Nonspecific Resistance to Intraocular Infection

I. Elicitation of Resistance by Neisseria meningitidis

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Received for publication 3 October 1973

Both eyes of rabbits whose right eyes had previously been injected with Neisseria meningitidis were injected with Diplococcus pneumoniae. The pneumococci failed to grow in the right eye of most rabbits challenged 2 and 4 days after the injection of the meningococci, but grew well in all left eyes. The "protective" effect was less pronounced in the rabbits challenged on days 6 and 9 and virtually absent when the interval between the two injections was 14 or 22 days. Pneumococci grew equally well in both eyes of rabbits that had received nitrogen mustard treatment prior to the intravitreal injection of meningococci, indicating a possible role for the polymorphonuclear leukocyte in the protective effect.

Previous studies (18, 19) have shown that the inflammatory response produced in the rabbit eye by intravitreally injected Neisseria meningitidis or its stable L-phase variant was predominantly polymorphonuclear (during its initial phases) and gradually became mononuclear. No overt meningococcal infection was established and the eyes appeared clinically normal within 2 to 3 weeks. Specific antimenin-gococcal antibody was not demonstrated in the serum or ocular fluids of most rabbits.

The presence of large numbers of inflammatory cells in the eye and its relatively isolated position suggested that the inflamed eye might be used for in vivo studies on nonspecific resistance to infection. This paper presents observations on the effect of a previous injection of meningococci on the response to intravitreal injection of Diplococcus pneumoniae. The pneumococcus produces severe intraocular infection when injected intravitreally and is known to be phagocytized primarily by polymorphonuclear phagocytes. The possible mechanisms for the nonspecific resistance are discussed.

MATERIALS AND METHODS

Intravitreal injections. Fifty-six New Zealand white rabbits weighing approximately 2 kg were injected in the right vitreous with N. meningitidis. The procedures for preparing the organisms and for the intravitreal injections were as previously described (18). Approximately 10⁴ meningococci were injected into the right eye of each rabbit.

The strain of D. pneumoniae used in these experiments was a type 23, initially isolated from the eye of a human patient with shrinkage of the conjunctival sac. The lyophilized organisms were reconstituted and plated on sheep blood agar 24 h prior to challenge. Washed pneumococci were suspended in Hanks balanced salt solution (HBSS), and the concentration was adjusted so that each eye would receive approximately 1,000 to 3,000 organisms. The actual number of organisms injected was determined by plating dilutions of the suspension on blood agar.

Both eyes of six groups of rabbits were challenged with the pneumococci. The right eyes of these rabbits had been injected intravitreally with meningococci 2, 4, 6, 9, 14, or 22 days previously. At least two rabbits from each group were sacrificed at 24, 48, and 72 h and at 7 days after the injection of the pneumococci.

Recovery of pneumococci. The procedures used for recovery of the pneumococci were essentially the same as those used for recovering meningococci (18). However, the diluted vitreous humor was plated on blood agar rather than on Mueller-Hinton agar. A separate pipette was used for each dilution step in these recovery experiments.

Treatment with nitrogen mustard. One group of eight rabbits was treated with nitrogen mustard prior to injection of the meningococci. The nitrogen mustard (NH₂)₂ (Mustargen, meclohexamine hydrochloride; Merck, Sharp and Dohme) was injected intravenously at a dose of 0.75 mg/lb. The number of leukocytes in peripheral blood was determined from day 0 until the rabbits were killed. The nitrogen mustard injections were given on days 0, 2, and 6 of the experiment. The meningococci were injected into the right eyes of the rabbits on day 4, and the pneumococci were injected into both eyes on day 8 (4 days after meningococci). Four rabbits were killed at
24 h, three were killed at 48 h, and 1 was killed at 72 h after the pneumococci injections.

**Growth of pneumococci in vitreous humor in vitro.** The multiplication of pneumococci in vitreous humor was determined by aspirating vitreous humor from normal rabbit eyes or from eyes that had been injected with meningococci 4 or 8 days previously. Half of the aspirated material was inoculated directly with pneumococci. The remainder was centrifuged at 1,500 rpm in an attempt to remove any cells and then inoculated. Samples were withdrawn 24, 48, and 72 h postinoculation and plated on blood agar. Dilutions were done with a single pipette in this experiment.

