Immunochemical analysis of vitreous and subretinal fluid

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Immunochemical analysis of human and bovine vitreous has shown that these structures contain many serum proteins. One of the alpha globulins appears to be present in human vitreous in concentrations higher than the serum itself. Pooled human vitreous revealed a precipitating antibody to an extracellular streptococcal antigen. With the most potent antisera available, human vitreous was shown to contain a minimum of 5 to 6 tissue antigens not shared with the serum, whereas bovine vitreous has shown 9 or more such antigens. It is thus clear that the soluble antigens of the vitreous are not entirely derived from plasma. Fifteen subretinal fluids from patients with idiopathic retinal detachments have been shown to contain vitreous "tissue" substances. These findings furnish proof that the vitreous is an integral part of the subretinal fluid, and thus may play an important role in the formation and progression of idiopathic retinal detachment.

Despite the fact that the vitreous occupies somewhat more than two-thirds of the intraocular volume, little is known of its detailed composition and function. Two of the major macromolecular components are collagen and hyaluronic acid. The latter, which is present in the vitreous in relatively high concentration, may form complexes with the collagen framework and thus aid in the maintenance of the gel. Until recently, there was relatively little information about the macromolecular components of the vitreous, other than collagen and hyaluronic acid. Soluble proteins have been demonstrated in low concentrations, but their identities in the past have not been clearly established despite the use of electrophoretic, ultracentrifugal, and ultraviolet absorption methods.

The total protein content of the vitreous is very small, in the range of 0.4 to 0.8 mg. per milliliter, as compared to serum with 65 mg. per milliliter or more. Recently, independent studies of human and bovine vitreous in this laboratory, and of bovine vitreous by Laurent and Howe, have shown that a large number of serum proteins are present in the vitreous. Significantly, additional antigenic substances not shared by serum were also detected. These latter findings were used in this investigation to test the hypothesis of many ophthalmologists that the vitreous body plays an indispensable role in the formation and progression of idiopathic retinal detachment. It is believed that a hole or tear appears in the retina, and then the vitreous percolates through this hole to float the retina away from its attachment.
to the pigment epithelium. The vitreous is then believed to become an integral part of the subretinal fluid, which forms between the two embryonic layers of the retina. If this hypothesis is correct, analysis of subretinal fluid should disclose the presence of vitreous "tissue" antigens. Previous reported studies of the subretinal fluid have stated that this fluid resembles serum and not vitreous.10,12

Immunodiffusion methods now allow for more precise qualitative and quantitative determinations of most of the vitreous soluble macromolecules. This report describes the further results obtained with these methods in the analysis of human and bovine vitreous, and of subretinal fluids from patients with idiopathic retinal detachments.

Materials and methods

Beef eyes were obtained from freshly killed animals and were frozen. The eyes were then immersed briefly in saline, the sclera incised equatorially, and the frozen vitreous body removed. Any adherent ciliary epithelium was removed by dissection. The vitreous was then allowed to thaw, and homogenized at 4°C for 2 to 3 seconds in a high-speed blender. The pooled vitreous was dialyzed at 4°C against large volumes of distilled water with two changes per day for 6 to 7 days. The dialysate was centrifuged in the cold at 10,000 r.p.m. (Spinco Preparative Ultracentrifuge, Rotor No. 21) for one hour. The supernate was lyophilized, and stored in tightly taped vessels at -20°C. The dried powder was uniformly white, fluffy, and readily soluble. Attempts to lyophilize undialyzed vitreous resulted in a glazed residue which was largely insoluble.

Human vitreous was obtained either by dissection of eyes available at autopsy, or by aspiration of other postmortem eyes (average time after death, 6 hours). This vitreous was homogenized, dialyzed, centrifuged, lyophilized, sealed, and stored as above.

For immunization of rabbits, the vitreous was reconstituted with 0.85 per cent saline, the bovine vitreous at a concentration of 20 mg. per milliliter, and the human vitreous at 10 mg. per milliliter. Following preimmunization bleedings, rabbits weighing approximately 3 kilograms were repeatedly immunized with the reconstituted vitreous (human or bovine) and homogenized to complete Freund's adjuvant.13 A total of 1 ml. was administered in five intradermal sites for each dose, for a total of 5 to 12 immunizations, at weekly or biweekly intervals. The total amount of vitreous given each rabbit ranged from 25 to 60 mg. dry weight for the human and 50 to 120 for the bovine. After three immunizations, the animals were bled approximately 10 to 12 days after each subsequent dose. Most bleedings were done from the central ear artery, but, occasionally, cardiac punctures were made. The serum was collected aseptically, centrifuged in the cold, and stored steriley at 4°C. The equine antisera to human serum proteins were obtained from the Pasteur Institute, Paris (Lots 13459, 13482, and 511). The Ouchterlony technique was performed according to modifications used previously.15 The method of Wadsworth was used for the micromodification of the two directional immunodiffusion tests. Immunoelectrophoresis was performed according to the micro-technique of Scheidegger.17 On four-inch slides, with 1 per cent Bacto (Difco) agar, veronal acetate buffer M 0.05 at pH 8.2, 6 volts per centimeter were applied for 2 to 2½ hours.

