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Activity of Antibiotics in Chronic Granulomatous Disease Leukocytes

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Summary

Patients with chronic granulomatous disease (CGD) have recurrent infections with catalase-producing organisms, particularly staphylococci, that survive within their leukocytes. To be most effective, antibiotics used to treat infections in CGD patients may need to kill both intracellular and extracellular organisms. We studied the ability of certain antibiotics to penetrate normal and CGD neutrophils and to kill intracellular staphylococci. Trimethoprim and clindamycin were concentrated in normal and in CGD neutrophils; maximum cellular-to-extracellular concentration ratios of clindamycin and of trimethoprim were ~30 and ~4, respectively. In contrast, penicillin was excluded from normal neutrophils. Clindamycin, trimethoprim/sulfamethoxazole, rifampin, and trimethoprim/rifampin significantly reduced the number of viable intracellular staphylococci in normal and CGD neutrophils. After 24 h of incubation in the presence of these antibiotics, the number of viable intracellular staphylococci in normal and CGD neutrophils was similar. In contrast, dicloxacillin, gentamicin, and cephalothin had no significant effect on the number of intracellular staphylococci in normal or CGD neutrophils.

Abbreviations

C, clindamycin
C/E, cellular-to-extracellular concentration ratio
cfu, colony forming units
CGD, chronic granulomatous disease
D, dicloxacillin
MBC, minimum bactericidal concentration
PMNs, polymorphonuclear leukocytes
Rif, rifampin
SMZ, sulfamethoxazole
TMP, trimethoprim

CGD is a clinical syndrome characterized by recurrent infections. PMNs from these patients do not kill phagocytosed microorganisms that produce catalase (1). Consequently, continual antibiotic administration has been employed by some to prevent and to treat chronic and recurrent infection in these patients. Certain investigators have warned that continual antibiotic administration may lead to the selection of more virulent organisms or antibiotic-resistant organisms (2); others have reported beneficial results without recognizable adverse effects (3, 4).

In CGD patients, to be effective, antibiotic therapy may need to act both against intracellular and extracellular organisms. Persistence of viable intracellular staphylococci has been suggested as an explanation for treatment failures with penicillins; these antibiotics are hydrophilic and do not penetrate into PMNs (5). We, therefore, studied the penetration and antimicrobial activity of certain lipophilic and hydrophilic antibiotics in CGD and normal PMNs.

MATERIALS AND METHODS

MBC. MBCs were determined by the tube dilution method (6) in Medium 199 (M199; Grand Island Biological Co., Grand Island, NY), using the same concentration of bacteria and the same culture conditions as were used in the bactericidal assay. Under these conditions, the MBCs of *Staph. aureus*, strain 502A, were the following: TMP, 1 μg/ml; SMZ, 4 μg/ml; D, 0.4 μg/ml; Rif, 0.2 μg/ml; TMP/SMZ, 0.25/5 μg/ml; TMP/Rif, 0.5/1.0 μg/ml. The MBCs for the Sudra strain of *Staph. aureus*, a recent isolate from a patient with bacteremia, were the following: TMP, 8 μg/ml; SMZ, >80 μg/ml; D, 4 μg/ml; Rif, 1 μg/ml; TMP/SMZ, 4/50 μg/ml; TMP/Rif, 4/1 μg/ml; C, 1 μg/ml; gentamicin, 1 μg/ml; and cephalothin, 2 μg/ml. The antibiotic concentrations used in the bactericidal assays are indicated in the results.

