PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 169, 301–305 (1982)

Increased Phagocytosis of *Escherichia coli* Pretreated with Subinhibitory Concentrations of Cyclacillin or Ampicillin (41347)

HERMAN FRIEDMAN AND GEORGE H. WARREN

Department of Microbiology and Immunology, College of Medicine, University of South Florida, Tampa, Florida 33612, and Wyeth Laboratories, Incorporated, Radnor, Pennsylvania 19087

Abstract. When incubated with subinhibitory concentrations of cyclacillin or ampicillin *E. coli* became more susceptible to subsequent phagocytosis by normal mouse monocytes (peritoneal exudate cells). Incubation for as little as 1 hr markedly increased the phagocytosis, which in turn was associated with increased intracellular killing of antibiotic-treated bacteria. Regardless of the length of time of incubation of the penicillin-resistant *E. coli* with potassium penicillin G there was no significant increase in phagocytosis.

Previous studies have shown that bacteria exposed to subinhibitory concentrations of some antibiotics develop abnormal morphologic and ultrastructural features (1, 2) and become more vulnerable to serum antibody and lysozyme-mediated lysis (3–6), but whether such exposure makes them more susceptible to host defense mechanisms such as phagocytosis has not been fully established. Although there have been some reports that organisms exposed to sublethal doses of some antibiotics became more susceptible than untreated organisms to bactericidal effects of phagocytic cells, it is not known whether this effect is specific for certain antibiotics and organisms or is a more general phenomenon. In this regard, studies in this laboratory have shown that penicillin-resistant staphylococci became more susceptible to phagocytosis by normal mouse peritoneal exudate cells *in vitro* when exposed to a small dose of a semisynthetic penicillin, nafcillin (7). Such noninhibitory concentrations of nafcillin had no detectable influence on viability, but the bacteria became more susceptible to ingestion and killing by peritoneal monocytes. Similarly, *Escherchia coli,* when exposed to small amounts of cyclacillin but not penicillin, an antibiotic to which they are resistant, became more susceptible to antibody-mediated bacteriolysis *in vitro* (8). In the present study, the susceptibility of *E. coli* after exposure *in vitro* to a sublethal concentration of the broad-spectrum semisynthetic penicillins, cyclacillin and ampicillin, was examined in regard to phagocytosis by normal mouse peritoneal exudate cells *in vitro*.

Material and Methods. *Antibiotics.* Cyclacillin, 6-(1-aminocyclohexanecarboxamido)penicillanic acid, a semisynthetic penicillin with a broad bacterial spectrum, was freshly prepared as a solution containing 100 μg antibiotic per milliliter saline. As a control, either ampicillin, another broad spectrum semisynthetic penicillin, or potassium penicillin G, was similarly used.

Bacteria. E. coli strain 0127:B8 was used for the study. This organism is susceptible to *in vitro* agglutination and lysis by specific anti-*E. coli* serum (8). The bacteria were highly resistant to penicillin but susceptible to both cyclacillin and ampicillin. Bacteria were cultured for 18 hr in brain–heart infusion (BHI) broth and harvested by centrifugation at approximately 10,000*g* for 30 min. The organisms were washed by several centrifugations alternating with resuspensions in sterile saline.

Phagocytic studies. Normal adult BALB/c mice were used as a source of peritoneal exudate (PE) cells. For this purpose, the mice were injected ip with 0.5 ml sterile thioglycollate (Difco Laboratories, Detroit, Mich.) 24 hr earlier. The cells were obtained by aspiration and washed several times by centrifugation at 4° with sterile minimal essential medium (MEM) fortified with fetal calf serum without antibiotics. The resulting washed cell suspensions were

