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Effect of Clindamycin on Neutrophil Killing of Gram-Negative Periodontal Bacteria

PAMELA J. BAKER AND MARK E. WILSON*

Department of Oral Biology and Periodontal Disease Clinical Research Center, State University of New York at Buffalo, Buffalo, New York 14214

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Periodontal diseases are infections of the tissues supporting the dentition. Recognition that relatively specific microfloras are associated with distinct clinical forms of periodontal disease has prompted the use of antimicrobial agents as adjuncts in periodontal therapy. Clindamycin is one of several antibiotics known to concentrate in bioactive form in neutrophils and to potentiate phagocyte bactericidal activity against certain bacteria. Neutrophils appear to play a key role in host defense against periodontopathic gram-negative bacteria. In the present study, we evaluated the effect of preincubation of neutrophils with therapeutically achievable concentrations of clindamycin upon subsequent in vitro bactericidal activity against three species of gram-negative periodontal bacteria, including *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, and *Capnocytophaga ochracea*. In each instance, clindamycin neither enhanced nor inhibited the kinetics of bactericidal activity at low bacterium-neutrophil multiplicities. Further, this antibiotic had no demonstrable effect upon neutrophil bactericidal capacity, as assessed at bacterium-neutrophil ratios as high as 50:1. Our results indicate that clindamycin does not potentiate neutrophil bactericidal activity against the species of gram-negative periodontal organisms tested.

Bacteria resident in dental plaque appear to play a major role in the etiology and pathogenesis of human periodontal disease, a major cause of tooth loss in the adult population. Further, relatively specific microfloras, often including gram-negative organisms, are associated with distinct clinical forms of periodontal disease (19). Recognition of the bacterial etiology of periodontitis has fostered attempts to institute appropriate antimicrobial therapy as an adjunct to conventional forms of periodontal treatment (10).

Traditionally, selection of antimicrobial agents in the treatment of specific infections has been predicated upon information regarding the in vitro susceptibility of a given pathogen to various antibiotics. However, evidence amassed in recent years indicates that certain antibiotics can influence host-bacterial interactions at a number of distinct levels, exclusive of any direct bactericidal or bacteriostatic action upon the target bacterium (5, 15).

In view of the protective role the polymorphonuclear neutrophil plays in host defense against periodontopathic bacteria (16), antimicrobial agents which may augment neutrophil bactericidal activity against periodontopathic organisms may have particular utility in the treatment of human periodontal diseases. Clindamycin is one of several antibiotics exhibiting a significant propensity to concentrate within neutrophils (13, 14, 18) and to potentiate intraphagocytic bactericidal activity against certain organisms (8, 24). In the present investigation, we assessed the effect of clindamycin on in vitro neutrophil bactericidal activity against selected gram-negative periodontal bacteria, including *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, and *Capnocytophaga ochracea*, which vary in their in vitro susceptibility to clindamycin (2).

MATERIALS AND METHODS

Neutrophil isolation. Peripheral blood was collected by venipuncture from adult volunteers who gave informed consent and who did not have overt periodontal or other disease. The volunteers indicated that they had not taken antibiotics for at least 6 months prior to the study and that they had refrained from the use of over-the-counter medication for 2 weeks and from alcohol or tobacco products for 48 h prior to blood donation. Neutrophils were partially purified from anticoagulated blood (acid citrate-glucose [1:6, vol/vol]) by unit gravity sedimentation in 2% gelatin (Difco Laboratories, Detroit, Mich.) in saline, as described previously (23), followed by centrifugation of the neutrophil-enriched fraction through a discontinuous gradient of 55 and 74% Percoll (Pharmacia AB, Uppsala, Sweden), as described by Hjorth and co-workers (12). Leukocyte preparations thus prepared contained >95% neutrophils and exhibited >98% viability by trypan blue dye exclusion.

Human serum. Serum was obtained by standard procedures from five individuals who also served as leukocyte donors. The sera were pooled, divided into aliquots, and frozen at −70°C until used as a source of normal human serum opsonins in neutrophil bactericidal assays.

Clindamycin. Clindamycin hydrochloride was a gift from The Upjohn Co. (Kalamazoo, Mich.). A stock solution of clindamycin was prepared in phosphate-buffered saline at 1 mg/ml and stored in aliquots at −20°C until used in bactericidal assays (G. Gerard, The Upjohn Co., personal communication). For determinations of MICs and MBCs, a concentrated solution of clindamycin was freshly prepared in phosphate-buffered saline and then diluted 1:100 into thioglycolate broth.

