

Enhanced Phagocytosis, Killing, and Serum Sensitivity of *Escherichia coli* and *Staphylococcus aureus* Treated with Sub-MICs of Imipenem

LUIGI E. ADINOLFI¹ AND PETER F. BONVENTRE^{2*}

Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524,² and Clinic of Tropical and Subtropical Diseases, 1st Medical School, University of Naples, 80135 Naples, Italy¹

Received 21 December 1987/Accepted 8 April 1988

The influence of pretreatment of *Escherichia coli* and *Staphylococcus aureus* with sub-MICs of the new β -lactam antibiotic imipenem on phagocytosis and killing by murine peritoneal macrophages and the susceptibility of these organisms to serum bactericidal activity were studied. The effects of imipenem, a round form inducer in gram-negative rods, and piperacillin, a filamentous form inducer, were compared. Bacteria grown in the presence of sub-MICs of imipenem or piperacillin were incubated for 30 min with macrophage monolayers in the absence of antibiotic. Phagocytosis, killing, and survival within macrophages were evaluated by microbiological and fluorescence microscope assays. Bacteria grown in the presence of a sub-MIC of imipenem were phagocytized and killed in numbers significantly higher than untreated or piperacillin-treated bacteria were. Intracellular bacteria pretreated with a sub-MIC of imipenem were also readily killed by lymphokine-activated macrophages. Prior treatment with a sub-MIC of imipenem resulted in an increased susceptibility of *E. coli* but not *S. aureus* to the bactericidal activity of immune serum. Imipenem treatment and immune serum acted synergistically to enhance phagocytosis and killing. The data indicate that exposure of *E. coli* and *S. aureus* to a sub-MIC of imipenem enhances the susceptibility of these potential pathogens to cellular and humoral host defense mechanisms.

The activity of antibiotics against clinically significant bacteria is usually expressed in terms of concentrations that either inhibit growth or kill microorganisms in vitro. Concentrations of antibiotics which are less than the MIC are defined as sub-MICs (18). Evidence is accumulating that antibiotics may be beneficial to infected hosts even when present at sub-MICs (8, 15, 23). This effect appears to be independent of the bactericidal potential of the antibiotic but is related to structural or metabolic changes which augment the susceptibility of bacterial pathogens to humoral and cellular defenses. Therefore, antibiotics at sub-MICs that act in concert with host defenses may be clinically significant.

We undertook this study to determine whether pretreatment of *Escherichia coli* and *Staphylococcus aureus* with sub-MICs of imipenem altered their susceptibility either to serum bactericidal activity or to phagocytosis and killing by murine macrophages. Imipenem, a β -lactam antibiotic and member of a new class, the carbapenems, is the first clinically available derivative of thienamycin (11). Imipenem possesses an unusually broad antibacterial spectrum, is biologically active at extremely low concentrations, and exhibits no cross resistance with other β -lactam antibiotics (13). Comparative studies indicate that there is a greater potency and wider spectrum of activity for imipenem than for other β -lactams against both gram-positive and gram-negative species (12). Another interesting attribute of imipenem is that at sub-MICs, the drug fails to induce the filamentation of gram-negative bacteria, as is the case with other penicillin derivatives. Imipenem, instead, induces round forms (22). Therefore, as another facet of this study, we compared the effects of imipenem at sub-MICs with that of piperacillin, which induces filamentous forms of *E. coli*.

MATERIALS AND METHODS

Chemicals. Imipenem (*N*-formimidoyl thienamycin monohydrate) was provided by Merck & Co., Inc. (Rahway, N.J.), and piperacillin (Lederle Laboratories, Pearl River, N.Y.) was obtained from the University Hospital pharmacy (University of Cincinnati College of Medicine). Minimal essential medium (MEM) was purchased from GIBCO Laboratories (Grand Island, N.Y.). In all experiments, MEM was supplemented with 10 μ M essential amino acids, 10 μ M nonessential amino acids, and 10 μ M sodium pyruvate (GIBCO). Lysostaphin was purchased from Sigma Chemical Co. (St. Louis, Mo.). A stock solution of 200 μ g/ml in phosphate-buffered saline (PBS) was prepared and stored at -20°C . All other chemicals were reagent grade and were obtained from commercial sources.

Bacteria. *E. coli* 775 and *S. aureus* 682 were recent clinical isolates obtained from J. Staneck, University Hospital, University of Cincinnati College of Medicine. The isolates were maintained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) slants stored at 4°C .

MICs of imipenem and piperacillin on test organisms. MICs of the antibiotics against *E. coli* 775 and *S. aureus* 682 were determined by a microtiter broth dilution method by using serial twofold dilutions of the individual antibiotic in Mueller-Hinton broth (GIBCO), and this broth was inoculated with 10^5 log-phase organisms per 0.1 ml of medium. The microtiter plates were incubated at 37°C for 18 h before the results were read. The MIC was defined as the lowest concentration of antibiotic resulting in the complete inhibition of bacterial growth. Under the conditions described, the MICs of imipenem for *E. coli* and *S. aureus* were 0.06 and 0.0075 mg/liter, respectively, while the MICs of piperacillin for the *E. coli* and *S. aureus* isolates were 0.97 and 3.90 mg/liter, respectively.

* Corresponding author.

