Spontaneous Inhibition of Bacterial Growth in Experimental Gram-Negative Endophthalmitis

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We compared the growth patterns of gram-negative bacilli in the vitreous humor (experimental endophthalmitis in rabbits) and in a subcutaneous site of infection (croton oil pouches in rats). In untreated animals, inoculation of *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* into either site was followed by a period of rapid bacterial multiplication. Thereafter, the numbers of bacteria in the vitreous humor fell spontaneously, whereas those in the subcutaneous site remained stable. During treatment with antibiotics, there was a decline in the numbers of bacteria in both sites. However, once the drug had been eliminated, the numbers of bacteria remained low in the vitreous humor but increased in the subcutaneous site. These findings suggest that during the course of infection, there was depletion of an essential bacterial nutrient or accumulation of an antibacterial substance in the vitreous humor but not in the subcutaneous site. To examine some of these possibilities, we made biochemical measurements during the course of untreated infection. In general, the biochemical changes in the two sites were similar except that the pH fell to about 6.6–6.8 in the vitreous humor but remained above 7.0 in the subcutaneous site. None of the biochemical changes that we observed seemed likely to account for the spontaneous decline in bacterial numbers in the vitreous humor. Further study is warranted to determine the cause of the antibacterial effect in the vitreous humor during the course of experimental bacterial endophthalmitis. Invest Ophthalmol Vis Sci 28:867-873, 1987

During studies of antibacterial treatment for experimental endophthalmitis caused by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (unpublished data), we noted that the density of both species in untreated eyes reached a maximum by 48 hr after intravitreal inoculation and then declined spontaneously. Following intravitreal injection of antibacterial drugs, there was a sharp decline in bacterial numbers and no regrowth of bacteria as vitreal concentrations of the drug declined. By contrast, in the rat croton oil pouch model, bacterial densities remain stable for up to 2 weeks after infection, and there is rapid regrowth of bacteria as the concentrations of antibacterial drugs in the pouch fluid fall. One explanation for the discrepancy between the two models is that an inhibitor of bacterial growth accumulates during infection in the vitreous humor but not in the subcutaneous pouch.

Another possibility is that the supply of one or more nutrients necessary for bacterial growth becomes depleted during infection in the vitreous humor but not in the subcutaneous pouch.

As a first step in elucidating possible reasons for differences in bacterial growth patterns between the two models, we have compared the changes in selected biochemical measurements during infection with *P. aeruginosa* and *K. pneumoniae*. We also have studied the regrowth of bacteria in eyes treated with a single intravitreal injection of antibiotic. Administration of treatment before the bacterial density reaches a maximum should result in sufficient nutrient being left in the eye to support regrowth of bacteria after clearance of the antibacterial drug from the vitreous humor.

Materials and Methods

This study was performed in accordance with the ARVO Resolution on the Use of Animals in Research.

Drugs

Solutions of cefoperazone (Pfizer Inc; New York, NY) and gentamicin (Schering Plough; Kenilworth, NJ) were prepared fresh on the day of use from powders obtained from the manufacturers.
Bacterial Isolates

*K. pneumoniae* 4479 and *P. aeruginosa* 5385 were clinical isolates obtained from a patient with vertebral osteomyelitis and a patient with septicaemia, respectively.

In-Vitro Susceptibility Tests

Minimum inhibitory concentrations (MIC) were measured by a standard, broth-dilution microtiter method in Mueller-Hinton broth supplemented with calcium and magnesium according to the recommendations of the National Committee for Clinical Laboratory Standards. The inoculum was 10⁵ colony-forming units (cfu) per 0.1 ml well (10⁶ cfu/ml).