**Histological procedures.** Smears were made from vitreous humor and stained with the Gram and the Giemsa stains. Formalin-fixed eyes were processed according to standard histological techniques. Hematoxylin and eosin and MacCallum-Goodpasture stains were used on most sections.

**Antibody determinations.** Sera from 56 rabbits were tested for antimeningococcal antibody by passive hemagglutination and bacterial agglutination methods described previously (18). Antibody to pneumococci was determined by a passive hemagglutination test using sheep erythrocytes coated with an extract prepared from the type 23 pneumococcus strain used for the intravitreal injections.

**RESULTS**

**Fate of intravitreally injected meningococci.** No meningococci were recovered from the right eyes of any of the rabbits. Meningococci were only rarely seen in Gram-stained smears of vitreous humor from rabbits challenged 2 days after the injection of meningococci. Previous experiments (18, 19) indicated that meningococci did not survive in the vitreous for longer than 48 h.

**Recovery of viable pneumococci. Challenge on day 2.** The intravitreally injected pneumococci did not multiply in 7 of the 11 right eyes of rabbits sacrificed from 24 h to 7 days postinjection (Table 1). In two additional eyes, fewer organisms were recovered from right eyes than from the left eyes of the same rabbits. The pneumococci grew well in all of the left eyes.

**Challenge on day 4.** This group showed an even more striking “protective” effect produced by the previous meningococcal injection (Table 1). Viable pneumococci were recovered from only 1 of the 10 right eyes, but were recovered from 8 left eyes. (No organisms were recovered from either eye 7 days postinjection.)

**Challenge on day 6.** No viable pneumococci were recovered from 11 of 16 right eyes but were recovered from all 16 left eyes (Table 1). Fewer organisms were recovered from right eyes than from the corresponding left eyes of the other five rabbits.

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*Too many organisms were present on plate of highest dilution for an accurate count to be made.

*Eye had collapsed. Insufficient amount of vitreous humor aspirated to dilute for plate counts.
Challenge on day 9. Some protection was noted in the three rabbits sacrificed at 24 h and in the four rabbits sacrificed at 48 h (Table 1). Few viable pneumococci were recovered from four of seven right eyes, and in the other three rabbits there was a 10- to 100-fold difference between the number of organisms recovered from right and left eyes. The recovery after 72 h in this group was variable, and little protection was noted.

Challenge on days 14 and 22. The protective effect in the seven rabbits in these two groups was negligible. In many cases pneumococci multiplied as well in right eyes as in left eyes.

Recovery of pneumococci from nitrogen mustard-treated rabbits. The nitrogen mustard injection schedule was designed to produce profound leucopenia at the time the right eyes were injected with meningococci. The average leukocyte count at the time of the meningococci injection was 943/ml of blood (range, 550 to 1,250), and the average count at the time of the injection of the pneumococci (4 days later) was 1,081 per ml (300 to 3,050). The average number pretreatment was 12,375/ml.

Figure 1 shows the average number of organisms recovered from the right and left eyes of treated rabbits compared with the numbers recovered from control rabbits challenged 4 days after the injection of meningococci. The lack of protection in the nitrogen mustard-treated group is evident.

Clinical manifestations. Almost all of the eyes showed severe iritis and conjunctivitis. Many of the left eyes were swollen shut, and the inflammation was accompanied by a whitish discharge. The right eyes were somewhat less severely inflamed.

Examination of vitreous smears. Numerous inflammatory cells, predominantly polymorphonuclear leukocytes (PMN), were observed in the vitreous smears from both right and left eyes. Gram-positive extracellular diplococci were seen in smears from left eyes and in some of the right eyes. Occasional intracellular diplococci and rare meningococci were found in the material from some right eyes.