Bacterial strains. *A. actinomycetemcomitans* Y4 and 75 (both leukotoxic) and strain 67 (nonleukotoxic), *E. corrodens* SUNYAB 5 and D3846, and *C. ochracea* B2906 and B5473 are maintained in the culture collection of the State University of New York at Buffalo (SUNYAB) Periodontal Disease Clinical Research Center and are stored in liquid N_2. These strains were grown on tryptic soy agar (Difco) supplemented with 1 g of yeast extract (Difco) per liter, 1 mg of vitamin K_1 (Sigma Chemical Co., St. Louis, Mo.) per liter, 5

* Corresponding author.

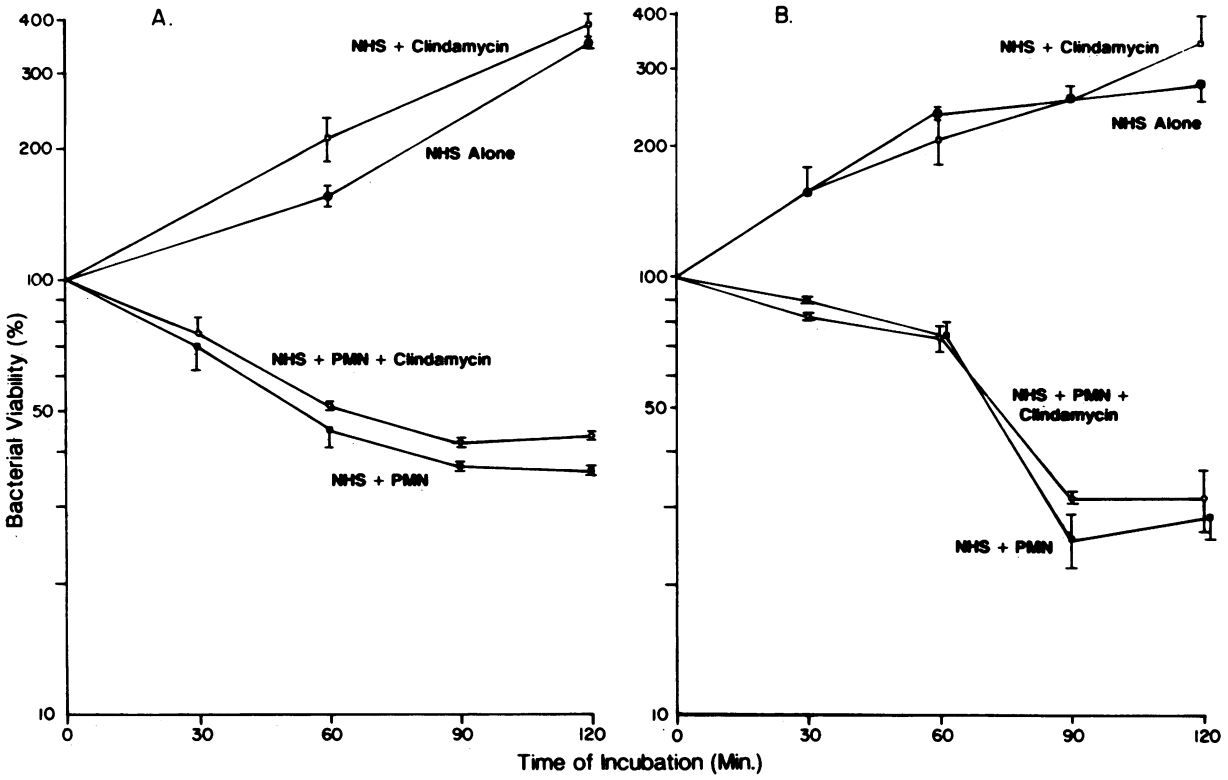

FIG. 1. Effect of clindamycin on the kinetics of neutrophil bactericidal activity against *A. actinomycetemcomitans* Y4 (A) and 67 (B). Neutrophils were preincubated for 30 min in the presence or absence of clindamycin (10 μg/ml) prior to the addition of bacteria at a ratio of approximately 3 CFU per leukocyte. The means ± standard deviations of triplicate samples at each interval are shown, and viability is expressed as a percentage of the starting inoculum. NHS, Normal human serum; PMN, polymorphonuclear leukocytes.

mg of hemin (equine type III; Sigma) per liter, and 5% defibrinated sheep blood (Crane Laboratories, Inc., Syracuse, N.Y.). For experiments with *A. actinomycetemcomitans*, bacteria were grown to mid-logarithmic or stationary phase. Briefly, a loopful of growth from a 1- to 2-day-old blood agar plate (incubated no more than 24 h) was transferred to 10 ml of NIH thioglycolate broth (Difco) supplemented with 5 g of yeast extract per liter and 1 g of sodium bicarbonate per liter. Following brief vortexing, serial 10-fold dilutions were prepared in thioglycolate broth and incubated statically overnight at 37°C in humidified 5% CO_2 (Forma Scientific, Marietta, Ohio). Aliquots of dilutions remaining in the desired growth phase, as determined by optical density at 650 nm, were used in bactericidal and in vitro antimicrobial susceptibility assays.

