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Experimental Bacterial Keratitis in Neutropenic Guinea Pigs: Polymorphonuclear Leukocytes in Corneal Host Defense

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Quantitative techniques were used to determine the relative concentrations of viable bacteria and polymorphonuclear leukocytes (PMNs) in the corneas of neutropenic and non-neutropenic guinea pigs with experimental bacterial keratitis induced with three strains of *Pseudomonas aeruginosa*. Neutropenia was produced by whole-body X-irradiation 1 week before infection. Significantly greater numbers of bacteria were present in the corneas of neutropenic animals 48 h after infection than were present in the corneas of non-neutropenic animals. The same was true 24 and 48 h after infecting animals with *Staphylococcus aureus*. Examination of histological sections showed that fewer PMNs were present in the corneas of infected neutropenic animals than in the corneas of infected non-neutropenic animals. Radiolabeling of PMNs confirmed a significant reduction in PMN concentration in the corneas of infected neutropenic animals. Tears and the corneal epithelium appear to be the most important elements protecting the cornea against local invasion by bacteria. However, once bacterial keratitis is established, PMNs play a role in limiting bacterial multiplication.

Little is known regarding the role of the polymorphonuclear leukocyte (PMN) in the defense of the cornea against bacterial infection. Intracorneal administration of bacterial or chemical agents causes the accumulation of many PMNs at the site of injection (8). Mice made neutropenic with cyclophosphamide and then infected intracorneally with *Pseudomonas aeruginosa* develop rapidly progressive ocular infections and are more likely to die of sepsis than are non-neutropenic animals similarly challenged (6). Corticosteroids inhibit the entry of PMNs into inflammatory corneal foci (8). Patients receiving such agents may be more susceptible to bacterial or fungal corneal infection (4).

The effect of neutropenia on the progress of bacterial keratitis or on the inflammatory response in the cornea has not been quantitatively assessed. By using analytic methods which allowed measurement of the ingress of PMNs into experimentally infected corneas and assay of the number of viable bacteria present, we demonstrated that PMNs play a major role in the response of the cornea to bacterial infection and that neutropenia alters that inflammatory response.

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MATERIALS AND METHODS

Induction of neutropenia. Male Hartley strain guinea pigs weighing about 300 g received 450 rads over a 2-min period from a 280 kV X-ray unit (Picker X-ray Corp., Pittsburgh, Pa.). The half-value layer was 1.0 mm copper. The target distance was 43 cm and the field size was 35 cm in diameter. The animals were lightly anesthetized and placed in a plexiglass holder; their eyes were shielded during irradiation with lead strips. Their position were reversed after one-half dose. White blood cell and differential counts on peripheral blood were performed prior to and daily for 9 days after irradiation of the animals.

Induction and quantitation of bacterial keratitis. Keratitis was established by intracorneal injection of bacteria as described previously (3). In brief, bacteria from an overnight culture were injected into the corneas of animals using a 30-gauge needle. The three strains of *P. aeruginosa* used in these studies (strains 105, 107, and 111) had been originally isolated from human corneal ulcers. Strains 107 and 111 had been previously demonstrated to be virulent in guinea pig corneas, i.e., capable of producing progressive corneal infection in normal animals after inoculation of about 10 viable bacteria. Strain 105 was a less virulent organism, with larger inocula producing a more indolent corneal infection (S. D. Davis, unpublished data). *Staphylococcus aureus* 503A was used in some experiments. Animals were sacrificed at intervals after infection, and their corneas were removed. The corneas were homogenized in Mueller-Hinton broth and portions of serial broth dilutions were streaked on agar plates. Bacterial colonies were counted after overnight incubation. Numbers of viable bacteria are presented

as means and standard deviations in common logarithms ($\log_{10}$). Statistical significance was calculated by Student's t test.

In vivo chemotaxis. Chemotaxis of PMNs into experimentally infected corneas was assayed as described by Leibowitz et al. with some slight modifications (8). Briefly, neutropenic and nonneutropenic animals were injected, 48 and 72 h before experimental infection, with 50 μCi of tritiated thymidine per kg (New England Nuclear Corp., Boston, Mass., specific activity 6.7 Ci/mmol). Animals were then infected with *P. aeruginosa* strain 107 as described above and sacrificed 24 h later. Control animals were not infected. A 6-mm trephine was used to obtain standard samples of corneal tissue. The corneas were dissolved overnight in 1.5 ml of NCS (Amersham Searle, Arlington Heights, Ill.). Neutrality of the solution was reestablished through the addition of hydrochloloric acid. Radioactivity was assayed with a scintillation counter (Packard, Tricarb Scintillation Counter, model 3390, Downers Grove, Ill.). Background counts were subtracted from the experimental groups, and quench correction was made by internal standardization.

