenting cells, can serve as targets for cytotoxic lymphocytes, and are, in turn, themselves modified by culture with soluble products obtained from activated lymphocytes. It is reasonable to question, therefore, whether both endothelial cell and lymphocyte activities are related and affected by similar factors.

We previously demonstrated that normal bovine vitreous contains both a heat stable growth inhibitor specific for corneal and vascular endothelium, and a heat-labile nonspecific mitogen. The present report extends these studies to examine the effect of this inhibitor on normal human lymphocyte responses to soluble antigen, alloantigen, and mitogen.

Materials and Methods. Preparation of bovine vitreous protein (BVP): BVP was prepared by a method previously reported. Briefly, adult bovine vitreous was homogenized and centrifuged at 40,000 X g for 30 min. at 4°C. The supernatant was dialysed against water at 4°C and lyophilized. Dried vitreous was then dissolved in 0.1 M sodium acetate-0.15 M sodium chloride, and glycosaminoglycans (GAG) were digested with bovine testicular hyaluronidase for 24 hr at room temperature. The digest was dialysed against water, precipitated with ammonium sulfate, centrifuged, and the pellet dissolved in water and dialysed again. The dialysate was lyophilized and stored desiccated at -20°C. Protein concentration was determined by the method of Bradford. Samples were resuspended to 5 mg/ml in distilled water and divided into 2 aliquots. One aliquot was heated at 95°C for 10 min and both aliquots were filter sterilized (0.22 μm) before making final dilutions in culture medium (see below).

Cell isolation and medium: After obtaining informed consent, peripheral blood mononuclear leukocytes (PBML) were obtained from 12 normal healthy volunteers by centrifugation of freshly drawn, defibrinated blood over Ficoll-hypaque gradients. PBML were washed, counted and resuspended at 1 X 10^6/ml in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% pooled human serum (complete medium). Viability was determined to be better than 98% by trypan blue exclusion.

Antigens and mitogens: Partially purified tetanus toxoid (TT, Massachusetts State Department of Public Health, Lot LP430PM) was prepared in complete medium so that the final dilution represented 2.0 LfU/ml (Limit of flocculation units). Concanavalin A (Con A, Sigma) was prepared to a final concentration of 80 μg/ml. Pokeweed mitogen (PWM, Gibco) was prepared as a 1:50 dilution of stock solution. Stimulator cells for one-way mixed lymphocyte culture (MLC) assays were prepared by irradiating random allogeneic donor PBML with 1500 rad from a cesium source prior to coculture.

Cell cultures: Assays were carried out in round bottom microtiter wells (Linbro, McLean, VA) using 50 μl PBML (5 X 10^4) and 50 μl of mitogen, antigen, or
irradiated allogeneic cells (5 × 10⁴) per well. One hundred microliters of heated or unheated vitreous protein was added to appropriate wells for final concentrations of 100, 50, 25, and 10 µg/ml. Control wells received 100 µl complete medium. Triplicate cultures were incubated in 5% CO₂ at 37°C for either 4 days (Con A, PWM) or 6 days (MLC, TT), at which time 1 µCi/well of tritiated thymidine was added. Cells were harvested 18 hr later and counted in a liquid scintillation counter. Percent inhibition was determined by the formula:

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1 - \frac{(cpm \ test \ wells + inhibitor)}{(cpm \ control \ wells + inhibitor)} \times 100
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\[
\frac{(cpm \ test \ wells - inhibitor)}{(cpm \ control \ wells - inhibitor)}
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Results. Heated BVP inhibited lymphoproliferative responses of all normal human PBML to allogeneic cells, TT and Con A, but not to PWM. In a representative individual (Fig. 1), allogeneic (Fig. 1A) and TT responses (Fig. 1B) were reduced to 50% normal with 25 to 40 µg/ml medium BVP. Higher concentrations of BVP up to 100 µg/ml did not significantly inhibit responses further. Responses to Con A (Fig. 1C) were similarly reduced with less than 10 µg/ml BVP. In contrast, the response to PWM (Fig. 1D) was increased significantly over wells containing no inhibitor, reaching 50% enhancement at 10 µg/ml. At no concentration up to 100 µg/ml was heated BVP mitogenic or toxic to unstimulated cells as determined by tritiated thymidine uptake and trypan blue exclusion of control wells.

Because heat treatment eliminated endothelial cell mitogenic activity of BVP, unheated material was also evaluated. Some variation occurred in PBML responses among individuals when the mitogenic activity of BVP was not destroyed by heating. Generally, unheated BVP inhibited lymphoproliferative responses of normal human PBML to TT and to Con A, but not to allogeneic cells or PWM as shown in Figure 2. In a representative individual, allogeneic responses were actually enhanced by 50% in the presence of 20 µg/ml unheated BVP (Fig. 2A). Higher concentrations up to 100 µg/ml did not enhance proliferation significantly over this. Responses to TT (Fig. 2B) and Con A (Fig. 2C) were inhibited at BVP concentrations of 25 to 50 µg/ml. Inhibition of proliferation in response to TT was weak,
never reaching 50%. Responses to PWM (Fig. 2D) in the presence of unheated BVP were not significantly different from responses in the absence of BVP. Unheated BVP was slightly mitogenic at all concentrations as determined by tritiated thymidine uptake of control wells (Fig. 2).

