PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 301-304 (1976)

Antibody-Mediated Bacteriolysis: Enhanced Killing of Cyclacillin-Treated Bacteria (39533)

HERMAN FRIEDMAN AND GEORGE H. WARREN

Departments of Microbiology, Albert Einstein Medical Center, Philadelphia, Pennsylvania 19141, and Wyeth Laboratories, Inc., Radnor, Pennsylvania 19087

Variations in the susceptibility of a microorganism to various antibiotics have generally been attributed to differing effects of the agents on bacterial metabolism. Recent studies, in this and other laboratories, have indicated significant differences in the susceptibility of a microorganism to a single antibiotic when tested *in vitro* and *in vivo*. For example, cyclacillin, a semisynthetic penicillin, is much more effective against *Escherichia coli in vivo* than *in vitro* (1, 2). Earlier studies in this laboratory suggested that the difference may be due to a synergistic effect of host factors present *in vivo* and absent *in vitro* (3, 4). For example, *E. coli* incubated with sublethal doses of cyclacillin *in vitro* were more susceptible to phagocytosis by rodent leukocytes than untreated bacteria or bacteria treated with an agent which had no effect on the organism.

The present study was designed to determine the extent of influence of cyclacillin on host factors associated with specific lymphoid cell activation i.e., to an antibody responsiveness *in vivo*. It was found that antibiotic-treated *Escherichia coli*, although not directly killed by a low dose of cyclacillin, nevertheless became highly susceptible to complement-dependent antibody-mediated bacteriolysis.

Materials and methods. E. coli strain 0127:B8, previously used for immunologic assays concerning responses at the single cell level, was passaged for 18 hr at 37° on brain heart infusion (BHI) agar slants (Difco Laboratories, Detroit, Mich.). For *in vitro* testing the bacteria were cultured overnight at 37° in 10 ml of BHI broth, harvested by centrifugation, and washed several times in sterile saline at 4°. The minimal inhibitory concentration (MIC) of cyclacillin and the reference standards, ampicillin and penicillin G, against the *E. coli* was determined by standard serial twofold dilution assays. Antibiotic dilutions were prepared in 0.5-ml volumes of sterile BHI broth in 13 × 75-mm-diameter tubes closed with plastic stoppers, using a stock solution containing 100 μg of each antibiotic per milliliter of saline. For MIC determinations, 0.1 ml of a washed overnight culture of the *E. coli* (2×10^7 bacteria) was added to each antibiotic dilution and the tubes incubated at 37° for 12 to 18 hr. The antibacterial concentration was determined qualitatively by examining the tubes for visual growth and quantitatively by transferring 0.1 ml to 10 ml of melted nutrient agar which was then poured rapidly onto the surface of a petri plate. After incubation at 37° for 18 hr the numbers of colonies on the plates were counted to determine the 50% inhibitory point for bacterial growth.

To determine the effect of the antibiotic on *in vitro* susceptibility of *E. coli* to antibody and complement, 0.5 ml of a 10^8 concentration of viable bacteria was incubated with an equal volume of a sublethal concentration of cyclacillin, or, as a control, with a similar or 10-fold higher concentration of penicillin G. After an incubation of 30–120 min at 37°, the bacteria were washed several times with saline and used as the target for complement-dependent antibody-mediated bacteriolysis. For this purpose hyperimmune rabbit serum was obtained from animals injected three to four times over a 2-month period with heat-killed *E. coli* (10^8 bacteria per injection). Sera obtained on the 10th day after the last injection were heated for 20 min at 56°. These sera showed a bacteriolytic titer of 1:2048 when added in 0.1 volumes to an equal volume of complement (guinea pig serum diluted 1:20) and 10^8 *E. coli* in 0.1 ml of saline until no killing was evident without complement. In addition, dispersed cell suspensions of the spleens of mice immunized 5 to 6 days ear-

lier by ip injection with heat-killed *E. coli* (10^8 bacteria) were prepared in sterile Hanks' solution by the usual "teasing" technique. These cells were used in a direct bacteriolytic plaque assay for enumerating specific anti-*E. coli* plaque forming cells (PFC) exactly as described elsewhere (5). PFCs appearing in agar plates containing spleen cells from immunized mice and viable *E. coli* were considered due to 19S IgM-secreting immunocytes. No PFCs appeared without complement.

