Potentiation of Opsonization and Phagocytosis of *Streptococcus pyogenes* following Growth in the Presence of Clindamycin

CURTIS G. GEMMELL, PHILLIP K. PETERSON, DAVID SCHMELING, YOUNGKI KIM, JOHN MATHEWS, LEWIS WANNAMAKER, and PAUL G. QUIE,
Departments of Pediatrics and Medicine, University of Minnesota, Minneapolis, Minnesota 55455; Pathology and Toxicology Research Unit, The Upjohn Company, Kalamazoo, Michigan 49001

ABSTRACT *Streptococcus pyogenes*, bearing M-protein on its surface, resists opsonization by normal human serum and subsequent phagocytosis by human polymorphonuclear leukocytes. Previous studies have shown that M-protein positive organisms are poorly opsonized by the alternate pathway of complement. In an attempt to define further the role of the surface components of *S. pyogenes* in this process, we examined the ability of clindamycin, an antibiotic that inhibits protein biosynthesis, to alter bacterial opsonization.

An M-protein positive strain of *S. pyogenes* was grown in varying concentrations of clindamycin at levels lower than those which inhibited growth, i.e., at levels less than the minimal inhibitory concentration. These bacteria were incubated with purified human polymorphonuclear leukocytes and peripheral blood monocytes. Significant enhancement of bacterial opsonization, phagocytosis, and killing resulted. Measurement of complement consumption and binding of the third component of complement (C3) onto the bacterial surface demonstrated that organisms grown in the presence of clindamycin activated complement more readily and fixed more C3 on their surface. Electron microscopy revealed the probable basis for these findings. Streptococci exposed to clindamycin during growth were largely denuded of surface "fuzz," the hairlike structures bearing M-protein.

We conclude that the incorporation of clindamycin at concentrations that fail to inhibit growth of *S. pyogenes* nevertheless causes significant changes in the capacity of these bacteria to resist opsonization by serum complement. These findings support the hypothesis that M-protein inhibits bacterial opsonization by interfering with effective complement activation on the bacterial surface.

INTRODUCTION

The effects of low concentrations of antimicrobial agents on bacteria in vitro have been recognized for several decades. Gardner (1) showed that *Clostridium perfringens* formed elongated unsegmented filaments in the presence of penicillin in concentrations below the minimal inhibitory concentration (sub-MIC).[1] Similar changes were seen with gram-negative bacilli, whereas *Staphylococcus aureus* and *Streptococcus pyogenes* cells became enlarged, with imperfect or incomplete fission. Since then there have been numerous reports of the structural effects of antibiotics on a variety of bacteria, and these have recently been reviewed by Lorian (2). In other studies, sub-MIC levels of antibiotics have been shown to interfere with adherence of bacteria to epithelial cell surfaces (3, 4) and to enhance the bactericidal activity of human serum (5). The significance of these in vitro effects of sub-MIC levels of antibiotics on interactions between bacteria and the human host is not fully understood.

For *S. pyogenes*, preliminary evidence has indicated that its virulence can be modified by sub-MIC of certain antibiotics (6); various toxins and enzymes (in-

Address reprint requests to Dr. Gemmell, Bacteriology Dept., Medical School, University of Glasgow, Royal Infirmary, Glasgow, Scotland, U. K.

Received for publication 8 September 1980 and in revised form 26 November 1980.

[1] *Abbreviations used in this paper*: cfu, colony-forming units; HBSS, Hanks' balanced salt solution; MIC, minimal inhibitory concentration; MN, monocyte; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; THB, Todd Hewitt broth.

cluding the streptolysins O and S and DNAase) are inhibited when streptococci are grown in the presence of clindamycin or lincomycin, but not when exposed to chloramphenicol, erythromycin, or penicillin. In addition, the expression of the surface antigens M and T proteins was reduced in the antibiotic-exposed organisms. In subsequent studies (7), sub-MIC levels of clindamycin and lincomycin were shown to enhance phagocytosis of *S. pyogenes* by polymorphonuclear leukocytes (PMN).

S. pyogenes strains that possess cell-surface M-protein have long been known to resist phagocytosis (8), and recent studies suggest that the antiphagocytic characteristic of M-protein may be related to its ability to inhibit bacterial opsonization by the serum complement system (9, 10). In the present investigation we used sub-MIC levels of clindamycin to alter the streptococcal surface and thereby to examine the hypothesis that M-protein inhibits opsonization by interfering with effective complement activation on the bacterial surface. Phagocytosis and bacterial killing by highly purified populations of human PMN and blood monocytes (MN) were evaluated.

METHODS

Bacteria and radioactive labeling. *S. pyogenes* (M type 6) S43 was chosen for use in this study. Its ability to elaborate M-protein was enhanced by rotation in whole blood by the method of Becker (11). A single colony from a horse blood agar plate was used to inoculate 50 ml Todd Hewitt broth (THB) and was incubated at 37°C for 8 h. The cells were harvested at this time, resuspended as a thick suspension in sterile THB, and stored in 0.5-ml amounts of −70°C until required as inocula. At this time a single vial was thawed, inoculated into 10 ml THB, and incubated at 37°C for 15 h. 0.5 ml of this culture was then used to inoculate 10 ml of prewarmed THB containing 20 μCi of thymidine (50 Ci/mmol sp act, Research Products International Corp., Elk Grove Village, Ill.) and was incubated for 4 h at 37°C. The bacteria were washed three times with sterile distilled water and resuspended in distilled water to a final concentration of 5×10^8 colony-forming units (cfu)/ml distilled water using a spectrophotometric method.

