

Antibiotic Inhibition of the Respiratory Burst Response in Human Polymorphonuclear Leukocytes

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Recently we found that certain antibiotics which are markedly concentrated by human polymorphonuclear leukocytes (PMN) failed to kill susceptible, intraphagocytic *Staphylococcus aureus*, even though cellular drug levels were quite high. The possibility that specific antibiotics might adversely affect phagocyte antibacterial function was considered. Thus, we studied the effects of multiple antibiotics and adenosine, a known modulator of the PMN respiratory burst response, on neutrophil antibacterial function. At nontoxic concentrations, these drugs had no effect on degranulation in stimulated PMN. Adenosine was a potent inhibitor of formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated superoxide and hydrogen peroxide generation in PMN but produced less inhibition of microbial particle-induced respiratory burst activity. Three of the tested antibiotics, all of which reach high concentrations in phagocytic cells, had a marked modulatory effect on the PMN respiratory burst. Clindamycin, which enters phagocytes by the cell membrane adenosine (nucleoside) transport system, had only a modest effect on FMLP-mediated superoxide production but inhibited the microbial particle-induced response by ~50%. Roxithromycin and trimethoprim were efficient inhibitors of PMN superoxide generation stimulated by FMLP and concanavalin A (also inhibited by erythromycin) but had less effect on zymosan-mediated respiratory burst activity. Antibiotics which entered phagocytes less readily had no effect on the respiratory burst response in PMN. These results, as well as those of experiments with inhibitors of cell membrane nucleoside receptors, indicated that the antibiotic effect is mediated through intraphagocytic pathways. The possibility that antibiotic-associated inhibition of the PMN respiratory burst response might alter leukocyte antimicrobial and inflammatory function deserves further evaluation.

The interaction of antimicrobial agents with leukocytes, and especially any influence on the fate of bacteria ingested by these phagocytic cells, may be of therapeutic importance. Entry of antibiotics into phagocytes is obviously a prerequisite for activity against intracellular organisms, but we recently demonstrated a discrepancy between uptake of certain antibiotics by human neutrophilic polymorphonuclear leukocytes (PMN) and the subsequent effect of these drugs on intraphagocytic bactericidal activity (18). Thus, clindamycin and erythromycin, which were markedly concentrated by PMN, exhibited poor activity against intraphagocytic *Staphylococcus aureus*, even though the intracellular drug levels exceeded the MBCs for this organism (18). The possibility that certain antibiotics might adversely influence phagocyte antibacterial function was considered. In the case of clindamycin, a potential modulatory role has been identified. We have shown that clindamycin enters PMN and macrophages by means of the cell membrane nucleoside (adenosine) transport system (16, 36). Since adenosine (by binding external cell membrane nucleoside receptors) regulates the generation of superoxide by activated human PMN (9), clindamycin potentially could inhibit oxidative antibacterial function in a similar manner.

In the present study we investigated the influence of antibiotics and nucleosides on phagocyte antimicrobial mechanisms. Human PMN incubated in the presence or absence of antibiotics or nucleosides were exposed to substances which stimulate respiratory burst activity and degranulation. We found that specific antibiotics and nucleosides inhibited oxidative metabolism, but not under the same conditions and not by a single pathway.

MATERIALS AND METHODS

Preparation of human PMN. Peripheral venous blood from normal human volunteers was obtained by venipuncture. Granulocytes were isolated by dextran sedimentation and Ficoll-Hypaque density gradient centrifugation (5, 27, 36), washed, and suspended in Hanks balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, N.Y.).

Activators of PMN oxidative metabolism. A clinical isolate of *S. aureus* was stored at -70°C and then grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for experiments. Zymosan A (Sigma Chemical Co., St. Louis, Mo.) was boiled, opsonized by incubation with fresh human serum, frozen at -70°C until needed, and used at a concentration of 0.1 mg/ml in the experiments described below. Formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-7} M), concanavalin A (ConA) (30 $\mu\text{g}/\text{ml}$), and phorbol myristate acetate (PMA) (1 $\mu\text{g}/\text{ml}$) (all from Sigma) were utilized as soluble membrane stimulating agents.