C. ochracea cultures were prepared in a similar manner in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). After overnight incubation, tubes exhibiting a suitable optical density at 650 nm were used to inoculate fresh Trypticase soy broth and incubated for an additional 4 h.

E. corrodens broth cultures were prepared as described for *C. ochracea*, except that the broth used was Todd-Hewitt broth (BBL) supplemented with 2 g of potassium nitrate per liter, 5 mg of hemin per liter, and 50 mg of L-cysteine hydrochloride (pH 7.2) per liter.

Determinations of MICs and MBCs. The MIC for each strain was determined by broth microdilution (1, 20). Serial twofold dilutions of antibiotic were prepared in microtiter plates so that each well contained 100 μl of a doubly concentrated solution of clindamycin in thioglycolate broth. Equal volumes of logarithmic-phase *A. actinomycetemcomitans* (2×10^6 CFU/ml in thioglycolate) were then added to each well. The plates were incubated overnight at 37°C in an atmosphere of 5% CO_2. The MIC was the lowest antibiotic concentration showing no visible growth after 24 h. The MICs for the other species were determined similarly, but in Trypticase soy broth or Todd-Hewitt broth, as appropriate.

After 2 h of incubation, 10-μl samples were removed from each well and plated onto one quadrant of a blood agar plate. Additionally, samples were removed from wells exhibiting no visible growth after 24 h and similarly plated onto blood agar. The MBCs (the lowest concentrations of antibiotic resulting in one colony or fewer per quadrant) were determined after 48 h of incubation (1, 20).

Neutrophil bactericidal assay. Bactericidal assays were performed in 1.5-ml polypropylene microcentrifuge tubes containing complete Dulbecco phosphate-buffered saline supplemented with 1 g of glucose per liter, 2 g of bovine serum albumin per liter (plus 1 g of potassium nitrate per liter for experiments with *E. corrodens*), freshly thawed normal human serum (20%, vol/vol), neutrophils (4×10^6), and the indicated bacteria, in a final volume of 1.0 ml. These reaction conditions yielded an intermediate rate of leukocyte bactericidal activity, thereby enabling detection of putative inhibitory or potentiating effects of clindamycin. Reaction tubes containing all constituents except bacteria were equilibrated to 37°C, after which clindamycin was added when appropriate. The reaction tubes were subjected to continuous end-over-end rotation in a 37°C incubator for 20 to 40 min to facilitate uptake of the antibiotic by neutrophils. Clindamycin remained present in the extracellular medium throughout

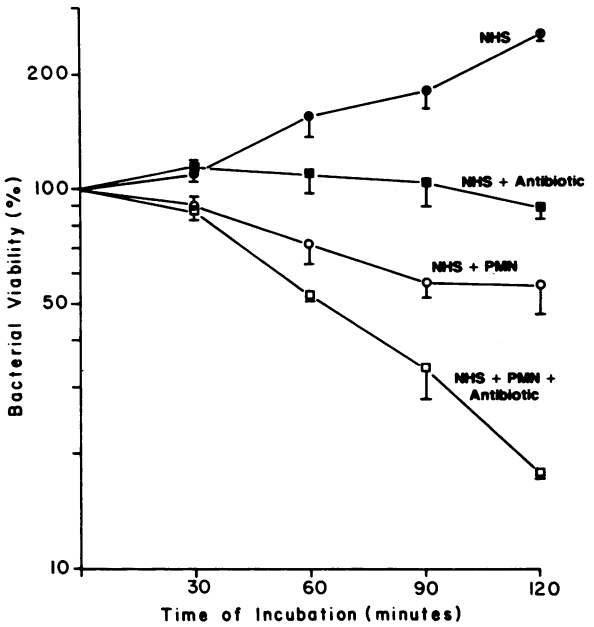

FIG. 2. Effect of clindamycin on the kinetics of neutrophil bactericidal activity against *A. actinomycetemcomitans* 75. Bacteria were incubated in the presence of untreated or clindamycin-treated (10 μg/ml, 30 min) neutrophils for various intervals up to 2 h. The means ± standard deviations of triplicate samples at each interval are shown, and viability is expressed as a percentage of the starting inoculum. Note that, in contrast to the results presented in Fig. 1, the bacteriostatic effect of clindamycin was demonstrable in the presence of normal serum (NHS) alone. PMN, Polymorphonuclear leukocytes.

the bactericidal assay, since it has been shown that removal of antibiotic from the medium enhances the rate of diffusion of the antibiotic from the leukocyte (14).