Eyes from selected animals were removed at 24 h and then fixed, sectioned, and stained by standard histological techniques.

RESULTS

Induction of neutropenia. Neutropenia developed in animals within 4 days after X-irradiation (Fig. 1). Neutropenia of less than 200 PMNs/mm^3 persisted for at least 9 days after irradiation. A transient leukocytosis was noted 6 h after irradiation. Animals tolerated the irradiation well. Daily weights taken after irradiation revealed no significant weight loss, and the animals did not develop vomiting or diarrhea.

Keratitis in neutropenic animals. Significantly more bacteria were present in the corneas of neutropenic guinea pigs infected with the three different strains of *P. aeruginosa* 48 h before sacrifice than were present in the corneas of non-neutropenic animals (Table 1). This was true of both the more virulent (strains 107 and 111) and less virulent strains (strain 105) of *Pseudomonas*. A larger inoculum of strain 105 was required to produce an infection of a magnitude equivalent to smaller inocula of the more virulent strains 107 and 111. There were significantly greater numbers of viable staphylococci in the corneas of neutropenic guinea pigs than in the corneas of non-neutropenic animals 24 and 48 h after experimental infection (Table 2).

Chemotaxis of neutrophils into corneas. Histological sections of corneas taken from non-neutropenic and neutropenic guinea pigs 24 and 48 h after experimental infection with *P. aeruginosa* 107 demonstrated large numbers of bacteria and a paucity of PMNs in the corneas of neutropenic animals. Non-neutropenic animals had numerous granulocytes in their corneas within 24 h of experimental infection (Fig. 2).

Neutrophils were labeled in vivo by injection of tritiated thymidine 2 and 3 days before the induction of experimental keratitis with *P.*

TABLE 1. *Number of viable P. aeruginosa in corneas of non-neutropenic and neutropenic guinea pigs with experimental keratitis*[a]

Strain	Inoculum (CFU)[b]	Corneal colony count ($\log_{10} \pm$ SD)[c]		
		Non-neutropenic animals	Neutropenic animals	*P*
105	3,200	5.79 ± 1.14 (6)	7.49 ± 0.42 (6)	<0.025
107	35	5.37 ± 0.46 (4)	7.22 ± 0.41 (4)	<0.005
111	35	3.66 ± 2.93 (6)	6.91 ± 0.11 (4)	<0.05

[a] Animals were sacrificed 48 h after intracorneal infection.
[b] CFU, Colony-forming units.
[c] SD, Standard deviation. Numbers in parentheses indicate number of corneas.

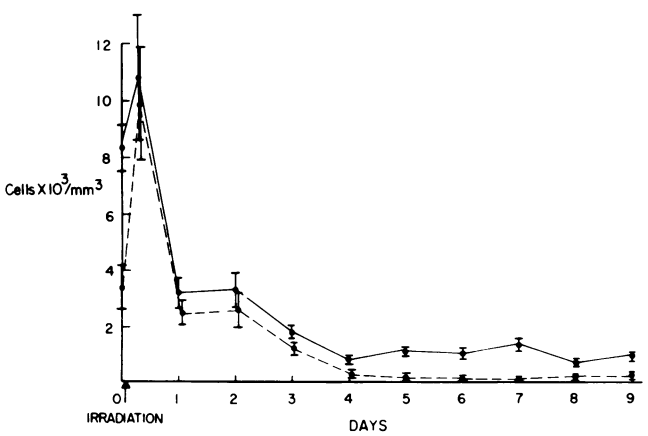

FIG. 1. *Total peripheral leukocyte (solid line) and PMN (broken line) counts (± standard error of the mean) of guinea pigs after whole-body irradiation (n = 4).*

TABLE 2. *Number of viable S. aureus in corneas of non-neutropenic and neutropenic guinea pigs with experimental keratitis*[a]

Time after infection	Corneal colony count ($log_{10} \pm SD$)[b]		P
	Neutropenic	Non-neutropenic	
24 h	5.86 ± 0.29 (4)	7.79 ± 0.45 (4)	<0.005
48 h	6.39 ± 0.46 (4)	8.74 ± 0.32 (4)	<0.005

[a] Animals infected intracorneally with 880 CFU, sacrificed 24 and 48 h later.

