ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1994, p. 886–888
0066-4804/94/$04.00+0

Vol. 38, No. 4

Killing Acanthamoebae with Polyaminopropyl Biguanide: Quantitation and Kinetics

RICHARD M. BURGER,[1]* ROBERT J. FRANCO,[2]† AND KARL DRLICA[1]

Public Health Research Institute, New York, New York 10016,[1] and Personal Products Division, Bausch & Lomb Company, Rochester, New York 14692[2]

Received 2 August 1993/Returned for modification 13 January 1994/Accepted 25 January 1994

The two *Acanthamoeba* species most often implicated in corneal keratitis, *A. castellanii* and *A. polyphaga*, were exposed as cysts to polyaminopropyl biguanide (PAPB), a commonly used antimicrobial agent. Killing of amoeba cysts was rapid and extensive, with fewer than 2% of either species surviving 30 s of exposure to ≥45 ppm of PAPB. Killing kinetics were biphasic, and further exposures of 15 min to 1 h killed greater than 90% of those surviving initial killing. This potency of PAPB, together with its low toxicity to humans when ingested or applied topically, underscores the potential of PAPB as an antiamoebic agent.

Acanthamoebae are ubiquitous, free-living, cyst-forming protozoa of soil, water, and air that cause a corneal keratitis associated with contact lens use (85% of infected patients have been contact lens users [9]). The rise in contact lens use and the drug resistance of acanthamoebae are bringing increasing attention to these organisms (11). In an encouraging study, Larkin et al. (5) reported therapeutic activity for polyaminopropyl biguanide (PAPB, also known as polyhexamethylene biguanide). This could be a major advance toward controlling acanthamoebae, since PAPB is already known to be relatively harmless (it is used as a disinfectant in swimming pools and as a preservative in cosmetic and pharmacological formulations [1]). To determine whether the compound works rapidly enough and at concentrations low enough to be prophylatically useful, we have adapted a quantitative bacterial plaque assay of *Acanthamoeba* spp. (4) and used it to monitor the killing of cysts by concentrations of PAPB suitable for contact lens cleaning and disinfection procedures. These experiments indicate that cysts of both *A. castellanii* and *A. polyphaga*, the *Acanthamoeba* species responsible for most cases of amoebic corneal keratitis, are vulnerable to rapid killing by safe concentrations of PAPB.

Amoeba cultures, obtained from the American Type Culture Collection (*A. castellanii* [8] ATCC 30010, *A. polyphaga* [13] ATCC 30461), were grown in PYG medium (12) at 29°C in polypropylene culture tubes (Falcon) for at least 4 weeks to obtain encysted forms. By 2 weeks (*A. castellanii*) or 3 weeks (*A. polyphaga*) growth had ceased. At that time, almost all cells that excluded trypan blue (0.2%) were encysted, as judged by microscopic observation of round, thick-walled, highly refractile cells.

Amoeba cultures, 1 to 2 months old and consisting of ≥90% cysts (1×10^6 to 2×10^6/ml), were treated with PAPB by adding 9 volumes of filter-sterilized solutions of 5, 10, 25, 50, and 100 ppm of PAPB in borate-buffered saline (137 mM H_3BO_3, 2.4 mM $Na_2B_4O_7 \cdot 10H_2O$, 77 mM NaCl, pH 6.4) at room temperature, thus giving final PAPB concentrations of 4.5, 9, 22.5, 45, and 90 ppm, respectively (the culture medium had a marked protective effect against killing by PAPB, so all treatments were made at the same culture:PAPB solution ratio). After periods of 30 s to 24 h, the treatments were terminated by the addition of amoeba samples (≤0.4 ml) to 3 ml of either melted Top Agarose (1% tryptone, 0.8% NaCl, and 0.6% agarose [6]) or half-strength, melted Difco D/E Neutralizing Agar (D/E Top Agar [3]). Also added was 0.05 ml of an *Enterobacter aerogenes* (ATCC 13048) suspension (4×10^{10} cells/ml) that was prepared from cells grown on LB agar plates (7) and suspended in 0.15 M KCl. These mixtures, which were at 50°C, were poured immediately onto 100-mm plates of SM/5 agar (10) and incubated for 4 to 8 days at 29°C. Plaques were then scored visually. Assays were performed in triplicate, and plates were reexamined 1 to 3 days after scoring to ensure that killing, rather than delay in growth, was being observed. The volumes assayed were chosen to give about 100 plaques per plate, except where prevented by extensive killing. The numbers of surviving amoebae were compared with those in aliquots from the same amoeba culture diluted in borate-buffered saline, which gave the same plaquing efficiency as did dilution in PYG.