Antibiotic treatment of S. pyogenes. Clindamycin HCl was obtained as a powder from Upjohn International, Kalamazoo, Mich., and a stock solution containing 1.0 mg/ml was prepared in distilled water, sterilized by membrane filtration, and stored at 4°C. This stock solution was diluted aseptically as appropriate for the growth studies.

The MIC of clindamycin HCl was found to be 0.05 μg/ml for *S. pyogenes* S43 as determined by a tube dilution method (12). The following concentrations of clindamycin were chosen for study: 1/2, 1/4, and 1/40 of the MIC. These drug concentrations were incorporated into 10-ml amounts of THB containing [^{3}H]thymidine, inoculated with 0.5 ml of streptococcal culture, and incubated for 4 h at 37°C. The bacteria were collected and washed as previously noted.

Opsonic sources and opsonization procedure. Serum from a single healthy donor who lacked anti-*S. pyogenes* M6 antibodies was used throughout this study. Serum was stored in 1.0-ml portions at −70°C. Heat-inactivated serum was prepared by heating thawed serum at 56°C for 30 min. Normal and heat-inactivated sera were diluted in Hanks' balanced salt solution containing 0.1% gelatin (gel-HBSS) to a final concentration of 10% before use.

Bacteria were opsonized before use by incubating 0.1-ml portions of bacteria at 37°C in polypropylene vials (Bio-vials, Beckman Instruments Inc., Fullerton, Calif.) containing 1.0 ml of the desired opsonic source for 15 min followed by centrifugation at 4,000 *g* for 15 min (4°C). The supernate was discarded, and the bacterial pellet was resuspended in 1.0 ml gel-HBSS for phagocytosis experiments.

Preparation of phagocytic cells. Blood was drawn into heparinized syringes (10 U heparin/ml blood) and 10-ml volumes of this blood were then mixed with 3 ml 6% dextran 75 in 0.9% sodium chloride (Travenol Laboratories, Deerfield, Ill.) in 16 × 150-mm plastic tubes (Bio-Quest, BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) and allowed to gravity-sediment at ambient temperature for 45–60 min. After sedimentation, the leukocyte-rich plasma was removed with a pasteur pipette, and 8 ml of this suspension was carefully layered onto 3 ml Ficoll-Isopaque (LSM, lymphocyte separation medium, Litton Bionetics, Kensington, Md.). After centrifuging at 200 *g* for 30 min, the mononuclear cell layer was removed with a pasteur pipette. Purified MN were then prepared by the method of Ackerman and Douglas (13). First, $20–50 \times 10^6$ mononuclear cells were suspended in 20 ml medium 199/20% fetal calf serum, and introduced into 75-cm^2 Falcon tissue culture flasks that had been prepared as follows: baby hamster kidney cells (American Type Culture Collection, Rockville, Md.) were grown to confluence (usually 4–6 d after passage), and then detached with 10 mM EDTA in phosphate-buffered saline (PBS) followed by vigorous rinsing of the flask to remove all baby hamster kidney cells, leaving behind an extracellular microexudate (14). Following introduction of the MN into the microexudate-coated flasks, the flasks were incubated at 37°C in an atmosphere of 5% CO_2 for 45 min to allow MN to attach. Nonadherent cells, primarily lymphocytes, were then decanted, and the flasks were rinsed with three changes of PBS, pH 7.4, prewarmed to 37°C. MN were detached by addition of 3.5 ml each of 10 mM EDTA/PBS and medium 199/20% fetal calf serum and incubation at 37°C (5% CO_2) for 15 min. The flasks were then removed from the incubator and shaken vigorously, after which the detached MN were decanted into 17 × 100-mm plastic tubes (Bioquest). The flasks were then rinsed vigorously once with 5 ml of 199/20% fetal calf serum, and this rinse was also transferred to the aforementioned tubes. These tubes were then centrifuged for 10 min at 160 *g*; the MN pellet was washed twice with gel-HBSS, and the cells were counted and resuspended to a final volume of 5×10^6 MN/ml gel-HBSS. Light microscopic observation of these cells generally revealed >95% purity and >90% viability as assessed by trypan blue exclusion. Following removal of adherent MN, the microexudate flasks were rinsed three times with PBS and stored, containing 10 ml of 10 mM EDTA/PBS at 4°C, for reuse.

To obtain purified PMN, a previously described method (15) was used, with minor modifications. Briefly, the remaining medium from the Ficoll-Isopaque separation tubes was withdrawn and discarded, and the residual erythrocytes were subjected to cold hypotonic lysis, followed by centrifugation of this PMN-lysed erythrocyte suspension for 10 min at 160 *g*. The PMN pellet was then washed an additional two times with gel-HBSS before quantification and resuspension to a final concentration of 5×10^6 PMN/ml gel-HBSS. Contamination by MN was evaluated by Wright-stained smears and generally did not exceed 1–2%. Cell viability, as assessed by trypan blue exclusion, was generally >90%.

Phagocytosis mixtures and assays. Phagocytosis was determined by modification of a previously published method (16). Briefly, 0.1 ml from a suspension of opsonized of nonopsonized bacteria was added to duplicate polypropylene vials followed by the addition of 0.1 ml of the appropriate phagocytic cell type. The vials were then incubated for 5, 15, and 60 min at 37°C in a gyrotory shaker incubator (New Brunswick Scientific Co. Inc., Edison, N. J.) at 250 rpm. Most experiments were carried out using a 10:1 cfu to phagocyte ratio. In one experiment, ratios of 10, 100, and 200 cfu to one phagocyte were used.