0037-9727/82/020301-05$01.00/0

then diluted to a concentration of approximately 10^7 nucleated monocytes/ml medium. Cell viability and cell numbers were determined by hemacytometer count using the standard dye exclusion trypan blue technique. The PE cell population consisted of approximately 75–80% macrophages, as determined by morphologic examination and the ability to ingest heat-killed yeast particles or colloidal carbon. The remainder of the cells were morphologically lymphocytes or other cell types. For the phagocytic test with control or antibiotic-treated bacteria, graded concentrations of the test antibiotic were first added to 1.0 ml BHI broth containing a standard inoculum of the freshly harvested and washed *E. coli* at a concentration of approximately $2-5 \times 10^7$ bacteria/ml. Following incubation at 37° for varied lengths of time, 0.1 ml of the antibiotic–bacteria mixture was added to 13 × 75-mm sterile glass tubes containing 5×10^6 PE washed cells in 1 ml MEM containing 10% sterile calf serum but no additional antibiotics. Control tubes contained either antibiotic alone, *E. coli* alone, or *E. coli* plus antibiotic without PE cells. Following incubation for 0.5 to 6 hr at 36° the number of bacteria ingested by the monocytes was determined by placing 0.1 ml of each cell suspension on a glass slide and air drying and staining it with either Giemsa or Gram stain. The numbers of bacteria present in 100 monocytes and the percentage of cells with more than two organisms each were determined by microscopic examination. In some experiments the numbers of *E. coli* in the supernatants of the PE cell cultures were determined after low-speed centrifugation by plating 0.1-ml aliquots of cell-free supernatants on BHI agar plates. In addition, washed suspensions of PE cells exposed to *E. coli* were sonicated for 30 sec to 1 min or frozen and thawed several times and the lysate plated onto agar plates.

Experimental Results. The *E. coli* cultures were essentially resistant to penicillin in that greater than 50 μg was required to kill approximately one-half the bacteria (unpublished results). In contrast, the bacteria were much more sensitive to cyclacillin, since the MIC for 2×10^7 inoculum was calculated as 6–7 μg/ml. The MIC for ampicillin was 1–2 μg/ml. As is evident in Table I, *E. coli* incubated for 1–2 hr with graded doses of cyclacillin, ampicillin, or penicillin G showed essentially no loss of

TABLE I. EFFECT OF GRADED CONCENTRATIONS OF CYCLACILLIN, AMPICILLIN, AND PENICILLIN G ON PHAGOCYTOSIS OF *E. coli* BY NORMAL MOUSE PERITONEAL EXUDATE CELLS

Antibiotic[a]	Concentration	Percentage viable bacteria[b]	Phagocytosis[c]	
			Percentage PE cells positive	Average number bacteria/PE cell
None (controls)	—	97.8	8.1 ± 0.8	3.9 ± 1.5
Cyclacillin	1.0 μg	94.5	19.6 ± 2.5	10.6 ± 3.5
	5.0	86.8	13.2 ± 3.1	9.5 ± 2.9
	10.0	39.3	9.6 ± 3.2	11.6 ± 2.8
	20.0	15.2	12.9 ± 2.7	6.7 ± 3.1
Ampicillin	1.0 μg	96.4	14.5 ± 3.1	8.5 ± 2.4
	5.0	10.8	12.6 ± 4.2	7.3 ± 2.9
	10.0	4.1	10.5 ± 2.1	4.9 ± 2.1
Penicillin G	10 U	96.5	7.6 ± 1.2	5.4 ± 1.8
	50	93.1	10.2 ± 2.9	6.6 ± 2.2
	100	94.8	10.8 ± 3.3	4.3 ± 1.2

[a] Indicated antibiotic added to cultures of 2.5×10^7 *E. coli*/ml BHI broth for 2 hr prior to washing and incubation with 2×10^6 PE cells from normal BALB/c mice.

[b] Average percentage of *E. coli* CFU obtained upon plating on agar after incubation with antibiotics.

[c] Average number of phagocytizing mononuclear PE cells ± SE, present after 2 hr incubation with *E. coli*.

TABLE II. ALTERED PHAGOCYTOSIS OF ANTIBIOTIC-TREATED *E. coli* BY NORMAL MOUSE PERITONEAL EXUDATE CELLS

Antibiotic[a]		Average number of PE cells having two or more *E. coli* after incubation at 37° for				
		0.5 hr	1 hr	1.5 hr	2 hr	4 hr
None (controls)		<5	2.6	5.8	7.3	11.2
Cyclacillin	1.0 μg	6.5	12.9	22.8	23.7	30.5
	5.0	6.9	15.0	28.5	31.2	33.6
	10.0	5.2	11.3	18.9	28.0	30.5
Ampicillin	1.0 μg	4.8	9.3	12.8	18.5	—
	5.0	2.3	8.1	9.2	—	20.5

[a] Incubated with 3×10^7 *E. coli*/ml BHI broth for 2 hr prior to washing and exposure to 2.5×10^6 PE cells for indicated length of time prior to assay for phagocytosis.