[b] SD, Standard deviation. Numbers in parentheses indicate number of corneas.

aeruginosa 107. Significantly more radioactivity was present in the infected corneas of both neutropenic and non-neutropenic animals than was present in noninfected corneas (Table 3). Non-neutropenic infected animals had significantly greater corneal radioactivity than did neutropenic infected animals. Radioautography demonstrated essentially all radioactivity to be cell associated. These findings were consistent with decreased ingress of PMNs into the infected corneas of neutropenic animals compared with non-neutropenic animals.

DISCUSSION

This study provides further evidence that infiltration by PMNs constitutes a major response of the cornea to bacterial infection. Many PMNs were present in the corneas of non-neutropenic animals after experimental infection as judged by routine histological sections. Results of a study of radioactive labeling of PMNs in vivo as well as routine histological studies confirmed a decreased concentration of PMNs in the corneas of neutropenic animals. Finally, neutropenic animals had significantly more bacteria in infected corneas than did non-neutropenic animals.

Previous studies have emphasized the importance of local mechanisms in the defense of the cornea against infections. Tears contain lysozyme (muramidase) and other poorly defined agents thought to inhibit bacterial invasion of the cornea (4). An intact corneal epithelium has been demonstrated to play an important role in preventing corneal infection. Trauma to the corneal epithelium may result in bacterial infection (2). Experimental animals whose corneal epithelia have been removed are more susceptible to developing bacterial keratitis (5). There is no documented increased incidence of bacterial keratitis in patients with systemic host defense defects such as neutropenia, agammaglobulinema, or complement deficiency. Local factors therefore seem to be of paramount importance in protection of the eye against bacterial infection.

Once the corneal epithelium has been broached and bacterial invasion of the cornea has occurred, it appears that PMNs play a significant but limited role in the defense of the eye. Histopathological studies have demonstrated that leukocytes in limbal blood vessels migrate into the corneal stroma. The corneal endothelium is not an effective barrier to PMN invasion from the anterior chamber (10). Neutrophils are present in the precorneal tear film and can pass directly into corneal wounds, but only if Descemet's membrane has been ruptured (2, 11).

The studies presented here on established bacterial keratitis demonstrate that PMNs play a significant role in host defense against intracorneal bacterial multiplication. Experimental keratitis was significantly worse in terms of number of viable organisms present within the corneas of neutropenic guinea pigs than it was in control animals. This was true with three different strains of *Pseudomonas* as well as staphylococci. Hazlett et al. have demonstrated previously that mice made transiently neutropenic by administration of cyclophosphamide developed disseminated infection after intracorneal infection with *Pseudomonas* and died, whereas similarly challenged non-neutropenic animals localized their infections (6).

In our studies, PMNs were only rarely noted in the corneal stroma of infected neutropenic animals. In addition, there did not appear to be as much corneal damage in the corneas of neutropenic animals 24 h after infection as there was in non-neutropenic animals. This may relate to the fact that PMNs have been implicated in the destruction of the cornea during bacterial infections (12, 14). In neutropenic animals a decrease in the number of PMNs early in the inflammatory process may slow this destructive process.

As a means for the induction of neutropenia, whole-body X-irradiation has some advantages over the use of cytotoxic agents such as cyclophosphamide. When single doses of such drugs are used, marrow suppression is only transitory with rapid return of marrow function (1, 6). Our animals remained neutropenic for more than a week. In addition, cyclophosphamide may have an effect upon rapidly multiplying corneal epithelial cells, confounding experimental results. We avoided this problem by shielding the animals' eyes during irradiation. The transient leukocytosis observed in our animals 6 h after irradiation has been previously noted, but its mechanism is unknown (13).

