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Aqueous Microbiology: The Possible Role of Cell Wall Deficient Bacteria in Uveitis

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Samples of aqueous humor were obtained from the anterior chamber of patients with uveitis. Bacterial L-forms, or cell wall deficient forms, were recovered from 11 of 19 eyes with unexplained uveitis. These bacterial variants were cultured on newer, highly specialized media and stained with acridine orange. This stain demonstrates fluorescence of nucleic acids under UV microscopy.

This is a preliminary report of a study on the microbiology of the aqueous in uveitis. It was designed to determine whether cell wall deficient bacterial forms (CWDF) are associated with this disease.

Among the CWDF are the bacterial L-forms of Klieneberger1,2 and the pleuropneumonia-like organisms (PPLO)3 of Nocard and Roux.4 Though much work has been done on the aqueous5 in uveitis6,7 and though L-forms8,9 and mycoplasma9,10 have been incriminated in a variety of human infections, they have not been reported in the aqueous in anterior uveitis.

Other variant bacterial forms, such as spheroplasts,11 protoplasts,12,13 and microplasts exist and may have independent significance. They are, however, so closely related to either the PPLO or the L-forms that they are considered here only as members of the family of “variant bacteria” or “cell wall deficient forms,” CWDF.

Materials and Methods

Included in this preliminary report are some of the microbiological studies done on aqueous samples from 26 patients who represent a variety of types of uveitis and controls.

Twelve aqueous samples were obtained from eyes with active anterior uveitis (iritis).

Five samples were from the eyes of four patients with active posterior uveitis (chorioretinitis) as well as active anterior uveitis.

Two aqueous specimens were obtained from eyes with active posterior uveitis but without evidence of anterior uveitis.
Four aqueous samples fall into a heterogeneous group. One of these patients had a penetrating diathermy procedure for malignant melanoma of his only eye. Several months following this, when the eye failed to regain vision and maintained intraocular inflammation, it was enucleated. The anterior chamber was tapped for aqueous just prior to this procedure. The second patient suffered an intraocular foreign body in an industrial accident. Aqueous was obtained before extraction of the cataractous lens containing the steel particle. The third sample was obtained from an eye donated for transplant purposes by a man who had died of pneumonia occurring as a complication of influenza. Aqueous was aspirated prior to use of the donor tissues. A fourth patient, with uveitis, was found to have spirochetes in the aqueous. Serving as controls were three aqueous samples obtained prior to cataract extraction and one obtained just before a strabismus procedure.

To minimize the possibility of contamination occurring between obtaining and seeding the samples, laboratory personnel were present in the operating room. Non-commercial autoclaved 4% cocaine was filtered through a Swinnex filter* and used locally to anesthetize the cornea. Extensive conjunctival and cul-de-sac sterilization measures were not used since fragile CWDF could be destroyed; nor were commercially available anesthetics, containing preservatives or germicidal agents, because such agents might kill the microorganisms. If any organisms were introduced from the conjunctival sac into the aqueous of the anterior chamber, they would have been seen as classical growth in the initial liquid cultures or on the sheep blood agar subculture plates.

Aqueous was obtained using the technique of Goldman and Girard. A sterile, disposable, one-inch No. 27 gauge needle on a sterile, disposable tuberculin syringe was used to withdraw 0.15 to 0.25 ml of aqueous. These fine sharp needles easily bore into the anterior chamber when the syringe is gently rocked between the finger tips. The needle was introduced tangential to the limbus so as not to endanger the lens.

1. Initial cultures: Immediately after obtaining aqueous, the following media were inoculated:

a. Thioglycollate broth: Five or six drops of aqueous were inoculated into an 8.0 ml screw cap tube containing 1.0 ml of Thioglycollate broth. This broth which contained a trace of agar will support CWDF as well as classical organisms. Aerobes grow well on the surface, anaerobes deep within the broth and the occasional microaerophilic organism causes a haze just beneath the surface. It is expected to remain almost clear if harboring only CWDF but becomes clouded by classical organisms occurring as contaminants or as isolates from the initial inoculum.

b. Medill-O’Kane (Mattman)** medium: Five or six drops of aqueous were inoculated into a sterile 8.0 ml screw cap tube containing 1.0 ml of Medill-O’Kane (Mattman) media. This synthetic medium is particularly useful in the culture of CWDF and is a complex, specialized, synthetic amino acid medium as well.