Bactericidal reactions were initiated via addition of bacteria at densities sufficient to yield the indicated CFU/neutrophil ratio. Immediately following bacterial addition and at specified intervals thereafter, 50-μl samples were withdrawn and diluted into 5 or 10 ml of sterile H_2O. Neutrophils were lysed to release intracellular bacteria by vortexing the water tubes for 10 s and allowing them to stand for 2 to 3 min. Neutrophil lysis was confirmed by visual inspection. After a further dilution into saline or thioglycolate broth, 75-μl aliquots were plated onto blood agar and incubated for 48 h at 37°C in 5% CO_2, after which CFU per plate were enumerated. Percent survival at time = t under each condition was calculated as [(CFU/ml at time = t)/(CFU per ml at time = 0)] × 100.

The methods described above yielded plating efficiencies of 95 to 115% for all bacterial strains. Dilution of samples in distilled water (necessary for lysis of neutrophils) did not impair the viability of the bacteria, since samples taken from tubes without neutrophils at either the beginning or the end of an assay yielded comparable numbers of CFU whether diluted into sterile H_2O or broth.

RESULTS

Antimicrobial susceptibility. The MICs and MBCs of clindamycin for the oral gram-negative strains included in this study were determined. The results (MIC and MBC at 24 h, respectively) were as follows: *A. actinomycetemcomitans* Y4, 16 and 64 μg/ml; *A. actinomycetemcomitans* 67, 32 and 128 μg/ml; *A. actinomycetemcomitans* 75, 8 and 32 μg/ml; *E. corrodens* SUNYAB 5 and D3846, >128 and >128 μg/ml; *C. ochracea* B2906, <1 and 8 μg/ml; and *C. ochracea* B5473, <1 and 2 μg/ml.

Pharmacokinetic studies indicate that the peak concentrations in serum following administration of single oral doses of 300 and 450 mg of clindamycin hydrochloride to adults are approximately 3.5 and 6 μg/ml, respectively (6). Hence, with the exception of *C. ochracea*, MICs exceeded the achievable concentration of clindamycin in serum for the strains tested. The MBCs were approximately four times greater than the corresponding MICs, as would be expected for a bacteriostatic antibiotic such as clindamycin.

Neutrophil bactericidal assays, including those described in this paper, are typically performed over short-term incubation periods (30 to 120 min), whereas MICs and MBCs are traditionally evaluated by using an 18- to 24-h endpoint. To make any comparison between direct effects of clindamycin on the organisms under study and indirect effects via potentiation of neutrophil bactericidal activity, MBCs were also determined following a 2-h incubation of *A. actinomycetemcomitans* with this antibiotic. The 2-h MBCs for both strains exceeded 512 μg of clindamycin per ml. Clindamycin is actively transported into neutrophils, resulting in intracellular/extracellular ratios varying from 11:1 to 40:1, given an extracellular concentration of 10 μg/ml (15, 18, 21). Given a therapeutically achievable level of 4 μg/ml, the maximum anticipated intracellular concentration of clindamycin (160

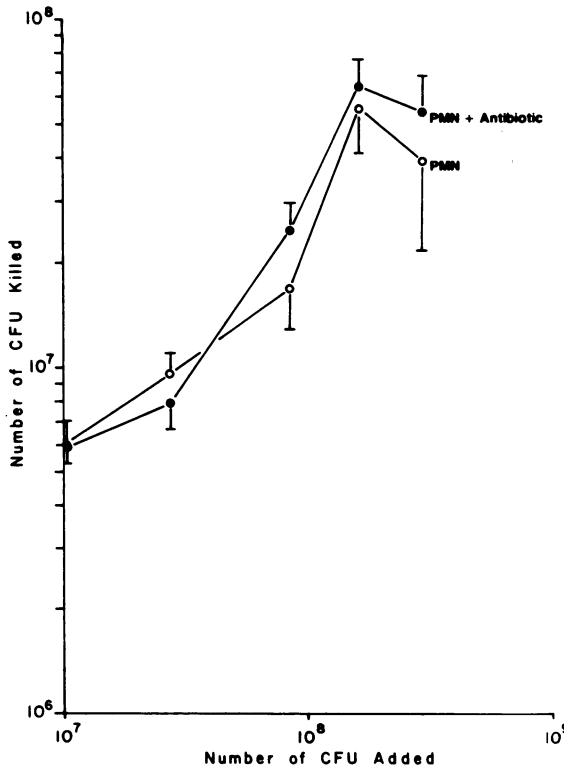

FIG. 3. Effect of clindamycin on neutrophil bactericidal capacity with respect to *A. actinomycetemcomitans* Y4. The indicated numbers of bacteria were incubated for 2 h in the presence of untreated or clindamycin-pretreated (4 μg/ml) neutrophils (4×10^6). Data are expressed as the mean number of bacteria killed ± standard deviation of triplicate samples. PMN, Polymorphonuclear leukocytes.