Exposure of PMN to nucleosides and antimicrobial agents. Nucleosides and their inhibitors used in these studies included adenosine, adenosine deaminase, nitrobenzylthioinosine (NBTI), dipyridamole (all from Sigma), and erythro-9-(2-hydroxy-3-nonyl)-adenosine HCl (EHNA) (Burroughs Wellcome Co., Research Triangle Park, N.C.). Antibiotics (standard susceptibility powders), kindly provided by pharmaceutical companies for these experiments, were clindamycin and lincomycin (The Upjohn Co., Kalamazoo, Mich.), roxithromycin (Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.), erythromycin (Eli Lilly & Co., Indianapolis, Ind.), trimethoprim (Hoffmann-La Roche, Inc., Nutley, N.J.), chloramphenicol (Parke, Davis & Co., Div. Warner-Lambert Co., Morris Plains, N.J.), gentamicin

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TABLE 1. Effect of clindamycin and adenosine on stimulated superoxide production in human PMN

Exptl group	Superoxide generation—% of control—at the following drug concn ^a :					
	10 ⁻³ M	10 ⁻⁴ M	5 × 10 ⁻⁵ M	2.5 × 10 ⁻⁵ M	10 ⁻⁵ M	10 ⁻⁶ M
Clindamycin						
FMLP	7.6 ± 1.6 (7) ^b	76.1 ± 13.7 (9)	89.7 ± 4.0 (2)	87.6 (1)	85.2 ± 12.4 (5)	108.8 ± 9.8 (3)
ConA	2.1 (2)	47.9 ± 7.6 (8) ^b	63.9 ± 12.1 (5) ^b	82.3 ± 9.2 (5)	93.1 ± 5.3 (4)	
Zymosan	3.7 ± 1.7 (15) ^b	48.9 ± 4.2 (29) ^b	68.9 ± 10.4 (7) ^b	69.5 ± 9.9 (7) ^b	98.8 ± 10.5 (13)	
<i>S. aureus</i>	2.6 ± 2.6 (6)	53.4 ± 5.3 (16) ^b	76.1 ± 4.2 (13) ^b	80.7 ± 3.4 (10) ^b	94.6 ± 4.9 (14)	
PMA		79.1 ± 3.7 (9) ^b	85.3 ± 2.5 (5) ^b	94.0 ± 4.0 (4)	94.1 ± 1.6 (9) ^b	
Adenosine						
FMLP	24.2 ± 9.6 (3) ^b	32.3 ± 4.7 (19) ^b	39.4 ± 8.7 (2)		34.5 ± 10.4 (6) ^b	55.9 ± 12.7 (7) ^b
ConA	33.0 (1)	35.6 ± 3.9 (6) ^b	44.0 ± 4.1 (4) ^b	42.0 ± 7.7 (4) ^b	49.2 ± 5.0 (5) ^b	55.6 (1)
Zymosan		43.4 ± 4.7 (22) ^b	55.2 ± 14.8 (5) ^b	54.1 ± 17.1 (4) ^b	56.9 ± 13.0 (9) ^b	85.3 (1)
<i>S. aureus</i>	59.6 ± 17.3 (5) ^b	74.2 ± 6.1 (15) ^b	74.8 ± 2.8 (9) ^b	77.4 ± 5.0 (6) ^b	89.1 ± 7.0 (16) ^b	96.5 ± 23.2 (2)
PMA		105.3 ± 2.5 (5)	109.5 ± 2.3 (2)	110.0 ± 0.5 (2)	107.1 ± 2.0 (5)	

^a Data are means ± standard errors of the mean of observations (number of experiments) for each group, 30-min incubation.

^b Significant difference between control (no clindamycin or nucleoside) and experimental group, $P < 0.05$.

(Schering Corp., Kenilworth, N.J.), and penicillin G (Pfizer Inc., New York, N.Y.).

Effects of nucleosides and antibiotics on PMN oxidative metabolism. (i) Superoxide generation by human PMN was determined as superoxide dismutase-inhibitable reduction of ferricytochrome *c* (7, 17, 21). PMN (2×10^6 cells per 3 ml) in HBSS containing 100 μ M ferricytochrome *c* were preincubated in the presence or absence of antibiotics or nucleosides for 15 min at 37°C. Cytochalasin B (5 μ g/ml) and the stimulating agent (zymosan, *S. aureus*, FMLP, ConA, or PMA) or control media were then added. Duplicate samples also contained superoxide dismutase (50 μ g/ml). After incubation, the cells were removed by centrifugation at 4°C. The optical density of the supernatants was determined at 550 nm in a spectrophotometer (Carey 219; Varian, Sunny Vale, Calif.).