Pretreatment of bacteria with sub-MICs of antibiotics. *E. coli* and *S. aureus* were inoculated into 10 ml of BHI broth or BHI broth containing a concentration of antibiotic at one-fifth of the MIC of imipenem or piperacillin. After 5 h of growth at 37°C, the bacteria were centrifuged, washed twice in PBS (pH 7.35), and suspended in PBS. The bacterial suspensions were adjusted to 2.0×10^8 CFU/ml for the control and antibiotic-treated organisms. Enumeration of viable units in both cases was accomplished by colony counts on BHI agar plates. Alterations of normal bacterial morphology as a result of antibiotic treatment were noted by light microscopy of Gram-stained smears. Round forms induced by imipenem and filamentous forms induced by piperacillin reverted to normal morphology when the antibiotic-treated *E. coli* was cultured on BHI agar plates. *S. aureus* grown in the presence of a sub-MIC of imipenem or piperacillin maintained its original spherical form but was ca. 2 times larger than the control bacteria.

Immune sera. BALB/c mice (Harlan, Indianapolis, Ind.) were immunized with the isolates to obtain immune sera. Mice received three injections of *E. coli* or *S. aureus* over a period of 1 month. On days 1 and 15, they were given 10^8 heat-killed organisms intraperitoneally in incomplete Freund adjuvant (GIBCO). Ten days later the mice received 10^7 viable bacteria intraperitoneally. One week later the mice were anesthetized and bled by cardiac puncture. Immune sera were pooled and stored in portions at -70°C . Normal heat-inactivated fetal bovine serum (FBS) was obtained from GIBCO and kept at -20°C .

Lymphokines. The production and assay of macrophage-activating lymphokines from C3H/HeN mice were done as described previously (16). In brief, samples (20 ml) of spleen cell suspension (5.0×10^6 /ml) with or without 5 μg of concanavalin A per ml were incubated in tissue culture flasks (75 cm²) in an upright position for 48 h at 37°C in 5% CO₂. At the end of the incubation period, concanavalin A was added to the unstimulated control culture, and then the cells were removed by centrifugation at $400 \times g$ for 10 min. Sephadex G-10 (10 mg/ml; Pharmacia Fine Chemicals, Piscataway, N. Y.) was added to the supernatants and mixed for 10 min to absorb the residual concanavalin A. The lymphokine-rich supernatants were filter sterilized and stored at -20°C .

Collection and culture of peritoneal macrophages. Thioglycolate-elicited mouse peritoneal macrophages were obtained by injecting 2.0 ml of 3% Brewer thioglycolate medium (BBL Microbiology Systems, Cockeysville, Md.) in BALB/c mice intraperitoneally. After 3 days, peritoneal exudate cells were collected with MEM containing 10 U of heparin per ml. Peritoneal exudate cells were centrifuged at $650 \times g$ for 10 min, erythrocytes were lysed with cold 0.83% NH₄Cl, and the exudates were washed twice with MEM. The cell pellets were suspended in 5 ml of MEM-10% FBS. Cell enumeration and viability were determined by counting cells diluted in 0.05% trypan blue with a hemacytometer. Cell viability was routinely greater than 95%. Adherence was used to separate macrophages from nonadherent cells; 2.0×10^6 cells in 0.4 ml of culture medium were applied to 18-mm-diameter circular glass cover slips (Arthur Thomas Co., Philadelphia, Pa.) and allowed to adhere for 1 h at 37°C in 5% CO₂. Nonadherent cells were washed away, and the macrophage cultures were placed in 12-well culture plates (Linbro; Flow Laboratories, Inc., McLean, Va.).

Experimental protocol. In order to study phagocytosis and killing of microbes by phagocytic cells, reliable in vitro assays are essential. It is particularly difficult to measure rates of phagocytosis and killing with the assurance that the

data generated are not subject to misinterpretation. We have previously described standardized assays, in part here and elsewhere (1), whereby phagocytosis and killing of bacteria by mononuclear phagocytes can be measured accurately.

(i) Phagocytosis and killing. Phagocytosis and killing of *E. coli* and *S. aureus* (untreated or treated with sub-MICs of antibiotics) were evaluated in vitro by adding 2 ml of MEM-10% FBS containing an inoculum of 2.0×10^7 CFU/ml to macrophage monolayers. Bacteria and macrophages were allowed to interact for 30 min, after which the number of bacteria in the cover slip culture supernatants of all groups was determined. Several cover slips in each group were washed with a jet of 30 ml of cold PBS. In experiments with *E. coli*, they were placed directly into sterile scintillation glass vials containing 4 ml of cold PBS. In experiments with *S. aureus* the cover slips were treated with 10 mg of lysostaphin per liter for 5 min at 4°C to kill residual extracellular bacteria, rewashed, and placed into scintillation vials. Incubation at 4°C prevented any effects on intracellular *S. aureus*, since lysostaphin does not enter cells at low temperatures but does eliminate extracellular bacteria (21). Cover slips were sonicated at 18 kcycles/s for 20 s. This brief burst disrupted macrophages without affecting bacterial viability. Viable bacteria in supernatants and cell lysates were determined by plating 10-fold serial dilutions on BHI agar plates, and CFU were determined after 24 h. Bacterial multiplication in the medium over a 30-min period was eliminated as a factor by counting the bacterial inoculum incubated in MEM-10% FBS in the absence of macrophages; no significant increase in control or antibiotic-treated bacteria occurred during the 30-min period. After washing and lysostaphin treatment for the experiments with *S. aureus*, infected cover slip cultures were reincubated in bacterium-free medium (MEM-FBS) for 3 h at 37°C in 5% CO₂. At 60, 120, and 180 min, samples were taken in triplicate, rewashed, and processed as described above to determine the number of intracellular viable *E. coli* and *S. aureus*.