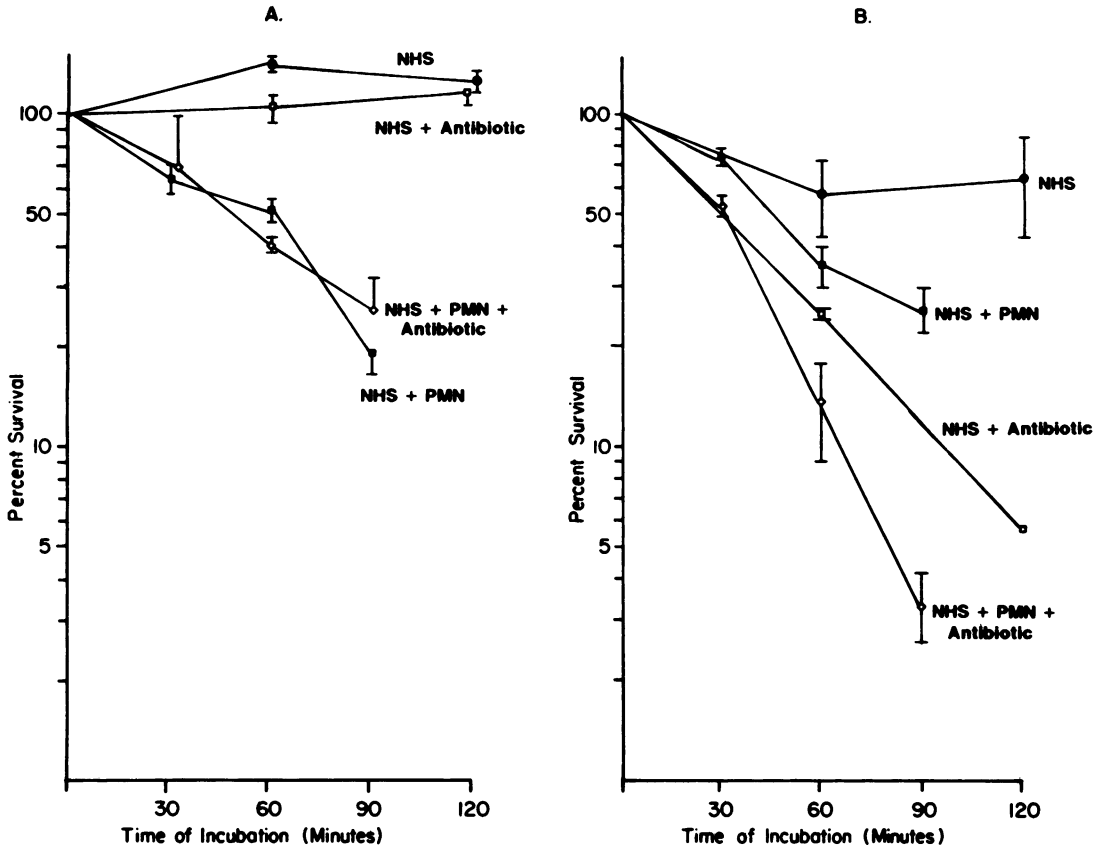

FIG. 4. Effect of clindamycin on the kinetics of neutrophil bactericidal activity against two serum-resistant isolates of *C. ochracea*. Untreated or clindamycin-pretreated (4 μg/ml) neutrophils were incubated with *C. ochracea* B2906 (A) and B5473 (B) at a ratio of approximately 3 CFU per leukocyte. Bacterial viability is expressed as a percentage of the starting inoculum. The means ± standard deviations of triplicate samples for each time interval are shown. NHS, Normal human serum; PMN, polymorphonuclear leukocytes.

μg/ml) would be well below the concentration required for a direct intracellular effect of clindamycin on phagocytized *A. actinomycetemcomitans* during a 1- to 2-h bactericidal assay.

