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Effect of Antimicrobial Agents on Human Polymorphonuclear Leukocyte Microbicidal Function

WILLIAM D. WELCH,[1]* DAVE DAVIS,[2] AND LAURI D. THRUPP[1,3]

Departments of Medicine[1] and Obstetrics-Gynecology,[3] University of California, Irvine, California 92668, and Department of Microbiology, California State University at Long Beach, Long Beach, California 90801[2]

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The effect of 19 antimicrobial agents on human polymorphonuclear leukocyte function was evaluated by chemiluminescence assays, yeast phagocytosis and killing, and lactate dehydrogenase release. Tetracycline and trimethoprim inhibited chemiluminescence and reduced killing at therapeutic concentrations of 2 μg/ml. Cephalothin inhibited yeast killing at a concentration of 20 μg/ml, but a significant depression of polymorphonuclear leukocyte chemiluminescence was encountered only at higher levels of 200 μg/ml. The inhibition shown by these drugs was reversible. None of the other antimicrobial agents tested demonstrated inhibition of chemiluminescence, phagocytosis, or killing at usual clinical serum levels. No antimicrobial agent tested caused release of lactate dehydrogenase from polymorphonuclear leukocytes. The results suggest that therapeutic concentrations of tetracycline, trimethoprim, and cephalothin may inhibit optimal polymorphonuclear leukocyte microbicidal function.

Several antimicrobial agents have been reported to inhibit human polymorphonuclear leukocyte (PMNL) function. For example, tetracycline, gentamicin, and cefamandole reduce either phagocytosis, bacterial killing, Nitro Blue Tetrazolium reduction, or chemotaxis by PMNLs (4, 9, 11, 14). The present study was undertaken to explore the nature of the inhibition of PMNL microbicidal function by these and other widely used antimicrobial agents. PMNL functions assessed included PMNL chemiluminescence (CL), yeast phagocytosis and killing, and PMNL viability.

Enzyme systems in PMNLs, such as myeloperoxidase or reduced nicotinamide adenine dinucleotide phosphate oxidase, which contribute significantly to bacterial killing, are also partly responsible for the emission of light or CL by PMNLs (2, 8). Demonstration of a defect in the generation of CL could thus provide insight into the mechanism of drug-caused reduction in PMNL microbicidal function. Our results showed that tetracycline, trimethoprim, and cephalothin depressed the CL response of human PMNLs, although their effects were reversible. Decreased phagocytosis and yeast killing by PMNLs also correlated with the CL results. Because none of the antimicrobial agents was found to induce cell damage as measured by the release of the cytoplasmic enzyme lactate dehydrogenase, the observed inhibition of the phagocytic and killing function of PMNLs by certain antimicrobial agents in vitro may be due to an altered PMNL oxidative microbicidal system.

MATERIALS AND METHODS

Preparation of human PMNLs and pooled human serum. Human PMNLs from normal, healthy, adult donors were separated from heparinized venous blood by sedimentation with plasmagel (Roger Bellon Laboratories, Neuilly, France) in a plasmagel-to-blood ratio of 1:4 for 30 min at room temperature and processed as previously described (17). For preparation of pooled human serum, equal volumes of serum obtained from six normal, healthy donors, aliquoted, and kept at −80°C until needed.

CL assay. The CL reaction mixture consisted of 5×10^5 PMNLs, 2×10^{-3} M luminol (Sigma Chemical Co., St. Louis, Mo.), 0.1 ml of the desired antimicrobial agent, and 0.05 mg of preopsonized zymosan. Opsonized zymosan was prepared by washing zymosan A (Sigma) twice with Hanks buffer and incubating it with 50% pooled human serum in a mixture of 2.5 mg of zymosan per ml for 30 min at 37°C in a water bath. All reaction components were diluted in Hanks buffer, pH 7.4, to a total volume of 2 ml in dark-adapted polypropylene vials.