Bacterial Counts

For enumeration of viable bacteria in the vitreous humor, the entire vitreous was removed and homogenized by passage through a 27-gauge needle; for enumeration of viable counts in the pouches an 0.5 ml sample of fluid was aspirated through a 25-gauge needle. A series of 10-fold dilutions were made in phosphate-buffered saline (PBS) (pH, 7.2); 10 μl of each dilution and of the undiluted pouch fluid or vitreous humor were plated on tryptic soy agar (TSA, Difco Laboratories; Detroit, MI). After overnight incubation at 37°C, the number of colonies from each dilution was counted, and the result was used to estimate the number of cfu/ml in the original sample. In addition, 100 μl of the undiluted pouch fluid were plated to give a lower limit of detection of 10 cfu/ml. All samples were plated in duplicate, and the average of the results was used for calculations.

Drug Assays

Concentrations of cefoperazone and gentamicin in pouch fluid and vitreous humor were measured with a disc-diffusion bioassay using *Bacillus subtilis* ATCC 6633 in TSA as the indicator system.

Endophthalmitis Model

Unilateral endophthalmitis was induced in Dutch belted rabbits by direct intravitreal injection of 0.1 ml of saline containing 500 cfu of either organism as previously described. At the intervals noted below, animals were killed by an intravenous injection of pentobarbital; the eyes were promptly enucleated, and the whole vitreous humor was removed.

Formation of Rat Pouches

Pouches were formed by a modification of the method of Selye on male Wistar rats, weighing 200–250 g and housed three to a cage with free access to a pelleted standard diet and water. The skin over the dorsum of the rat was shaved, and 30 ml of filter-sterilized air was injected through a 23-gauge needle. Following this 0.5 ml of croton oil (Sigma Chemical Co.; St Louis, MO) diluted to 1% in tricaprylin oil (Fluka; Hauppauge, NY) was injected through the same needle. Two days later all remaining air was aspirated through a 25-gauge needle. The pouches were infected 7 days after formation by an intrallesional injection of 0.1 ml saline containing 500 cfu of either organism.

Measurement of Biochemical Variables in Infected Pouch Fluid and Vitreous Humor

Samples of 1.0 ml of pouch fluid or vitreous humor were obtained from six animals before and 24 hr, 48 hr, and 5 days after they had been infected with *K. pneumoniae* 4479 or *P. aeruginosa* 5385. The pouch fluid samples were obtained by repeated aspiration from the same group of animals, whereas the vitreous humor samples were obtained after killing the animals, and a different group was used for each time point. After removal of 300 μl of fluid for enumeration of viable counts, all air was expelled from the syringe, which was then sealed and transported on ice for measurement of the pO₂ and pH on a blood gas analyzer (ABL 2, Radiometer; Copenhagen, Denmark). The remaining sample was centrifuged and the concentrations of calcium, iron, magnesium, phosphate, and total protein in the supernatant were measured with an automatic analyzer (Ektachem 700, Kodak; Rochester, NY).

The Effect of Treatment on Subsequent Growth of Bacteria in the Vitreous Humor

Endophthalmitis was induced with *K. pneumoniae* 4479 (40 rabbits) or *P. aeruginosa* 5385 (44 rabbits). After infection the rabbits were divided into two groups. One group received a single intravitreal injection of saline 24 hr after infection (control), and the other group received either a single intravitreal injection of 1000 μg of cefoperazone (rabbits infected with *K. pneumoniae*) or 50 μg of gentamicin (rabbits infected with *P. aeruginosa*). These drugs and doses were chosen on the basis of the results of earlier experiments (unpublished data), which indicated that they would lower the bacterial counts in the vitreous humor without sterilizing the eyes. Groups of four control and four treated animals were killed at intervals up to 5 days after infection for enumeration of viable bacteria and measurement of drug concentrations in the vitreous humor. In addition, because of the long half-life of gentamicin in the vitreous humor, four animals infected with *P. aeruginosa* and treated with gentamicin...
Table 1. Bacterial density in the vitreous humor at intervals after infection with *Klebsiella pneumoniae* 4479 or *Pseudomonas aeruginosa* 5385*.