(ii) Hydrogen peroxide release was measured by the extinction of scopoletin fluorescence during its oxidation by horseradish peroxidase (28, 29). Scopoletin, dissolved in phosphate buffer, was added at a final concentration of 4 μ M to HBSS containing 2.5×10^6 PMN per ml. Nucleosides or antibiotics were added to half of the tubes in each assay system. Cuvettes were held at 37°C in a constant-temperature water bath attached to a spectrofluorometer (model SF-330; Varian). The excitation wavelength was 350 nm, and the emission wavelength was 460 nm. Horseradish peroxidase was added in a concentration of 22 nM. Baseline recordings were made for several minutes before addition of membrane-stimulating agents.

Assays of phagocyte granular and cytoplasmic enzymes. Stimulus-induced degranulation was evaluated by assaying the extracellular release of phagocyte granular enzymes, i.e., β -glucuronidase, a marker for azurophil granules, and lysozyme, an enzyme found in both azurophil and specific granules. Release of lactic dehydrogenase (LDH), a cytoplasmic enzyme, was monitored as an indicator of cellular injury. PMN (10^7 /ml) in HBSS were preincubated in the presence or absence of antibiotics or nucleosides for 15 min, followed by addition of cytochalasin B (5 μ g/ml) and the stimulating agent (FMLP or zymosan). After an additional incubation (with rotation) for 20 min at 37°C, the cells were recovered by centrifugation, resuspended, and lysed in 0.2% Triton X-100. Enzyme activity was determined in cell pellets, supernatants, and whole cell suspensions (without centrifugation and after Triton X-100 disruption). β -Glucuronidase activity was assayed by the release of phenolphtha-

lein from phenolphthalein (β) glucuronide (13). Lysozyme was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* (33). LDH was assayed by measuring the increase in A_{340} resulting from the reduction of NAD to form NADH (1).

Antibiotic and nucleoside uptake by human PMN. The uptake of radiolabeled clindamycin and adenosine by PMN was determined by means of a velocity gradient centrifugation technique which we have described in detail elsewhere (18, 27, 36). Human PMN in HBSS, at a concentration of 10^7 cells per ml, were incubated at 37°C with or without a stimulating agent and either [³H]clindamycin hydrochloride (Upjohn) or [2,8-³H]adenosine (Dupont, NEN Research Products, Boston, Mass.). Concentrations of these substances were $\sim 2.5 \times 10^{-5}$ M (clindamycin, 10 μ g/ml; adenosine, 7 μ g/ml). At intervals, uptake of antibiotic or nucleoside was determined by velocity gradient centrifugation in a microcentrifuge. In some experiments, NBTI and dipyrindamole, potential inhibitors of clindamycin and adenosine uptake, were preincubated with PMN for 20 min prior to determination of radiolabeled clindamycin or nucleoside uptake. Antibiotic uptake was expressed as the ratio of the cellular concentration of antibiotic to the extracellular concentration (C/E).

Statistical analysis of data. Differences between experimental groups were determined by means of two-sample or paired *t* tests by using a Tektronic 4051 computer and an appropriate statistical program.

RESULTS

Characteristics of the cell population. The granulocyte population was $\geq 97\%$ neutrophils. Since $>90\%$ of these cells were mature polymorphonuclear neutrophilic leukocytes, the cells will be referred to as PMN. Greater than 95% of the cells were viable, as judged by exclusion of trypan blue.

Superoxide generation. When human PMN were exposed to soluble substances (FMLP, ConA, or PMA) or microbial particles (zymosan or *S. aureus*) which trigger cell membrane activity, the respiratory burst with superoxide production was stimulated. Adenosine was an efficient inhibitor of superoxide production stimulated by FMLP and ConA but had less effect on microbial particle (especially *S. aureus*)-induced production of superoxide (Table 1).

Three of the tested antibiotics had a marked effect on stimulated superoxide production by neutrophils (Table 2).