In experiments in which fewer PMNLs were used, the CL reaction mixture consisted of 10^5 PMNLs, 10^{-7} M luminol, 0.1 ml of the desired antimicrobial agent, and 0.05 mg of preopsonized zymosan in a total volume of 2 ml in Hanks buffer. The PMNL-drug-luminol solution and appropriate controls were incubated at room temperature for 30 min. The CL reaction was initiated by the addition of 0.1 mg of opsonized zymosan, and subsequent CL was measured in an

LS100C scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) in the out-of-coincidence mode for 60 min at 0.1-min intervals.

The patterns of CL responses were reviewed for initial slopes, peaks, slopes of declining response, duration of response, and areas under the curve. Reproducibility of replicate assay vials was excellent (see Fig. 1, 2, and 3). Each experimental run included control reaction mixtures without an antimicrobial agent. It was apparent that the peak counts per minute correlated with the other parameters and reflected the experimental differences accurately enough so that analyses are presented simply in terms of peak counts per minute. To analyze the results from a series of experiments, the percent inhibition of peak counts per minute was calculated for each reaction mixture with the antimicrobial agent compared with its own control as follows: {(peak counts per minute without antimicrobial agent [control] − peak counts per minute with antimicrobial agent)/peak counts per minute without antimicrobial agent} × 100.

Antimicrobial agents. The antimicrobial agents tested included penicillin G, ampicillin, carbenicillin, cephalothin, cephalexin, cefazolin, cephaloridine, ceforanide, cefamandole, clindamycin, erythromycin, kanamycin, gentamicin, amikacin, sisomicin, tetracycline, doxycycline, trimethoprim, and sulfisoxazole. With the exception of trimethoprim, all antimicrobial agents were diluted in Hanks buffer and adjusted to a pH of 7.4. For trimethoprim, initial dilutions were made with distilled water, and then 0.1 M HCl was added until the drug went into solution. The solution was then further diluted with Hanks buffer and adjusted to a pH of 7.4. For initial screening, the drugs were tested in high concentrations within or beyond the usual pharmacological range. Thus, the penicillins and cephalosporins were screened at 200 μg/ml, and the remaining drugs were screened at 20 μg/ml. Additional low and intermediate concentrations were tested for selected drugs. No significant light absorption was detected for any of the drug solutions by testing in the range of 350 to 600 nm with a Beckman 34 spectrophotometer with recorder.

Yeast phagocytosis assay. The yeast phagocytosis assay was performed as previously described (15). Briefly, the reaction mixture contained 0.25 ml of 1×10^6 PMNLs per ml, 0.125 ml of 1×10^7 yeast (*Saccharomyces cerevisiae*) cells, 0.25 ml of 8×10^{-4} M methylene blue, and the desired antimicrobial agent for a final volume of 1 ml. This suspension was incubated in no. 2054 plastic tubes (Falcon Plastics, Oxnard, Calif.) on a tilt table for 30 min at 37°C and then centrifuged at 800 × *g* for 10 min. A wet mount was made from the pellet, and the numbers of live (nonblue) and dead (blue) intracellular yeast cells in 50 PMNLs were counted by direct microscopy at a ×400 magnification.

Lactate dehydrogenase assay. The release of lactate dehydrogenase from PMNLs after incubation with selected antimicrobial agents was assayed by the method of Wacker et al. (16). A total of 1.5×10^5 PMNLs per ml in the antimicrobial solution were incubated for 30 min. A 0.2-ml amount was then added to the lactate dehydrogenase reaction mixture and measured spectrophotometrically for 5 min at 340 nm.

Trypan blue staining of human PMNLs. After preincubation with the desired antimicrobial agent, 50 PMNLs were counted in the presence of 0.15% trypan blue (Sigma). The percent dead was determined by dividing the number of dead (blue) PMNLs by the total visible PMNLs and multiplying this number by 100.

Statistics. Mean ± standard deviation and the unpaired Student *t* test were calculated with a Monroe 1860 computer.

