ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1993, p. 1856–1859
0066-4804/93/091856-04$02.00/0

Vol. 37, No. 9

Age and Therapeutic Outcome of Experimental *Pseudomonas aeruginosa* Keratitis Treated with Ciprofloxacin, Prednisolone, and Flurbiprofen

JEFFERY A. HOBDEN,[1] JAMES M. HILL,[1,2]* LEE S. ENGEL,[2] AND RICHARD J. O'CALLAGHAN[2]

Lions Eye Research Laboratories, LSU Eye Center,[1] and Department of Microbiology, Immunology, and Parasitology,[2] Louisiana State University Medical Center School of Medicine, 2020 Gravier Street, Suite B, New Orleans, Louisiana 70112*

Received 2 April 1993/Returned for modification 3 June 1993/Accepted 25 June 1993

This study was conducted to determine whether the age of the host influences the pathogenesis and therapeutic outcome of drug-treated *Pseudomonas aeruginosa* keratitis. Young (3- to 5-month-old) and old (1.5- to 3-year-old) rabbits were intrastromally infected with *P. aeruginosa* ATCC 27853. Sixteen hours later, rabbits in both age subpopulations were divided into three groups and treated topically as follows: group 1, phosphate-buffered saline; group 2, 0.3% ciprofloxacin; and group 3, 0.3% ciprofloxacin, 1.0% prednisolone, and 0.03% flurbiprofen. Drops were given every 15 min for 1 h and then every 30 min for 9 h. At 27 h postinfection, ocular pathology was graded with a slit lamp examination (SLE) scoring system. Aqueous humor was collected for ciprofloxacin quantitation, and corneas were harvested for bacterial enumeration and estimation of polymorphonuclear leukocytes. Young rabbits had more severe inflammation and pathology than old rabbits. At 27 h postinfection, SLE scores and polymorphonuclear leukocyte numbers were significantly higher for young rabbits than old rabbits ($P < 0.02$), regardless of treatment. Prednisolone and flurbiprofen significantly reduced SLE scores in both age groups ($P < 0.03$) without affecting the antimicrobial efficacy of ciprofloxacin.

While conducting a previous study investigating the concurrent administration of ciprofloxacin (7.5 mg/ml) and prednisolone (10 mg/ml) for the therapy of experimental *Pseudomonas aeruginosa* keratitis (6), we noticed that, all other factors being equal, older rabbits appeared to develop less severe pathology compared with younger rabbits, whether the infection was treated or not. If, in fact, age is a factor in the development of corneal pathology resulting from *P. aeruginosa* infection, this variable could contribute to the inconsistencies seen in the results of animal studies on the use of combinations of antibiotics and anti-inflammatory agents for the therapy of bacterial keratitis (4, 6, 11). To test this theory, we designed a study involving young and old rabbits infected and treated identically to determine the influence of age on disease characteristics and the response to treatment regimens.

MATERIALS AND METHODS

Experimental *Pseudomonas aeruginosa* keratitis. Twenty-four New Zealand White rabbits were used in this study. Twelve of these rabbits, a subpopulation designated as young rabbits, were between 3 to 5 months of age and weighed 1.74 ± 0.10 kg (mean ± standard error of the mean [SEM]). The remaining 12 rabbits, the subpopulation designated as old rabbits, were between the ages of 1.5 and 3 years of age and weighed 4.00 ± 0.21 kg. The terms young and old were arbitrarily chosen; however, the young rabbits were sexually immature, whereas the old rabbits were retired breeders. All rabbits were anesthetized with a subcutaneous injection of a 1:5 mixture of xylazine (100 mg/ml; Miles, Shawnee, Kans.) and ketamine hydrochloride (100 mg/ml; Aveco, Fort Dodge, Iowa). Topical corneal anesthesia was achieved by instilling a drop of proparacaine hydrochloride (0.5%, Ophthaine; Squibb, Princeton, N.J.). Each eye then received an intrastromal injection, as previously described (12), of 10 μl of tryptic soy broth (Difco, Detroit, Mich.), containing 10^3 CFU of logarithmic-phase *P. aeruginosa* ATCC 27853. This strain has a minimum inhibitory concentration for ciprofloxacin of 0.24 μg/ml. Because the infections were to be stopped at a relatively early stage (i.e., before significant stromal edema and stromal infiltrate-occluded vision), both eyes of the rabbits were infected.