Effect of clindamycin on neutrophil killing of *A. actinomycetemcomitans*. In previous studies, we reported that *A. actinomycetemcomitans* is effectively killed by neutrophils (17). Since this organism is resistant to achievable extracellular and intracellular concentrations of clindamycin, we were particularly interested in determining whether clindamycin uptake into neutrophils would potentiate intraphagocytic killing of this organism. Exposure of neutrophils to clindamycin (4, 10, or 20 μg/ml in selected experiments) for 30 to 60 min prior to bacterial addition and throughout the remainder of the assay failed to influence the rate of killing of *A. actinomycetemcomitans* Y4 (Fig. 1A) or strain 67 (Fig. 1B) during a 2-h incubation period. Incubation of these organisms with clindamycin (10 μg/ml) and normal human serum in the absence of neutrophils did not alter bacterial viability of this serum-resistant organism during this period (Fig. 1) and for intervals up to 5 h (data not shown). Bactericidal activity of clindamycin was evident only after exposure of these strains to 20 μg/ml for 4 h or to 80 to 160 μg/ml for 3 h.

A. actinomycetemcomitans 75 exhibited greater susceptibility to clindamycin, with an MIC of 8 μg/ml. Neutrophils incubated with 10 μg of clindamycin per ml were more effective in killing this strain than untreated neutrophils (Fig. 2), suggestive of a potentiating effect of the antibiotic on neutrophil bactericidal activity. In this figure, bacterial viability was calculated as a percentage of the $t = 0$ inoculum. However, as also indicated in Fig. 2, 10 μg of clindamycin per ml had a perceptible bacteriostatic effect on this strain during a 2-h incubation period. The extent to which the bacteriostatic effect of this antibiotic contributed to the apparent increase in neutrophil killing of strain 75 was determined by recalculation of the data as follows: % viability = (CFU/ml with neutrophils)/(CFU/ml without neutrophils) at each indicated time interval, and in the presence or absence of clindamycin. Reconsideration of the data in this fashion demonstrated that the apparent increase in killing of strain 75 by neutrophils exposed to clindamycin was attributable to a direct bacteriostatic effect of the antibiotic on this strain.

Effect of clindamycin on neutrophil bactericidal capacity. The results of the aforementioned experiment indicated that, at a bacterium/neutrophil ratio of 3:1, clindamycin failed to augment the rate of bacterial killing of *A. actinomycetemcomitans*. However, it is possible that clindamycin uptake into neutrophils may alter bactericidal capacity (number of bacteria killed per leukocyte), rather than bactericidal rate. Accordingly, experiments were performed in which killing of *A. actinomycetemcomitans* by untreated and clindamycin-treated (4 μg/ml) neutrophils was compared at various CFU/neutrophil ratios. Raising the CFU/neutrophil ratio from 2.5:1 to approximately 50:1 resulted in inoculum-dependent