RESULTS

Effect of antimicrobial agents on PMNL CL response. Of the 19 antimicrobial agents tested, only ampicillin, cephalothin, cephalexin, tetracycline, doxycycline, gentamicin, and trimethoprim were shown to have an inhibitory effect on the PMNL CL response. The percent inhibition of the peak CL response compared with controls (no antimicrobial agent) for various concentrations of antimicrobial agents is shown in Table 1. Cephalothin, cephalexin, and ampicillin demonstrated an inhibitory effect only at concentrations of 200 μg per 2.5×10^5 PMNLs per ml, and gentamicin, trimethoprim, tetracycline, and doxycycline showed an inhibition at 2 or 20 μg per 2.5×10^5 PMNLs per ml. In experiments with fewer PMNLs, i.e., 2.5×10^4 per ml, the same antimicrobial agents and concentrations showed similar patterns of inhibition of the CL response (data not shown). Because tetracycline, trimethoprim, and cephalothin

TABLE 1. *Effect of selected antimicrobial agents on CL response PMNL (2.5×10^5/ml)*

Antimicrobial agent	μg/ml	No. of experiments	% Inhibition of control CL peak response[a]	*P* value[b]
None		8		
Ampicillin	200	2	16.2 ± 5.2[c]	<0.01
Cephalothin	200	3	19.3 ± 3.0	<0.005
Cephalexin	200	3	17.3 ± 5.8	<0.01
Tetracycline	2	4	10.5 ± 4.4	<0.01
	20	5	25.0 ± 3.1	>0.005
Doxycycline	2	2	6.1 ± 4.0	>0.3
	20	2	11.3 ± 2.2	<0.01
Trimethoprim	2	3	9.8 ± 3.4	<0.05
	20	5	26.0 ± 4.9	<0.005
Gentamicin	20	2	4.0 ± 1.1	<0.05

[a] Percent inhibition = [(peak control counts per minute − peak counts per minute with antimicrobial agent)/peak control counts per minute] × 100.

[b] Compared with PMNL CL response without antimicrobial agents (unpaired Student *t* test).

[c] Mean ± standard deviation.

demonstrated an inhibition of PMNL CL at therapeutic levels, these antimicrobial agents were titrated from 2 to 200 μg/ml for their effect on PMNL CL (Fig. 1, 2, and 3). A dose response was seen for these three drugs on the PMNL CL response.

Removal of inhibition of the response by antimicrobial agents by washing antimicrobial agent-treated PMNLs. To determine whether the inhibitory effect of antimicrobial agents on the PMNL CL response was reversible, PMNL-antimicrobial agent suspensions were washed twice by gentle centrifugation (200 × *g*) and suspension in Hanks buffer and then examined for CL. Washing completely removed the previous inhibitory effect on CL seen in the presence of the antimicrobial agents (Table 2).

Effect of antimicrobial agents on yeast phagocytosis and killing by PMNLs. To compare the CL response with a direct measurement of PMNL microbicidal function, yeast phagocytosis and killing by PMNLs were assayed. Cephalothin, tetracycline, doxycycline, and gentamicin showed no significant effect on the number of yeast cells ingested as compared with controls without antimicrobial agents. However, of the phagocytosed intracellular yeast cells, the proportion killed was less in the presence of these antimicrobial agents (Table 3). Cephalexin and ampicillin, in contrast, reduced the number of yeast cells ingested by PMNLs but showed no significant effect on the percentage of yeast cells killed (Table 3).