Slit lamp examination (SLE). Eyes were examined with a slit lamp biomicroscope (Topcon SL-5D; Tokyo, Japan) prior to infection, before the initiation of therapy (16 h postinfection), and immediately before sacrifice (27 h postinfection). The scoring system has been previously described (6, 8). Briefly, scores of 0 (absent) to 4 (severe) were assigned to seven parameters: conjunctival injection, conjunctival chemosis, iritis (cell and flare), fibrin in the anterior chamber, hypopyon, stromal infiltrate, and stromal edema. The sum of the scores from each of the seven parameters reflects the degree of inflammation observed. A normal eye has a score of 0. The highest possible score is 28. At no point in this study were the infections allowed to progress beyond a score of 20.

Bioassay for ciprofloxacin concentration. Immediately after the rabbits were sacrificed, the eyes were proptosed and any mucopurulent discharge was removed with a sterile Dacron swab. The eyes were then rinsed with sterile phosphate-buffered saline (PBS), and aqueous humor was collected by anterior chamber paracentesis from two rabbits (four eyes) in each of the three treatment groups within both age subpopulations. Ciprofloxacin was quantitated in aqueous humor by a bioassay (7).

Quantitation of viable bacteria per cornea. Corneas were

* Corresponding author.

aseptically excised at the corneoscleral limbus and homogenized as previously described (12). Aliquots (0.5 ml) of the homogenates were serially diluted 1:10 in sterile PBS, and 0.1 ml of each dilution was plated in triplicate onto tryptic soy agar (Difco). All agar plates were incubated for 48 h at 37°C, and CFU were counted in a masked fashion. All colony counts were expressed as base 10 logarithms.

Estimation of corneal PMN. The number of polymorphonuclear leukocytes (PMN) in corneal tissue was estimated by quantitating myeloperoxidase activity in an assay similar to that described by Williams et al. (17). Corneal homogenates used for quantitation of CFU per cornea were further homogenized in the presence of hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 0.5%. Tissue debris was removed from the homogenate by centrifugation (40,000 × *g* for 15 min), and 0.1 ml of the supernatant was mixed with 2.9 ml of potassium phosphate buffer (50 mM, pH 6.0) containing *o*-dianisidine dihydrochloride (16.7 mg/100 ml; Sigma) and hydrogen peroxide (0.0005%). The change in optical density with time at 460 nm was measured every 12 s with a system 2600 recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) for 15 min at room temperature. One unit of myeloperoxidase activity was equivalent to approximately 100,000 PMN. The lowest detectable myeloperoxidase activity was 0.01 U, which is equivalent to approximately 1,000 PMN. For corneas with less than 0.01 U, a value of 0 PMN per cornea was used to calculate the average number of PMN per group. PMN determinations are expressed as the log base 10 number of PMN per cornea.

Experimental design. Therapy was initiated at 16 h after inoculation. Twelve young and 12 old rabbits were used. Within the age subpopulations, rabbits were randomly assigned to one of three treatment groups (four rabbits [eight eyes] per group). In both young and old rabbits, group 1 was treated with PBS (pH 7.2), group 2 was treated with topical drops of 0.3% ciprofloxacin hydrochloride (Ciloxan; Alcon Laboratories, Fort Worth, Tex.), and group 3 was treated with 0.3% ciprofloxacin, 1.0% prednisolone acetate (Pred Forte; Allergan Pharmaceuticals, Hormigueros, Puerto Rico), and 0.03% flurbiprofen sodium (Ocufen; Allergan Pharmaceuticals). The ciprofloxacin was applied first, and then the prednisolone and flurbiprofen drops were added at 5-min intervals thereafter. All drops were administered every 15 min during the first hour of treatment, and then every 30 min for an additional 9 h. One hour after the final treatment (27 h postinfection), all eyes were examined, the rabbits were sacrificed and corneas and aqueous humor were collected.

Statistical analysis of data. Results were analyzed by means of the Statistical Analysis Systems (13). Analysis of variance was performed on the base 10 logarithm of the number of colonies, PMN per cornea, and concentration of ciprofloxacin per eye expressed as micrograms of drug per milliliter of aqueous humor. Where analysis of variance indicated significant differences, *t* tests between the least-squares mean from each treatment group were conducted. Comparisons between treatment groups were also conducted by specific contrast statements (13). For slit lamp scores, a nonparametric one-way analysis of variance (Kruskal-Wallis test) was used. For comparison among groups in this analysis, Wilcoxon scores were used.