(ii) Calculations of bacterial phagocytosis and killing. Phagocytosis was expressed as the decrease in the initial number of viable extracellular bacteria in the inoculum by the following equation: $P(t) = N_0 - N_1$, in which P is phagocytosis at time t , N_0 is the number of untreated or sub-MIC-treated viable bacteria incubated in medium for 30 min at 37°C in the absence of macrophages, and N_1 is the number of viable extracellular bacteria in macrophage monolayer supernatants after 30 min of incubation. Bacterial killing was calculated by the following equation: $K(t) = P(t) - V(t)$, in which $K(t)$ is killing at time t , $P(t)$ is phagocytosis at time t , and V is the number of viable intracellular bacteria at time t . In our previous study (1), we established that the decrease in the number of viable bacteria at time t is a valid measure of the antibacterial effect exerted by the macrophages between time t_0 and t .

(iii) Differentiation between intracellular and extracellular bacteria. Intracellular viable bacteria were determined by standard colony counts (CFU) and by a sensitive acridine orange fluorescence method in which crystal violet was used to quench the fluorescence of noningested organisms (9, 10, 19). Acridine orange-labeled bacteria fluoresce apple green if they are viable and brick red if they are nonviable. Cultures of infected monolayers were washed and stained with acridine orange (0.1 mg/ml in 0.1 M PBS [pH 7.2]) for 1 min and washed in cold PBS to remove excess stain. To distinguish intracellular organisms from extracellular or adherent bacteria, the cells were flooded with crystal violet solution (1

mg/ml in 0.1 M PBS [pH 7.2]) for 1 additional min. The cover slips were washed in Hanks balanced salt solution (pH 7.2), and each cover slip was wet mounted onto a glass slide and examined under a fluorescence microscope with a $\times 100$ oil immersion objective. The number of viable bacteria was scored for a minimum of 300 macrophages. The percentage of viable intracellular bacteria was calculated by the following equation: $(Ib/Tb) \times 100$, in which *Ib* is the number of intracellular viable bacteria scored by the combined acridine orange, crystal violet fluorescence method, and *Tb* is the total number of viable bacteria (intracellular plus extracellular) scored only by the acridine orange method.

After the washing procedure, the percentage of extracellular *S. aureus* was also ascertained by counting the number of organisms in control and lysostaphin-treated samples. Results of previous experiments showed that 0.6 mg (10 times the MIC) of imipenem per liter for *E. coli* 775 had no measurable effect on intracellular viability but that the same concentration of drug was bactericidal for extracellular bacteria in numbers several orders of magnitude greater than were present intracellularly (data not shown). Therefore, we conducted experiments in which residual extracellular bacteria were eliminated by treating macrophage monolayers with 0.6 mg of imipenem per liter for 45 min. Elimination of extracellular *S. aureus* with lysostaphin or *E. coli* with imipenem permitted an accurate determination of intracellular killing, survival, or multiplication within macrophages.

(iv) **Effects of lymphokines on intracellular bacteria.** Macrophages infected with untreated bacteria or bacteria treated with a sub-MIC of imipenem were incubated with 20% (vol/vol) lymphokine-containing spleen cell supernatants over an 18-h period. Temporal samples were sonicated, and the number of intracellular bacteria was determined as described above. Residual extracellular bacteria were eliminated with lysostaphin for the experiments with *S. aureus* and with imipenem (10 times the MIC for 45 min) for the experiments with *E. coli*. No extracellular bacteria were detected by culture or microscopy during the prolonged incubation with the lymphokines.

(v) **Effects of immune serum on survival of *E. coli* and *S. aureus* pretreated with sub-MICs of imipenem.** Untreated and sub-MIC-treated (2×10^7 CFU/ml) bacteria were incubated in the presence of 10% immune serum at 37°C for 90 min, after which appropriate dilutions were plated onto BHI agar. As experimental controls, growth curves of untreated and sub-MIC-treated bacteria in the absence of immune serum were made. No difference was observed in the *in vitro* growth rates in broth cultures of untreated and sub-MIC-treated bacteria.

(vi) **Effects of immune serum on phagocytosis and killing by macrophages of *E. coli* and *S. aureus* pretreated with sub-MICs of imipenem.** Protocols similar to those described above for experiments in which phagocytosis and killing of *E. coli* and *S. aureus* were measured in the presence of FBS were used to determine the effects of immune serum on phagocytosis and killing by macrophages of *E. coli* and *S. aureus* pretreated with sub-MICs of imipenem. The only difference between the two protocols was that FBS was substituted by 10% immune serum both during phagocytosis and during subsequent reincubation of infected macrophages in bacterium-free medium.

Statistics. All data are the means \pm standard deviations of nine single results for each group (three experiments run in triplicate). Bacterial counts were made in triplicate for each point. Student's *t* test was used to compare the means, and a level of 5% was taken to be significant (20).

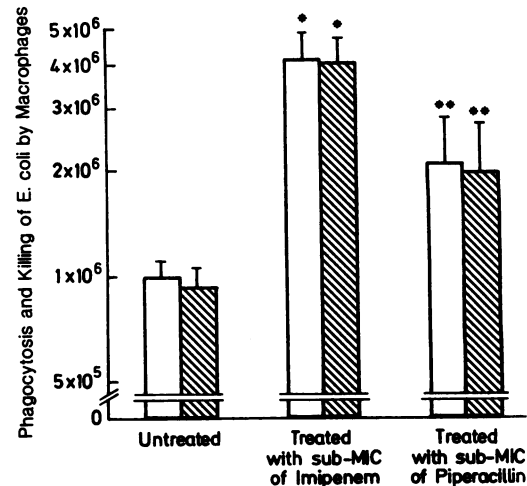


FIG. 1. Phagocytosis (□) and killing (▨) of *E. coli* pretreated with sub-MICs of imipenem or piperacillin by peritoneal murine macrophages. Untreated or sub-MIC-treated *E. coli* (2×10^7 /ml) was incubated with macrophage (2×10^6) monolayers at 37°C in 5% CO₂ for 30 min in the presence of 10% FBS. Phagocytosis and killing were calculated as described in the text. The data are expressed as means \pm standard deviations. Symbols: *, $P < 0.001$ versus results for untreated *E. coli* and $P < 0.01$ versus results for bacteria treated with sub-MICs of piperacillin; **, $P < 0.05$ versus results for untreated *E. coli*.