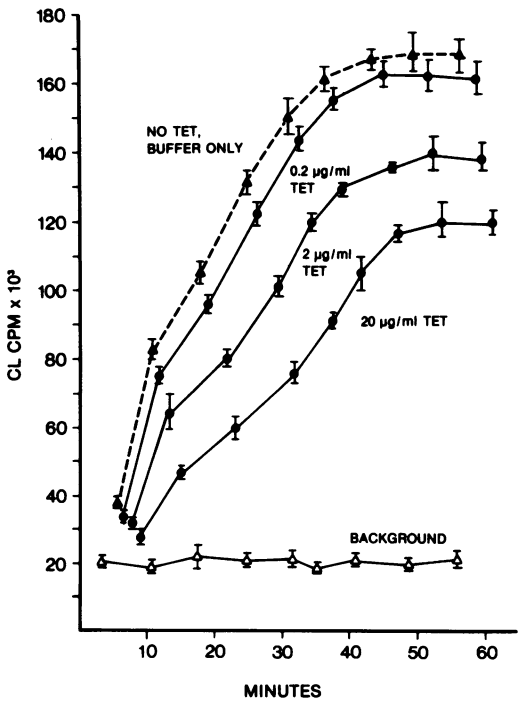

FIG. 1. *Effect of preincubating human PMNLs with 0.2, 2, and 20 μg of tetracycline (TET) per ml on the CL response. Points and bars indicate mean ± standard deviation.*

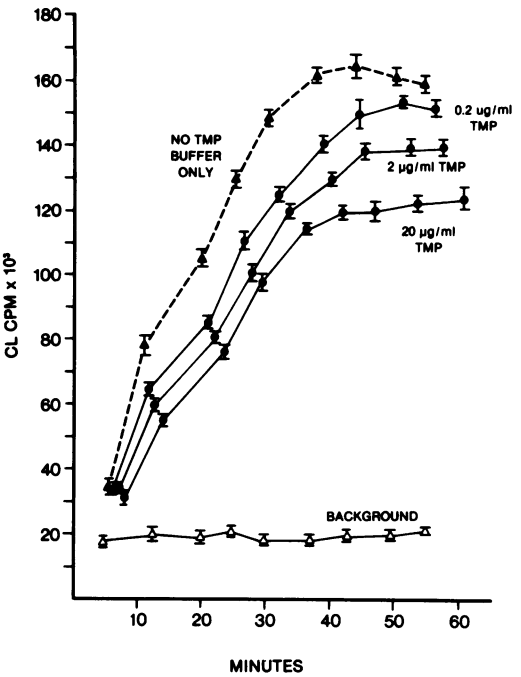

FIG. 2. *Effect of preincubating human PMNLs with 0.2, 2, and 20 μg of trimethoprim (TMP) on the CL response. Points and bars indicate mean ± standard deviation.*

Determination of PMNL cellular integrity after incubation with antimicrobial agents by release of lactate dehydrogenase and PMNL death by uptake of trypan blue. To determine whether the reduced CL response and yeast phagocytosis or killing were due to drug-induced damage of the PMNLs, release of the cytoplasmic enzyme lactate dehydrogenase was measured after incubation with selected antimicrobial agents (Table 4). None of the antimicrobial agents which inhibited CL or yeast phagocytosis or killing or both caused significant lactate dehydrogenase release.

As another measure of possible PMNL damage, uptake and exclusion of the vital dye trypan blue by PMNLs preincubated with and without antimicrobial agents were measured (Table 5). Only preincubation with ampicillin and cephalexin demonstrated significantly more dead PMNLs than controls with no antimicrobial agents present (14.5 and 17.5 versus 3.8%, respectively).

