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Enhanced Susceptibility of Penicillin-Resistant Staphylococci to Phagocytosis after *in Vitro* Incubation with Low Doses of Nafcillin (38177)

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It is widely accepted that the effectiveness of antibiotics against susceptible bacteria is due to a direct action of the antimicrobial agent on the target microorganism. Nevertheless, it seems likely that host factors may also contribute to the efficacy of an antimicrobial agent *in vivo,* although probably by an indirect means. For example, earlier studies (1–3) showed that the *in vitro* treatment of penicillin-resistant strains of *Staphylococcus aureus* with subinhibitory levels of nafcillin significantly increased the susceptibility of these bacteria to lysis by lysozyme, a ubiquitous enzyme present in most tissues and secretions of higher organisms. This work suggested that a very low *in vivo* concentration of the semisynthetic penicillin may effectively operate through host response mechanisms, rendering the bacterial organisms more vulnerable to body defenses than they would be if no penicillin had been administered. In the present study it was found that similar subinhibitory concentrations of nafcillin affected staphylococci *in vitro* so that the bacteria became more sensitive to phagocytosis by peritoneal exudate cells from normal mice. As in previous studies, the small concentrations of the nafcillin used had no direct effect on the viability of the bacteria studied.

Materials and Methods. A stock solution of sodium nafcillin monohydrate (100 μg/ml) was prepared and stored at 4° until used. Appropriate ten-fold dilutions of the drug were made in saline just before use. Comparable concentrations of penicillin G, sodium methicillin and sodium oxacillin were prepared and used as control antibiotics.

Test bacteria. Freshly isolated *S. aureus* strains were cultured from clinical wounds from hospitalized patients and shown to be highly virulent for mice. Each of the bacterial isolates secreted coagulase, was hemolytic on blood agar, and was resistant to penicillin G as determined by tube dilution assays. At least 10 different isolates were individually tested in this study and the results obtained were the same regardless of strain. For each experiment a fresh isolate of the *S. aureus* was grown for 18 hr at 37° in brain heart infusion (BHI) broth. The bacteria were collected by centrifugation and washed several times in broth; the organisms were then resuspended in sterile saline to a standard concentration of 5×10^8 viable bacteria per ml broth.

Experimental animals. Normal NIH albino (A) mice were obtained from Huntington Farms, Philadelphia, Pennsylvania, and used as the source of peritoneal exudate (PE) cells. For this purpose the mice were injected intraperitoneally with 0.1 ml sterile thioglycollate (Difco Laboratories, Detroit, Michigan) 24 hr earlier. The PE cells were obtained by aspiration and washed several times by centrifugation in the cold with sterile physiological Hanks' solution and then suspended to a concentration of 5×10^7 nucleated monocytes per ml Hanks' solution. Cell viability and total cell numbers were determined by hemocytometer count and trypan blue stain technique. The PE cell population consisted of

approximately 65–75% macrophages, the remainder being lymphocytes and other cell types.

Phagocytic tests. The PE cells readily phagocytized carbon and yeast particles, as well as staphylococci, *in vitro*. For the phagocytic test with antibiotic-treated bacteria, graded concentrations of the test antibiotic were first added to sterile BHI (2 ml) containing a standard inoculum of the freshly harvested and washed staphylococci. Following incubation at 37° for varying length of time, 0.5 ml of the antibiotic-bacteria mixture was added to test tubes (13 × 75 mm) containing 2 ml of a washed suspension of PE cells. In general, either 5×10^6 or 5×10^7 viable PE cells were present in each tube. Control tubes contained either antibiotic alone, staphylococci alone, or staphylococci plus antibiotic without PE cells. After incubation for 1–3 hr at 37° the number of viable bacteria remaining in each tube was determined by standard pour plate technique, using 0.1 ml volumes in triplicate. In addition, the number of staphylococci in the supernatants after sedimenting the PE cells by low speed centrifugation was determined by additional pour plates. Also, the average number of staphylococci ingested by individual macrophages was determined by appropriate Giemsa and Gram-stain of cell smears. The average number of bacteria present in 100 monocytes, and the percent of cells with more than 5 organisms each, was determined by microscopic examination.