Immediately after removal from the incubator at the indicated times, the duplicate sets of vials were separated, and to one set of vials was added 3.0 ml of ice-cold PBS, and to the second set 2.0 ml of scintillation liquid (Aquasol-2; New England Nuclear, Boston, Mass. was added. Radioactivity in this second set of vials, representing the total bacterial population, was counted using a Beckman LS-7500 liquid scintillation counter (Beckman Instruments, Inc.). Phagocyte-associated radioactivity was determined using the first vial from each duplicate set by washing the phagocytic cells three times in cold PBS by differential centrifugation at 160 *g* (4°C). The final phagocytic cell pellets were resuspended in 3.0 ml of scintillation liquid and were counted in the liquid scintillation counter. All experiments were repeated on at least 3 separate d with phagocytes from different normal donors. The uptake of bacteria by PMN or MN at a given sampling time was calculated with the following formula: % uptake = (cpm in washed phagocytic cell pellet/ total cpm) × 100.

Morphologic evaluation of phagocytosis. Phagocytosis was routinely assessed visually by examination of Wright-stained smears of phagocytic cells incubated with either opsonized or nonopsonized bacteria. Smears were made from phagocytic mixtures, which were constituted as indicated above, by removing a 50-μl sample from each vial after the defined incubation period, and adding it to a chamber of a Shandon Elliott Cytocentrifuge (Shandon Scientific Co. Ltd., London, England). The samples were then centrifuged for 3 min at 750 rpm, and the slides were removed, air dried, and stained with Wright's stain. Microscopic examination was done under oil immersion.

Bactericidal assay. Bacterial killing was measured by constituting mixtures as for the phagocytosis assay above and determining cfu at specific incubation times. Three sets of vials were made up for each assay, one to be sampled immediately upon addition of phagocytic cells (zero time), and the other two to be sampled at 15 and 60 min. Volumes of 0.1 ml of opsonized or nonopsonized bacteria were added to each vial, followed by the addition of 0.1 vol of the appropriate phagocytic cells. Then, following the desired incubation interval (0, 15, or 60 min), 1.0 ml of cold sterile water was added to each vial; the vials were mixed thoroughly, and appropriate dilutions were made. Samples from these dilutions were added to sterile petri dishes to which 10 ml of nutrient agar had already been poured and solidified followed by the addition of 10 ml nutrient agar containing 5% horse blood, and the culture was mixed well. After overnight incubation at 37°C, cfu were counted. The percent change in colony counts at 15 and 60 min, relative to cfu at zero time, was calculated. These experiments were performed on at least 3 separate d using phagocytic cells from three different donors.

Electron microscopy. Washed bacteria were exposed to 2% glutaraldehyde in PBS buffer, pelleted by centrifugation, and washed three times with distilled water. Portions of each aqueous suspension were spread on 8-mm glass discs and allowed to air dry. The discs bearing dried bacteria were cemented to stubs, sputtered with gold, and examined by scanning electron microscopy. Representative photographs were taken.

The remainder of each aqueous suspension was collected by centrifugation, and the bacterial pellet was resuspended in warm 1% agar contained in warm, conical centrifuge tubes. These tubes were immediately spun in a centrifuge to allow the bacteria to be pelleted before the agar hardened. The resulting agar-embedded bacterial pellet was minced and processed through osmium tetroxide fixation, dehydration, and final Epon 812 resin embedment. After curing, these blocks were sectioned, stained with uranyl acetate and Reynolds lead nitrate, and examined in a Philips 201 transmission electron microscope (Philips Electronic Instruments Inc., Mahwah, N. J.). In photographing these specimens the principal area of interest was the bacterial surface. Proper exposure to see the surface usually resulted in overexposure of the bacterium itself.

Measurement of complement activation. 0.1-ml samples of bacteria at specific concentrations, grown in the presence of clindamycin, or in its absence (control) were added to polypropylene vials containing 1 ml of the indicated serum source. After incubation for 15 and 60 min in the incubator shaker (250 rpm, 37°C), the vials were centrifuged at 2,000 *g* for 15 min (4°C), and the supernates were stored at −70°C until measurement of complement components C3-9 (17, 18). Haemolytic cellular intermediate EAC$\overline{14}$ was prepared according to a previously described method (19). Test samples were serially diluted from 1/20 to 1/640 in 0.01 M EDTA-gelatin-glucose-Veronal buffer without magnesium and calcium. 1 ml of 0.01 M EDTA-gelatin-glucose-Veronal buffer was added to 1.0 ml of each dilution of test samples. EAC$\overline{142}$ were obtained by incubation of EAC$\overline{14}$ with guinea pig complement (C2) for T_{max} at 30°C, and 1.0 ml EAC$\overline{142}$ (5×10^7/ml) was then added to each tube. After incubation at 37°C for 60 min, 4.5 ml of cold normal saline was added, and the tubes were centrifuged. The optical density of the supernates was read at 412 nm, and results were expressed as 50% lysis. Appropriate controls and complete blanks were run simultaneously.

Staining of bacteria with fluorescein-conjugated anti-C3 antibody. 0.1-ml samples (10^7 or 10^8 cfu) of *S. pyogenes*, grown in the presence or absence of clindamycin at various sub-MIC levels, were individually added to polypropylene vials containing 1.0 ml of normal serum, heat-inactivated serum, or gel-HBSS. After incubation at 37°C in the incubator shaker (250 rpm) for 15 and 60 min, the vials were centrifuged at 2,000 *g* for 15 min (4°C). The bacterial pellets were then washed three times with cold PBS. From the final bacterial pellets 5-μl samples were taken, deposited on glass slides, and air dried at room temperature. These bacterial preparations were then stained with fluorescein-conjugated monospecific antiserum to human C3, and the slides were examined under an immunofluorescence microscope.