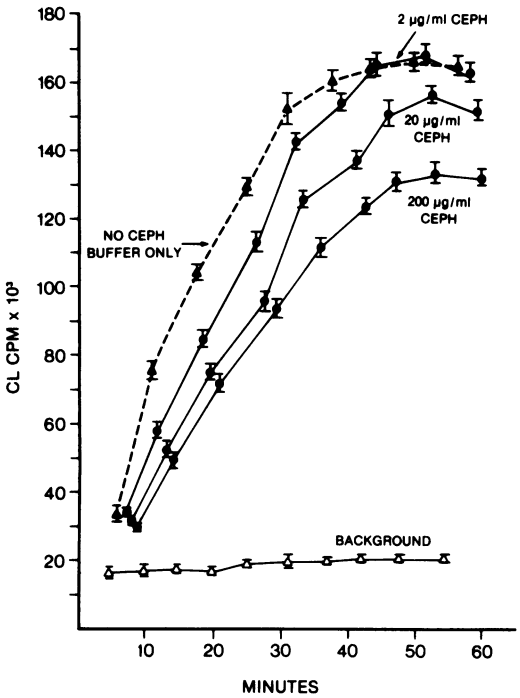

FIG. 3. *Effect of preincubating human PMNLs with 2, 20, and 200 μg of cephalothin (CEPH) per ml on the CL response to zymosan stimulation. Points and bars indicate mean ± standard deviation.*

DISCUSSION

CL by phagocytosing PMNLs results from the generation of highly reactive microbicidal oxygen species such as superoxide anions (O_2^-), hydroxyl radicals, and hydrogen peroxide (2). Inhibition of the CL response was observed after incubation of PMNLs with several antimicrobial agents in concentrations within or beyond the upper limits of the usual range of serum levels, i.e., ampicillin (200 μg/ml), cephalothin (100 μg/ml), cephalexin (200 μg/ml), gentamicin (20 μg/ml), and doxycycline (20 μg/ml). The other antimicrobial agents tested, penicillin G, carbenicillin, cephalexin, cephaloridine, cefamandole, cefazolin, ceforanide, erythromycin, sisomicin, clindamycin, amikacin, doxycycline, and sulfisoxazole, did not inhibit CL at concentrations higher than the usual therapeutic levels.

PMNL integrity and viability were tested after preincubation with several antimicrobial agents by the release of cytoplasmic lactate dehydrogenase and exclusion of trypan blue. No difference in the release of lactae dehydrogenase was seen, but the number of dead (blue) PMNLs after preincubation with 200 μg of ampicillin and cephalexin per ml was significantly higher than that of PMNLs preincubated without antimicrobial agents. Since no alteration in CL or yeast killing was observed in the presence of these antimicrobial agents, the uptake of trypan blue by PMNLs preincubated with ampicillin or cephalexin may be due to a binding beween the dye and the antimicrobial agents, one or both being bound to the surface of the PMNLs. The binding of ampicillin or cephalexin to the PMNL membrane could also account for the lower number of yeast cells phagocytosed in the presence of these antimicrobial agents by blocking C3 or Fc receptors.

Tetracycline and trimethoprim, however, both reduced the CL response at 2 μg/ml, a level within the range of common clinical use. Anderson et al. (3) reported that a high level of trimethoprim (100 μg/ml) inhibited Nitro Blue Tetrazolium reduction (a measurement of O_2^- formation), hexose monophosphate shunt activity, H_2O_2 production, and myeloperoxidase-mediated iodination in human PMNLs. In our study, the inhibition of the CL response with 2 μg of trimethoprim per ml could thus have been due to an interference of these oxidative processes which are integral to PMNL CL. The inhibition of oxidative activity demonstrated in our study by a much lower level of trimethoprim also suggests that the CL assay may be a more sensitive indicator of drug-related interference of PMNL oxidative function than the methods used by Anderson et al. (3).

Majeski et al. (9) have reported the inhibition of human neutrophil chemotaxis (elicited by lipopolysaccharide-activated serum) in the presence of 100 μg of cefamandole per ml. However,

TABLE 2. *Removal of inhibition of LDCL by washing PMNLs previously incubated with antimicrobial agents*

Antimicrobial agent[a] (μg/ml)	No. of experiments	% Inhibition of control peak LDCL values[b]		P value[c]
		Unwashed	Washed	
Ampicillin (200)	2	15.2 ± 5.6[d]	None	<0.005
Tetracycline (20)	2	26.5 ± 4.9	None	<0.005
Trimethoprim (20)	2	23.0 ± 1.4	None	<0.005
Gentamicin (20)	2	6.5 ± 2.3	None	<0.005
Cephalothin (200)	2	21.0 ± 1.0	None	<0.005
Cephalexin (200)	2	18.3 ± 4.9	None	<0.005

[a] Incubated with 2.5 × 10^5 PMNLs per ml for 30 min at room temperature.