The initial cultures (liquid and solid) were kept for seven to ten days. If growth of the organisms occurred, the media were examined for characteristic colonial形态, biochemical tests, and serology.

The data were compiled and analyzed by the Statistical Course (Galbraith) at the University of Kentucky in the summer of 1965.

* Tradename for filter, Millipore Filter Corporation, Bedford, Massachusetts.
** Tradename for synthetic medium, Cook Laboratories, Inc., Bloomington, Indiana.
acid medium which can be prepared as either a liquid or a solid.

The original thioglycollate and Medill-O'Kane (Mattman) broth cultures were incubated anaerobically (GasPak®) for thirty days. After five days and again at about thirty days, the initial liquid cultures were subcultured to solid media.

Though this was primarily a search for "fastidious" or "cell wall deficient organisms," appropriate media and tests were also utilized for classical bacteria, spirochetes, fungi, and mycobacteria. Results of this work will not be included in this paper. They were mainly negative.

2. Subcultures: After five days and again at thirty days, the following solid media were inoculated:

a. Chanock's medium: A solid CWDF medium prepared in small 6.0 ml plates. Areas thought to be suspicious by low-power microscopy for L-growth were subjected to certain confirmatory tests.

b. Sheep Blood Agar: This medium, inoculated for classical organisms, was held for at least thirty days. Colonies seen on the sheep blood agar plates were gram stained and were studied with routine bacteriological tests. If we were isolating classical bacteria from the aqueous, sheep blood agar plates or the initial liquid cultures would be expected to grow them.

In most routine bacteriology laboratories, culture plates negative for classical bacteria are discarded after five days. It has been shown that L-forms are usually not seen this quickly. Plates should be held for thirty days or more; a situation analogous to holding slants for six weeks when the acid fast bacillus is suspected. In aseptic meningitis, cultures negative for classical organisms after five days may be positive for CWDF if held for several weeks longer.

Media used for the culture of CWDF are suited for other forms of microbiological life as well. Great care must be exercised to avoid contamination.

Common artificial and natural media are known to have suppressing effects on the CWDF. Thus, though aqueous samples have been put on a wide variety of classical media in the past, the chances of recovering CWDF were poor, even if these media were held for long periods. Only recently have sophisticated media, such as those of Medill-O'Kane (Mattman), Chanock and Abram, been defined well enough to make their use practical in routine microbiology laboratories.

3. Confirmatory tests for L-growth:

At intervals, the solid Chanock's medium plates (for CWDF) were scanned with the low power (100x) of a standard laboratory microscope. The small size of variant bacteria colonies allows them to be missed by the naked eye. Unless microscopic study of solid media is done CWDF colonies will be missed. Suspicious areas were subjected to further tests for "variant" or CWDF growth.

a. Micrographs of suspect areas were made with a Zeiss photomicroscope, using high speed, type B, Ektachrome film at 160x.
b. Acridine Orange Test:17-20

Suitable areas on the Chanock's plates, after their location microscopically, were cut out with a sterile spatula. These blocks of agar were pushed across a sterile slide which was then stained with acridine orange stain and McIlvaine's Phosphate buffer (pH 3.8). Acridine orange working stain and buffer were both autoclaved and filtered through an 0.2 micron Millipore Swinnex filter. Specimens were studied at 540x (High Dry) under UV.*

The staining difficulties encountered with CWDF are legendary. Conventional staining methods distort morphology.9 The usual bacterial stains convert these already amorphous forms into aggregations almost impossible to differentiate from artifact.