Bactericidal activity against intracellular Staph. aureus. Intracellular killing of *Staph. aureus* 502A (three experiments) and the Sudra strain of *Staph. aureus* (two experiments) by PMNs from CGD and normal subjects were evaluated in parallel. None of the subjects were receiving antibiotics, and all were free of signs or symptoms of infection at the time of the assays. Purified PMNs (>95%) were isolated as previously described (7); viability was >97% by trypan blue dye exclusion. Reaction mixtures (3.5 ml total volume) contained PMNs (6×10^6/ml), 15% human type AB serum stored at −70°C to preserve complement, 25% heat-inactivated pooled human serum, and *Staph. aureus* (1.2×10^7 organisms/ml) in M199. The assay was initiated by the addition of organisms to the suspension; immediately after mixing, duplicate 0.1-ml aliquots were removed, diluted initially in 0.9 ml of distilled water and then serially diluted in phosphate-buffered saline with 0.1% gelatin. Aliquots of these dilutions were cultured on Trypticase soy agar at 37°C for 18–20 h, then viable organisms were enumerated. The remaining 3.4 ml of the reaction mixture was incubated at 37°C in a water bath shaking at 100 rpm; after 30 min this was centrifuged at $125 \times g$ and the PMN pellet was resuspended in M199 containing 10 units of lysostaphin per ml for 10 min. The PMN pellet was then washed twice in M199 by centrifugation at $250 \times g$ for 10 min, and resuspended in 3.4 ml of M199. This procedure effectively removed extracellular organisms because (*1*) cultures of the final wash supernatant were sterile and (*2*) filtration of the PMN pellet through 1-μ pore size or 2-μ pore size filters (Nuclepore Corp., Pleasanton, CA) removed all PMNs (as determined by phase-contrast microscopy) and decreased organisms to an undetectable density (<10 cfu/ml). A second 0.1-ml aliquot of the reaction mixture was added to distilled water, diluted, and cultured. The remaining reaction mixture was then aliquoted into 1-ml volumes containing M199 alone or M199 plus one of the antibiotics or antibiotic combinations. The tubes were incubated at 37°C in a water bath shaking at 100 rpm. At 2, 6, and 24 h, an aliquot of the reaction mixture was processed for quantitation of viable PMN-associated organisms.

Certain antibiotics are rapidly degraded in solution; therefore, the bioactivity of each antibiotic in the reaction mixtures after

24 h of incubation was determined by assay against *Staph. aureus* (Sudra) (6). Assays for the residual activity included, as controls, supernatants from PMNs in M199 and M199 alone to determine if reaction-mixture components inhibited the growth of organisms.

Antibiotic uptake by PMNs. TMP and C penetration into PMNs was assayed by a previously described method (8). Reaction mixtures containing PMNs in M199 plus [^3H_2O] (25 mCi/ml, New England Nuclear, Boston, MA), Na_2 [$^{35}SO_4$] (50 mCi/mmole, New England Nuclear, Boston, MA), [^{14}C]-TMP (10 mCi/mmole; Ciba-Geigy, Summit, NJ), or [^{3}H]-C (67.6 mCi/mmole; Upjohn, Kalamazoo, MI) were incubated for the indicated intervals, layered over inert silicone oil (Versilube, General Electric Co., Schenectady, NY), and centrifuged at 10,000 × *g* for 2 min. Radioactivity of the PMN pellet and an aliquot of the supernatant were determined in a liquid scintillation spectrometer. The total and extracellular water content of the PMN pellet was determined by relating the cpm in the PMN pellet to the cpm in the known volume of the supernatant for [^3H_2O] and Na_2 [$^{35}SO_4$], respectively. The extracellular water content was always <10% of the total water content (normal, 0.42 ± 0.27 μl/10^6 PMN, and CGD, 0.35 ± 0.13 μl/10^6 PMN of the PMN pellet). The cellular water content of the PMN pellet was determined by subtracting the extracellular water content from the total water content of the PMN pellet. The C/E of TMP and C was determined as previously described (8). PMN viability was 95% ± 0.7% at 10 min and 88.9% ± 2.4% at 24 h.

Statistics. Variance is expressed as standard error. The significance of differences between means was determined by the Student's *t* test (9).

RESULTS

Bactericidal activity of antibiotics against intracellular Staph. aureus. Intracellular killing of the Sudra strain of *Staph. aureus* by CGD and normal PMNs is shown in Figures 1A and B. These results were obtained with antibiotic concentrations that were equal to or twice the MBC for the organism and within the range of clinically achievable serum concentrations. Rif, and the combinations TMP/SMZ and TMP/Rif reduced the intracellular bacterial density in CGD and normal PMNs more effectively than any of the other drugs evaluated in these studies.