Fig. 1. Immunoelectrophoretic pattern of normal human serum when tested with antihuman vitreous serum. The well contains human vitreous at 20 mg. dry weight per milliliter.
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Fig. 2. Immunoelectrophoretic demonstration of serum proteins in human vitreous, with equine antiserum to normal human serum (Pasteur Institute). The top well contains human vitreous at a protein concentration of 10 mg. per milliliter; the bottom well contains human serum, 80 mg. per milliliter.

For immunodiffusion tests, the lyophilized vitreous was reconstituted in veronal acetate (pH 8.2) at a concentration of 20 mg. dry weight per milliliter. Except for traces of insoluble residues, the human vitreous went into solution readily when stored at 4° C. overnight. The bovine vitreous was more viscous, and usually required somewhat longer storage.

Absorption of the antivitreous sera was carried out by the addition of lyophilized pooled human or bovine serum. For complete absorption to be achieved, a concentration of lyophilized serum of 60 mg. per milliliter was found to be necessary. The absorption mixture had to be stored for a minimum of 24 hours at 4° C. to be complete.

For immunodiffusion tests with other tissues, extracts of beef heart, liver, kidney, skeletal muscle, brain, lens, and human lens were lyophilized and reconstituted with saline at a concentration of 50 mg. dry weight per milliliter. Human heart, liver, kidney, skeletal muscle, cornea, optic nerve, and beef cornea and optic nerve were used as extracts without previous lyophilization.

The subretinal fluids were collected from patients with idiopathic retinal detachments. These were new, old, or recurrent cases. For aspirating the subretinal fluid, the points were removed from 30 gauge, 1/8 inch needles, by careful filing.

A scleral incision was made at the selected site of drainage, then the blunt needle, fitted to a 2 c.c. syringe, was introduced through this site. The subretinal fluid was very gently aspirated, sealed tightly in small test tubes, and stored at -20° C. All were completely clear, and direct microscopic examination of the test samples of subretinal fluids collected in this manner revealed no red blood cells. If any evidence of gross contamination with blood was apparent, the specimens were discarded.

Table I. Subretinal fluids investigated

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<th>Subretinal fluid samples</th>
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*We are deeply indebted to Dr. Graham Clark for his assistance in obtaining these specimens, and for making the records of the patients available.

Results

Human vitreous and subretinal fluids.

In the analysis of human vitreous, serum proteins were demonstrated in two ways: by tests with antibodies to human serum proteins against vitreous; and by tests with the antivitreous antibodies against human serum. That numerous serum proteins must be present in human vitreous was clearly demonstrated by the complexity of the latter reactions. As shown in Fig. 1, human vitreous must contain at least 14 serum proteins, since antibodies to these were induced by immunization of rabbits with vitreous.

When serum proteins in the human vitreous were found with standard anti
human serum antibodies, the number of components detectable was considerably less, as shown in Fig. 2. Only 4 or 5 precipitin arcs could be seen. It seems virtually certain that quantitative factors were responsible for this apparent discrepancy.

When serial dilutions of the vitreous were tested with the standard equine antihuman serum, and compared to serum proteins at equal concentrations, it appeared that at least one of the serum components may be present in the vitreous in concentrations higher than the serum itself (Fig. 3). The identity of this antigen is not known, but it clearly resides in the alpha globulin region.

Preliminary attempts to detect antibodies in pooled human vitreous have demonstrated precipitating antibodies to an extracellular streptococcal antigen (Fig. 4). It may be pointed out that the total protein concentration of the vitreous solution which could be achieved was about 35 mg. per milliliter, only an undetermined small portion of which was gamma globulin. Other studies had shown numerous precipitating antibodies in purified pooled normal human gamma globulin at very high concentrations (180 mg. per milliliter) against these bacterial concentrates. The failure to detect more antibodies in the vitreous may be caused by the low concentrations of vitreous employed.

To determine whether components other than serum proteins were present, tests were carried out with antihuman vitreous
serum from which antibodies to serum proteins were removed by complete absorption with lyophilized normal pooled human serum. With the most potent antiserum, a minimum of 5 to 6 nonserum components were found, as shown in Fig. 5. The clearly different electrophoretic mobilities of the components visualized make it unequivocal that different molecular species are involved. Completeness of absorption was verified by the absence of immune precipitates with normal human serum.