Acridine orange is a cytological stain which produces fluorescence of nucleic acids. Acridine orange staining was described by Bertalanffy, et al in 1956.17 It was applied to the in vitro study of L-forms by Hui, et al9 in 1969. Deoxyribonucleic acid (DNA) stains bright, light green.20 Ribonucleic acid (RNA) stains orange to fire red. Young colonies fluoresce red (RNA).20 As they age, fluorescence becomes greener as DNA increases.

c. Thioglycollate broth: Though able to maintain life and reproduction of many of the CWDF, thioglycollate broth is also one of the better reversion media and was used as such at this stage. Small blocks of agar, cut from promising areas of the Chanock's plates were dropped into the media, minced with a spatula, vortex shaken and incubated.

To say with any assurance that we had isolated CWDF, we held that photographs of cultural characteristics on Chanock's media had to resemble those published in the field; that acridine orange stains had to reveal nucleic acids; that the sheep blood agar plates had to remain negative for classical bacterial growth; and that the initial thioglycollate and Medill-O'Kane (Mattman) liquid media had to remain relatively unclouded. When these criteria were met, we felt we had "grown something," perhaps a CWDF.

Results

Table I illustrates the results obtained. Typical photographic studies of the findings are illustrated in figures 1-9.

Discussion

The Microbiology of Aqueous in Uveitis:

Woods defined nongranulomatous uveitis as a “sterile nonpurulent inflammation resulting from a probably toxic or allergic insult to the tissues.” In discussing the sterility of the aqueous in uveitis, he stated, “All the bacteriological work we have points towards the sterility of these eyes.”6 Kolmer28 was among the early authors to suggest that non-granulomatous uveitis was a toxic or allergic disease and that the aqueous was sterile. This feeling was enhanced by the negative bacteriological studies of a group of authors,20-32 who
Aqueous Microbiology

TABLE 1

SUMMARY OF THE ISOLATION OF CELL WALL DEFICIENT FORMS FROM AQUEOUS:

<table>
<thead>
<tr>
<th></th>
<th>No. of Eyes</th>
<th>No. of Patients</th>
<th>Positive for C.W.D.F.</th>
<th>Negative for C.W.D.F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Anterior Uveitis</td>
<td>12</td>
<td>12</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>(Iritis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II Anterior and Posterior Uveitis</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>(Iritis and Chorioretinitis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III Posterior Uveitis</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(Chorioretinitis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV Miscellaneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postop. Uveitis</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Intraocular Foreign Body</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Donor Eyes</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>(Pneumonia Death)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spirochetal Uveitis</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>V Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cataract Extraction</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Strabismus Operation</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

concluded that the aqueous was generally sterile.

When one considers the frequency of this disease, only a relatively small number of cases have had microbiological study of the aqueous. Von Sal- man reported 103 paracenteses in 88 patients with anterior uveitis. He cultured organisms from four patients. Two were thought to be conjunctival contaminants, one a postoperative infection, and the last an Aerobacter. He felt that the aqueous was usually sterile in anterior uveitis.

Spencer reviewed 490 "uveitis" eyes received since 1956 at the University of California. Of these, 340 had "isolation studies." There were 39 bacterial, 2 mycotic, 1 viral, and 5 toxoplastic isolations. Eighteen of the 39 bacterial isolations were thought to be contaminants. Twenty-one isolates
Hessburg, Mattman, Barth and Dutcheshen


Aqueous Microbiology

At left:

Figure 1
CWDF colony growth on Chanock's Agar from the aqueous of a 42-year-old male who suffered an intraocular foreign body. Aqueous was removed one week after injury and just prior to cataract extraction. (160x)

Figure 2
Red fluorescing (RNA) acridine orange stain of CWDF colony growth from Chanock's plate of same patient shown in Figure 1. (540x)

Figure 3
Red fluorescing (RNA) acridine orange stain of CWDF colony growth of same patient shown in Figure 1. (540x)

Figure 4
Green fluorescing (DNA or old RNA) acridine orange stain of CWDF colony growth from a Chanock's plate. Same patient as Figure 1. (540x)

Figure 5
Red fluorescing (RNA) acridine orange stain of CWDF growth from a Chanock's plate inoculated with aqueous from a 32-year-old male with anterior (iritis) uveitis and proven sarcoid. (540x)

were considered bona fide pathogens.*

Spencer stated, “Most could be traced to exogenous causes . . . only two were from eyes with endogenous uveitis.”