In contrast, dicloxacillin, a hydrophilic antibiotic, had little effect on intracellular bacterial density in either cell type. In two additional experiments, the effects of the hydrophilic antibiotics, cephalothin and gentamicin, were also evaluated. At 2 and 6 h (not shown) and at 24 h, values for cephalothin, gentamicin and no drug were comparable in normal PMNs (5.0 ± 2.8 × 10^4, 1.7 ± 2.9 × 10^4 and 4.1 ± 1.6 × 10^4 cfu/ml, respectively) and in CGD PMNs (1.1 ± 3.0 × 10^7, 4.8 ± 4.1 × 10^6 and 1.6 ± 2.1 × 10^7 cfu/ml, respectively) whereas results with C were significantly different ($P < 0.001$) (normal, 2.0 ± 1.3 × 10^6 cfu/ml and CGD, 4.1 ± 3.8 × 10^2 cfu/ml). Results with strain 502A using antibiotic concentrations equal to or 4–10 times the MBC were comparable; gentamicin, and cephalothin were not tested with this strain. In the assay supernatants, bioactivity of each antibiotic decreased less than 25% after 24 h incubation. PMN viability was 85.6 ± 1.2% after 24 h of incubation; values for control and CGD PMNs were comparable. Under these conditions, in the presence of clinically achievable serum concentrations of C, TMP/SMZ, TMP/Rif, or Rif the bactericidal activity of CGD and normal PMNs was comparable at 24 h.

Antibiotic uptake by PMNs. As shown in Figure 2, TMP and C were rapidly taken up by normal PMNs. Maximum C/E concentration ratios were observed at 10 min. Thereafter, the C/E concentration ratio of TMP declined up until 120 min (0.44 ± 0.11) whereas the C/E concentration ratio of C continued to decline throughout the assay and was lowest at 24 h (2.75 ± 0.62). This decline was not associated with: (*1*) PMN clumping (10) because by phase contrast microscopy little clumping was observed at any time point, (*2*) a comparable change in PMN viability, which was 95 ± 1% at 10 min and 89 ± 2% at 24 h, and (*3*) a change in the extracellular or total water content of the PMN pellet (data not shown). Nevertheless, C/E concentration ratios of both drugs were greater at all time points than the C/E concentration ratio of penicillin G, which we have previously reported to be less than 0.2 and stable for up to 120 min of incubation (8). At the time points studied, penetration of TMP (120 min, C/E = 0.38 ± 0.11, $n = 3$) and C (10 min, C/E = 30.9 and 120 min C/E = 8.18 ± 1.24, $n = 2$) into CGD PMNs was similar to that in normal PMNs.

DISCUSSION

Continuous antibiotic administration to patients with CGD has been advocated since 1967 (1); various reports have suggested that nafcillin (3), C (4), sulfonamides (1), and TMP/SMZ (11, 12) (Harvey Cohen, M.D., personal communication, 1981) may be effective in preventing recurrent or recrudescent staphylococcal infections in such patients. Without a controlled clinical trial, as has been done in Chediak-Higashi syndrome (13), the efficacy of such therapy is unclear. Before such a clinical trial is conducted, the antibiotic(s) most likely to be effective may be suggested by *in vitro* studies of antimicrobial activity in CGD PMNs. The effectiveness of antibiotics for prophylaxis and treatment in CGD patients may be determined in part by their activity against intracellular organisms. If antibiotics that do not enter PMNs are employed, persistence of viable intracellular organisms may contribute to treatment failures in normal patients (5) and in CGD patients.

Our results indicate that C and the combinations TMP/SMZ and TMP/Rif were most effective in killing intracellular *Staph. aureus in vitro* and that C and TMP penetrated into normal and CGD PMNs and were at least transiently concentrated in these cells. Other studies on the interaction between antibiotics and CGD PMNs have studied either penetration or antimicrobial activity of one or two antibiotics in these cells. The results of these limited studies are compatible with ours: (*1*) TMP and SMZ penetrate into normal and CGD PMNs (11) and, (*2*) Rif penetrates into normal PMNs (14) and is active against *Staph. aureus* in normal and CGD PMNs (15).