<table>
<thead>
<tr>
<th>Time after infection (hr)</th>
<th>Log10 cfu/ml K. pneumoniae mean ± SEM</th>
<th>Log10 cfu/ml P. aeruginosa mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>8.3 ± 0.2 (n = 12)</td>
<td>5.7 ± 0.2 (n = 50)</td>
</tr>
<tr>
<td>48</td>
<td>8.4 ± 0.2</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td>72</td>
<td>7.6 ± 0.2†</td>
<td>6.8 ± 0.1†</td>
</tr>
<tr>
<td>96</td>
<td>6.0 ± 0.3‡</td>
<td>6.2 ± 0.1‡</td>
</tr>
</tbody>
</table>

* The data were obtained from control groups in studies on the effects of treatment on experimental endophthalmitis; some of the data for *P. aeruginosa* have been published previously.1,2

† Significantly lower than 48-hr value (P < 0.05, unpaired t-test).
‡ Significantly lower than 48-hr value (P < 0.01, unpaired t-test).

were killed 10 days after infection in order to measure bacterial numbers after gentamicin had been cleared from the eyes. It was considered unethical to include a control group of animals untreated for 10 days after intraocular infection with *P. aeruginosa*.

**Results**

**Growth Curves of Bacteria in Untreated Ocular and Pouch Infections**

Our previous experiments suggested that, with either organism, bacterial counts reached a peak by 2 days after intraocular infection and fell thereafter. The counts of both organisms were significantly lower 3–4 days versus 2 days after infection (Table 1). This pattern of growth was confirmed in our prospective comparative study. The decline in bacterial counts that occurred between days 2–5 after infection of the rabbit eyes contrasted markedly with the slight increase in bacterial counts that occurred over the same interval in the rat pouches (Fig. 1). *K. pneumoniae* grew more rapidly than *P. aeruginosa* in the rabbit eyes, whereas the opposite was true in the rat pouches (Fig. 1).

Three of six rats whose pouches were infected with *P. aeruginosa* died between 2–5 days after infection. At postmortem examination one animal had necrotizing pneumonia. The remaining two animals had macroscopically visible infarcts in the kidneys, and one of them had infarcts in the liver. *P. aeruginosa* was isolated from all of these lesions. The three surviving animals were killed 6 days after infection; their kidneys, livers, and lungs appeared normal, and blood cultures were sterile.

**Biochemical Changes Owing to Infection in the Rabbit Eye and Rat Pouch**

We had difficulty studying biochemical changes in samples obtained more than 48 hr after the onset of infection in either model. Three of six animals with pouch infections caused by *P. aeruginosa* died between 2–5 days after infection. Vitreous humor obtained more than 2 days after infection with either bacterial species was either too viscous to aspirate or too viscous to analyze with the Ektachem 700 analyzer.

We were unable to obtain reproducible measurements of pO2 in the infected vitreous humor with the ABL blood gas analyzer because of the viscosity of the samples. However, in the pouch fluid, the mean ± SEM of the pO2 was 30.4 ± 2.3 mm Hg before infection; this fell to 17.6 ± 4.1 and 11.5 ± 3.5 mm Hg 48 h after infection with *K. pneumoniae* and *P. aeruginosa*, respectively. The changes observed in the remaining biochemical variables are shown in Figures 2–4. Figure 2 shows the measurements that increased during infec-
Fig. 2. Measurements that increased in infected eyes and pouches. Each point represents the mean ± SEM of data derived from six animals. Open symbols and dashed lines indicate measurements in vitreous humor; closed symbols and solid lines indicate measurements in pouch fluid.

Fig. 3. Measurements that decreased in infected eyes and pouches. Each point represents the mean ± SEM of data derived from six animals. Open symbols and dashed lines indicate measurements in vitreous humor; closed symbols and solid lines indicate measurements made in pouch fluid.
Inhibition of Bacterial Growth in Endophthalmitis

The concentrations of total protein, phosphate, and iron rose after infection with either species in both sites (Figure 2). The single exception was the concentration of iron in pouch fluid after infection with *K. pneumoniae*; this measurement remained stable for 2 days after the onset of infection but by 5 days had risen to 456 ± 70 µg/dl.