Results. Incubation of the staphylococci with varying concentrations of nafcillin, methicillin or oxacillin resulted in varying degrees of inhibition of bacterial viability. Ten micrograms of each of the antibiotics markedly inhibited growth. A 1.0 μg concentration of nafcillin was also inhibitory. Methicillin and oxacillin were less inhibitory. Incubation of the staphylococci with the same or higher concentrations of penicillin G failed to inhibit growth. As shown previously with stock laboratory cultures of staphylococci (1, 4), a 0.1 μg concentration of nafcillin, methicillin or oxacillin was noninhibitory for all of the clinical isolates of staphylococci used in this study and resulted in no detectable growth inhibition; identical numbers of staphylococci remained when the bacterial cultures were incubated at 37° for a period up to 5 hr in either medium alone or medium containing a 0.1 μg dose of any of the three semisynthetic penicillins.

Treatment of staphylococci with nafcillin (0.1 μg, 1–3 hr, 37°) prior to exposure to PE cells (5×10^7, 1–5 hr, 37°) resulted in a significantly greater inhibition of staphylococcal viability over that observed when staphylococci were exposed to medium alone or PE cells alone (Table I); the additional inhibition compared to that observed in the case of medium alone

TABLE I. Effect of Subinhibitory Concentrations of Antibiotics on Altered Susceptibility of *Staphylococcus aureus* to Phagocytosis by Normal Mouse PE Cells.

Antibiotic tested[a]	Incubation period (hr)				
	0	1	2	3	5
	Bacteria surviving/culture[b]				
None (controls)	4.2	4.8	5.9	8.7	9.9
Nafcillin	4.5	3.2	1.1	0.8	0.1
Methicillin	4.7	5.1	5.3	7.8	8.3
Oxacillin	4.5	4.7	5.8	6.9	8.3
Penicillin G	4.1	4.7	4.9	7.5	8.6

[a] Dose of antibiotic = 0.1 μg; each antibiotic incubated with staphylococci for indicated period of time at 37° before addition of PE cells.

[b] Number of staphylococci ($\times 10^7$) per culture after additional 1 hr incubation with PE cells at 37°.

amounted to approximately 30%–40%. Preincubation of the staphylococci for 1 hr with similar low doses of methicillin, oxacillin or penicillin G had no detectable effect on such phagocytosis; in fact, the number of bacteria in these culture tubes actually increased.

Examination of the average number of organisms ingested by the PE cells during incubation *in vitro* revealed that prior treatment with nafcillin, but not with the other penicillins, resulted in an increased uptake of the bacteria (Table II). For example, when PE cells were incubated with the nafcillin-treated staphylococci, many more macrophages showed intracellular staphylococci; most of these macrophages had, on the average, 2–3 times as many organisms as did those incubated with untreated staphylococci. This effect seemed due to the nafcillin treatment of the bacteria and not to mere incubation at 37° for several hours before addition of the PE cells, since increased phagocytosis did not occur with any of the other antibiotics under similar conditions. Similar effects were noted with all of the individual isolates of the coagulase positive penicillin-resistant staphylococci treated in this manner, regardless of patient source. Furthermore, in additional experiments incubation of the staphylococci and PE cells in medium fortified with 10% sterile isologous mouse or normal rabbit serum resulted in essentially the same findings, i.e., incubation with low doses of nafcillin, but not methicillin, oxacillin or penicillin G, markedly increased the relative phagocytosis of test staphylococci by normal mouse PE cells as compared to the phagocytosis of the same staphylococci incubated in serum-containing medium without antibiotics.

Discussion and Conclusion. The results of these studies showed that incubation of penicillin-resistant staphylococci with nafcillin, at a noninhibitory concentration, increased the susceptibility of the bacteria to subsequent phagocytosis *in vitro* by PE cells from normal mice. Larger doses of nafcillin, as well as of methicillin or oxacillin, inhibited the growth of the staphylococci. However, only the low dose of nafcillin, i.e., 0.1 μg, affected the subsequent phagocytosis by the normal mouse PE cells. The level of phagocytosis by individual macrophages was markedly enhanced when the staphylococci were first exposed to this low dose of nafcillin.

It is noteworthy that a relatively similar study with lysozyme suggested that nafcillin could profoundly affect the integrity of the *S. aureus* cell wall (1, 4). The data obtained in that study, as well as in the present one, indicate that the effect is dose dependent, i.e., low doses of nafcillin potentiate lysis of the bacteria by lysozyme, whereas larger doses result in direct growth inhibition. Approximately 1/80th the con-

TABLE II. Effect of Antibiotic on Presence of Staphylococci in Mouse PE Cells Incubated with Antibiotic-Treated Bacteria.