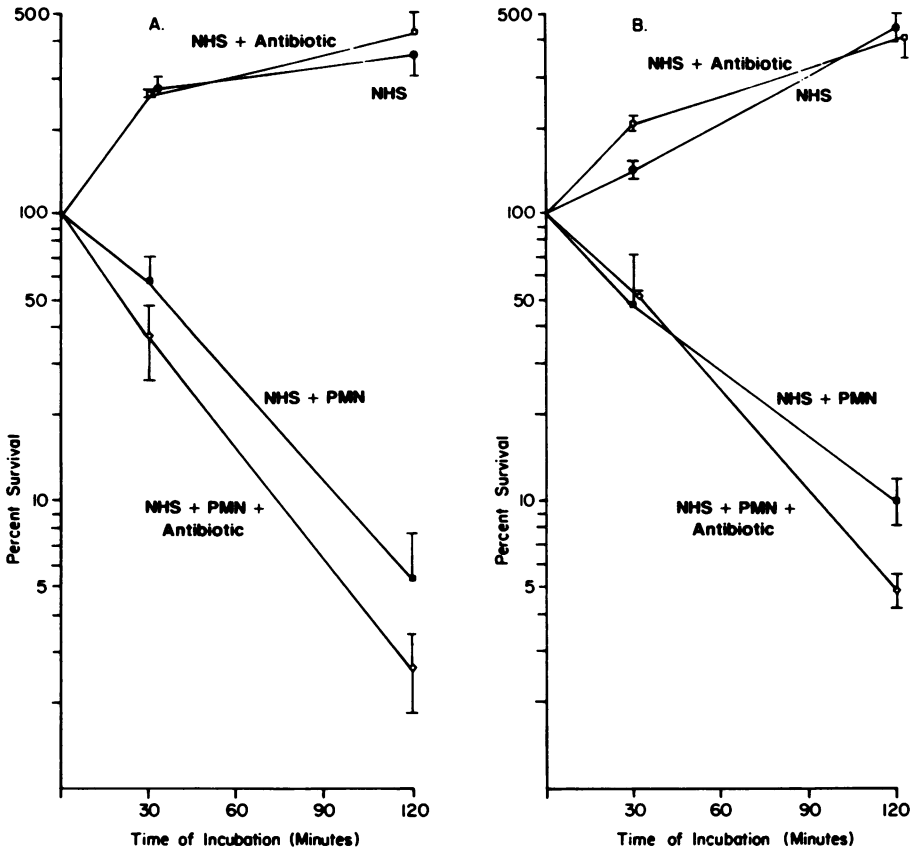

FIG. 5. Effect of clindamycin on the kinetics of neutrophil bactericidal activity against *E. corrodens*. Strains SUNYAB 5 (A) and D3846 (B) were incubated with or without untreated or clindamycin-treated (4 μg/ml) neutrophils at a ratio of 3:1 bacteria per leukocyte. Bacterial viability is expressed as a percentage of the starting inoculum. The means ± standard deviations of triplicate samples at each time interval are shown. NHS, Normal human serum; PMN, polymorphonuclear leukocytes.

increases in the number of bacteria killed during a 2-h incubation period (Fig. 3). Bactericidal capacity peaked at a ratio of 50:1, corresponding to approximately 15 organisms killed per neutrophil per 2 h. Incubation of neutrophils with clindamycin failed to alter the number of bacteria killed at CFU/neutrophil ratios lower than 50:1, nor did it promote bacterial killing at ratios in excess of 50:1. Hence, pretreatment of neutrophils with clindamycin at therapeutically relevant concentrations failed to augment either neutrophil bactericidal rate or bactericidal capacity with respect to *A. actinomycetemcomitans*.

Effect of clindamycin on neutrophil killing of other gram-negative periodontal bacteria. The ability of clindamycin to augment neutrophil killing of other species of gram-negative periodontal bacteria was also examined. Initial experiments were conducted to evaluate the effect of clindamycin on neutrophil killing of two strains of *C. ochracea* recovered from blood cultures, each of which exhibited MICs of <1 μg of clindamycin per ml. Both strains were resistant to serum bactericidal activity (Fig. 4), consistent with our previous findings (22). Clindamycin (4 μg/ml) showed a modest bacteriostatic effect on strain B2906 (Fig. 4A) and a bactericidal effect on *C. ochracea* B5473 (Fig. 4B). Results for the latter strain were the same with or without serum (data not shown), indicating that this was a direct effect of the antibiotic and was not the result of increased susceptibility of this strain to serum bactericidal action. Clindamycin did not augment neutrophil killing of B2906 during a 2-h incubation. Although killing of B5473 was increased in reaction tubes containing clindamycin, neutrophils, and serum (Fig. 4B), this effect appeared to be attributable to an increase in serum sensitivity rather than to any potentiation of neutrophil bactericidal activity.

Additional studies were conducted with two strains of *E. corrodens*. Both strains were highly resistant to clindamycin, with MICs and MBCs of >128 μg/ml. Neither strain of *E. corrodens* was rendered more susceptible to serum bactericidal activity in the presence of clindamycin (Fig. 5). Further, incubation of neutrophils with clindamycin (4 μg/ml) for 30 min prior to bacterial addition had little effect on the rate of neutrophil killing of these two strains (Fig. 5).

DISCUSSION

The likelihood that periodontal diseases are infections has prompted the use of antibiotics as adjuncts to conventional periodontal therapy (10). Traditionally, selection of appropriate antibiotics in the treatment of periodontal disease has been based largely upon in vitro susceptibility testing results. However, there is growing recognition that antibiotics may profoundly influence host-bacterial interactions via mechanisms which are independent of any direct bacteriostatic or bactericidal action upon the target organism (5, 15). Such recognition prompted our interest in studying antibiotics which may have a beneficial effect on host defense, particularly as regards neutrophil bactericidal activity.

Certain classes of antibiotics have been shown to exhibit a capacity to concentrate in phagocytic cells. Uptake of clin-

damycin into neutrophils via the nucleoside transport system has been reported to result in a cellular-extracellular ratio of 11 following exposure of these cells to 10 μg of clindamycin per ml (11, 18). Other groups reported as great as a 40-fold increase in clindamycin concentration in the cytoplasm and a 50-fold increase within isolated neutrophil lysosomes following incubation of neutrophils with this antibiotic at 10 μg/ml (14). In vivo studies indicate that the concentration in serum achieved after oral administration of 300 mg of clindamycin four times per day for 2 days may range from 0.4 to 2.6 μg/ml, with a resultant intraphagocytic concentration of 8 to 9 μg/ml (8). This represents a threefold increase in the cellular-extracellular ratio.