In the present study, intracellular antimicrobial activity in normal and CGD PMNs of antibiotics from different classes was assessed; this activity was related to penetration into PMNs by certain of these antibiotics. Our results indicate that lipophilic antibiotics, which penetrate PMNs, reduce the bacterial density in CGD PMNs to levels comparable to those in normal PMNs. In contrast, hydrophilic antibiotics, such as dicloxacillin, gentamicin, and cephalothin are not effective against intracellular *Staph. aureus*. We suggest that this is due, at least in part, to poor penetration of such drugs into PMNs, which we and others have previously observed (8, 11).

Others have suggested that certain lipophilic antibiotics kill intracellular *Staph. aureus* by a direct antibacterial effect. Klempner and Styrt (10) demonstrated that C retained its bioactivity intracellularly. Gmünder and Seger (11) observed that TMP/SMZ did not inhibit bacterial catalase, enhance PMN oxidative metabolism, or synergistically affect non-oxygen dependent killing by PMNs. They concluded that its effect was direct and dependent on intracellular penetration. Utilizing a strain of *Staph. aureus* that was resistant to C (MBC, 128 μg/ml) and Rif (MBC, 2 μg/ml), we obtained data that support a similar mechanism of action for C and Rif. With this strain, the intracellular bacterial density was not reduced by concentrations of these drugs that were effective against the 502A and Sudra strains (not shown). Our data suggest that the reduction of intracellular bacterial density by these antibiotics was due to a direct antibacterial effect.

Eradication of intracellular organisms may contribute to resolution of infection and prevention of recrudescent infection in