The plaquing efficiency of both species was 50 to 100% of the microscopically scored, trypan blue-excluding amoebae. D/E Top Agar produced a slightly higher efficiency and a 2-day-shorter plaque development period than Top Agarose. However, the scoring of plates with D/E Top Agar was complicated by a tendency to form small satellite plaques around the primary plaques. This made it necessary to deliver fewer amoebae per plate and to score the primary plaques promptly. In either medium, *A. castellanii* plaques developed about 2 days sooner than those of *A. polyphaga*. Plaques of *A. castellanii* were circular and smooth edged and had a tendency to merge with adjacent plaques. Plaques of *A. polyphaga* were less regular and contained lobes; they often formed boundaries with abutting plaques.

Two characteristics of the assay were assessed: sensitivity and kinetic accuracy. The limit of sensitivity is a single PFU per plate. Scoring of rare plaques was unambiguous, since amoebae within plaques were easily seen by microscopic examination. Since the most concentrated test mixtures (0.4 ml of amoebae:PAPB solution [1:9]) initially contained about 0.5×10^5 PFU, we were able to detect fewer than one survivor per 10^4 PFU. Survivors were observed in every incubation except those of *A. castellanii* at 90 ppm of PAPB for ≥1 h; consequently, the determination of killing kinetics almost never depended on maximum estimates. Top Agarose and D/E Top

* Corresponding author. Mailing address: Public Health Research Institute, 455 First Ave., New York, NY 10016. Phone: (212) 578-0813. Fax: (212) 578-0804. Electronic mail address: burger@phri.nyu.edu.

† Present address: Pattiglio Rabin Todd & McGrath, Weston, MA 02193.