The right eye smears from the nitrogen mustard-treated rabbits showed the presence of gram-positive diplococci and a small number of inflammatory cells, mostly mononuclear. More inflammatory cells and many organisms were observed in the left eyes.

Examination of stained sections. Both right and left eyes of the challenged rabbits showed mild to severe inflammation in the vitreous, retina, choroid, limbus, and anterior uvea. Inflammatory cells were occasionally seen in the anterior chamber. The cells were mostly PMN, but some mononuclear cells were seen at the later intervals. The inflammation in the left eyes was generally more severe. The results of the MacCallum stains in both right and left eyes correlated with the results of the recovery experiments. In the left eyes the diplococci appeared to be concentrated along the posterior surface of the lens.

Right and left eyes of the nitrogen mustard-treated rabbits showed only a minimal inflammatory reaction by 24 h post-pneumococci injection. The exudate in the vitreous was predominantly mononuclear. Many gram-positive diplococci were present. By 48 h the inflammation was more severe, especially in the left eyes. More mononuclear cells than PMN were present.

Growth of pneumococci in vitreous humor in vitro. Pneumococci multiplied for at least 48 h in the vitreous humor aspirated from normal rabbit eyes (Fig. 2). The small volume of vitreous fluid available precluded sampling at later intervals.

The survival of pneumococci in the vitreous humor removed 4 days after the eyes had been
injected with meningococci is shown in Fig. 3. The pneumococci did not multiply in uncentrifuged vitreous humor, and the number of viable organisms decreased over the 48-h observation period. The pneumococci appeared to survive in the centrifuged vitreous humor from these eyes, but failed to multiply.

The pneumococci were able to grow in vitreous humor that was removed 8 days after injection of viable meningococci (Fig. 4). The growth pattern was very similar to that observed in normal vitreous humor.

Antibody determinations. None of the 56 sera tested were positive for antibody to meningococci as determined by passive hemagglutination. Two serum samples were positive in the bacterial agglutination test. Both samples were from rabbits challenged 9 days after injection of meningococci. Only one of the serum samples showed antibody to type 23 pneumococcus. This sample was from a rabbit killed 7 days after the pneumococcus injection.

DISCUSSION

These experiments demonstrated that prior injection of the right eyes of rabbits with meningococci protected the eyes against subsequent infection with viable pneumococci. The degree of protection depended on the interval between the two injections, and appeared to be most pronounced when the interval was 4 days. However, some effect was noted as late as 9 days. The term "protection" is used to indicate failure of the pneumococci to multiply or survive in the eyes and does not imply absence of clinically or histologically evident ocular damage.

The type of nonspecific resistance that follows infection of immunization with intracellular pathogens (4, 5, 20, 23) and is mediated by "activated macrophages" (13, 20, 22) was probably not responsible for the resistance to pneumococcal infections in the right eyes of our rabbits. Pneumococci are not readily phagocytized by macrophages (26), and macrophages were not predominant in the eyes when protection was maximal.

Cross-reacting antibody could theoretically have protected the rabbits against pneumococcal challenge. Miller et al. (15) and Bankneider (3) have reported cross-reactions between meningococci and unrelated organisms. Antibody is present in vitreous humor in rabbits injected with protein antigens (8), but we found antimeningeococcal antibody in only two serum samples tested. In previous experiments, no antibody was found in vitreous or aqueous humors (18). In any case, maximal protection occurs earlier than antibody could have been produced by ocular cells (4 days after meningococci injection). For the same reason, it is unlikely that antipneumococcal antibody would have had an effect, since protection was noted as early as 24 h after pneumococci injection.

![Fig. 2. In vitro Growth of pneumococcus in normal rabbit vitreous humor. Normal = vitreous humor; normal C = vitreous humor that had been centrifuged to remove cells.](http://iai.asm.org/)

![Fig. 3. In vitro Growth of pneumococcus in vitreous humor from rabbit eyes injected 4 days previously with meningococci. 139 = vitreous humor; 139 C = vitreous humor that had been centrifuged to remove cells.](http://iai.asm.org/)
Only one rabbit had antipneumococcal antibody in its serum.