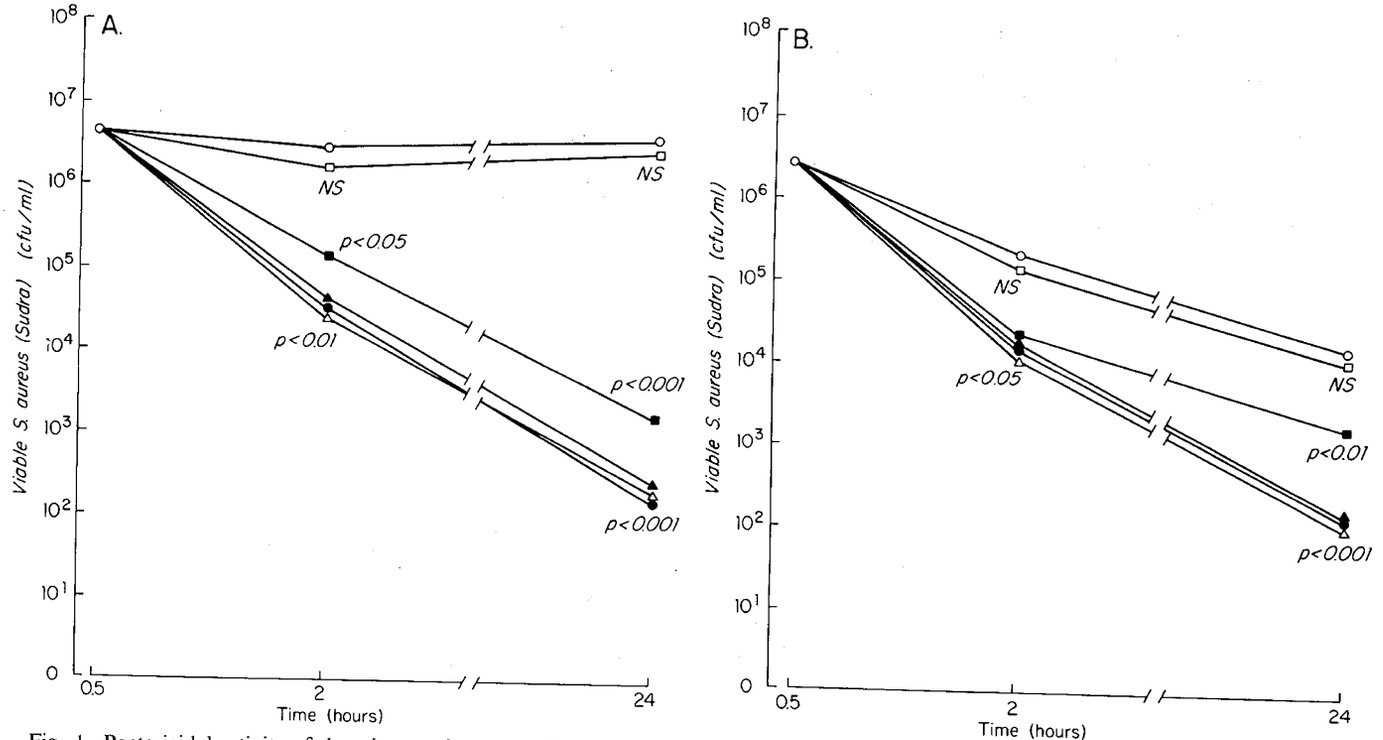

Fig. 1. Bactericidal activity of chronic granulomatous disease polymorphonuclear leukocytes (*A*) and normal polymorphonuclear leukocytes (*B*) alone and in the presence of antibiotics against a clinical isolate (Sudra strain) of *Staph. aureus*. Viable intracellular *Staph. aureus*, expressed as the $\log_{10}$ colony forming units (cfu/ml), are shown on the ordinate; time in h is shown on the abscissa. The bactericidal activity is shown with polymorphonuclear leukocytes in the absence of antibiotics (○); and in the presence of 8 μg/ml dicloxacillin (□), 1 μg/ml rifampin (■), 4/80 μg/ml trimethoprim/sulfamethoxazole (△), 4/1 μg/ml trimethoprim/rifampin (▲), and 1 μg/ml clindamycin (●).

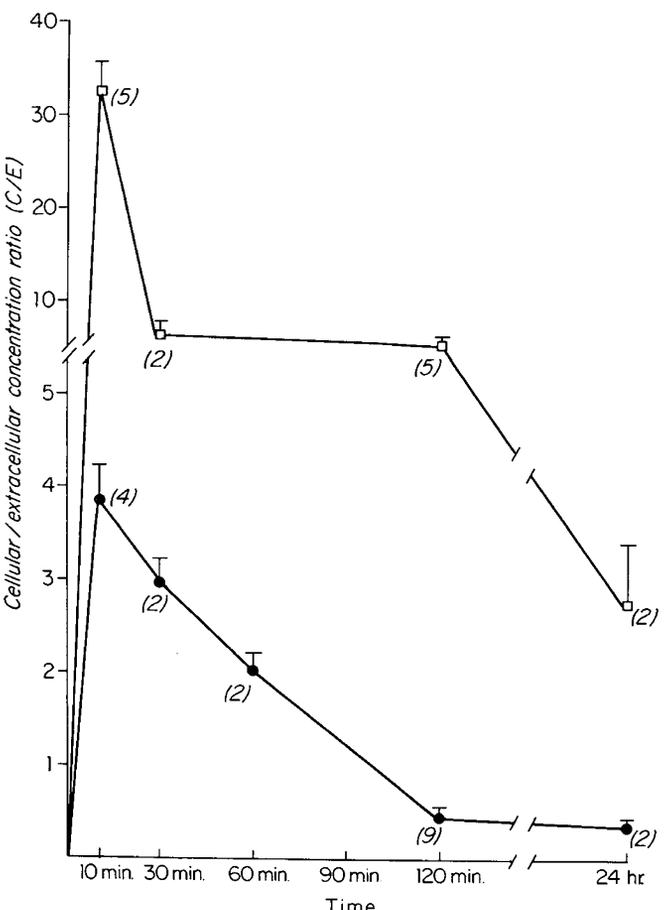

Fig. 2. Kinetics of trimethoprim and clindamycin uptake by normal polymorphonuclear leukocytes. Cellular to extracellular concentration ratio (C/E) of trimethoprim (●) and clindamycin (□) is expressed on the ordinate and time on the abscissa. (*n*) is the number of experiments.