Results. Increased susceptibility of *E. coli* to antibody-complement-mediated bacteriolysis was evident after incubation with 1–2 μg of cyclacillin for a time as short as 60–120 min. The concentration of cyclacillin was one-fifth to one-tenth the MIC. As can be seen in Table I, use of untreated *E. coli* as the target bacteria for the serologic tests gave a titer of approximately 1:1600 (50% inhibition) when incubated with antiserum and guinea pig complement. Use of the cyclacillin-treated *E. coli* as the target antigen in the serologic bacteriolytic tests gave a titer of 1:12,800 to 1:25,600 when incubated in the same manner with the antiserum and complement. *E. coli* incubated with the same doses of ampicillin also showed markedly increased susceptibility to antibody-mediated lysis; in general, these treated bacteria *in vitro* gave average titers slightly lower than those obtained with the cyclacillin-treated bacteria. *E. coli* incubated for 60 min with either saline or penicillin G at the same or a 10- to 50-fold higher concentration resulted in a bacterial preparation which gave essentially the same titer as untreated *E. coli*. Increased lysis occurred when the *E. coli* were incubated for longer time periods with the 1- to 2-μg dose of cyclacillin or ampicillin, but not penicillin G. Maximum lysis occurred after 4–8 hr of incubation at 37°.

E. coli incubated with cyclacillin for 60–240 min at 37° prior to washing and incorporation into agar plates for the PFC assay resulted in many more bacteriolytic plaques. As is evident in Table II, splenocytes from immunized mice showed approximately 300–400 PFCs per million cells tested. When the same numbers of splenocytes were incubated in agar plates containing cyclacillin-treated bacteria, the number of PFCs was usually at least three- to fourfold higher (1500 to 2000 PFCs per million splenocytes). Although many of the bacteriolytic plaques were about the same size as those observed in plates containing untreated *E. coli*, it is important to note that about 30% were relatively smaller (approximately one-half to one-third the size of those observed on plates containing untreated bacteria). However, even if only

TABLE I. EFFECT OF ANTIBIOTICS ON SUSCEPTIBILITY OF *Escherichia coli* TO ANTIBODY-MEDIATED KILLING IN THE PRESENCE OF GUINEA PIG COMPLEMENT.[a]

Antibiotic	Concentration (μg)	Antiserum dilution[b]								
		1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:25,600	0
None		0	0	±	2+	4+	4+	4+	4+	4+
Cyclacillin	1.0	0	0	0	0	0	±	1+	3+	4+
	5.0	0	0	0	0	0	0	0	±	3+
	10.0	0	0	0	0	0	0	0	0	2+
Ampicillin	1.0	0	0	0	±	2+	4+	4+	4+	4+
	5.0	0	0	0	0	±	2+	4+	4+	4+
	10.0	0	0	0	0	0	0	±	3+	3+
Penicillin G	10.0	0	0	±	2+	4+	4+	4+	4+	4+
	50.0	0	0	±	2+	4+	4+	4+	4+	4+

[a] Bacteria (10^8) were incubated with indicated dose of antibiotic at 37° for 2 hr, washed, and then incubated with serial dilutions of rabbit anti-*E. coli* antibody plus guinea pig complement diluted 1:20 for 18 hr at 37°; cultures with antibiotic only (no serum or complement) resulted in bacterial growth comparable to that in last column (3+ or 4+).

[b] 0 = no growth; ± = trace growth; 2+ or 3+ = slight to moderate growth (20–50% inhibition); 4+ = turbid growth (no inhibition).

TABLE II. INFLUENCE OF ANTIBIOTICS ON SUSCEPTIBILITY OF *Escherichia coli* TO ANTIBODY-MEDIATED LYSIS IN BACTERIOLYTIC PLAQUE ASSAY IN AGAR GEL CONTAINING SPLENOCYTES FROM IMMUNIZED MICE AND COMPLEMENT.

Antibiotic	Number of PFCs per 10^6 spleen cells[a]		
	Large	Small	Total
None (controls)	315 ±31	58 ±8	373 ±46
Cyclacillin (1–2 μg)	1410 ±295	455 ±48	1865 ±342
Ampicillin (1–2 μg)	868 ±214	305 ±76	1173 ±292
Penicillin G (10–20 μg)	325 ±28	56 ±9	381 ±36

[a] Average number of bacteriolytic plaques for five to six or more agar plates, each containing 10^6 spleen cells pooled from mice immunized 5 days earlier with 10 μg of *E. coli* LPS; small plaque = smaller than 0.05 mm in diameter; agar plates contained inoculum of viable *E. coli* incubated for 120 min with indicated antibiotic before washing and plating. No PFCs appeared in control plates with bacteria only and no spleen cells.

large PFCs were counted, the increase in number was still approximately three times higher than that observed with untreated bacteria. *E. coli* incubated with a 10 times higher dose of penicillin G still resulted in the same number of PFCs as that observed with bacteria incubated in saline only.