RESULTS

Phagocytosis, killing, and intracellular fate of *E. coli* and *S. aureus* that were untreated and treated with sub-MICs of imipenem or piperacillin. Experiments to determine the phagocytosis, killing, and intracellular fate of *E. coli* and *S. aureus* were carried out with 10% FBS in the medium since bovine serum at this concentration had no direct bactericidal activity on either organism and provided adequate opsonization of bacteria for efficient uptake. Thus, by avoiding extracellular killing by FBS, the disappearance of bacteria from the medium could be attributed exclusively to phagocytosis and killing by the macrophages.

A correlation of 0.9 between CFU and fluorescence assay values established the validity of both the microbiological and microscopic assays in the assessment of intracellular viable bacteria. The percentage of extracellular bacteria after the washing procedure was less than 10%, as ascertained by the fluorescence assay and by comparing the results obtained in the control with those obtained in the lysostaphin- or imipenem-treated preparations. This small number of residual extracellular bacteria did not affect the interpretation of data since they did not increase significantly during the 3-h period.

(i) *E. coli*. The results obtained with *E. coli* that was untreated and treated with sub-MICs of imipenem and piperacillin are given in Fig. 1. During a 30-min incubation of 2.0×10^7 CFU of bacteria per ml with macrophage (2.0×10^6) cover slip cultures, the parameters of phagocytosis and killing of the three groups of *E. coli* were calculated by using the CFU and microscopic assays. By using the numbers of untreated *E. coli* phagocytized and killed as base-line values, valid comparisons could be made; the number of untreated *E. coli* phagocytized and killed were calculated as $1.06 \times 10^6 \pm 0.24 \times 10^6$ and $9.45 \times 10^5 \pm 0.20 \times 10^5$, respectively. The antibiotics at subinhibitory concentrations altered the bacteria so that they were phagocytized and killed during the same

TABLE 1. Proportion of killed to surviving bacteria phagocytized by murine macrophages during a 30-min period

Bacterium	Killed bacteria treated with sub-MICs of ^a :		
	Untreated	Imipenem	Piperacillin
<i>E. coli</i>	18.8	65.8	32.6
<i>S. aureus</i>	2.75	5.0	1.95

^a Values are the proportion of killed bacteria per 1.0 residual viable bacterium.

30-min period in far greater numbers. The enhancement of phagocytosis and killing after treatment with sub-MICs of imipenem, however, was significantly greater than that after treatment with sub-MICs of piperacillin (Fig. 1). In addition, it is shown in Table 1 that the imipenem-treated *E. coli* was more readily eliminated than the groups that were treated differently. If the ratio of *E. coli* killed to the number of viable units remaining after the 30-min period in the differently treated groups is calculated, it is evident that sub-MICs of antibiotic predispose the bacteria to the bactericidal action of the macrophages. If the ratios are compared, the rate of killing of imipenem-treated *E. coli* was ca. 2 times that of the piperacillin-treated organisms and ca. 4 times that of untreated controls.

(ii) *S. aureus*. The data obtained for *S. aureus* treated with sub-MICs of antibiotics were similar to those obtained for *E. coli*. The methodology was the same except that after the 30 min of incubation of 2×10^7 CFU of *S. aureus* per ml with macrophage monolayers, extracellular bacteria were eliminated with lysostaphin before the number of viable intracellular *S. aureus* was determined. Data for *S. aureus* comparable to those obtained for *E. coli* were obtained (Fig. 2 and Table 1). Again, it is apparent that pretreatment with the β -lactam antibiotics rendered *S. aureus* more amenable to phagocytosis and intracellular killing. Imipenem appeared to be more effective than piperacillin in both respects, although the differences were not statistically significant. The rates of killing, as calculated from the ratios of killed to viable

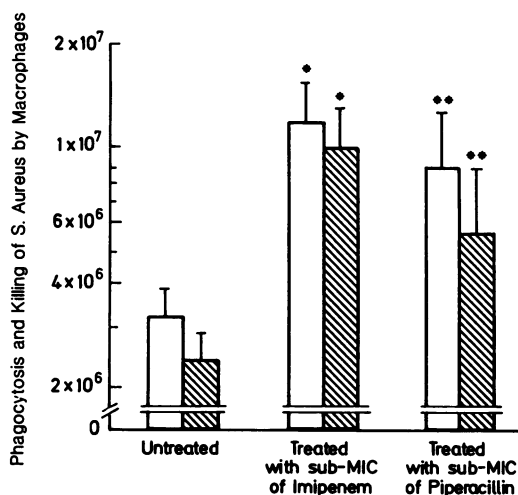


FIG. 2. Phagocytosis (□) and killing (▨) of untreated *S. aureus* or *S. aureus* (2×10^7 /ml) treated with sub-MICs of antibiotic by peritoneal macrophages in the presence of FBS. Experimental conditions and data are expressed as described in the legend to Fig. 1. Symbols: *, $P < 0.001$ versus results for untreated *S. aureus*; **, $P < 0.01$ versus results for untreated *S. aureus*.