[b] Percent inhibition = [(peak control counts per minute − peak counts per minute with antimicrobial agent)/peak control counts per minute] × 100.

[c] Unwashed PMNL LDCL response compared with control (no antimicrobial agent) response (unpaired Student *t* test).

[d] Mean ± standard deviation.

TABLE 3. *Effect of selected antimicrobial agents on S. cerevisiae phagocytosis and killing by PMNLs (2.5×10^5/ml)*

Antimicrobial agent	μg/ml	No. of experiments	Total no. of intracellular yeast cells per 50 PMNLs	P value[a]	% Dead intracellular yeast cells in 50 PMNLs	P value[a]
None		6	165.3 ± 4.5[b]		23.4 ± 3.5	
Penicillin	200	2	165.0 ± 7.1	>0.2	22.7 ± 1.4	>0.4
Ampicillin	200	3	127.0 ± 6.4	<0.005	22.0 ± 0.8	>0.4
Cephalothin	20	4	170.3 ± 7.7	>0.3	15.8 ± 1.0	<0.05
	200	4	174.0 ± 17.5	>0.3	12.0 ± 1.8	<0.01
Cephalexin	200	3	129.3 ± 2.5	<0.005	23.2 ± 4.4	>0.3
Tetracycline	5	2	168.4 ± 6.0	>0.2	17.0 ± 4.5	<0.05
	20	4	173.3 ± 11.4	>0.2	16.3 ± 3.8	<0.025
	200	4	163.7 ± 14.7	>0.2	12.8 ± 2.1	<0.01
Doxycycline	20	2	169.0 ± 7.0	>0.2	16.5 ± 3.5	<0.025
Trimethoprim	5	2	169.2 ± 4.2	>0.2	17.2 ± 2.0	<0.05
	20	4	172.3 ± 8.0	>0.2	15.8 ± 3.3	<0.025
	200	4	169.0 ± 2.2	>0.3	13.0 ± 2.1	<0.025
Gentamicin	20	2	172.5 ± 6.1	>0.3	18.5 ± 0.7	<0.01

[a] Compared with reaction mixtures without antimicrobial agents (unpaired Student *t* test).
[b] Mean ± standard deviation.

TABLE 4. *Lactate dehydrogenase release from PMNLs incubated with selected antimicrobial agents*

Antimicrobial agent[a]	μg/2.5×10^5 PMNLs per ml	LDH units[b]
0.2% Triton X-100 (control)		15.3 ± 3.0
Hanks buffer (control)		0
Tetracycline	20	0
Doxycycline	20	1.2 ± 1.1[c]
Trimethoprim	20	0
Cephalothin	200	0
Cephalexin	200	2.3 ± 1.2[c]
Gentamicin	20	0
Ampicillin	200	0
Penicillin	200	0

[a] Antimicrobial agents or Triton X-100 was incubated with PMNLs 30 min before lactate dehydrogenase testing.
[b] One unit of lactate dehydrogenase (LDH) activity is defined as the change in absorbance of 0.001 per min.
[c] Not statistically different from buffer control or other antimicrobial agents tested.