Responses of all donors are represented in Figure 3 as percent inhibition or enhancement of proliferation. As in the representative individual, heated BVP inhibited proliferation in response to PBML, TT, and Con A, but not PWM. Nonheated material consistently inhibited only responses to TT and Con A.

Discussion. We previously demonstrated that a heat stable antiangiogenic substance in normal vitreous is capable of inhibiting in vivo neovascularization induced by either tumor or retinal-derived growth factor. Subsequent studies demonstrated that this antiangiogen was a protein that specifically inhibited the proliferation of corneal and vascular endothelium, and smooth muscle cells in vitro. An increase in inhibitory activity after heating was attributed to the heat lability of a nonspecific mitogen also present in vitreous. In the current study, we demonstrate the capacity of this heat stable bovine vitreous inhibitor (BVP) to modulate the proliferative response of human lymphocytes to various mitogens and antigens.

It is not clear why responses to PWM were strongly augmented by heated BVP while other responses were inhibited, and current experiments in our laboratory are directed at this question. Our results however are in agreement with previous studies which showed that poorly vascularized tissue such as cartilage and aorta possess both immunosuppressive activity and endothelial cell growth inhibitors. In those studies it was found that T cells in particular were inhibited by a factor isolated from the aorta.

Our results are also in general agreement with recently published data showing that crude bovine vitreous contains an inhibitor of in vitro mitogen-driven proliferation of mouse spleen cells. In that report, however, the authors found a B cell mitogenic response (LPS) to be more inhibited than a T cell mitogenic (Con A) response. This discrepancy may be due to differences in experimental protocol and species or may indicate more than one inhibitor is present in vitreous.

Those authors found that trypsin treatment of crude vitreous did not result in significant loss of endothelial cell growth inhibitory activity, suggesting that inhibition was not due to a protein. Our study, however, used only the proteinaceous fraction of vitreous. Crude preparations of vitreous contain significant quantities of glycosaminoglycan or GAG (mostly hyaluronic acid and chondroitin sulfate) and we have found that chondroitin sulfate inhibits proliferation of lymphocytes in vitro assays (unpublished results). Our treatment of BVP with testicular hyaluronidase eliminated 98% of both chondroitin sulfate and hyaluronic acid, the major GAG in vitreous.

The inhibition by BVP of both vascular endothelial cell and lymphocyte proliferation indicates that these two different cell types are similarly affected by the same endogenous substance. This suggests that some endothelial cell and lymphocyte activities may be related and may occur concurrently in disease.

Key words: vitreous, anti-angiogenesis, lymphoproliferation, bovine vitreal protein

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References
Vitreous Fluorophotometry in Patients With Senile Macular Degeneration

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Twenty-two phakic eyes of 16 patients with varying stages of senile macular degeneration (SMD) underwent vitreous fluorophotometry. The upper tolerance limit of the penetration ratio in 17 eyes of 17 age-matched controls was 6.33 × 10⁻⁴ min⁻¹. Ten of the 22 eyes with SMD had values exceeding this, indicating abnormal blood–retinal barrier function. When the fluorescence recorded in the vitreous but not related to the local intravitreal dye was evaluated, it was greater in those eyes with more severe forms of the disease. All ten eyes with drusen alone and no SMD had normal fluorophotometry values. Vitreous fluorophotometry may be helpful in the diagnosis and classification of SMD. Invest Ophthalmol Vis Sci 28:756–759, 1987

Vitreous fluorophotometry is a relatively new technique that was developed to measure minute amounts of fluorescein in the vitreous. It is most useful in evaluating the permeability of the blood–retinal barriers. The recent development of a vitreous fluorophotometer for clinical use has made measurements of the concentration of fluorescein in the vitreous simple, reliable, and reproducible.¹,²

Cunha-Vaz points out that there are two blood–retinal barriers.³ The inner barrier is located at the endothelial cell membrane of the retinal blood vessels, and the outer barrier is found at the level of the retinal pigment epithelium (RPE). Most vitreous fluorophotometric studies concerned with the impairment of these barriers have been performed on patients with diseases affecting retinal blood vessels at the inner barrier. Few studies have examined the permeability of the outer barrier. Argon laser photocoagulation⁴ and sodium iodate⁵ have been used to produce an abnormal RPE, which impaired the outer barrier severely. Patients with fundus flavimaculatus, a disease presumably affecting the RPE, and therefore the outer barrier, have demonstrated normal fluorescein levels.⁶

The early stages of senile macular degeneration (SMD) affect the RPE and the subretinal space. These alterations include drusen, subretinal newvascular nets, leakage (hot spots) observed by fluorescein angiography, accumulation of subretinal fluid, and spots of atrophy, hypertrophy, and hyperpigmentation of the RPE. The current study explores how the evolution of SMD and drusen affects the blood–retinal barrier, as detected by vitreous fluorophotometry, and whether any particular stage of the disease can be implicated if such impairment exists.

Materials and Methods. For the purpose of this study, (1) SMD was defined as a macular lesion that became symptomatic (patient noticed decreased or distorted vision) after age 50; and (2) ophthalmoscopy revealed macular drusen and one or more of the following: subretinal neovascularization in the macular area, serous detachment of the RPE, and subretinal fibrous or fibrovascular membrane. We studied 22 phakic eyes of 16 patients (ten men, six women) affected by SMD. Their ages were 69.6 ± 9.5 yr (mean and SD). Also examined were ten fellow eyes with drusen only. Informed consent was obtained after the nature of the procedures had been explained. For an