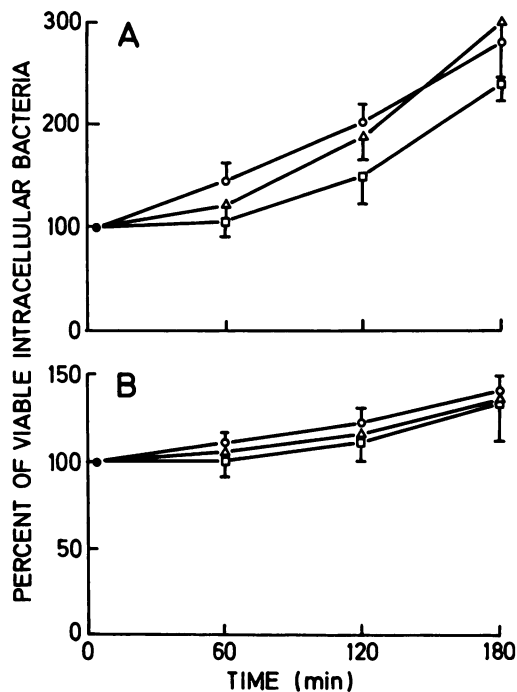


FIG. 3. Fate of residual intracellular viable bacteria that were untreated or treated with sub-MICs of antibiotics. After 30 min of phagocytosis, infected macrophages were reincubated in bacterium-free medium in the presence of 10% FBS. The initial numbers of viable intracellular bacteria were calculated by the difference between phagocytosis and killing values for each group shown in Fig. 1 and 2. (A) *E. coli*. (B) *S. aureus*. Symbols: ○, untreated bacteria; △, bacteria treated with sub-MICs of piperacillin; □, bacteria treated with sub-MICs of imipenem. Data are expressed as a percentage of the mean \pm standard deviation of the initial number of intracellular viable bacteria. Data were calculated as described in the legend to Fig. 1.

intracellular units (Table 1), revealed that the imipenem-treated *S. aureus* was killed at a rate that was ca. 2 times that for piperacillin-treated and untreated *S. aureus*.

(iii) Fate of bacteria within macrophages. The dynamics of phagocytosis and killing described above occurred during an initial 30-min period. It was of interest to know whether the bacteria treated with sub-MICs of antibiotics differed from the untreated bacteria in their susceptibilities to killing on prolonged intracellular residence. In our previous studies (1), we showed that under some conditions, residual viable bacteria can multiply within the phagocytic cells. Intracellular *E. coli* increased measurably in numbers over a 3-h period, while the number of *S. aureus* increased only modestly (Fig. 3). It is interesting that prior treatment with antibiotics did not significantly enhance the killing of the bacteria that remained after the initial 30-min period, during which greater than 90% of phagocytized *E. coli* or *S. aureus* was eliminated. The microscopic assay coupled with the CFU determinations on extracellular fluids performed at the end of the experiment confirmed an absence of significant extracellular bacterial growth.

Fate of bacteria within lymphokine-treated macrophages. In view of the inability of the elicited macrophages to eliminate residual *E. coli* or *S. aureus*, it became of interest to know whether activation with lymphokines (17) altered the intracellular fate of residual viable bacteria. Macrophages were infected as described above and then incubated in bacterium-

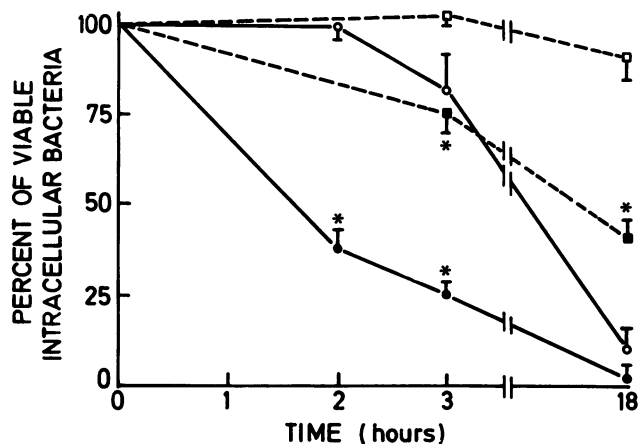


FIG. 4. Effect of T-cell-derived lymphokines on the survival of untreated bacteria or intracellular bacteria treated with sub-MICs of imipenem. Infected macrophages were incubated in bacterium-free medium in the presence of lymphokines (20% [vol/vol]). The initial numbers of viable intracellular bacteria for each group were similar to those calculated by differences (see legends to Fig. 1 and 2). Symbols: □, untreated *S. aureus*; ■, *S. aureus* treated with sub-MICs of imipenem; ○, untreated *E. coli*; ●, *E. coli* treated with sub-MICs of imipenem; *, $P < 0.01$ versus results for the respective untreated bacteria. Data are expressed as a percentage of the mean \pm standard deviation of the initial number of intracellular viable bacteria. Data were calculated as described in the legend to Fig. 1.

free medium containing lymphokine at a concentration of 20% (vol/vol). The presence of lymphokines in the medium significantly altered the kinetics of killing (Fig. 4). The viability of intracellular *E. coli* was reduced by ca. 75% during the first 3 h of incubation with lymphokine and by >95% after 18 h of incubation of the sub-MIC-treated organisms. The kinetics of killing of untreated *E. coli* were considerably retarded, but the eventual clearance of *E. coli* by the lymphokine-treated macrophages occurred after 18 h. Cells not activated with lymphokine permitted the intracellular multiplication of *E. coli* 775 and the eventual destruction of the phagocytic cells after 18 h of incubation (data not shown). In contrast to the results obtained with *E. coli*, *S. aureus* was not eliminated by lymphokine-treated macrophages. However, prior treatment of *S. aureus* with sub-MICs of imipenem permitted a 50% reduction of intracellular *S. aureus* by activated macrophages after 18 h of incubation.