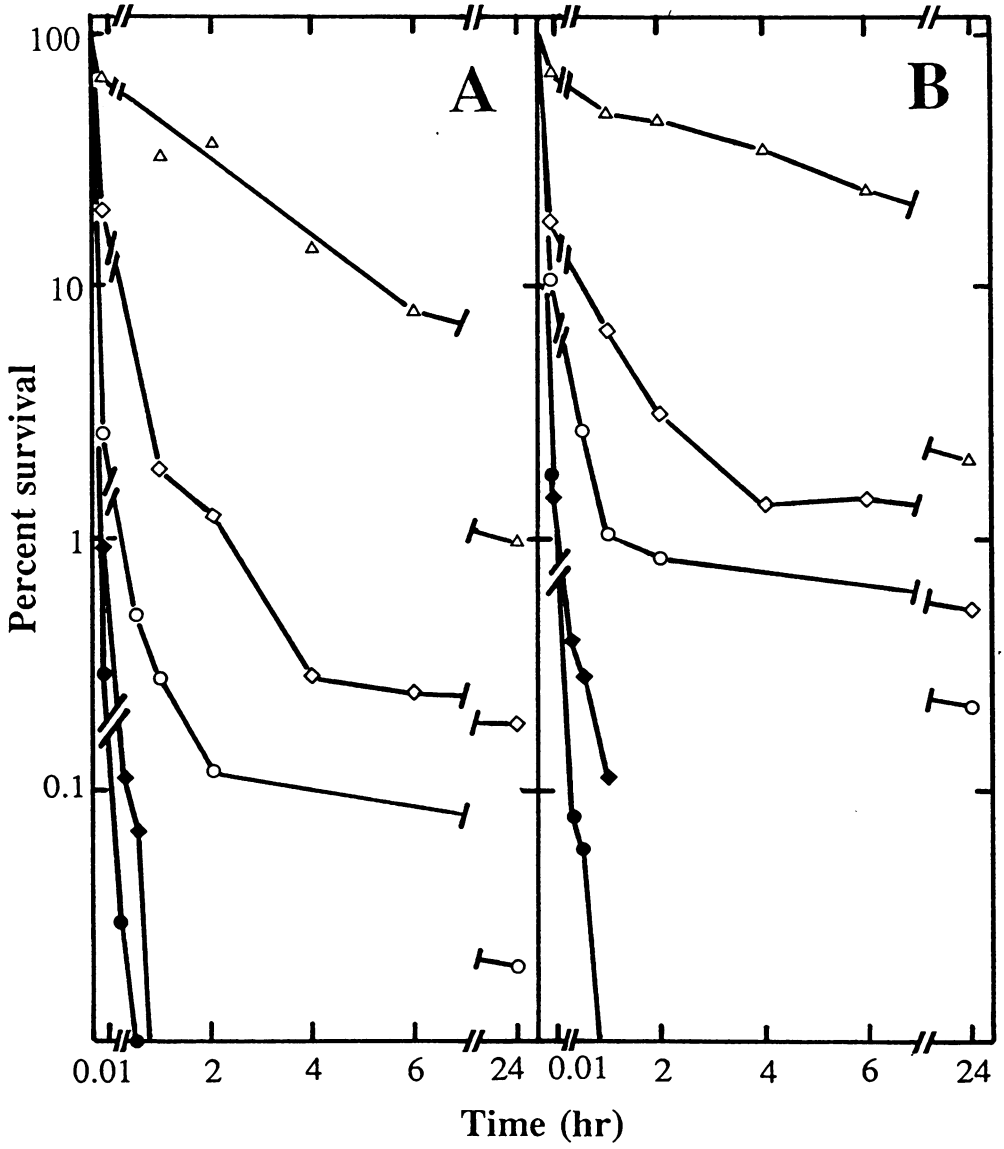

FIG. 1. Kinetics of killing amoebae with PAPB. Encysted amoeba cultures were exposed to various concentrations of PAPB for the indicated times. Treatments were terminated by addition of aliquots to ≥7.5 volumes of D/E Top Agar followed by plating with *E. aerogenes* for plaque assays as described in Materials and Methods. For each experiment, the number of amoebae initially present was determined by diluting the suspension with borate-buffered saline lacking PAPB. For each incubation, the initial experimental point represents exposure to PAPB for 0.01 h. (A) *A. castellanii*; (B) *A. polyphaga*. PAPB concentrations were 4.5 ppm (△), 9 ppm (◇), 22.5 ppm (○), 45 ppm (◆), and 90 ppm (●).

Agar gave comparable results. The accurate determination of killing kinetics depends on PAPB action ceasing at the time of plating. This was ascertained by adding PAPB separately to Top Agarose and assaying its ability to kill both species of *Acanthamoeba* subsequently added to the agarose with bacteria. Killing in Top Agarose was barely detected (50%) at the highest PAPB concentration used (0.36 ml of 100 ppm of PAPB added to Top Agarose). No killing was detected in D/E Top Agar, which contains lecithin, a compound known to neutralize biguanides.

Killing of amoebae by PAPB occurred very rapidly. When PAPB was neutralized by D/E Top Agar within 30 s after addition of PAPB to amoeba cultures, we observed irreversible killing of 30 to 99.7%, values that depended on the amoeba species and PAPB concentration. These results are shown as the shortest time points in Fig. 1. Killing of >99% of *A. castellanii* was observed after 30 s of exposure to either 90 or 45 ppm of PAPB; >98% killing of *A. polyphaga* was observed under these conditions. With 22.5 ppm of PAPB, 30 s of exposure killed 97% of *A. castellanii* and 89% of *A. polyphaga*. Since 30 s approaches the practical lower limit for initiating and terminating a PAPB incubation, the rate of killing in the initial 30-s phase was considered too rapid to measure reliably.

After the initial rapid phase, killing of the amoebae continued at a decreasing rate for 1 to 2 h (Fig. 1). The times necessary to kill half the survivors ranged from 5 h (4.5 ppm, *A. polyphaga*) to 5 min (90 ppm, *A. castellanii*), as determined from the killing curves shown in Fig. 1. For the remainder of the observation period, up to 24 h, killing was slower. Except at the lowest concentrations, most killing occurred in the first 2 h

TABLE 1. PAPB exposure times sufficient to kill *Acanthamoeba* cysts

Organism and amount (ppm) of PAPB	Time (h) to 99.9% killing[a]	
	Assay 1	Assay 2
A. castellanii		
4.5	>24	>24
9	>24	4.6, 5
22.5	3	2, 3.3
45	0.3	ND
90	0.1	ND
A. polyphaga		
4.5	>24	>24, >24
9	>24	>24, >24
22.5	>24	>24, 3.3
45	1	ND
90	0.2	ND

[a] Assay 1 and assay 2 differed only by use of D/E Top Agar and Top Agarose, respectively, for the plaque assays. Where two numbers are given, they are the results of replicate kinetic experiments. ND, not determined because Top Agarose does not completely neutralize the toxicity of 90 ppm of PAPB.

(only 80% of the initial survivors died between 2 and 24 h of PAPB exposure). At every time and PAPB concentration, *A. castellanii* was 2- to 10-fold more sensitive than *A. polyphaga*. The reason for this difference is unknown. The times necessary to kill 99.9% of the amoebae are summarized in Table 1. The kinetic results obtained by using Top Agarose are also abstracted in Table 1. They were similar to those obtained with D/E Top Agar over the lower PAPB range for which both plating formulations are suitable. Since the approach to 99.9% killing was quite gradual at low PAPB concentrations, estimates of the time to reach that point varied considerably (see *A. polyphaga* treated with 22.5 ppm of PAPB, Table 1).