The concentrations of calcium and glucose fell after infection in both sites (Fig. 3). Glucose concentrations became undetectable (<10 mg/dl) 2 days after infection in all instances except for the vitreous humor in eyes infected with *P. aeruginosa*. The pH declined to about 6.6–6.8 in the vitreous humor but remained above 7.0 in the pouch fluid (Fig. 3).

Magnesium concentrations consistently rose in the infected pouch fluid and fell in the infected vitreous humor (Fig. 4). However, the concentrations of magnesium in the vitreous humor 2 days after infection remained higher than the concentration in the pouch fluid before infection.

Effect of Early Treatment on Bacterial Regrowth in the Vitreous Humor

Treatment was administered to animals with eyes infected with *K. pneumoniae* or *P. aeruginosa* at least 12 hr before the bacterial density peaked (Fig. 5). Ceferazone was undetectable (<0.1 µg/ml) in the vitreous humor 2 days after administration of the drug. Although the MIC of ceferazone for this strain is 0.25 µg/ml, there was no regrowth of bacteria in the treated eyes; in fact, the numbers of bacteria fell parallel to those for control eyes (Fig. 5). The concentrations of gentamicin had fallen below the MIC for *P. aeruginosa* (1.0 µg/ml) 4 days after administration of the drug (5 days after infection). Nevertheless there was no regrowth of *P. aeruginosa* even as late as 9 days after treatment (10 days after infection) when there was no detectable gentamicin (<0.1 µg/ml) in the vitreous humor (Fig. 5).

Discussion

The growth curves of bacteria in vitro tend to follow a well-defined and reproducible pattern. The population density increases exponentially to a maximum, remains stationary for a period, and then declines when the supply of a critical nutrient in the medium is exhausted.

Over the course of several experiments, the authors noted that the density of *K. pneumoniae* and *P. aeruginosa* in the rabbit's eye appears to follow a similar pattern (Table 1). In the present studies we confirmed this pattern of growth in the rabbit eye and contrasted it with the pattern of growth in the rat croton oil pouch, in which the density of bacteria remained stable or increased slightly after the period of exponential growth (Fig. 1). Others have reported the stability of bacterial counts in the croton oil pouch.

The spontaneous decrease in bacterial counts in the vitreous humor could be caused by a depletion of critical bacterial nutrients or to the accumulation of an antibacterial substance. That such changes should occur in the vitreous humor but not in the subcutaneous pouches could be explained by the fact that the retinal capillaries have tight junctions, which might limit the ingress of nutrients or the egress of antibacterial substances.
substances from the vitreous humor, whereas the capillaries of the subcutaneous pouches are presumably fenestrated and able to allow the passage of molecules up to a molecular weight of about 1,000.

Our biochemical investigations were designed to study changes in the concentration of substances that might influence bacterial growth in the vitreous humor and in the pouch. We measured the concentration of the more important nutrients, including sources of carbon (glucose), nitrogen (protein) and phosphate, and common cofactors for bacterial growth (calcium, iron, and magnesium). We also measured the pH and pO2, derangements of which are known to inhibit bacterial growth.

We were able to compare only biochemical changes over the first 2 days after the onset of infection because of technical difficulty in making measurements in the infected vitreous humor and because of death of rats with disseminated P. aeruginosa infection at later intervals. These data suggest that the spontaneous decline in bacterial density in the vitreous humor was not due to changes in concentration of any of the nutrients or growth cofactors. The amounts of iron, protein, and phosphate actually increased after infection in both sites. Of course, we measured only the total concentrations in pouch fluid after infection of rat croton oil derangements of which are known to inhibit bacterial growth.