RESULTS

Phagocytosis of S. pyogenes S43 grown in the presence and absence of clindamycin. After incubation of the different suspensions of bacteria (grown in the presence and absence of various sub-MIC levels of clindamycin) in 1 ml normal serum, heat-inactivated serum, and gel-HBSS, phagocytosis mixtures were constituted, and uptake by PMN and MN was measured at 3, 10, and 15 min (Fig. 1*a* and *b*). When normal serum was used as an opsonic source, phagocytosis of strepto-

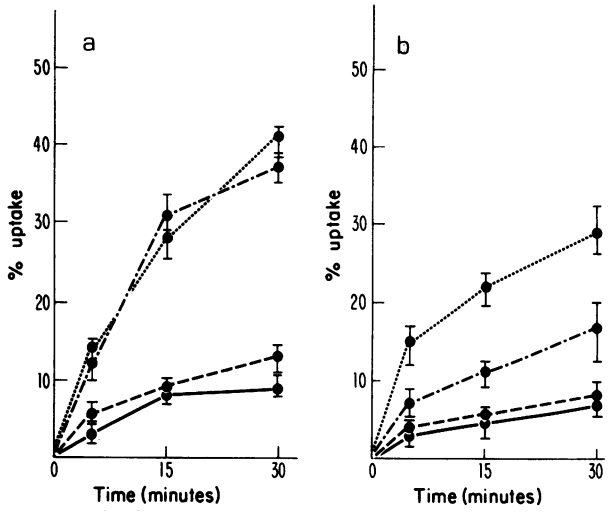


FIGURE 1 Phagocytosis of *S. pyogenes* S43 by PMN and MN. 4-h logarithmic-phase cultures grown in the presence or absence of clindamycin (1/2, 1/4, and 1/40 MIC) were used as targets for phagocytosis measured by following uptake of radiolabeled bacteria by PMN (*a*) and MN (*b*). —, streptococci grown in absence of clindamycin; ----, streptococci grown in presence of 0.025 μg/ml clindamycin (1/2 MIC); -·-·, streptococci grown in presence of 0.01 μg/ml clindamycin (1/4 MIC); - - - -, streptococci grown in presence of 0.001 μg/ml clindamycin (1/40 MIC).

cocci grown in the absence of antibiotic was poor compared with that of organisms grown in the presence of clindamycin. The respective values at 15 min for PMN were 9 vs. 42% and for MN, 6 vs. 30%. These values were obtained when the bacteria were grown in the presence of 0.025 μg/ml clindamycin (1/2 MIC); reduced levels of uptake were measured when the organisms were grown in lower concentrations of the drug. At 0.001 μg clindamycin/ml, no significant potentiation of uptake was detected. Heat-inactivated serum was a poor opsonic source for both control and drug-grown streptococci (<5% uptake), suggesting that the enhanced opsonization in normal serum was mediated by complement. There was virtually no leukocyte-associated radioactivity of streptococci when no opsonin (gel-HBSS) was used. These experiments were performed with a cfu to phagocyte ratio of 10:1. When higher ratios (100:1 and 200:1) were studied, similar results were obtained.

The enhanced phagocytosis of the drug-grown organisms was confirmed morphologically by Wright-stained smears. When normal serum was used as an opsonic source, >50% of the PMN and MN was found to contain several intracellular streptococci. In contrast, only a very occasional PMN or MN contained bacteria when organisms grown in the absence of clindamycin were studied.

Bacterial killing by PMN and MN. After incubation of the different suspensions of bacteria in 1 ml normal serum, heat-inactivated, serum and gel-HBSS, phagocytosis mixtures were constituted as before and bacterial killing by PMN and MN was measured at 30 and 60 min. As in the uptake experiments, a greater degree of streptococcal killing was demonstrated when the bacteria had been grown in the presence of clindamycin (75 vs. 42% for PMN and 75 vs. 50% for MN, Fig. 2a and b).

Complement activation by S. pyogenes grown in the presence and absence of clindamycin. To study the capacity for complement activation of *S. pyogenes* grown in the presence and absence of clindamycin, bacteria were incubated in normal serum at a concentration of 10^8 cfu/ml of serum for two time-intervals (15 and 60 min). After centrifugation, the haemolytic titer of C3-9 was measured in these sera and in simultaneous controls (normal serum incubated with an equal volume of distilled water for the same time intervals). Consumption of C3-9 in the sera incubated with bacteria was expressed as a percentage relative to the C3-9 levels in the control sera. A clear time vs. C3-9 consumption relationship was established for both the drug-grown and control streptococcal suspensions. Bacteria grown in the presence of clindamycin were capable of greater complement activation than control organisms (Table I). Drug-grown (1/2 MIC) streptococci consumed 31% of the available complement compared with 19% consumption by bacteria without drug. The differences were more apparent when the bacteria were incubated for 15 min (the time used for opsonization prior to use in the phagocytosis experiments) than when incubation was continued for 60 min.