The actual rate of killing is potentially even faster than is shown in Fig. 1, since the PYG medium used for encystment and storage of amoebae has a protective effect: survival of amoebae diluted 10-fold with 4.5 ppm of PAPB for 15 min was complete, whereas only half the cells survived this treatment when diluted 99-fold. The difference was eliminated by adding more PYG to the 99-fold amoeba dilution, thus making the two dilutions equivalent with respect to PYG. The protective effect can be quite dramatic at higher PAPB concentrations. At 25 ppm, the disinfectant became 100 times more effective when the culture:PAPB ratio was lowered by a factor of 10. A previous study (2) on antagonism of PAPB action by organic material also showed a 10- to 100-fold reduction in *Acanthamoeba* killing at high PAPB concentration. Such an effect might be important for therapeutic use of PAPB, since patients' eye fluids may similarly protect *Acanthamoeba* spp. from PAPB toxicity. However, it should be negligible for in vitro lens cleaning and disinfection procedures, since very small volumes of eye fluids are associated with contact lenses (0.03 ml) and since relatively large volumes of disinfectant and/or rinse are customarily used for lens care.

PAPB has been used primarily as a preservative and an environmental biocide. At the concentrations used in lens care solutions, it is bacteriocidal but almost innocuous to amoeba cysts. The minimal cystocidal concentration for clinical *Acanthamoeba* isolates is 1 to 4 ppm of PAPB with exposure for 2 days (5). Concentrations in the range of 45 to 90 ppm are necessary to kill 99.9% in less than 1 h of treatment (Fig. 1, Table 1). This concentration range is below that used either in swimming pools or therapeutically (5). Thus, PAPB is well suited for antiamoebic disinfection.

We thank David Proud for stimulating discussions.

These studies were supported in part by the Bausch & Lomb Company of Rochester, N.Y.

REFERENCES

1. **Block, S. S.** 1991. Disinfection, sterilization, and preservation, 4th ed. Lea & Febiger, Philadelphia.
2. **Dawson, M. W., T. J. Brown, C. J. Biddick, and D. G. Till.** 1983. The effect of Baquacil on pathogenic free-living amoebae (PFLA). 2. In simulated natural conditions—in the presence of bacteria and/or organic matter. N. Z. J. Mar. Freshwater Res. **17**:313–330.
3. **Engley, F. B., and B. P. Dey.** 1970. A universal neutralizing medium for antimicrobial chemicals, p. 100–106. *In* Proceedings of the 56th Mid-Year Meeting of the Chemical Specialties Manufacturers Association, New York.
4. **Hugo, E. R., W. R. McLaughlin, K. Oh, and O. H. Tuovinen.** 1991. Quantitative enumeration of *Acanthamoeba* for evaluation of cyst inactivation in contact lens care solutions. Invest. Ophthalmol. Visual Sci. **32**:655–657.
5. **Larkin, D. F. P., S. Kilvington, and J. K. G. Dart.** 1992. Treatment of *Acanthamoeba* keratitis with polyhexamethylene biguanide. Ophthalmology **99**:185–191.
6. **Lech, K., and R. Brent.** Media preparation and bacteriological tools, p. 1.1.4. *In* F. Ausubel, R. Brent, R. Kingston, J. Smith, and J. Seidman (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
7. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Neff, R. J.** 1957. Purification, axenic cultivation, and description of a soil amoeba, *Acanthamoeba sp.* J. Protozool. **4**:176–182.
9. **Stehr-Green, J. K., T. M. Bailey, and G. S. Visvesvara.** 1989. The epidemic of Acanthamoeba keratitis in the United States. Am. J. Ophthalmol. **107**:331–336.
10. **Sussman, M.** 1987. Cultivation and synchronous morphogenesis of *Dictostelium* under controled experimental conditions. Methods Cell Biol. **28**:9–29.
11. **Symposium.** 1991. International Symposium on *Acanthamoeba* and the Eye. Rev. Infect. Dis. **13**:S367–S450.
12. **Visvesvara, G. S., and W. Balamuth.** 1975. Comparative studies on related free-living and pathogenic amoebae with special reference to *Acanthamoeba*. J. Protozool. **22**:245–256.
13. **Visvesvara, G. S., D. B. Jones, and N. M. Robinson.** 1975. Isolation, identification, and biological characterization of *Acanthamoeba polyphaga* from a human eye. Am. J. Trop. Med. Hyg. **24**:784–790.