viability except with the higher doses of the semisynthetic antibiotics. When these antibiotic-treated bacteria were washed and added to the cultures of normal mouse PE cells, varying numbers of *E. coli* were phagocytized. Incubation of *E. coli* in saline resulted in only relatively minimal phagocytosis in that fewer than 10% of the PE cells were found to have ingested two or more bacteria during this incubation period. In contrast, incubation of *E. coli* with 1 to 5 μg cyclacillin resulted in a higher level of phagocytosis with approximately 20 and 13% positive, respectively, for the 1- and 5-μg doses. These cultures also showed approximately three times as many organisms ingested per monocyte as controls. The *E. coli* incubated with either 10 or 20 μg cyclacillin showed less viability and fewer monocytes phagocytized these cells. However, those monocytes which did phagocytize the *E. coli* after the 10-μg dose of cyclacillin showed a consistently higher number of bacteria per phagocyte. Similarly, monocytes which phagocytized the *E. coli* exposed to 20 μg cyclacillin also showed enhanced numbers of bacteria per cell but not as many as with the other doses of cyclacillin. The bacteria treated with ampicillin also were phagocytized at a greater rate than control bacteria, especially with the lower doses of the antibiotic. Penicillin G-treated *E. coli* were phagocytized at essentially the same rate as *E. coli* incubated with saline only.

Although the uptake pattern of cyclacillin- or ampicillin-treated *E. coli* was essentially similar to that which occurred when phagocytes were incubated with untreated or penicillin-treated bacteria (Table I), the total number of monocytes ingesting two or more microorganisms increased to a greater degree at each time period examined during the 1–4 hr of incubation (Table II).

There was no apparent shift in the rate of

TABLE III. EFFECT OF TIME OF INCUBATION OF *E. coli* WITH ANTIBIOTIC ON SUBSEQUENT PHAGOCYTOSIS BY PE CELLS

Antibiotic[a]		Average percentage of PE monocytes that phagocytized *E. coli* after incubation at 37° for					
		0.2 hr	0.5 hr	1 hr	2 hr	4 hr	8 hr
None (controls)		4.8	5.3	6.9	5.3	6.8	8.1
Cyclacillin	2.0 μg	4.3	5.9	16.6	25.6	28.6	24.8
Ampicillin	1.0 μg	5.0	4.7	10.6	18.3	—	15.8
Penicillin G	100 U	5.9	6.2	8.3	7.6	8.7	7.9

[a] Indicated antibiotic added to cultures of 2×10^7 *E. coli*/ml BHI broth for indicated length of time at 37°.

TABLE IV. EFFECT OF LENGTH OF TIME OF INCUBATION OF *E. coli* WITH CYCLACILLIN OR PENICILLIN ON SUBSEQUENT KILLING BY PHAGOCYTOSIS

Incubation time[a]	Number of viable *E. coli*[b]					
	Cyclacillin incubated		Penicillin incubated		Control (untreated)	
	Extracellular ($\times 10^6$)	Intracellular ($\times 10^2$)	Extracellular ($\times 10^6$)	Intracellular ($\times 10^2$)	Extracellular ($\times 10^6$)	Intracellular ($\times 10^2$)
0 (controls)	19.3 ± 2.5	55.8 ± 2.0	18.5 ± 3.1	42.2 ± 3.8	20.5 ± 3.4	50.5 ± 6.2
1 hr	10.2 ± 3.6	28.6 ± 3.9	22.5 ± 5.3	36.5 ± 6.1	24.6 ± 5.2	32.5 ± 5.6
2 hr	8.4 ± 1.8	18.2 ± 4.2	34.3 ± 18.2	38.9 ± 8.3	—	—
4 hr	2.7 ± 0.9	9.5 ± 5.1	26.3 ± 4.6	31.4 ± 3.5	29.3 ± 7.2	36.8 ± 3.9

[a] *E. coli* (3.0×10^7/ml BHI broth) incubated with cyclacillin (2.0 μg) or penicillin (50 μg) for indicated length of time at 37° before washing and incubation with 2×10^6 PE cells.

[b] Mean ± SE number of *E. coli* in culture fluid or lysate of PE cells 1 hr after incubation with cyclacillin- or penicillin-treated bacteria or, as control, untreated bacteria.

phagocytosis regardless of the dose of antibiotic used to pretreat the bacteria. On the other hand, phagocytic activity was markedly influenced by the time of incubation of the bacteria with the antibiotic. As is evident in Table III, when bacteria were incubated with a subinhibitory dose of cyclacillin for 2–8 hr, maximum phagocytosis occurred upon subsequent exposure of the washed bacteria to normal PE cells. One-hour incubation of the bacteria with antibiotic still resulted in a significantly increased uptake by phagocytes, but a half-hour incubation had essentially no effect. Regardless of the length of time of incubation of the penicillin-resistant *E. coli* with penicillin G, there was no significant increase in phagocytosis.