TABLE 2. Influence of roxithromycin, trimethoprim, and erythromycin on stimulated superoxide production by human PMN

Exptl group	Superoxide generation—% of control—at the following drug concn ^a :				
	10 ⁻⁴ M	5 × 10 ⁻⁵ M	2.5 × 10 ⁻⁵ M	10 ⁻⁵ M	10 ⁻⁶
Erythromycin					
FMLP	84.9 ± 5.4 (3)			105.9 ± 7.2 (3)	
ConA	62.1 ± 5.6 (10) ^b	62.6 ± 13.4 (3)	58.5 ± 24.2 (2)	88.6 ± 6.2 (9)	
Zymosan	109.2 ± 17.1 (5)		92.7 ± 16.9 (3)	99.5 ± 23.4 (3)	
<i>S. aureus</i>	85.3 ± 12.8 (6)	104.3 ± 13.2 (5)	127.1 ± 25.5 (3)	100.7 ± 11.1 (6)	
PMA	118.9 (1)	126.8 (1)		111.1 (1)	
Roxithromycin					
FMLP	29.1 ± 6.5 (7) ^b	54.0 ± 10.1 (4) ^b	57.7 ± 5.3 (4) ^b	60.9 ± 4.0 (7) ^b	76.1 (1)
ConA	25.4 ± 1.6 (9) ^b			62.1 ± 2.6 (10) ^b	71.1 ± 11.0 (4) ^b
Zymosan	30.3 ± 4.7 (16) ^b	74.4 ± 5.1 (8) ^b	89.5 ± 7.6 (7) ^b	86.7 ± 6.0 (14) ^b	101.3 ± 7.7 (5)
PMA	58.9 ± 6.4 (8) ^b	91.3 ± 0.8 (3) ^b	91.5 ± 2.6 (3)	107.4 ± 6.4 (7)	110.3 ± 3.9 (4)
Trimethoprim					
FMLP	29.5 ± 4.3 (8) ^b	40.1 ± 4.8 (4) ^b	57.2 ± 2.0 (4) ^b	71.7 ± 3.4 (8) ^b	88.7 (1)
ConA	49.5 ± 5.0 (10) ^b			71.0 ± 4.6 (9) ^b	109.4 ± 4.9 (4)
Zymosan	67.2 ± 4.3 (17) ^b	83.6 ± 7.4 (8) ^b	93.4 ± 5.8 (8)	97.9 ± 3.9 (16)	114.3 ± 9.9 (7)
PMA	82.6 ± 2.5 (7) ^b	88.2 ± 7.1 (2)	90.2 ± 5.2 (2)	93.8 ± 1.2 (7) ^b	97.4 ± 1.6 (3)

^a Data are means ± standard errors of the mean of observations (number of experiments) for each group.

^b Significant difference between control (no antibiotic) and experimental group, $P < 0.05$.

These drugs, clindamycin, roxithromycin, and trimethoprim, are all highly concentrated by phagocytic cells, including human PMN (15, 16, 18–20, 27). In contrast with adenosine, clindamycin at therapeutic concentrations (10⁻⁵ to 10⁻⁴ M) had only a modest (15 to 24%) inhibitory effect on FMLP stimulation of superoxide production. However, in a dose-dependent fashion, clindamycin was a potent inhibitor (by ~50%) of particle-stimulated superoxide production. Since clindamycin at high concentrations ($\geq 10^{-3}$ M) is toxic to phagocytes (see below), the profound inhibition of superoxide generation observed at 10⁻³ M is of questionable significance. The effects of clindamycin or adenosine on PMN superoxide production stimulated by FMLP and zymosan were continuous throughout the study period (Fig. 1). Roxithromycin and trimethoprim were efficient inhibitors of PMN superoxide production induced by soluble agents (FMLP and ConA) but had less effect on zymosan-mediated respiratory burst activity (Table 2).

At a concentration of 10⁻⁴ M, clindamycin and trimetho-

prim inhibited PMA-induced superoxide generation by only 20%, whereas roxithromycin caused greater suppression (~40%) (Tables 1 and 2). At lower concentrations, these agents had little influence on the PMA response. Adenosine had no effect on the production of superoxide by PMN exposed to PMA.

Experiments were also carried out in the presence of other antibiotics—erythromycin, lincomycin, chloramphenicol, gentamicin, and penicillin G. Erythromycin inhibited ConA-induced superoxide generation (Table 2), but the other four antibiotics had no effect on the respiratory burst.

Next, we determined the consequences of combining adenosine and clindamycin in the incubation system (Table 3). This combination had an additive effect (greater inhibition than either agent alone) on zymosan-stimulated superoxide production. There was no change, as compared with adenosine alone, when both agents were present in studies with FMLP-exposed PMN.