The model of experimental bacterial keratitis

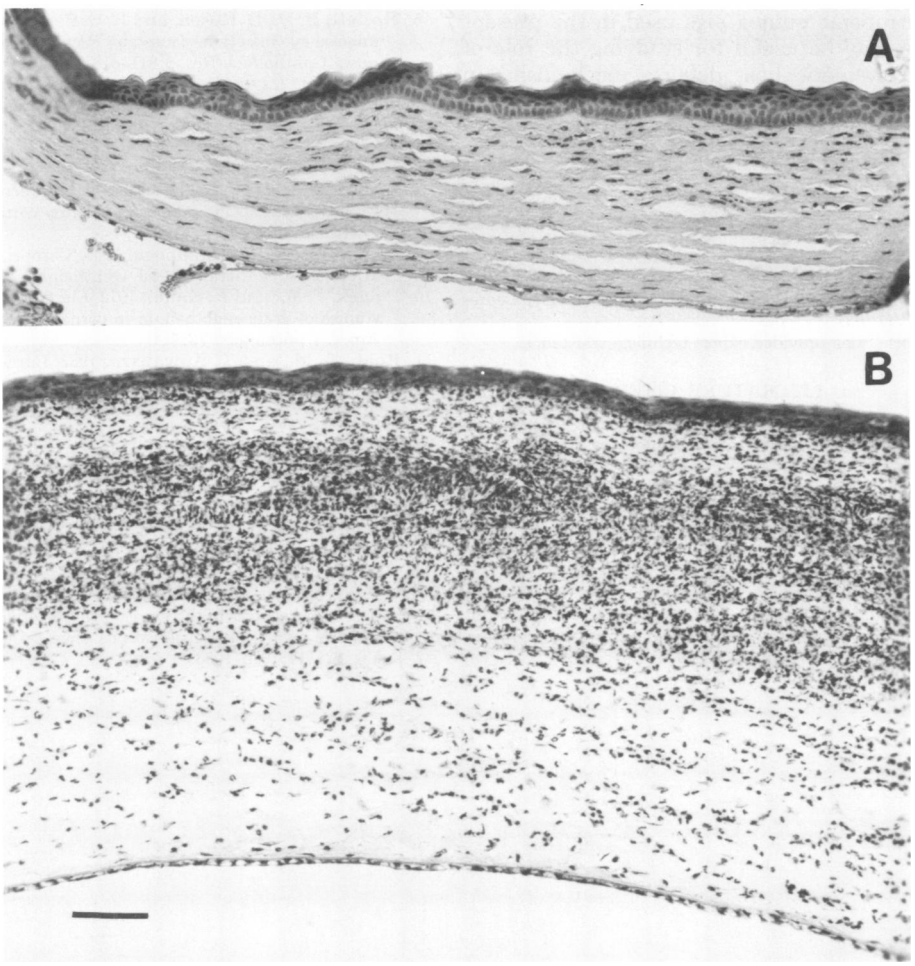

FIG. 2. *Sections of cornea from neutropenic (A) and nonneutropenic (B) guinea pigs obtained 24 h after intracorneal injection of P. aeruginosa (hematoxylin and eosin). (A) The cornea is slightly edematous and a sparse infiltrate is present. Rare PMNs are noted. (B) The cornea is very edematous and markedly thickened. A dense, predominantly PMN infiltrate is present. Necrosis and early abscess formation are evident. Bar = 100 μm.*

TABLE 3. *Corneal radioactivity after intravenous injection of tritiated thymidine and induction of experimental keratitis*[a]

Animal[b]	Material injected	No. of eyes	Corrected mean cpm ± SD (*P*)[c]	
Non-irradiated (non-neutropenic)	Saline	4	1,334 ± 16	(<0.05)
	Pseudomonas	6	18,576 ± 4,021	(<0.001)
Irradiated (neutropenic)	Saline	4	1,410 ± 125	(<0.05)
	Pseudomonas	6	6,475 ± 1,352	

[a] 50 μCi/kg of [^{3}H]thymidine administered 48 and 72 h before intracorneal injection of 10 to 20 *P. aeruginosa* or saline.

[b] Animals were sacrificed 24 h after intracorneal injection.

[c] SD, Standard deviation.

in neutropenic guinea pigs used in the present studies may be useful for studying the role of certain systemic host defense mechanisms in local infection. It allows for quantitative study of bacterial and host elements in infection since the entire infected site can be removed surgically for assay.

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