Staining of bacteria with fluorescein-conjugated anti-C3. After incubation of 10^8 cfu of the drug-grown

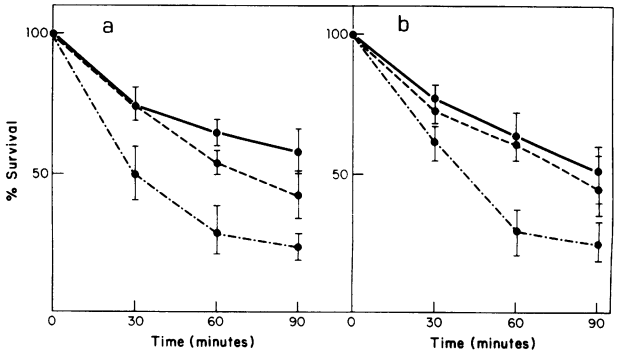


FIGURE 2 Killing of *S. pyogenes* S43 by PMN and MN. 4-h logarithmic-phase cultures grown in the presence or absence of clindamycin (1/2 and 1/4 MIC) were used as targets for killing by PMN (a) and MN (b). ——, streptococci grown in absence of clindamycin; -·-·, streptococci grown in presence of 0.025 μg/ml clindamycin (1/2 MIC); - - -, streptococci grown in the presence of 0.01 μg/ml clindamycin (1/4 MIC).

TABLE I
Complement Consumption by Cells of S. pyogenes S43, Grown in the Presence or Absence of Clindamycin, during Serum Opsonization

Bacterial inoculum	Opsonin	Complement consumption after 15 min	Complement consumption after 60 min
		%	
S43 grown in absence of drug	Normal serum	19	54
S43 grown in clindamycin (0.025 μg/ml)	Normal serum	31	100
S43 grown in clindamycin (0.01 μg/ml)	Normal serum	33	100

The human serum was used at a concentration of 10% in gel-HBSS to opsonize 1×10^8 cfu *S. pyogenes* in each part of the experiment.

and the control (no drug present) streptococci individually in 1.0 ml of normal serum, heat-inactivated serum, or gel-HBSS, the bacteria were washed, stained with fluorescein-labeled anti-C3 antibody, and examined. Whereas none of the bacterial specimens stained positively after incubation with heat-inactivated serum or gel-HBSS, both drug-grown and control bacteria showed some staining after incubation in normal serum (Table II). However, a clear difference in intensity of staining was observed with the drug-grown streptococci compared with the control organisms, indicating that the greater activation of the complement system corresponded to the increased fixation of C3 at the cell surface.

Ultrastructural appearance of streptococci grown in the presence and absence of clindamycin. 4-h cultures of *S. pyogenes* were used as source material for the investigation of the fine structure of the organisms. Using the scanning electron microscope, significant morphological changes were seen in the streptococci grown in the presence of clindamycin. Although chain length was not generally different, swollen cells, shrunken cells, and elongated cells were characteristic of the drug-grown organisms, especially when clindamycin was incorporated at 1/2 MIC. Fewer such cells were present (but still approaching 50% of the population) in the culture grown in 1/4 MIC of the drug (Fig. 3).

Thin sections of these preparations of bacteria as viewed under the transmission electron microscope also revealed significant differences between the drug-grown and control bacteria (Fig. 4). The surface "fuzz," which contains M-protein (8, 20), can be seen on the streptococci grown in the absence of clindamycin. This layer was essentially absent in the bacteria grown in the presence of 1/2 MIC clindamycin and partially present in those grown in 1/4 MIC. Such changes in cellular ultrastructure suggest that M-protein is absent in drug-grown bacteria.

TABLE II
C3 Binding to S. pyogenes Grown in the Presence or Absence of Clindamycin, during Serum Opsonization

Bacterial inoculum	Opsonin	C3 binding after 15 min	C3 binding after 60 min
S43 grown in absence of drug	Normal serum	1+	2+
S43 grown in clindamycin (0.025 μg/ml)	Normal serum	3+	4+
S43 grown in clindamycin (0.01 μg/ml)	Normal serum	2+	3+

The human serum was used at a concentration of 10% in gel-HBSS to opsonize 1×10^8 cfu *S. pyogenes* in each part of the experiment.
1+, trace reaction; 2+, weak reaction; 3+, strong reaction; 4+, intense reaction. The intensity of staining in each slide was evaluated in a blinded fashion by one of us (Y.K.).

DISCUSSION

Earlier studies (7) had shown that clindamycin, in contrast to several other antibiotics which affect bacterial protein synthesis, can impair the antigenicity of *S. pyogenes* in vitro and that such organisms are more readily phagocytosed by PMN. This effect was seen when clindamycin was incorporated into culture media at concentrations below those that inhibited growth of the organism. Our study has sought to investigate more closely how these alterations could affect the interaction of streptococci with phagocytic cells.

Phagocytosis by highly purified populations of human PMN and MN was quantified using preopsonized [^{3}H]thymidine-labeled bacteria. Our results have shown that growth in sub-MIC levels of clindamycin potentiates the susceptibility of *S. pyogenes* S43, an M-protein-containing strain, to phagocytosis by both cell types, and that this effect is related to enhanced opsonization of these organisms by serum complement. The degree of potentiation was related to the level of antibiotic incorporated in the growth medium. Clindamycin at 1/2 and 1/4 MIC was more effective than at 1/40 MIC. Bacteria grown in the presence of clindamycin were also killed more readily by both PMN and MN, with survival rates of 25 and 30%, respectively, compared with 60 and 52% for bacteria grown in the absence of the drug.