Rowley (21) and Shilo (24) described nonspecific resistance to infection induced by lipopolysaccharides. The meningococcal lipopolysaccharides could have induced nonspecific resistance to the pneumococci. Another possibility is that a toxic product of the meningococci was bactericidal for pneumococci. The failure of pneumococci to survive in centrifuged vitreous humor from eyes previously injected with meningococci might support this hypothesis. However, we cannot assert that all inflammatory cells were removed from the vitreous samples. The abnormally high population values shown in Fig. 2 and 4 probably reflect the fact that separate pipettes were not used for each dilution step.

The maximal protective effect was noted when PMN were still present in large numbers in the vitreous (4 days). When inflammation had subsided (14 and 22 days), the protection was less pronounced. Rabbits treated with nitrogen mustard to reduce the numbers of circulating PMN showed no protective effect at 4 days. The mere presence of PMN in the right eye at the time of pneumococcal challenge might explain, in part, the apparent destruction of the pneumococci. However, although many PMN were present in the left eye by 24 h post-pneumococcal injection, the organisms remained extracellular and were not eliminated. Neither extracellular nor intracellular organisms were seen in many right eyes. Increased phagocytosis and subsequent digestion of the ingested organisms could account for this.

C-reactive protein (CRP) is a substance found in serum during acute infections (2, 9, 14, 25) and after episodes of tissue damage (10). It reacts with the C (somatic) polysaccharide of the pneumococcus and its appearance has been associated with leukocytosis. A similar substance, the C-reactive protein (CXR) was found in rabbits (1, 11) and studied extensively (28, 29, 30, 31). It has been shown to cross-react with human CRP (7). Recent evidence indicates that CRP enhances the phagocytosis of several bacterial species by human leukocytes (6, 12). CRP appears in serum before antibody can be demonstrated (25).

Patterson et al. (16, 17) postulated that CRP might participate in the nonspecific resistance to infection induced by bacterial endotoxins. They also found that isolated CRP had antibacterial activity.

We might hypothesize that intravitreal injection of meningococci (into the right eye) induced production of CXR in addition to the acute inflammatory reaction. CXR could have reacted specifically with the polysaccharides of the injected pneumococci or have been nonspecifically bactericidal. CXR could also have acted nonspecifically to enhance the phagocytosis of the pneumococci. Although CXR could also be induced in the left eyes by the pneumococci, it would not have done so before the organisms would have been present already when the pneumococci were injected. Wolkomicz et al. (27) showed that CXR was present in the aqueous humor of rabbits with uveitis induced by typhoid vaccine, streptococci, and staphylococci. CXR appeared within 24 h, and titers were higher in the aqueous humor than in serum, suggesting that it was produced locally (within the eye). CXR was not demonstrated 14 days after intravitreal injection of streptococci. We did not observe protection in rabbits challenged 14 and 22 days after injection of meningococci. CXR could have been no longer present in these eyes. Preliminary experiments, done by using a commercially available test (the Sylvana Co.) for human serum CRP, indicated that CXR is present in the rabbit vitreous humor 2, 4, and 6 days after injection of meningococci. Further experiments are underway to elucidate the possible role of this substance in enhanced phagocytosis of intravitreally injected microorganisms. The effect of meningococcal infection on subsequent infection with an organism primarily phagocytized by
macrophages (Listeria) is also being investigated. As will be shown in another paper, the protective effect was not noted when right eyes were preinjected with heat-killed meningococci, bovine gamma globulin, or polystyrene latex particles.

The experimental model described would seem to provide an alternative method of studying antibacterial action of inflammatory exudates. It offers the advantage of being an in vivo system, yet isolated from many of the factors that complicate similar in vivo tests (presence of other cells, abundant blood supply). In addition, the demonstration of local production of a substance that enhances phagocytosis might be important in furthering understanding of the mechanisms of intraocular infection.

ACKNOWLEDGMENTS

This investigation was supported in part by the Office of Naval Research under a contract between the Office of Naval Research and the Regents of California and by Public Health Service grant no. EY00310 from the National Institutes of Health.

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