RESULTS

SLE and PMN. All eyes were normal (SLE score = 0) prior to bacterial inoculation. At 16 h postinfection (before treatment), the young rabbits did not have significantly greater corneal pathology compared with the old rabbits (mean SLE score, 5.63 ± 0.34 for young rabbits versus 4.96 ± 0.24 for old rabbits; $P = 0.08$).

TABLE 1. SLE scores

Group[a]	Treatment[b]	SLE score[c]	
		Young	Old
1	PBS	15.75 ± 1.01	11.12 ± 0.45
2	0.3% ciprofloxacin	16.87 ± 1.11[d]	11.12 ± 1.00[d]
3	0.3% ciprofloxacin, 1.0% prednisolone acetate, 0.03% flurbiprofen	12.25 ± 0.98[e]	8.87 ± 0.90[e]

[a] Each group consisted of four young (3- to 5-month-old) and four old (1.5- to 3-year-old) rabbits (eight eyes in each group).
[b] Drops were administered every 15 min for 1 h and then every 30 min for 9 h.
[c] SLE scores (SLE mean ± SEM) at 27 h postinfection. Scores of young rabbits were significantly higher ($P < 0.002$) than scores of old rabbits for all groups.
[d] Not significantly different from same age rabbits in group 1 ($P > 0.25$).
[e] Significantly different from groups 1 and 2 of same-age rabbits ($P < 0.001$ for young rabbits; $P < 0.03$ for old rabbits).

At 27 h postinfection (after treatment), all three groups of young rabbits had significantly greater SLE scores and numbers of PMN compared with the corresponding groups of old rabbits, regardless of treatment (Tables 1 and 2). In both young and old rabbits, the groups receiving the combined steroid–anti-inflammatory–antibiotic treatment (group 3) had significantly lower SLE scores than the groups receiving PBS (group 1) or antibiotic alone (group 2) (Table 1); SLE scores in groups 1 and 2 were not significantly different from each other at either age.

Viable bacteria per cornea. Age did not affect bacterial growth; the numbers of viable bacteria in PBS-treated young and old corneas were not significantly different (Table 3). All eyes treated with ciprofloxacin, regardless of age or treatment group, had significantly fewer bacteria than the PBS-treated eyes. All of the eyes from young rabbits treated with 0.3% ciprofloxacin, 1.0% prednisolone, and 0.03% flurbiprofen (group 3) were essentially sterile; i.e., fewer than 10 *P. aeruginosa* cells were recovered from corneal homogenates, whereas none of the similarly treated eyes of the old rabbits were sterile. Of the 16 eyes in group 2 (0.3% ciprofloxacin) 7 eyes of young rabbits and 6 eyes of old rabbits were sterile.

TABLE 2. Estimated numbers of corneal PMN

Group[a]	Treatment[b]	Corneal PMN[c]	
		Young	Old
1	PBS	5.04 ± 0.73	1.30 ± 0.85
2	0.3% ciprofloxacin	5.41 ± 0.08[d]	2.55 ± 1.00[d]
3	0.3% ciprofloxacin, 1.0% prednisolone acetate, 0.03% flurbiprofen	4.65 ± 0.67[d]	2.03 ± 1.00[d]

[a] Each group consisted of four young (3- to 5-month-old) and four old (1.5- to 3-year-old) rabbits (eight eyes in each group).
[b] Drops were administered every 15 min for 1 h and then every 30 min for 9 h.
[c] Corneal PMN at 27 h postinfection expressed as log base 10 mean ± SEM. Young rabbits had significantly greater numbers of PMN than old rabbits ($P < 0.02$).
[d] Not significantly different from group 1 of same age rabbits ($P > 0.35$).

TABLE 3. Viable bacteria per cornea

Group[a]	Treatment[b]	CFU/cornea[c]	
		Young	Old
1	PBS	7.40 ± 0.12[d]	7.52 ± 0.05
2	0.3% ciprofloxacin	0.06 ± 0.06[e]	0.45 ± 0.16[e,f]
3	0.3% ciprofloxacin, 1.0% prednisolone acetate, 0.03% flurbiprofen	0.00 ± 0.00[e]	1.46 ± 0.21[e,g]

[a] Each group consisted of four young (3- to 5-month-old) and four old (1.5- to 3-year-old) rabbits (eight eyes in each group).
[b] Drops were administered every 15 min for 1 h and then every 30 min for 9 h.
[c] CFU per cornea at 27 h postinfection expressed as log base 10 mean ± SEM.
[d] Not significantly different from group 1 of old rabbits ($P = 0.5$).
[e] Significantly less than group 1 ($P < 0.0001$).
[f] Not significantly greater than corresponding group in young rabbits ($P = 0.12$).
[g] Significantly greater than corresponding group in young rabbits ($P < 0.01$).