Accumulation of clindamycin within neutrophils has been noted to enhance killing of certain bacteria. For example, in vivo loading of neutrophils with clindamycin enhanced neutrophil bactericidal activity against a clindamycin-resistant strain of *S. aureus* (8). In vitro addition of clindamycin to neutrophils following ingestion of clindamycin-susceptible *Staphylococcus aureus* has also been reported to enhance intracellular killing (13). Similar results were obtained when neutrophils from normal subjects and from patients with chronic granulomatous disease were used (24).

The results of the present study failed to provide evidence that clindamycin at therapeutically achievable concentrations potentiates (or inhibits) in vitro neutrophil bactericidal activity against strains representative of three species of gram-negative periodontal bacteria, including *A. actinomycetemcomitans*, *C. ochracea*, and *E. corrodens*. In these studies, incubation of neutrophils with 4 to 20 μg of clindamycin per ml failed to influence either bactericidal rate or bactericidal capacity with respect to these strains.

The available evidence indicates that clindamycin does not alter phagocytosis per se (9). Any perceived alteration in intraphagocytic killing would, therefore, likely be due to (i) an effect of the antibiotic on a postphagocytic event relating to bactericidal activity or (ii) a direct interaction between the phagocytized bacteria and intracellular antibiotic. Recent studies indicate that the mere capacity of clindamycin to accumulate within phagocytes does not necessarily result in enhanced intraphagocytic killing of gram-positive and aerobic gram-negative bacteria. This has been demonstrated following either in vivo or in vitro exposure of neutrophils to clindamycin (3, 11).

It has been suggested that the concentration of clindamycin attained within the neutrophil must exceed the MBC in order for increased killing of *S. aureus* to be demonstrated (11). The results of the present study appear to support this hypothesis. The only conditions under which enhanced killing was evident involved those strains exhibiting direct susceptibility to clindamycin alone. In these instances, the extent of neutrophil killing in the presence of clindamycin was no greater than could be explained by the activity of intracellular clindamycin alone. Thus, we were unable to obtain evidence that clindamycin potentiates neutrophil bactericidal activity against these organisms, as was suggested for neutrophil killing of clindamycin-resistant *S. aureus* (8). It is possible, however, that in vivo loading of neutrophils with clindamycin potentiates neutrophils in a way not mimicked by in vitro loading.

On the basis of previous estimates regarding the extent of intracellular accumulation of clindamycin, it is likely that intracellular levels approached or exceeded the 24-h MBCs for the strains included in this study (with the possible exception of *E. corrodens*). Although the MBC measured after 24 h of exposure of the bacteria to clindamycin was 64 μg/ml for *A. actinomycetemcomitans* Y4, the MBC for a 2-h exposure (>512 μg/ml) was considerably higher. In addition, it was observed that at clindamycin concentrations achievable intracellularly, these slow-growing facultative anaerobes required 3 to 5 h of exposure for clindamycin to have a perceptible bactericidal effect. Hence, it is unlikely that any direct bacteriostatic or bactericidal effect of intracellular clindamycin would become manifest during the course of short-term (1- to 2-h) neutrophil bactericidal assays. It seems plausible that, for direct intracellular antibiotic activity to become manifest, not only must the intracellular concentration exceed the MBC (11), it must also exceed the MBC for the time period employed in the neutrophil bactericidal assay.

The results of this study indicate that clindamycin neither enhances nor inhibits neutrophil bactericidal activity against selected species of periodontal gram-negative bacteria, including *A. actinomycetemcomitans*, *E. corrodens*, and *C. ochracea*. In these studies, neutrophils were exposed to clindamycin prior to and during the bactericidal assay. Other studies have demonstrated, however, that exposure of bacteria to sub-MICs of antibiotics such as clindamycin during logarithmic-phase growth renders these organisms more susceptible to neutrophil phagocytosis and killing, possibly by promoting complement-mediated opsonization (4, 7, 9). In this context, it has been reported that peak clindamycin concentrations of 2 μg/ml have been achieved in gingival crevicular fluid following administration of a single oral dose of this antibiotic (21). This local concentration of clindamycin is within the range of MICs for a number of periodontal bacteria (2, 21). Thus, it is germane to consider possible direct effects of clindamycin on periodontal bacteria which may render these organisms more susceptible to phagocytosis and killing by infiltrating neutrophils. Such studies are in progress in this laboratory.

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