CGD patients and in certain normal patients; thus, C may be a logical drug for the treatment of staphylococcal infections in CGD patients. Because pseudomembranous colitis may complicate C therapy more frequently than it complicates therapy with other drugs, it may be less suitable for continuous treatment. TMP/SMZ or TMP/Rif, which also are active against many gram-negative organisms that are the major cause of fatal infections in CGD patients (2), may be more suitable for continuous therapy. Although these data indicate that TMP/SMZ and TMP/Rif may be suitable for continuous therapy in CGD patients, a prospective, controlled study to analyze the efficacy and potential complications of chronic antibiotic administration in patients with CGD is needed.

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Hemodynamics in Experimental Hypernatremic Dehydration with Special Reference to Individual Organ Blood Flow in Shock and after Rehydration

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Summary

Shock after hypernatremic dehydration in the mini-pig is characterized by low cardiac output but little reduction of arterial blood pressure. Maintenance of pressure is due to extensive arteriolar vasoconstriction in the splanchnic and renal vascular bed, as calculated from their markedly diminished blood flow. The expected preservation of flow to vital organs did occur in the brain and the adrenals, but not in the heart. Sufficient oxygen was probably provided by the elevated hematocrit.

After 24 h, intravenous fluid therapy produced adequate rehydration as seen from the correction of azotemia, metabolic acidosis, and hypernatremia; only serum creatinine remained elevated. Although cardiac output increased, it did not reach the initial value. Blood flow to most organs was back to normal, but gastrointestinal and particularly renal blood flow remained diminished.

Although diagnosis and treatment of hypernatremic dehydration in infancy follows established guidelines (10, 18), proper management of this disease remains a challenge for the clinician. Assessment of the circulatory state still represents a major problem because these infants are often in a state of pre-shock or shock. Little is known about the hemodynamic consequences of the perfusion of vital organs and the effect of treatment. We therefore developed an animal model where hemodynamic changes and particularly cardiac output, its distribution and individual organ blood flow could be investigated.

METHODS

In 20 young 2-month-old mini-pigs, weighing 3.0 kg (2.6–3.66), polyvinyl catheters were inserted into the right femoral vessels; through a left-sided thoracotomy a thin polyethylene PP 30 catheter was introduced into the left atrium via the left atrial appendage. The operation was performed under halothane/O_2 anesthesia with the animal intubated and ventilated. The atrial catheter went through a subcutaneous dorsal tunnel and the femoral catheters were fixed at the groin in order that the catheters could be manipulated in the unrestrained awake animal. Operation time was on average 240 min. After 3 days of recovery, measurements of blood chemistry and hemodynamics were performed. Sodium, potassium, and calcium were measured with flame photometry; chloride was determined amperometrically. Glucose was measured with the oxidase reaction, creatinine with the Jaffé's reaction, and urea with the urease digestion-method. Osmolarity was determined by the freezing point depression. Arterial blood gases were measured on an Acid-Base-O_2 Microanalyzer, model 939, AVL AG, Schaffhausen, Switzerland, and adjusted to the animals body temperature, which was measured continuously with a rectal probe. Cardiac output was calculated with the dye dilution method using cardiogreen on a Waters XC-Densitometer. Pressure measurements were done with a Statham P 23 Dc transducer and a Honeywell 2106 Visicorder. Distribution of cardiac output was measured with the left atrial injection of radioactive microspheres ([^{46}Sc], [^{85}Sr], [^{51}Cr], [^{141}Ce], and [^{125}I]) (9, 15, 17).

In five animals, spheres with a diameter of 50 μ and in 15 animals spheres with a diameter of 15 μ were used and each injection averaged 37,670 for the 50- and 351,000 for the 15-μ spheres. Studies on the use of microspheres in the mini-pig showed no difference in extraction for 50- or 15-μ spheres for all major organs (11).

The relatively small number of injected 50-μ spheres limited the accuracy of flow determination in the adrenal and partly in pancreas, liver artery, and spleen because 400 microspheres are required for a $\pm 10\%$ accuracy and this number could not always be reached (1). This problem was, however, compensated for by an adequate number of animals.

The gamma activity of the dissected animals was counted on