Two directional immunodiffusion tests with other tissue extracts and antihuman vitreous serum, from which antibodies to serum proteins had been absorbed, indicated that some of the vitreous tissue components were not present in any of the tissues tested, including heart, skeletal muscle, liver, kidney, brain, lens, cornea, and optic nerve. Different antihuman vitreous antisera showed variable results in tests with other tissues. Several of the antisera gave no reaction with any of the tissues tested; others showed a small number of reactions with one or two of the tissues, and one or two reacted with a number of them.

Our immunochemical studies have conclusively shown that a large number of serum proteins are present in the 15 subretinal fluids examined (Fig. 6). Furthermore, it was of considerable importance that there were vitreous "tissue" substances present in all of the samples of subretinal fluid tested, as judged by reactions with antihuman vitreous sera, absorbed free of antibodies to serum proteins (Fig. 7). Of the 15 subretinal fluids tested (Table 1), 13 revealed three such antigens, while two others showed two. These findings thus clearly show that the vitreous did become an integral part of the subretinal fluid in these patients with idiopathic retinal detachments.

Bovine vitreous. Similar analysis of bovine vitreous has also been carried out. The presence of bovine serum proteins could be readily demonstrated with the

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Fig. 6. Analysis of subretinal fluid from a patient with idiopathic retinal detachment by immunoelectrophoresis against equine antiserum to normal human serum. Top well, human vitreous, 10.4 mg. protein per milliliter; bottom well, subretinal fluid, No. 12, 7.54 mg. protein per milliliter; trench, equine antiserum to human serum (Pasteur Institute).

Fig. 7. Two-directional immunodiffusion assays of subretinal fluid from 5 patients with idiopathic retinal detachment, when tested against antihuman vitreous serum, absorbed free of antibodies to serum proteins. Peripheral wells 1 to 5 contain different samples of subretinal fluid from different patients. Peripheral well 6 contains human serum (60 mg. dry weight per milliliter). The center well contains absorbed antihuman vitreous serum.
antibovine vitreous sera in tests with bovine serum, as shown in Fig. 8.

After absorption of the antibodies to serum proteins with lyophilized normal bovine serum, as many as 9 or more vitreous "tissue" antigens could be visual-

**Fig. 8.** Immunelectrophoretic assay of normal bovine serum when tested against antibovine vitreous serum. Well, bovine serum, 60 mg. dry weight per milliliter; trench, antibovine vitreous serum.

**Fig. 9A.** Immunelectrophoretic assay of bovine vitreous against antibovine vitreous serum absorbed free of antibodies to normal bovine serum proteins. Top well, bovine vitreous, 20 mg. dry weight per milliliter; bottom well, normal bovine serum, 60 mg. dry weight per milliliter; trench, antibovine vitreous serum.

**Fig. 9B.** Two-directional immunodiffusion assay of bovine vitreous, 20 mg. dry weight per milliliter, against absorbed antibovine vitreous serum, as in A. Top well, bovine vitreous; bottom well, antivitreous serum.
ized with the most potent antisera (Fig. 9A). The completeness of absorption is also demonstrated here. In Fig. 9B, confirmation of the numbers of antigens involved is shown by means of the micro-Ouchterlony technique.

During our investigations, Laurent and Howe reported three bovine vitreous antigens not shared with serum. It is probable that the antisera used here, which revealed more vitreous "tissue" antigens, were more potent.

Immunodiffusion tests of antihuman vitreous sera (absorbed with bovine serum) against bovine tissue extracts showed results similar to those of the antihuman vitreous experiments mentioned above.

**Relationship of mammalian vitreous antigens.** Immunochemical analysis of the species specificity of the vitreous "tissue" antigens has been carried out. It was found with sheep, bovine, and human vitreous that varying numbers of these substances are immunochemically related. For example, antihuman vitreous sera, absorbed free of antibodies to bovine serum proteins, revealed large numbers of cross-reactions with sheep vitreous, and fewer with human vitreous. One such result is shown in Fig. 10. The similarity and complexity of the pattern with sheep vitreous to that seen with bovine vitreous (Fig. 9A) are striking. The human cross-reacting vitreous antigen showed an electrophoretic mobility comparable to the most intense precipitin arcs seen with sheep vitreous.

Interestingly, antihuman vitreous sera absorbed free of antibodies to human serum proteins showed several reactions with both sheep and bovine vitreous, as can be seen in Fig. 11. The mobilities, patterns, and numbers of these cross-reacting antigens were very similar.

**Discussion**

Immunochemical analyses of human and bovine vitreous have demonstrated a large number of serum proteins in each. Whether or not all the serum proteins are represented in the vitreous has not yet been determined. The hyaluronic acid content and viscosity of the vitreous made analysis by absorption techniques at concentrations higher than 20 mg. per milliliter very difficult. Serum proteins present in the vitreous, in amounts too small to be detected with antibodies to serum proteins, were evident by the fact that they evoked measurable antibody responses. The patterns obtained in these latter reactions were similar in their complexity to those with antisera produced by injection of human serum.