TABLE 5. *Trypan blue staining of PMNLs after incubation with selected antimicrobial agents*

Antimicrobial agent	μg/ml	PMNLs per ml	No. of experiments	% PMNLs dead[a]
Hanks buffer		5×10^4	5	3.7 ± 0.5
		2.5×10^5	3	3.8 ± 0.2
Tetracycline	20	2.5×10^5	2	3.5 ± 0.7[b]
Trimethoprim	20	2.5×10^5	2	4.0 ± 1.0[b]
Cephalothin	200	2.5×10^5	2	4.1 ± 0.6[b]
Cephalexin	200	5×10^3	2	14.5 ± 0.7[c]
		2.5×10^5	6	13.5 ± 1.1[c]
Gentamicin	20	5×10^4	4	3.9 ± 0.6[b]
		2.5×10^5	4	3.4 ± 0.8[b]
Ampicillin	200	5×10^4	2	17.5 ± 0.7[c]
		2.5×10^5	7	15.5 ± 2.6[c]
Penicillin G	200	2.5×10^5	2	4.0 ± 0.1[b]

[a] Fifty PMNLs counted after a 30-min incubation with antimicrobial agents.
[b] $P > 0.2$ compared with control values.
[c] $P < 0.005$ compared with control values (no antimicrobial agent present).

the observation by these authors of no reduction in bacterial killing by PMNLs in the presence of cefamandole is in agreement with our findings. Since certain chemotactic factors such as F-met-leu-phe are able to generate CL in human PMNLs (6), it would be of interest to examine F-met-leu-phe induction of CL by PMNLs in the presence of cefamandole.

Tetracycline has been shown to inhibit Nitro Blue Tetrazolium reduction by human PMNLs (14). This inhibition and the decrease in CL in

the presence of tetracycline reported here may result from the binding of PMNL intracellular calcium by tetracycline. Additional observations support this hypothesis: (i) tetracycline readily accumulates intracellularly in PMNLs (13), (ii) tetracycline is an effective Ca^{2+} chelator (1), and (iii) binding of PMNL intracellular Ca^{2+} by a specific calcium antagonist prevents the generation of O_2^- (10). Because our studies showed that the washing of tetracycline- or trimethoprim-treated PMNLs removes the inhibitory effect, the nature of the inhibition, by whatever mechanism(s), appears to be reversible. The failure of doxycycline to inhibit the CL response at levels similar to those of tetracycline may be due to different Ca^{2+} affinities, membrane permeabilities, or protein binding between the two antimicrobial agents.

Melby and Midtvedt (11) found no difference in phagocytic ingestion by human PMNLs after incubation with high concentrations of cephalothin (300 μg/ml) or trimethoprim (100 μg/ml). Anderson et al. (3) also showed no effect of phagocytosis with 100 μg of trimethoprim per ml. PMNL microbicidal activity was not examined in these reports. In our study, the direct assessment of PMNL microbicidal function was measured by the ability of PMNLs to phagocytose and kill yeast cells. Even though no interference with phagocytosis was observed with cephalothin or trimethoprim, in agreement with previous work (3, 11), or with tetracycline, inhibition of killing was demonstrated at therapeutic concentrations with cephalothin (20 μg/ml), tetracycline (5 μg/ml), and trimethoprim (5 μg/ml). Inhibition of phagocytosis and killing by PMNLs was seen with several other antimicrobial agents (Table 3), but the concentrations required were above the usual clinical range. Although cephalothin at 20 μg/ml reduced the ability of PMNLs to kill yeast cells, no decrease in CL response was seen at this concentration. A possible explanation for this discrepancy could be an enhanced susceptibility to cephalothin of nonoxidative microbicidal systems, such as lysozyme or cationic proteins, compared with oxidative microbicidal systems.

Inhibition of phagocytosis by tetracyclines (doxycycline and chlortetracycline) has been reported by several authors (4, 5, 12). Conversely, a lack of inhibition by tetracycline on PMNL phagocytosis has also been documented (7). These discrepancies may be due to the widely different methods used to quantitate phagocytosis.

In conclusion, of 19 antimicrobial agents studied, only tetracycline, trimethoprim, and cephalothin demonstrated an inhibition of CL or yeast phagocytosis by PMNLs in vitro at high therapeutic levels. The mechanism of reduced killing by PMNLs in the presence of trimethoprim or tetracycline may be due to an inhibition of PMNL oxidative metabolism. Whether these effects result in any impairment of PMNL function in vivo remains to be determined.

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