Ciprofloxacin concentrations in aqueous humor. Ciprofloxacin concentrations in aqueous humor were similar in young and old rabbits in both groups 2 and 3 (Table 4). In rabbits of both ages, the groups receiving antibiotic plus steroid–anti-inflammatory therapy (group 3) had ciprofloxacin concentrations higher than those receiving the antibiotic alone (group 2).

DISCUSSION

As *P. aeruginosa* multiplies and spreads within the stroma, an acute host inflammatory response ensues. The severity of this acute inflammatory response depends upon the virulence of the organism and the immune status of the host (1). Highly virulent organisms such as *P. aeruginosa* evoke a pronounced host response. In the immunosuppressed host, the hallmarks of ocular inflammation are relatively reduced (10). Because of a decrease in the nonspecific resistance mechanisms in geriatric patients, the signs and symptoms of infection can be less obvious (2). In this study, the SLE scores and corneal PMN numbers of young rabbits, presumably as a result of a more vigorous inflammatory response, were consistently higher than the scores for the older rabbits in the corresponding treatment groups.

The ocular damage in *Pseudomonas* keratitis depends to a large degree on the action of proteases (3, 9, 16). Three sources of proteases could contribute to the tissue destruction seen during *P. aeruginosa* keratitis: the bacteria, stromal fibroblasts, and degranulating PMN. *P. aeruginosa* alkaline protease and elastase are capable of degrading stromal proteoglycans (16). These enzymes could also activate an endogenous matrix metalloproteinase, MMP-2. This type I collagenase is expressed by stromal fibroblasts in normal corneas in an inactive proenzyme form. Recently, Matsumoto et al. (9) reported the ability of alkaline protease and elastase to activate MMP-2.

TABLE 4. Ciprofloxacin concentrations in aqueous humor 27 h postinfection

Group[a]	Treatment[b]	Ciprofloxacin concn (μg/ml)	
		Young	Old
1	PBS	None	None
2	0.3% ciprofloxacin	1.73 ± 0.36[c]	1.33 ± 0.11
3	0.3% ciprofloxacin, 1.0% prednisolone acetate, 0.03% flurbiprofen	3.11 ± 0.46[d]	4.00 ± 0.43

[a] Each group consisted of four young (3- to 5-month-old) and four old (1.5- to 3-year-old) rabbits (eight eyes in each group).
[b] Drops were administered every 15 min for 1 h and then every 30 min for 9 h.
[c] Not significantly different from similarly treated old rabbits ($P = 0.5$).
[d] Not significantly different from similarly treated old rabbits ($P = 0.1$).

The lysosomal enzymes of PMN (collagenase and cathepsins) contribute to corneal degradation (3). Preventing the influx of PMN into the *P. aeruginosa*-infected cornea with anti-inflammatory drugs appears to be an attractive adjunct therapy. This is true provided the bactericidal activity of the concurrently administered antibiotic is not compromised. The regimen that was associated with the least amount of pathology together with the fewest viable bacteria recovered from the cornea involved treatment with ciprofloxacin in addition to prednisolone and flurbiprofen (group 3). Ciprofloxacin efficiently eliminated 6 to 7 log units of *P. aeruginosa* from the cornea, and prednisolone and flurbiprofen reduced the amount of tissue damage from the host inflammatory response.

Cell-mediated immunity, humoral immunity, and nonspecific immune mechanisms decrease in functional capacity with age (5, 14, 15). Hazlett et al. (5) isolated PMN and macrophages from *P. aeruginosa*-infected mouse corneas and peripheral blood of uninoculated mice and noticed an impaired phagocytic function in cells of aged animals versus young adults. A loss in the functional capacity of PMN and macrophages of old rabbits could explain the lower number of corneal PMN in old rabbits during *Pseudomonas* keratitis.