One of these was a Staphylococcus aureus, the other a Nocardia.

There have been reports, however, of a wide variety of organisms isolated from the aqueous as occasional or “surprise” isolates.34 Clostridium perfringens,35 Neisseria gonorrhoeae,36 Staphylococcus,37 Leptospira,38 Actinomyces,39 Coccidioides,40 Pneumococcus,41 Listeria,42 Streptococcus, and Treponeme43 have each been reported. These sporadic reports of recovery of microbiological agents have not changed the impression in ophthalmology that non- granulomatous uveitis is some sort of sterile assault.

As an explanation for the consistent sterility of the aqueous, some have felt that aqueous and vitreous are self-sterilizing.44 others attribute bacterial or antimicrobial action to the aqueous humor.47 49

In summarizing the work on aqueous microbiology, Woods6 stated, “My present feeling on the existing evidence is that if organisms arriving in the eye are related to an iritis, they are either dead on arrival or they are promptly phagocytosed, and they owe their pathogenic activity to their antigenic or toxic rather than their infectious properties.” Coles7 stated, “The absence of bacteria in the aqueous during the attacks of iridocyclitis (anterior uveitis) seems to be well established in this country.”

In an interesting series of European papers,32 48 50 60 begun in 1945, Verrey claimed to find bodies believed to be bacteria in 4% to 8% of patients with anterior uveitis. The work of Verrey, and later of Verrey and Amsler,59 60 has been confirmed only by Offret and Saraux.63

**Biology of Cell Wall Deficient Forms:**

The rigid cell wall of classical bacteria accounts for their shape as cocci or bacilli, for their gram positive or gram negative staining, for their sensitivity or resistance to most antibiotics, and to a large extent, for their pathogenicity.

Bacterial L-forms64 have a non-rigid exterior surface65, 66 accounting for their marked pleomorphism.67 “Most L-forms have a limited cell wall69 and exist as soft protoplasmic bodies of varying morphology . . . making them
At left:

Figure 6
Red and green fluorescing (RNA and DNA or old RNA) acridine orange stain of CWDF growth from a Chanock's plate inoculated with aqueous from same patient as in Figure 5. (540x)

Figure 7
Red fluorescing (RNA) acridine orange stain of CWDF growth from a Chanock's plate inoculated with aqueous from a 54-year-old female with anterior (iritis) uveitis and Marie-Strumpell rheumatoid spondyloarthitis. (540x)

Figure 8
Red fluorescing (RNA) acridine orange stain of CWDF growth from a Chanock's plate inoculated with aqueous from a 47-year-old female with anterior (iritis) uveitis. (540x)

Figure 9
Yellow orange and slight red fluorescing (RNA) acridine orange stain of CWDF growth from a Chanock's plate prepared with aqueous from a 39-year-old male patient with anterior and posterior uveitis for five years. (540x)

extremely vulnerable to the solvents and osmotic pressures of conventional staining techniques."

L-forms are larger than viruses, though smaller than classical bacteria. They average 300 to 1,000 mu in size. When cultured from clinical material, they are frequently composed of small ovoid units giving a "granular" colony. Several other colony types are possible, depending upon culture medium. However, the "fried egg" colony, often seen when L-forms are made in vitro by antibiotic action, are only rarely seen when the organism originates in vivo. Unlike viruses, CWDF can multiply on artificial media and have long been known to occur in nature, especially in sea water.

Mycoplasma (PPLO) are small (125-200 mu) organisms with a non-rigid wall. On solid media, they penetrate into the agar so that the center of the colony appears more dense than the periphery, accounting for the "fried egg" appearance of the colonies. Their role in the etiology of human disease is better understood than that of the more recently discovered bacterial L-forms.

Change from the classical bacterial to the L-form is called conversion.