Effect of specific immune serum on bacteria, phagocytosis, killing, and subsequent intracellular fate. Before the effects of immune serum on subsequent phagocytosis and killing by macrophages were determined, it was necessary to determine the bactericidal activity of immune serum on *E. coli* and *S. aureus* in the absence of phagocytic cells. The *E. coli* strain was serum resistant (i.e., 100% survival after 90 min of incubation with 10% immune serum). *S. aureus* exposed to similar conditions was reduced in viability by approximately 33%. *E. coli* exposed to immune serum treated with sub-MICs of imipenem was reduced in viability by ca. 32%, while *S. aureus* treated with sub-MICs of imipenem was not rendered any more susceptible to the bactericidal action of immune serum than untreated *S. aureus* was (i.e., 33% reduction in viability in the presence of immune serum in both cases).

The phagocytosis and killing of *E. coli* and *S. aureus* by macrophages in the presence of immune serum were determined during a 30-min period of incubation. The experimental conditions were the same as those described above (Fig.

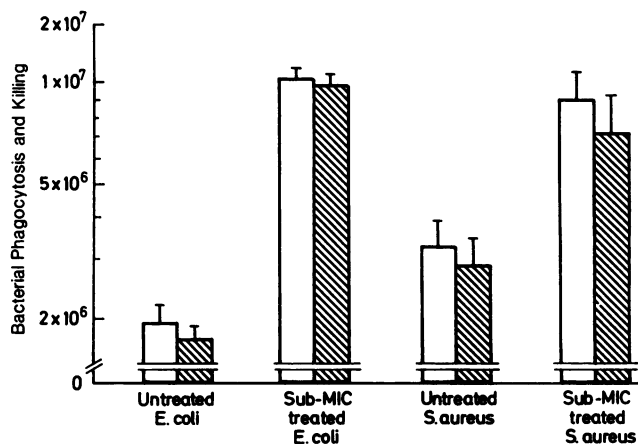


FIG. 5. Phagocytosis (□) and killing (▨) of untreated *E. coli* and *S. aureus* or *E. coli* and *S. aureus* treated with sub-MICs of imipenem by macrophages in the presence of 10% immune serum. Experimental conditions and data are expressed as described in the legend to Fig. 1.

1), except that 10% immune rat serum replaced FBS. With *E. coli* 775, the presence of immune serum augmented the phagocytosis and intracellular killing of untreated bacteria by a factor of 2 above the levels seen in the presence of FBS. The enhanced uptake and killing by immune serum seen with *E. coli* treated with sub-MICs of imipenem was even more pronounced (Fig. 1 and 5). On the other hand, immune serum did not enhance phagocytosis or killing of *S. aureus* treated with sub-MICs of imipenem above the levels seen with *S. aureus* treated with sub-MICs of imipenem and incubated with FBS.

Prolonged incubation of infected macrophages with immune serum (10%) in bacterium-free medium beyond the 30-min period of phagocytosis significantly inhibited the intracellular multiplication of *E. coli* treated with sub-MICs of imipenem. Untreated *E. coli* incubated with immune serum did not behave differently within macrophages than did *E. coli* incubated with FBS (Fig. 3 and 6). In both instances, intracellular multiplication of *E. coli* occurred. Immune serum reduced the intracellular survival of *S. aureus* over the 3-h period. This was true of untreated *S. aureus* and *S. aureus* treated with sub-MICs of imipenem. After 3 h, however, the killing of intracellular *S. aureus* treated with sub-MICs of imipenem was significantly higher ($P < 0.01$) than that observed with untreated staphylococci (Fig. 6).

DISCUSSION

Before we started our study we determined that it was important to develop assays that were reliable and accurate. One of the most troublesome aspects of the assays routinely used by investigators is the differentiation of extracellular and surface-adherent bacteria from those which are truly within phagocytic cells. Another inherent difficulty is the extracellular multiplication of bacteria which, if permitted to occur, would compromise the valid interpretation of data. The assays that we employed in this study addressed these problems successfully; the microbiological assay coupled with the sensitive fluorescence assay permitted us to dissect the kinetics of bacterial phagocytosis and killing by murine macrophages during a defined period and to follow the course of intracellular events thereafter (1).

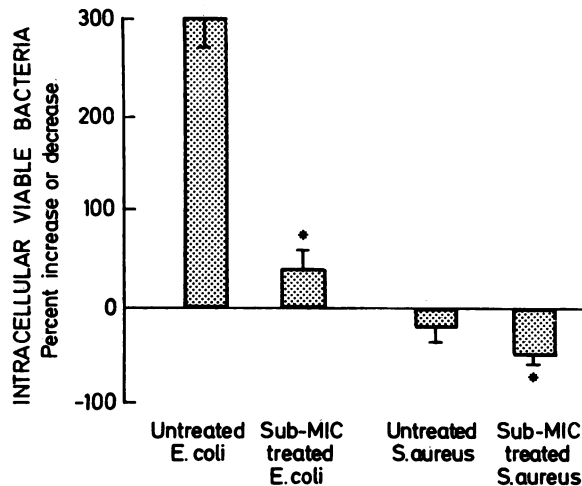


FIG. 6. Fate of residual intracellular bacteria that were untreated or viable bacteria that were treated with sub-MICs of imipenem. Infected macrophages were incubated in bacterium-free medium in the presence of 10% specific immune serum over a 3-h experimental period. The initial numbers of viable intracellular bacteria were calculated by determining the difference between phagocytosis and killing values for each group of bacteria for which results are given in Fig. 5. Data are expressed as the percent increase or decrease in the number of bacteria within macrophages as compared with the initial number of intracellular bacteria (zero base line represents the initial number of intracellular bacteria for each group). Symbol: *, $P < 0.01$ versus results for the respective untreated bacteria.