Ultrastructural studies using both scanning and transmission electron microscopy revealed that streptococci grown in the presence of clindamycin were changed morphologically and had greatly diminished

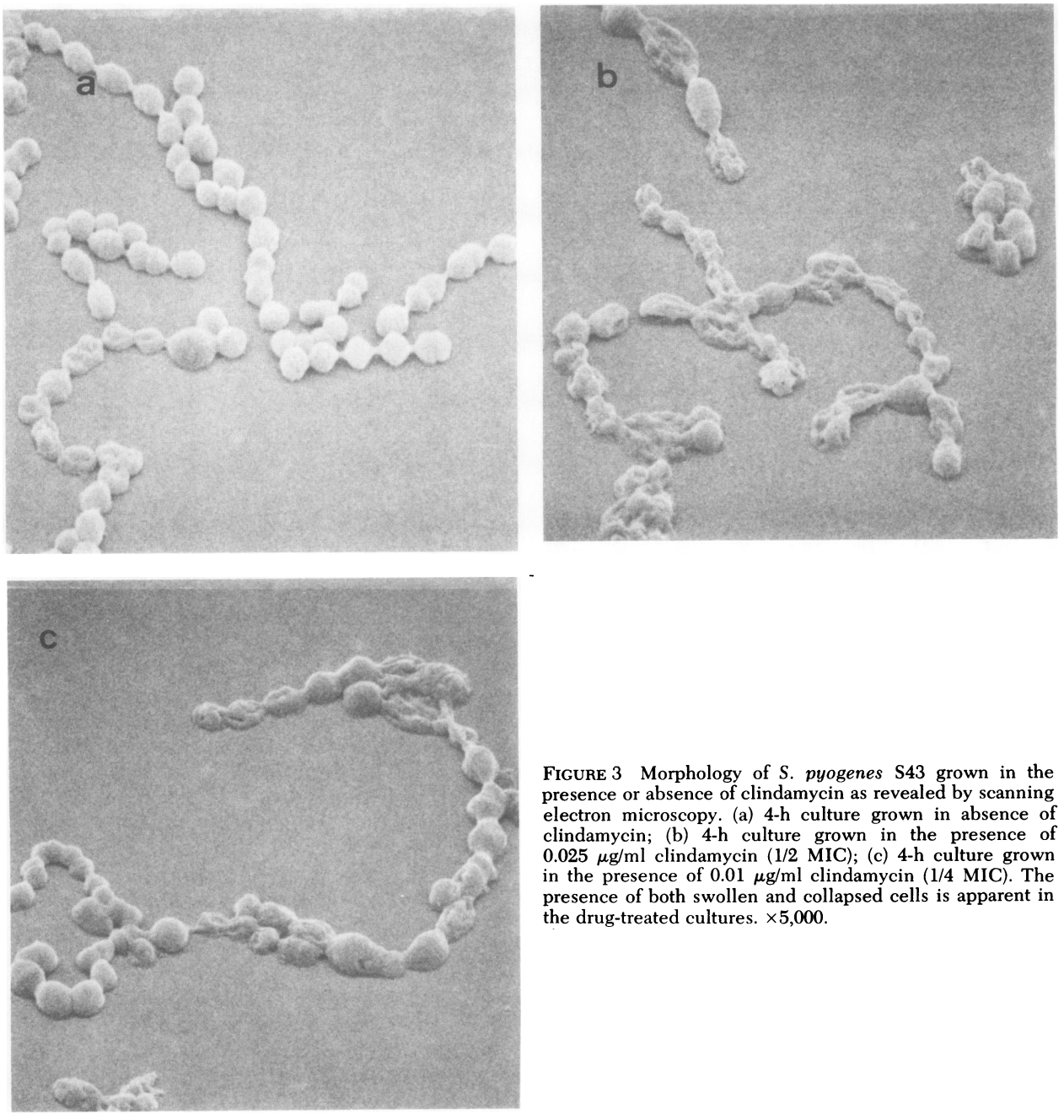
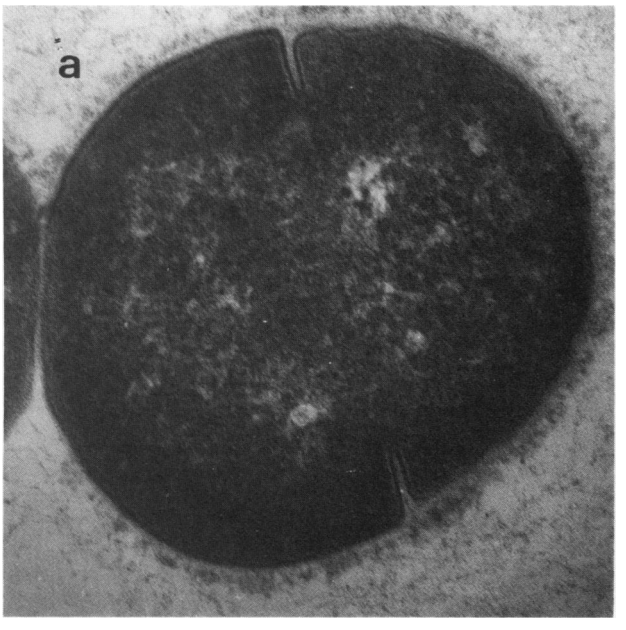
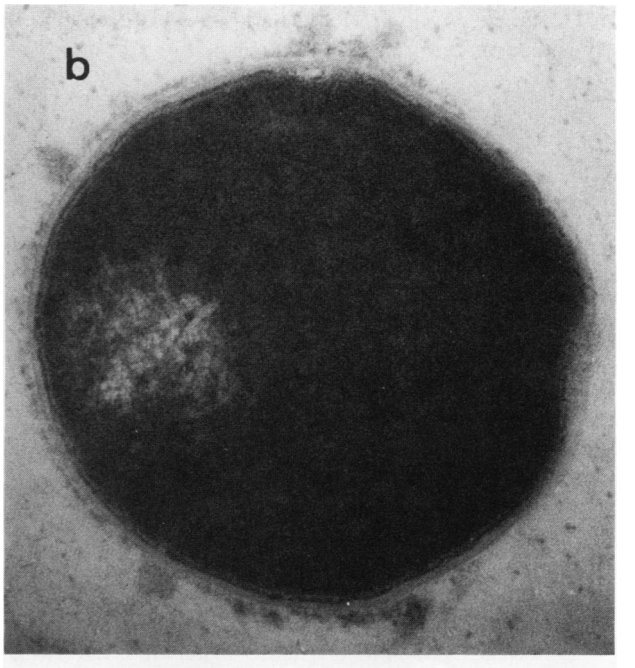