Change back to the classical form is called reversion. Conversion is usually produced by a deleterious stress on the cell wall. Agents such as penicillin, methicillin, bacitracin, cephalosporin, and cycloserine inhibit cell wall synthesis and act as converting agents. The amino acids phenylalanine and methionine, the alkaloid caffeine, and some naturally occurring compounds such as the mucopeptidase lysozyme are known effective converting agents. Antisera has been shown to convert Salmonella typhosa to its L-form. Pneumococci, Streptococci, and Staphylococci require high salt concentrations to convert to their L-form. A large, varied group of such agents is now known which will convert, in vitro, almost every bacterial species to its CWDF.

In vivo conversion of classical bacteria to their L-form was shown by Godzeski. In a group of patients with chronic staphylococcal infections treated with antibiotics, L-forms could be obtained from blood cultures after the disease symptoms had apparently cleared. Under certain cultural conditions, these could be reverted back to classical staphylococci. Mortimer inoculated mice with lethal doses of group A, type-14 streptococci and L-forms. The latter eventually reverted to group A, type-14 streptococci.

Thus, it has been shown that classi-
cal bacteria, under certain conditions, will convert both in vitro and in vivo to their L or cell wall deficient forms.  

Once converted, another group of compounds is needed to "stabilize" the L-forms. Among such stabilizers, the simple salts of inorganic cations, such as magnesium sulfate, are best known. They act by increasing the tonicity of the medium itself and by biochemically strengthening the cell membrane, so it can withstand increased osmotic pressure. Barile points out that polypeptides act this way in the body to stabilize cell membranes in osmotically disruptive situations.

When the forces which caused conversion to the L-form state are removed, some L-forms revert immediately to classical bacteria. L-forms which will not so revert are called stable L-forms. Kagan has said L-forms "do what they want to do"; nowhere is this truer than when one attempts to obtain reversion. Often, when reversion occurs, we can only guess at the forces which have induced it. Sometimes reversion will be caused by growth of the L-form in liquid rather than solid media, the addition of yeast to the media or the subtraction of blood serum.

Debates over whether classical bacteria and L-forms were the same bacteria in different states were settled when the laborious homology technique was used by McCarthy and Bolton to prove that DNA from the L-form could link up with DNA from classical bacteria of the same species.

Although it is generally believed that, by definition, a Mycoplasma cannot revert, such reversion to a classical bacterium may also occasionally occur in vitro and in vivo. Smith reported that white mice treated with cortisone while carrying PPLO in their upper respiratory tract as normal flora, died of a diphtheroid infection. Mice without such mycoplasma did not die when so treated.

Relationship of Cell Wall Deficient Forms to General Disease:

Though the exact role of PPLO is not fully known, their pathogenic role is well documented in atypical pneumonia, in meningitis, in other human respiratory and urogenital infections and in a wide variety of avian, porcine, and bovine diseases.

One criterion formerly used to distinguish mycoplasma from the L-forms was the latter's non-pathogenicity. Lack of an antigenic cell wall was thought to make the L-forms non-pathogenic. It was shown, however, that coagulase positive staphylococci retain this activity as L-forms and, furthermore, that the L-forms of Clostridium tetani produce tetanus toxin. Following early reports of L-form pathogenicity, they have been recovered as the sole isolates in a wide group of human infections.

The L-forms of streptococcus have been recovered as sole isolates from blood cultures in septicemia, from the cerebro-spinal fluid in meningitis, and as sole isolates in rheumatic fever, scarlet fever, and Whipple's disease.

Staphylococcal L-forms have been isolated as sole isolates from septicemia, meningitis, and subacute bacterial endocarditis.

The L-forms of Herellea, Klebsiella, E. coli, Proteus, Pseudomonas, Haemophilus, Listeria, Corynebacterium, Candida, Mycoplasma, and a number of other organisms have been recovered as sole isolates in a wide variety of human infections.