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It is of interest that Dalhoff also has reported a decline in calcium concentrations in pouch fluid after infection of rat croton oil pouches with Escherichia coli. Concentrations of magnesium were similar in the pouch fluid and the vitreous humor 2 days after infection, although they had declined from pretreatment values in the vitreous humor and had increased in the pouches. This discrepancy is probably because of the fact that the uninfected pouch fluid contains red cells that become haemolized after infection. Thus, infection would be expected to cause a release of intracellular ions, such as magnesium, into the pouch fluid.

The pH declined to about 6.6-6.8 in the vitreous humor but remained above 7.0 in pouch fluid during infection (Fig. 3). Bacterial growth is influenced by pH because of its effect on adsorption and transport of nutrients. However, most species of bacteria are more tolerant of acid pH than of alkaline pH: bacteria grow optimally over a range from 5-7.5, and growth does not cease until the pH falls below 2.5.

K. pneumoniae is a facultative anaerobe that grows equally well under aerobic and anaerobic conditions. P. aeruginosa is classed as an obligate aerobe. Although the pouch fluid was virtually anaerobic (mean pO2, 11.5 mm Hg) by 2 days after infection, the density of P. aeruginosa did not decline in the rat pouches. The authors found that this strain of P. aeruginosa is able to grow, albeit slowly, in an anaerobic chamber (unpublished data). We were unable to obtain reproducible measurements of pO2 in vitreous humor removed from infected rabbit eyes but polarographic studies in vivo have shown that the normal rabbit vitreous humor is virtually anaerobic. In summary, total exhaustion of oxygen would not affect the growth of K. pneumoniae and, although it might slow the growth of P. aeruginosa, it would not be expected to result in a decline in the numbers of the organism.

The results of early treatment of endophthalmitis further argue against the possibility that there is a critical depletion of bacterial nutrients. Because there were sufficient nutrients in the untreated eyes to sustain a 10-fold increase in bacterial numbers between 24 h and 36 hr after the onset of infection, there also should have been sufficient nutrients to sustain regrowth of bacteria after the antibiotics had been eliminated (Fig. 5). The so-called postantibiotic effect is not likely to account for the failure of bacteria to regrow because it does not last for more than a few hours and would be expected to be as evident in the pouch fluid as in the vitreous humor.

Our results with experimental endophthalmitis are in marked contrast to those in the rabbit meningitis model in which there is also a barrier between the site of infection and the systemic circulation. Bacteria regrow within 12 hr after clearance of antibacterial drugs from the cerebrospinal fluid. The vitreous humor of the normal rabbit has no inhibitory effect on the growth of bacteria in vitro and initially supports bacterial growth in vivo well. The results of these experiments suggest that a change occurs in the vitreous humor during infection that not only inhibits bacterial growth but leads to bacterial death. This change does not appear simply to be the result of nutrient depletion or of changes in the pH or pO2. The decline in bacterial counts in the rabbit's eye could occur because of the operation of host defenses. However, it is not clear why such defenses should operate much better in the vitreous humor than in the pouch fluid. Indeed, complement and other serum proteins have a strong inhibitory effect on the growth of serum-sensitive bacteria, such as Staphylococcus aureus in pouch fluid, yet this species grows well in the vitreous humor. This leaves the possibility that either a defi-
ciency develops of some nutrient as yet undefined, or that an inhibitor accumulates during infection in the vitreous humor but not in the granuloma pouch. An example of such an inhibitor is the fatty acid that has been isolated from abscess contents and that has a bactericidal action on *S. aureus* in vitro and in vivo.\(^\text{16}\) This substance is not secreted into the abscess until at least 24 hr after initiation of infection.\(^\text{17}\) Further work is warranted to explore the possibility that a natural inhibitor of the growth of gram-negative bacteria accumulates in the vitreous humor during experimental bacterial endophthalmitis.

**Key words:** gram-negative, endophthalmitis, host defense, nutrient depletion

### References