ACKNOWLEDGMENTS

We thank Ada Rivera for her secretarial skills in the preparation of the manuscript and Michelle C. Callegan for her editorial comments.

This work was supported in part by U.S. Public Health Service grant EY08871 and Core grant EY02377 from the National Eye Institute, National Institutes of Health, Bethesda, Md.

REFERENCES

1. **Arffa, R. C.** 1991. Grayson's diseases of the cornea, 3rd ed., p. 163–198. Mosby Year Book, St. Louis.
2. **Fox, R. A.** 1988. Atypical presentation of geriatric infections. Geriatrics **43**:58–68.
3. **Gray, L. D., and A. S. Kreger.** 1975. Rabbit corneal damage produced by *Pseudomonas aeruginosa* infection. Infect. Immun. **12**:419–432.
4. **Gritz, D. C., T. Y. Lee, S. Kwitko, and P. J. McDonnell.** 1990. Topical anti-inflammatory agents in an animal model of microbial keratitis. Arch. Ophthalmol. **108**:1001–1005.
5. **Hazlett, L. D., F. B. Kreindler, R. S. Berk, and R. Barrett.** 1990. Aging alters the phagocytic capability of inflammatory cells induced into the cornea. Curr. Eye Res. **9**:129–138.
6. **Hobden, J. A., R. J. O'Callaghan, J. M. Hill, M. Hagenah, M. S. Insler, and J. J. Reidy.** 1992. Ciprofloxacin and prednisolone therapy for experimental *Pseudomonas* keratitis. Curr. Eye Res. **11**:259–265.
7. **Hobden, J. A., J. J. Reidy, R. J. O'Callaghan, M. S. Insler, and J. M. Hill.** 1990. Ciprofloxacin iontophoresis for aminoglycoside-resistant pseudomonal keratitis. Invest. Ophthalmol. Vis. Sci. **31**:1940–1944.
8. **Johnson, M. K., J. A. Hobden, M. Hagenah, R. J. O'Callaghan, J. M. Hill, and S. Chen.** 1990. The role of pneumolysin in ocular infections with *Streptococcus pneumoniae*. Curr. Eye Res. **9**:1107–1114.

9. **Matsumoto, K., N. B. K. Shams, L. A. Hanninen, and K. R. Kenyon.** 1993. Cleavage and activation of corneal matrix metalloproteases by *Pseudomonas aeruginosa* proteases. Invest. Ophthalmol. Vis. Sci. **34**:1945–1953.
10. **Nanda, M., S. C. Pflugfelder, and S. Holland.** 1991. Fulminant pseudomonal keratitis and scleritis in human immunodeficiency virus-infected patients. Arch. Ophthalmol. **109**:503–505.
11. **Ohadi, C., K. L. Litwin, H. Moreira, S. Kwitko, W. J. Gauderman, D. C. Gritz, A. Gwon, R. Jones, and P. J. McDonnell.** 1992. Anti-inflammatory therapy and outcome in a guinea pig model of *Pseudomonas* keratitis. Cornea **11**:398–403.
12. **Rootman, D. S., J. A. Hobden, J. A. Jantzen, J. R. Gonzales, R. J. O'Callaghan, and J. M. Hill.** 1988. Iontophoresis of tobramycin for the treatment of experimental *Pseudomonas* keratitis in the rabbit. Arch. Ophthalmol. **106**:262–265.
13. **SAS Institute Inc.** 1985. SAS user's guide: statistics, version 5. SAS Institute, Cary, N.C.
14. **Simons, R. J., and H. Y. Reynolds.** 1990. Altered immune status in the elderly. Sem. Respir. Infect. **5**:251–259.
15. **Solana, R., J. L. Villanueva, J. Pena, and M. de la Fuente.** 1991. Cell mediated immunity in ageing. Comp. Biochem. Physiol. **99A**:1–4.
16. **Steuhl, K. P., G. Doring, A. Henni, H. J. Thiel, and K. Botzenhart.** 1987. Relevance of host-derived and bacterial factors in *Pseudomonas aeruginosa* corneal infections. Invest. Ophthalmol. Vis. Sci. **28**:1559–1568.
17. **Williams, R. N., C. A. Paterson, K. E. Eakins, and P. Bhattacherjee.** 1982/1983. Quantification of ocular inflammation: evaluation of polymorphonuclear leukocytes infiltration by measuring myeloperoxidase activity. Curr. Eye Res. **2**:465–470.