Relationship of PPLO to Viral Infections:

The relationship between PPLO and viruses has long been a matter of conjecture. PPLO is of the same species as PPLO and is not thought to be related to PPLO. PPLO is a microorganism with a broad host range and is not thought to be related to PPLO.
bacterium,\textsuperscript{102} and Brucella\textsuperscript{104} have been isolated from a variety of human diseases.\textsuperscript{9}

The facts known\textsuperscript{3, 8, 10, 67, 107} about these enigmatic forms suggests the role CWDF play in relapsing diseases. Without a cell wall, they are refractory to a variety of antibiotics,\textsuperscript{74} and to most of the antibodies\textsuperscript{106} produced by the host cells (even within the erythrocytes) from which the CWDF arose. Since CWDF often lie dormant within the host cells (even with the erythrocytes) they are further out of reach of both drugs and established defense mechanisms. The ability to lose pathogenicity under some conditions and regain it under others makes it reasonable to imagine them as possible pathogens in relapsing chronic inflammatory disease.\textsuperscript{108, 134} These concepts have been well reviewed elsewhere.\textsuperscript{67}

Relationship of Cell Wall Deficient Forms to Eye or Eye Related Disease:

The first description\textsuperscript{109} of a CWDF as an ophthalmic pathogen was a 1923 report that pleuropneumonia-like organisms (PPLO) were isolated from the conjunctiva and joints of sheep with agalactia. The eye involvement was called a "keratitis." In 1948,\textsuperscript{110} iritis was reported in three of nine patients with Reiter's syndrome, a disease thought to be of PPLO origin. Later, PPLO were found in the joint fluid of a migratory polyarthritis associated with mouse conjunctivitis.\textsuperscript{111}

Relationship between PPLO and uveitis was suggested by Holland and Worlton in 1957.\textsuperscript{112} They searched for PPLO in the prostatic and vaginal secretions of fourteen patients with uveitis. Seven of these cultures were positive for PPLO, although search for PPLO in conjunctival secretions was unsuccessful. Holland stated, "Review of the pertinent literature and the material presented...suggest a relationship of PPLO to uveitis and conjunctivitis that justified further investigation and evaluation. More comprehensive studies may relate the PPLO etiologically, directly or indirectly (through hypersensitivity) to a number of types of ocular inflammation."\textsuperscript{114}

Their work was corroborated by de Grosz\textsuperscript{115} who found PPLO in the prostatic secretions of 25% of patients with uveitis, but not by Catterall\textsuperscript{85} whose survey of 211 uveitis patients showed PPLO in the prostatic secretions of only 10%, about the same percentage as a control group.

Association between the PPLO and collagen diseases has been extensively studied.\textsuperscript{10} Bartholomew\textsuperscript{116} isolated PPLO from synovial fluid, bone marrow, kidney, or serum of 14 of 17 patients with either rheumatoid arthritis, lupus erythematosus or Reiter's syndrome.

A further association between PPLO and uveitis was made by Witmer.\textsuperscript{117} He stated that, "A serological study for complement fixation against Mycoplasma pneumonia (PPLO) was done in an additional group of 28 patients (with posterior chronic cyclitis).... From this study, we may draw the conclusion that PPLO infection may be the cause of some of the respiratory infections as well as the cyclitis found in this group. The incidence of 38% positives is much higher than the average of 10% to 20% found in a normal European population."

Association of some of these diseases or syndromes\textsuperscript{118, 123} with uveitis makes it possible that CWDF are related to uveitis.
Although the association between PPLO and uveitis has been suggested in the literature, and though mycoplasma have been sought in uveitis patients (especially in their prostatic secretions), we find no mention of either mycoplasma or L-forms being sought in the aqueous or iris tissue of patients with anterior uveitis.

Summary

In summary, we obtained specimens of aqueous from patients with uveitis and from some controls. These were cultured for cell wall deficient forms. Such micro-organisms were obtained from 11 of 19 eyes with unexplained uveitis. No positive cultures were obtained from four control eyes. Some of the pertinent literature has been reviewed.

We believe that we have isolated cell wall deficient microbiological forms from the aqueous of patients with inflammatory intraocular eye disease.

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Aqueous Microbiology


Aqueous Microbiology


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