FIGURE 3 Morphology of *S. pyogenes* S43 grown in the presence or absence of clindamycin as revealed by scanning electron microscopy. (a) 4-h culture grown in absence of clindamycin; (b) 4-h culture grown in the presence of 0.025 μg/ml clindamycin (1/2 MIC); (c) 4-h culture grown in the presence of 0.01 μg/ml clindamycin (1/4 MIC). The presence of both swollen and collapsed cells is apparent in the drug-treated cultures. ×5,000.

surface "fuzz," the hairlike structures which contain M-protein (8). This was especially noticeable when the bacteria had been grown in 1/2 MIC clindamycin; these cells were essentially devoid of surface "fuzz." At 1/4 MIC clindamycin, only parts of the cell surface lacked these structures, and the remainder of the cell surface appeared normal. Using a serological method to quantitate M-protein, Gemmell and Amir (6) had previously shown that clindamycin-exposed streptococci had significantly reduced amounts of M-protein. These ultrastructural changes in the drug-exposed organisms could explain their greater capacity to activate serum complement and to bind the opsonically active fragment of C3, i.e., C3b (21), to their surface. These findings are consistent with previous studies, which have shown that in contrast to M-protein positive strains, M-protein negative variants are readily opsonized by the alternate pathway of complement (9, 10).

Although the main therapeutic effect of antibiotics





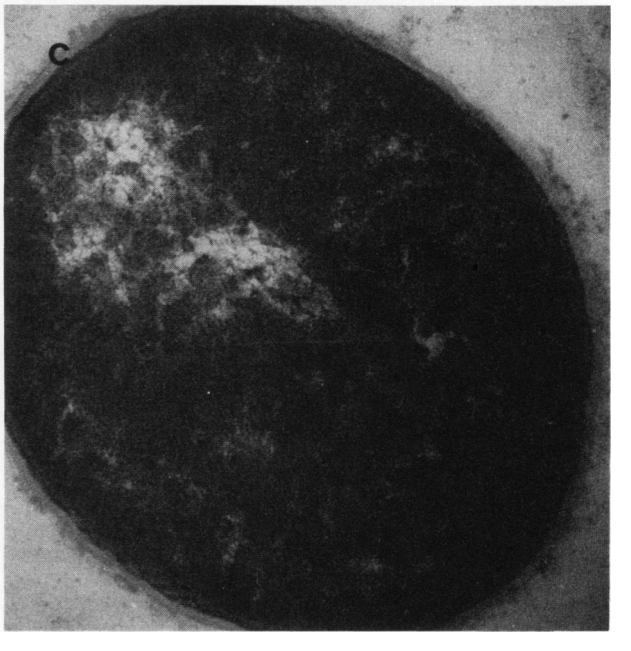


FIGURE 4 Morphology of *S. pyogenes* S43 grown in the presence or absence of clindamycin as revealed by transmission electron microscopy. (a) 4-h culture grown in the absence of clindamycin; (b) 4-h culture grown in the presence of 0.025 μg/ml clindamycin (1/2 MIC); (c) 4-h culture grown in the presence of 0.01 μg/ml clindamycin (1/4 MIC). The presence or absence of an electron-dense "fuzz" is demonstrated in these cells. ×5,000.

in vivo is undoubtedly related to their ability to inhibit growth and/or to kill bacteria directly, the results of this study and the work of other investigators (22, 23) suggest that even at sub-MIC levels, certain antibiotics might indirectly decide the fate of microorganisms by enhancing their uptake by phagocytic cells of the host.

Recently, Ofek et al. (24) investigated the effects of sub-MIC of both penicillin and streptomycin on the ability of growing or resting phase bacteria to adhere to oropharyngeal epithelial cells. With resting phase bacteria, the addition of penicillin resulted in diminished adherence of *S. pyogenes*, and this was associated with a loss of lipoteichoic acid, which normally binds *S. pyogenes* to specific receptor sites on the epithelial cell. Like M-protein, lipoteichoic acid is found in the surface "fuzz" of *S. pyogenes* (8). Given the ultrastructural changes brought about by growth in sub-MIC levels of clindamycin that we observed in the present study, it is possible that this

important mechanism in the pathogenesis of group A streptococcal infection might also be significantly altered.

ACKNOWLEDGMENTS

We are grateful to Marge Lindemann for excellent technical assistance and to Paulette Juin for typing the manuscript.

This work was supported by National Institutes of Health grant 2R01 AI-08821-02 and by the Wellcome Foundation, United Kingdom.