The objective of this study was to determine whether prior treatment with sub-MICs of imipenem or piperacillin influenced phagocytosis, intracellular killing, and sensitivity of *E. coli* and *S. aureus* to serum. The data showed that treatment with sub-MICs of the β -lactam antibiotics measurably increases the susceptibility of these clinical isolates to humoral and cellular defenses.

Both *E. coli* 775 and *S. aureus* 682 were phagocytized rapidly, and killing of the organisms by macrophages exceeded 90% during a 30-min period. A small percentage of ingested microbes survived and persisted. This may be of clinical significance, especially with *S. aureus* (6). Prior treatment with sub-MICs of imipenem or piperacillin enhanced phagocytosis and killing. Both antibiotics failed to augment the killing of residual viable organisms by macrophages after the initial burst of phagocytosis and killing. However, when the infected macrophages were subjected to treatment with T-cell-derived lymphokines, intracellular survival and multiplication of bacteria were inhibited. This was especially true for imipenem-treated *E. coli*, which was readily eliminated by activated macrophages. Untreated *E. coli* exhibited greater resistance but was also eventually killed by lymphokine-treated macrophages. The intracellular survival pattern of *S. aureus* was different. Residual staphylococci that were not treated with imipenem were resistant to killing by activated macrophages. Prior treatment with imipenem, however, permitted a 50% reduction of viable *S. aureus* in lymphokine-treated macrophages. The data suggest, therefore, that exposure of *E. coli* and *S. aureus* to sub-MICs of imipenem predisposes both species to killing by activated macrophages.

Since the phagocyte defense system acts in concert with serum factors in vivo, we examined the influence of immune sera on extracellular and intracellular killing using experimental protocols similar to those described above. We found

that *E. coli* in suspension was resistant to 10% immune rat serum but that prior treatment with sub-MICs of imipenem decreased serum resistance so that ca. 30% of the inoculum was killed during a 90-min incubation at 37°C. In contrast, the viability of untreated and sub-MIC-treated *S. aureus* was reduced by ca. 33% when incubated with immune serum. Phagocytosis and killing of both species were also modified by immune sera. A combination of immune serum and prior treatment with sub-MICs of imipenem acted synergistically to increase phagocytosis and killing of *E. coli* (Fig. 1 and 5). Phagocytosis and killing of *S. aureus*, on the other hand, were not modified by specific anti-staphylococcal serum (Fig. 2 and 5).

Immune serum also modified the fate of intracellular bacteria after the initial 30 min of phagocytosis. With FBS, antibiotic-treated *E. coli* multiplied over a 3-h period (Fig. 3). With 10% immune serum, however, intracellular multiplication was significantly inhibited ($P < 0.01$; Fig. 6). *E. coli* antibodies, on the other hand, did not reduce the capacity of untreated *E. coli* to multiply within macrophages (Fig. 3 and 6). The effects of immune serum on *S. aureus* were different. Specific antibody enhanced killing of *S. aureus* after 3 h (Fig. 3 and 6). *S. aureus* treated with sub-MICs of imipenem was killed in greater numbers than untreated bacteria ($P < 0.01$; Fig. 6).

The mechanisms by which antibiotics at sub-MICs enhance phagocytosis and killing of *E. coli* and *S. aureus* or modify sensitivity to antibody and complement probably involved bacterial surface alterations and perhaps other more subtle physiological changes. Although the sub-MIC-treated bacteria did not demonstrate changes in growth kinetics when they were transferred to antibiotic-free medium, they were visibly changed in morphology. Imipenem caused *E. coli* to assume a spherical shape, while at sub-MICs piperacillin induced elongated filamentous forms. The data indicate that the round forms of *E. coli* were more readily engulfed and killed. Altered cell surface characteristics of the antibiotic-treated bacteria may also increase the bactericidal action of antibody and complement. Others (7) have proposed this as an explanation to account for the increased susceptibility of *E. coli* treated with small amounts of semisynthetic penicillins to killing by immune serum.

In this study we showed that at sub-MICs the β -lactam antibiotics augment humoral and cellular defenses against bacterial pathogens. The significance of these in vitro observations is unclear. *Borrelia recurrentis* is cleared more efficiently from the circulation of patients treated with antibiotics (4). Additionally, aberrant bacterial forms isolated from clinical specimens have been noted in patients with low levels of antibiotics in the blood (3). Moreover, experimentally infected animals with sub-MICs of antibiotics in plasma survive longer and demonstrate a lower mortality (5,23). Sub-MICs of penicillin also increase the susceptibility of *S. aureus* to killing by phagocytic cells in vivo (14). These observations suggest that sub-MICs of antibiotics may be of potential value in the favorable outcome of bacterial infections. This would be of particular importance when infections are localized in poorly perfused tissues or in areas of severe inflammation. One can speculate that synergism between sub-MICs of antibiotics and host defenses can explain the apparent paradox that antibiotics may demonstrate greater efficacy in vivo than their poor in vitro activity would predict (6). Results of several investigations (2-7, 14, 23), in addition to the results of this study, suggest the possibility that the choice of antibiotic(s) should take into

account not only in vitro susceptibility data but also the potential facilitation of host defenses.