That uptake of bacteria by phagocytosis was associated with intracellular killing is evident in the data shown in Table IV. There was a rapid reduction in the number of viable *E. coli* recovered in the culture fluids one hour after incubation with PE cells treated for 1–4 hr with cyclacillin. Cultures containing bacteria treated for the longest period of time showed the fewest organisms in the extracellular medium. Furthermore, upon lysing of the monocytes by two cycles of freezing and thawing or by sonication, fewer living organisms were detectable in the intracellular lysate of cells incubated with cyclacillin-treated bacteria as compared to untreated ones (Table IV).

Discussion. The results of the present studies lend support to the view (3, 4, 7) that treatment of bacteria with subbactericidal concentrations of an antibiotic may render the organisms more susceptible to host defense mechanisms. Previous studies in this laboratory, as well as in a number of others, have now indicated that both Gram-positive and Gram-negative organisms, when incubated *in vitro* with subinhibitory concentrations of several different antibiotics, show enhanced susceptibility to either antibody-dependent inhibition *in vitro* or phagocytic killing (3–8). In the present study it was found that cyclacillin, which often seems more effective in inhibiting bacteria at relatively low doses *in vivo* than *in vitro*, interacted with *E. coli*, a typical

Gram-negative organism, making it more susceptible to phagocytosis. Relatively similar results were obtained with ampicillin, another semisynthetic penicillin to which the bacteria were also susceptible, especially *in vitro* (5, 6).

In previous *in vivo* studies it has been found that following treatment with low doses of cyclacillin, greater numbers of *E. coli* antibody-producing cells are observed, suggesting that the presence of the antibiotic increases the immunogenicity of microorganisms (8). This occurs presumably by alteration of the bacterial cell surface, resulting in the release of immunogenic moieties. *E. coli* incubated with small doses of cyclacillin *in vitro,* but not with penicillin G (an antibiotic to which it is resistant) also become more susceptible to higher dilutions of antibody and complement as compared to dilutions needed to kill the untreated organism (6).

The present data support the previous observations and show that incubation of *E. coli* with subinhibitory doses of cyclacillin or ampicillin results in increased susceptibility to phagocytosis by peritoneal exudate cell suspensions from normal mice. It is of interest that although the effective *in vitro* MIC of ampicillin is lower than for cyclacillin, the efficacy of similar doses of both drugs in enhancing the phagocytosis of *E. coli* was relatively the same. This is similar to earlier observations that the *in vivo* efficacy of both antibiotics against susceptible bacteria is the same despite apparently different *in vitro* susceptible activities. The enhanced uptake of microorganisms by PE cells was found to be reflected in enhanced killing of the *E. coli* by the phagocytes. Further investigation into the possible synergistic effect between antibiotics and host defense mechanisms should provide a better understanding of how antibiotic treatment of infectious diseases requires a functioning immune defense system for optimal efficacy.

1. Warren GH, Gray J. Production of a polysaccharide by *Staphylococcus aureus* III Action of penicillins and polysaccharides on enzymic lysis. Proc Soc Exp Biol Med 116:317–323, 1964.
2. Warren GH, Stasny JT. Electron microscopic study of the action of nafcillin on *Staphylococcus aureus*. Chemotherapy 13:356–361, 1968.
3. Dutcher BS, Reynard AM, Beck ME, Cunningham RK. Potentiation of antibiotic bactericidal activity by normal human serum. Antimicrob Agents Chemother 13:820–826, 1978.
4. Lorian V. Some effects of subinhibitory concentrations of penicillin on the structure and division of staphylococci. Antimicrob Agents Chemother 7: 864–870, 1975.
5. Friedman H, Warren GH. Antibacterial activity of cyclacillin in vivo and in vitro: Effects on gram-negative organisms. Prog Chemother 2:119–126, 1974.
6. Friedman H, Warren GH. Antibody-mediated bacteriolysis: Enhanced killing of cyclacillin-treated bacteria. Proc Soc Exp Biol Med 153:301–304, 1976.
7. Friedman H, Warren GH. Enhanced susceptibility of penicillin-resistant staphylococci to phagocytosis after in vitro incubation with low doses of nafcillin. Proc Soc Exp Biol Med 146:707–711, 1974.
8. Friedman H, Warren GH. Cyclacillin-induced potentiation of *Escherichia coli* immunogenicity in vivo and in vitro. Chemotherapy 23:324–336, 1977.

Received May 5, 1981. P.S.E.B.M. 1982, Vol. 169.