REFERENCES

1. Gardner, A. D. 1940. Morphological effects of penicillin on bacteria. *Nature (London)* **146:** 837–838.
2. Lorian, V. 1980. Effects of subminimum inhibitory concentrations of antibiotics on bacteria. *In* Antibiotics in Laboratory Medicine. V. Lorian, editor. Williams and Wilkins, Baltimore, Md. 342–408.
3. Eisenstein, B. I., I. Ofek, and E. H. Beachey. 1979. Interference with the mannose binding and epithelial cell adherence of *Escherichia coli* by sublethal concentrations of streptomycin. *J. Clin. Invest.* **63:** 1219–1228.
4. Sandberg, T., K. Stenquist, and C. Svanborg-Edén. 1979. Effects of subminimal inhibitory concentrations of ampicillin, chloramphenicol and nitro-furantoin on the attachment of *Escherichia coli* to human uroepithelial cells *in vitro*. *Rev. Infect. Dis.* **1:** 838–844.
5. Lorian, V., and B. A. Atkinson. 1979. Effect of serum and blood on Enterobacteriaceae grown in the presence of subminimal inhibitory concentrations of ampicillin and mecillinam. *Rev. Infect. Dis.* **1:** 797–806.
6. Gemmell, C. G., and M. K. A. Amir. 1978. Effects of certain antibiotics on the formation of cellular antigens and extracellular products by group A streptococci. *In* Pathogenic Streptococci. M. T. Parker, editor. Reedbooks, Ltd., England. 67–68.
7. Gemmell, C. G., and M. K. A. Amir. 1980. Antibiotic induced changes in streptococci with respect to their interaction with human polymorphonuclear leukocytes. *In* Current Chemotherapy and Infectious Disease. J. D. Nelson and C. Grassi, editors. American Society for Microbiology, Washington, D. C. **2:** 810–812.
8. Fox, E. N. 1974. M proteins of Group A streptococci. *Bacteriol. Rev.* **38:** 57–86.
9. Peterson, P. K., D. Schmeling, P. P. Cleary, B. J. Wilkinson, Y. Kim, and P. G. Quie. 1979. Inhibition of alternative complement pathway opsonization by Group A streptococcal M protein. *J. Infect. Dis.* **139:** 575–585.
10. Bisno, A. L. 1979. Alternate complement pathway activation by Group A streptococci: role of M protein. *Infect. Immun.* **25:** 1172–1176.
11. Becker, C. G. 1967. Enhancing effect of type specific anti-streptococcal antibodies on the emergence of streptococci rich in M protein. *Proc. Soc. Exp. Biol. Med.* **124:** 331–335.
12. Washington, J. A., and A. L. Barry. 1974. Dilution test procedure. *In* Manual of Clinical Microbiology. E. H. Lennette, E. H. Spaulding, and J. P. Truant. American Society for Microbiology, Washington, D. C. 2nd edition. 410–417.
13. Ackerman, S. K., and S. D. Douglas. 1978. Purification of human monocytes on microexudate coated surfaces. *J. Immunol.* **120:** 1372–1374.
14. Poste, G., L. W. Greenham, L. Mallucci, P. Reeve, and D. J. Alexander. 1973. The study of cellular "microexudate" by ellipsometry and their relationship to the cell coat. *Exp. Cell Res.* **78:** 303–313.
15. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97): 77–89.
16. Peterson, P. K., J. Verhoef, D. Schmeling, and P. G. Quie. 1977. Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. *J. Infect. Dis.* **136:** 502–509.
17. Gerwurz, H., A. R. Page, R. J. Pickering, and R. A. Good. 1967. Complement activity and inflammatory neutrophil exudation in man: studies in patients with glomerulonephritis, essential hypocomplementemia and agammaglobulinemia. *Int. Arch. Allergy Appl. Immunol.* **32:** 64–90.
18. McLean, R. H., H. Geiger, B. Burke, R. Simmons, J. Najarian, R. L. Vernier, and A. F. Michael. 1976. Recurrence of membranoproliferative glomerulonephritis following kidney transplantation. Serum complement component studies. *Am. J. Med.* **60:** 60–72.
19. Borsos, T., and H. J. Rapp. 1967. Immune haemolysis: a simplified method for the preparation of EAC'4 with guinea pig or with human complement. *J. Immunol.* **99:** 263–268.
20. Swanson, J., K. C. Hsu, E. C. Gotschlich, 1968. Electron microscopy studies on streptococci. I M antigen. *J. Exp. Med.* **130:** 1063–1091.
21. Stossel, T. P., R. J. Field, J. D. Gitlin, C. A. Alper, and F. S. Rosen, 1975. The opsonic fragment of the third component of human complement (C3). *J. Exp. Med.* **141:** 1329–1347.
22. Friedman, H., and G. H. Warren. 1974. Enhanced susceptibility of penicillin-resistant staphylococci to phagocytosis after IN VITRO incubation with low doses of nafcillin. *Proc. Soc. Exp. Biol. Med.* **146:** 707–711.
23. Pruul, H., and P. J. McDonald. 1980. Enhancement of antibacterial activity of human leukocytes against *Escherichia coli* pretreated with chloramphenicol. *In* Current Chemotherapy and Infectious Disease. J. D. Nelson and C. Grassi, editors. American Society for Microbiology, Washington, D. C. **4:** 796–797.
24. Ofek, I., E. H. Beachey, B. I. Eisenstein, M. L. Alkan, and N. Sharon. 1979. Suppression of bacterial adherence by subminimal inhibitory concentrations of β-lactam and aminoglycoside antibiotics. *Rev. Infect. Dis.* **1:** 832–837.