Discussion. Earlier studies showed that *in vitro* nafcillin treatment of *Staphylococcus aureus* results in enhancement of subsequent phagocytosis by normal mouse peritoneal exudate cells (6). This enhanced phagocytosis was not due to a direct synergism of the antibiotic with the phagocytic cells per se. This model system differed significantly from studies by other investigators in which addition of an antibiotic to an incubation mixture containing a target organism and phagocytes resulted in increased uptake of microorganisms. In such situations the antibiotic apparently served only to inhibit extracellular division of the bacteria. In the present study, as well as in previous studies in this laboratory with phagocytic cells (6), the antibiotic was incubated with the target organism for a specific length of time and the microorganisms were then washed. It seems likely that the exposure of the bacteria to the antibiotic *in vitro* at 37° resulted in subtle changes in bacterial surface structure and/or physiology. Since penicillin G and its derivatives are known to alter and affect mainly cell wall metabolism, resulting in altered cell wall structure, it seems likely that a sublethal dose of an antibiotic such as cyclacillin which shows *in vivo* and *in vitro* efficacy against *E. coli* may result in surface alterations. Thus lesions on the surface of the bacteria may occur which cannot be detected by usual means, including detecting inhibition of cell growth. Nevertheless, these subtle changes may make the organism more susceptible to lysis by antibody and complement and/or reduce the effectiveness of cell surface repair after incubation.

It is important to note that these results are relatively similar to a recent study with mammalian cells by Segerling *et al.* (7). In that system, the ascitic forms of two antigenically distinct guinea pig hepatomas showed marked enhancement of susceptibility to killing by antibody plus guinea pig complement after *in vitro* incubation with small doses of chemotherapeutic agents. The increased killing was dependent on the drug dose and not on increased antigenic expression or fixation of the early components of guinea pig complement. The beneficial effects of various chemotherapeutic agents, including antibiotics, in the treatment of microbial infections might be similar to that proposed for the treatment of hepatomas by drugs, i.e., increased susceptibility to killing by antibody plus complement occurs after prior incubation with small amounts of a chemotherapeutic agent (7). Thus the present study suggests a possible explanation for the earlier results concerning increased susceptibility of antibiotic-treated *E. coli* to host factors and provides a possible mechanism for the greater *in vivo* efficacy of some antibiotics as compared to *in vitro* efficacy. Further studies are in progress concerning the relationship of organism susceptibility to immune factors after treatment with antibiotics.

Summary. Incubation of *Escherichia coli* with sublethal concentrations of the semisynthetic penicillins cyclacillin and ampicillin substantially increased the susceptibility of the bacteria to subsequent lysis by serum

antibody or specifically immunized murine spleen cells in the presence of guinea pig complement. The effect was dependent on the dose of the antibiotic and the length of incubation time and appeared to be due to subtle alterations in the bacterial surface which rendered the organisms more susceptible to immunologic lysis. These results provide a possible mechanism for the greater *in vivo* efficacy of some antibiotics as compared to their *in vitro* activity.

1. Warren, G. H., Rosenman, S. B., Stone, J. L., and Yurchenco, J. A., *in* "Proceedings of an International Symposium," Aachen, Nov. 30–Dec. 1, 1972. Arzneimittel-Forsch., Beiheft No. 25, p. 14 (1973).
2. Yurchenco, J. A., Hopper, M. W., Vince, T. D., and Warren, G. H., Chemotherapy **15,** 209 (1970).
3. Friedman, H., and Warren, G. H., Experientia **27,** 1241 (1971).
4. Friedman, H., and Warren, G. H., Progr. Chemother. **2,** 119 (1974).
5. Schwartz, S. A., and Braun, W., Science **149,** 200 (1965).
6. Friedman, H., and Warren, G. H., Proc. Soc. Exp. Biol. Med. **146,** 707 (1974).
7. Segerling, M., Ohanian, S. H., and Borsos, T., Science **188,** 55 (1975).

Received May 10, 1976. P.S.E.B.M. 1976, Vol. 153.