ACKNOWLEDGMENTS

This study was supported by a grant from Merck Sharp & Dohme. L. E. Adinolfi was supported in part by grant 87.00638.52 from the Consiglio Nazionale delle Ricerche NR, Rome, Italy.

LITERATURE CITED

- Adinolfi, L. E., M. A. Dilillo, M. F. Tripodi, R. Utili, P. F. Bonventre, and G. Ruggiero. 1988. Kinetics of phagocytosis and killing of *E. coli* by macrophages in presence of different serum preparations. *Microbiologica* 11:13-20.
- Andreana, A., P. Perna, R. Utili, M. Dilillo, and G. Ruggiero. 1984. Increased phagocytosis and killing of *Escherichia coli* treated with subinhibitory concentrations of cefamandole and gentamicin in isolated rat liver. *Antimicrob. Agents Chemother.* 25:182-186.
- Atkinson, B. A., and L. Amaral. 1982. Sublethal concentrations of antibiotics: effects on bacteria and the immune system. *Crit. Rev. Microbiol.* 9:101-138.
- Butler, T., M. Aikawa, A. Habte-Michael, and C. Wallage. 1980. Phagocytosis of *Borrelia recurrentis* by blood polymorphonuclear leukocytes is enhanced by antibiotic treatment. *Infect. Immun.* 28:1009-1013.
- Comber, K. R., R. J. Boon, and R. Sutherland. 1977. Comparative effects of amoxicillin and ampicillin on the morphology of *Escherichia coli* in vivo and correlation with activity. *Antimicrob. Agents Chemother.* 12:736-744.
- Elliot, G. R., P. K. Peterson, H. A. Verbrugh, M. R. Freiberg, J. R. Hoidal, and P. G. Quie. 1982. Influence of subinhibitory concentrations of penicillin, cefalothin, and clindamycin on *Staphylococcus aureus* growth in human phagocytic cells. *Antimicrob. Agents Chemother.* 22:781-784.
- Friedman, H., and G. H. Warren. 1976. Antibody-mediated bacteriolysis enhanced killing of cyclacillin-treated bacteria. *Proc. Soc. Exp. Biol. Med.* 153:301-304.
- Gemmell, C. G. 1978. Effect of subinhibitory concentrations of antibiotics in experimental pyogenic infection in mice, p. 512-514. *In* W. Siegenthaler and R. Lüthy (ed.), *Current chemotherapy*. American Society for Microbiology, Washington, D.C.
- Goldner, M., H. Farkas-Himsley, A. Kormendy, and M. Skinner. 1983. Bacterial phagocytosis monitored by fluorescence and extracellular quenching: ingestion and intracellular killing. *Lab. Med.* 14:291-294.
- Hed, J. 1977. The extinction of fluorescence by crystal violet and its use to differentiate between attached and ingested microorganisms in phagocytosis. *FEMS Microbiol. Lett.* 1:357-361.
- Kahan, F. M., H. Kropp, J. Sundelof, and J. Birnbaum. 1983. Thienamycin: development of imipenem-cilastatin. *J. Antimicrob. Chemother.* 12(Suppl.):1-35.
- Kesado, T., T. Hashizuma, and Y. Asahi. 1980. Antibacterial activities of a new stabilized thienamycin, *N*-formimidoyl thienamycin, in comparison with other antibiotics. *Antimicrob. Agents Chemother.* 17:912-917.
- Kropp, H., L. Gerckens, J. G. Sundelof, and F. M. Kahan. 1985. Antibacterial activity of imipenem: the first thienamycin antibiotic. *Rev. Infect. Dis.* 7:S389-S410.
- Lam, C., A. Georgopoulos, G. Laber, and E. Schutze. 1984. Therapeutic relevance of penicillin-induced hypersensitivity of *Staphylococcus aureus* to killing by polymorphonuclear leukocytes. *Antimicrob. Agents Chemother.* 26:149-154.
- Lorian, V., M. Koike, O. Zak, U. Zanon, L. D. Sabath, G. G. Grassi, and W. Stille. 1978. Effects of subinhibitory concentrations of antibiotics on bacteria, p. 72-78. *In* W. Siegenthaler and R. Lüthy (ed.), *Current chemotherapy*. American Society for Microbiology, Washington, D.C.
- Nickol, A., and P. F. Bonventre. 1985. Visceral leishmaniasis in congenic mice of susceptible and resistant phenotypes: immunosuppression by adherent spleen cells. *Infect. Immun.* 50:160-168.
- North, R. J. 1978. The concept of the activated macrophages. *J. Immunol.* 121:806-809.
- Rolinson, G. N. 1977. Subinhibitory concentrations of antibiotics. *J. Antimicrob. Chemother.* 3:111-113.
- Smith, D. L., and F. Rommel. 1977. A rapid micro-method for the simultaneous determination of phagocytic-microbicidal activity of human peripheral blood leukocytes *in vitro*. *J. Immunol. Methods* 17:241-247.
- Snedecor, G. W. 1956. *Statistical methods*. Iowa State University Press, Ames.
- van den Broeck, P. J., L. F. M. Buys, H. Mattie, and R. van Furth. 1986. Effect of penicillin G on *Staphylococcus aureus* phagocytosed by human monocytes. *J. Infect. Dis.* 153:586-592.
- Williams, R. J., Y. J. Yang, and D. M. Livermore. 1986. Mechanisms by which imipenem overcomes resistance in gram-negative bacilli. *J. Antimicrob. Chemother.* 18(Suppl.):9-13.
- Zak, O., and F. Kradolfer. 1979. Effects of subminimal inhibitory concentrations of antibiotics in experimental infections. *Rev. Infect. Dis.* 1:862-879.