Human vitreous appears to contain at least one serum protein in the alpha globulin fraction in concentrations higher than the serum itself. This suggests a possible selectivity on the part of human vitreous for serum proteins.

The presence in human vitreous of a precipitating antibody to an extracellular streptococcal antigen, at low protein concentrations, is an interesting finding. The relationship of vitreous antibodies to those...

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**Fig. 10. Cross-reactions of sheep and human vitreous "tissue" antigens against antihuman vitreous serum absorbed free of antibodies to normal human serum proteins.** Top well, sheep vitreous, 20 mg. dry weight per milliliter; bottom well, human vitreous, 20 mg. dry weight per milliliter; trench, antihuman vitreous serum.

**Fig. 11. Cross-reactions of bovine and sheep vitreous "tissue" antigens against antihuman vitreous serum, absorbed free of antibodies to normal human serum proteins.** Top well, bovine vitreous, 20 mg. dry weight per milliliter; bottom well, sheep vitreous, 20 mg. dry weight per milliliter; trench, antihuman vitreous serum.
frequently found in serum, and their role in resistance mechanisms of ocular tissue should be further investigated.

Both human and bovine vitreous contain a number of antigens not shared with serum. The origin, chemical composition, localization, and function of these antigenic substances have not yet been determined. Balazs has demonstrated a rather specialized cell on the surface of the vitreous of several species. The possibility that the vitreous “tissue” antigens shown may be related to these cells should be considered. Preliminary attempts at purification of these substances by continuous flow electrophoresis have been made by Howe. Studies of the species specificity of the vitreous “tissue” antigens, thus far carried out, have indicated that some of these substances from several species are similar immunochemically. Sheep vitreous was more closely related to bovine than to human vitreous. However, both sheep and bovine vitreous do contain some antigens which are similar to antigens found in human vitreous. In these studies, rabbit vitreous was not tested. If the antisera are found to cross-react with rabbit vitreous, another potential autoimmune system would be available. Fundus and slit-lamp examinations of these rabbits have failed to reveal any pathologic changes.

Previous reports of subretinal fluid examinations have indicated that these are derived solely from the plasma. These findings were at odds with the postulated pathogenesis of idiopathic retinal detachment, as proposed by Gonin in 1904. The immunochemical studies described here demonstrate conclusively that there are vitreous “tissue” antigens in the subretinal fluids, and support this original hypothesis.

REFERENCES

Discussion

Dr. Theodore W. Sery, Philadelphia, Pa. The technique of immunoelectrophoresis and agar gel diffusion with immune reagents have made possible the detection of small quantities of antigen or antibody in tissue extracts. The sensitivity of these methods is of the order of 1.0 to 0.1 μg of material. Although the greater proportion of vitreal protein types were indistinguishable immunologically from serum proteins, several (5 or 6) were apparently unique to the vitreous gel. The finding that these vitreal proteins are present in subretinal fluid, in cases of idiopathic uveitis, establishes the important part the vitreous plays as a source of this fluid. It establishes, by a sensitive immunologic technique, that vitreous fluid can and does enter into the subretinal space through a retinal tear.

The presence of plasmalike proteins in the vitreous, as well as the cornea, as shown by Kawerau and Ott (Exper. Eye Res. 1: 137, 1961), poses a question as to whether they arrived in these avascular tissues by diffusion or whether they were formed in situ by local cells. In the case of corneal epithelium, some proteins might be those released by mechanical disruption of cells. For vitreous, however, there are essentially no cells, and protein sources would presumably involve diffusion from the peripheral tissues. It would be of value therefore to make similar tests on the inner vitreal material to determine whether there is a concentration gradient of protein decreasing from the outer vitreal cortex, where the vitreal cells are located, toward the central region.

The techniques used to detect these proteins have been profitable within their own limitations. Precipitation methods will of course reveal only antigens that can be made insoluble by antibodies as immune complexes. Additional proteins either in lower concentrations or as nonprecipitable proteins might be found by other methods. There are several new immunologic techniques that might be used for detecting soluble immune complexes. One in particular described by Boyden (Mechanisms of Hypersensitivity, Boston, 1959, Little, Brown and Company, chapter 7), making use of specific immune precipitates tagged with radioactive markers, might prove to be a valuable system in this type of study. Another procedure described by Sehon, in this book, discussion of chapter 7, as reversed hemagglutination, might also serve to detect the presence of a much lower order of protein concentrations. Sehon says that the technique, which uses specific antibody instead of antigen, adsorbed to red cells, can detect antigen at 1,000 to 10,000 times lower concentrations than can be found by the agar gel technique. This would mean that proteins at levels of about 10^-4 μg of material could be identified.