Antibiotic tested[a]	Staphylococci in PE cell lysate ($\times 10^3$) [b]	Average number of staphylococci per PE cell[c]	Percent PE cells with staphylococci
None (controls)	168 ± 23	5 ± 3	6.8 ± 1.9
Nafcillin	25 ± 5	18 ± 9	16.3 ± 3.5
Methicillin	112 ± 29	6 ± 3	3.9 ± 1.2
Oxacillin	173 ± 48	8 ± 3	5.8 ± 2.2
Penicillin G	62 ± 21	7 ± 3	6.1 ± 2.8

[a] Indicated antibiotic (0.1 μg dose) added to staphylococcal culture (5×10^8 bacteria per ml) for 2 hr at 37° before further incubation with 5×10^7 mouse PE cells *in vitro*.

[b] Average number of staphylococci present in lysate of PE cell cultures after incubation for 1 hr at 37° with antibiotic-treated bacteria (±S.D.).

[c] Average number of ingested staphylococci as determined by Gram-stain and microscopic examination (±S.D.).

centration of nafcillin required to inhibit growth of the organisms was effective in making the bacteria more susceptible to lysozyme destruction. Similarly, in the present study, an equally subinhibitory dose of nafcillin increased the susceptibility of staphylococci to phagocytosis by normal mouse PE cells.

The results of the present study, coupled with the results of the previous study with lysozyme (4), suggest that nafcillin interferes with some cellular mechanism necessary for maintenance of the surface integrity of the organism without affecting viability. Such alteration could influence the interaction of staphylococci with cells of the host defense mechanism, since even a subinhibitory dose of antibiotic, below that considered therapeutic, affects the ability of the staphylococci to resist phagocytosis. It seems likely that if a bacterium is first altered by the antibiotic, resulting in a subtle but significant change in cell wall integrity, the host may react to the microorganism in a more positive manner. Such a response could explain the many clinical and laboratory observations which show that an antibiotic may be effective against a microorganism even when present in body fluids at a concentration below that considered inhibitory.

There have been very few, if any, studies reported from other laboratories concerning the synergistic interaction between an antibiotic and host PE cells, either *in vitro* or *in vivo*. Most studies concerning antibiotics and lymphoid cells *in vitro* have dealt with the direct effects of either the antimicrobial agent or leukocytes on bacteria *per se* (5–8). Antibiotics have usually been incorporated into test mixtures containing bacteria and phagocytic white blood cells only to minimize the extracellular growth of the bacteria. Most investigators believe that antibiotics influence phagocytosis by directly diminishing the viability of the bacteria only at the point of contact with the phagocyte—either outside the phagocytic cell or inside the cell if the antibiotic is capable of penetrating it (7, 8). The present study, on the other hand, involves a different type of analysis, i.e., the effect of a subinhibitory concentration of an antibiotic on phagocytosis of staphylococci after treatment with the antimicrobial agent.

In the present study, the altered susceptibility of the penicillin-resistant staphylococci to macrophage-dependent phagocytosis after exposure to a subinhibitory dose of nafcillin *in vitro* probably reflects events which may also occur *in vivo*. This is further suggested by the additional experiments indicating similar results with serum supplements in the test medium, a factor known to be important for phagocytosis *in vivo*. Thus the antibiotic may affect a microorganism, short of actual killing, by increasing the likelihood of increased phagocytosis by macrophages or other phagocytes; this may be one of the mechanisms whereby the action of an antibiotic is potentiated by host factors.

Summary. Incubation of penicillin-resistant staphylococci with a subinhibitory concentration of nafcillin increased the susceptibility of the organisms to *in vitro* phagocytosis by normal mouse peritoneal exudate cells (macrophages). Methicillin and oxacillin were effective as inhibitors of bacterial growth only when used in relatively large doses; subinhibitory doses did not significantly affect subsequent phagocytosis by the PE cell. Penicillin G had no detectable effect on the staphylococci used in this system, either as a direct inhibitor of microbial growth or by influencing phagocytosis. The synergistic effect described here between nafcillin and macrophages may be related to the clinical efficacy reported for this antibiotic even when present only in low doses.

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