NBTI binds to cell membrane nucleoside transport recep-

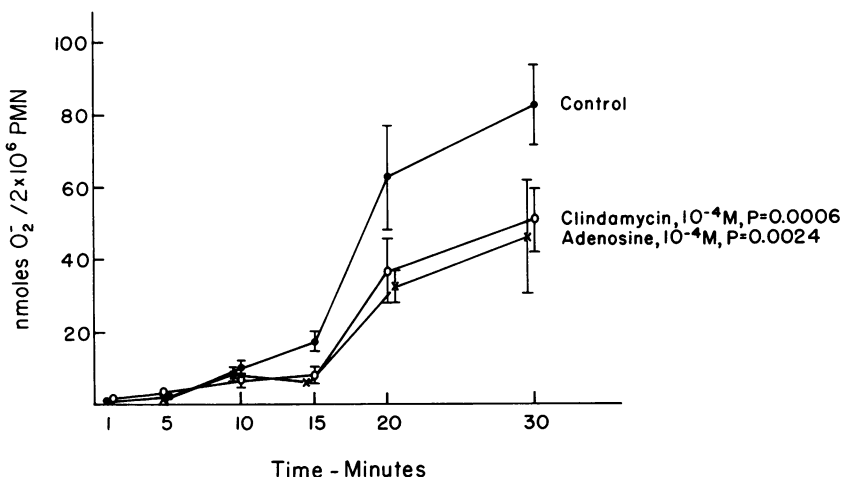


FIG. 1. The effects of clindamycin and adenosine on zymosan-stimulated superoxide production by human PMN during a 30-min observation period.

TABLE 3. Effect of clindamycin, adenosine, NBTI, and dipyridamole on FMLP- and zymosan-stimulated superoxide generation by human PMN

Exptl group	Superoxide generation—% of control—at the following drug concn ^a :	
	10 ⁻⁵ M	10 ⁻⁴ M
FMLP		
Clindamycin	89.6 ± 4.3 (5) <i>P</i> = 0.049 ^b	71.8 ± 5.6 (6) <i>P</i> = 0.002
Adenosine		28.1 ± 2.5 (11) <i>P</i> = 0.00001
Clindamycin (10 ⁻⁴ M) plus adenosine (10 ⁻⁴ M)		22.6 ± 4.3 (5) <i>P</i> = 0.0004
NBTI	61.4 ± 4.9 (7) <i>P</i> = 0.0001	
Dipyridamole	62.4 ± 6.1 (5) <i>P</i> = 0.002	
Zymosan		
Clindamycin	79.6 ± 3.7 (2)	55.2 ± 4.0 (6) <i>P</i> = 0.00006
Adenosine		48.2 ± 2.6 (8) <i>P</i> = 0.00001
Clindamycin (10 ⁻⁴ M) plus adenosine (10 ⁻⁴ M)		25.2 ± 4.9 (6) <i>P</i> = 0.0002
NBTI	140.7 ± 6.6 (6) <i>P</i> = 0.0006	
Dipyridamole	147.3 ± 13.6 (4) <i>P</i> = 0.026	

^a Data are means ± standard errors of the mean of observations (number of experiments) for each group.

^b *P* values reflect differences between control and experimental groups.

tors and inhibits nucleoside transport but is not itself transported into the cell (26). This agent reduced FMLP-stimulated superoxide production (Table 3). However, NBTI failed to inhibit (and appeared to augment) zymosan-stimulated respiratory burst activity. Dipyridamole, like NBTI, inhibits the membrane transport of nucleosides (26). As expected (because of increased extracellular adenosine), exposure to dipyridamole lowered the quantity of superoxide generated by FMLP-stimulated PMN. Dipyridamole did not inhibit zymosan-induced respiratory burst activity.

The possibility that antibiotics or nucleosides might directly inhibit the reduction of cytochrome *c* was considered. In a cell-free system, none of the tested agents had any effect on the reduction of cytochrome *c* by superoxide generated during the reaction between hypoxanthine and xanthine oxidase.

Adenosine deaminase blocked the modulatory effect of adenosine on FMLP-stimulated superoxide production. Furthermore, adenosine deaminase (in the absence of exogenous adenosine) increased superoxide production, due to breakdown of the adenosine which is endogenously produced by PMN and then released externally. In contrast,

adenosine deaminase did not influence the inhibition of superoxide production by clindamycin, roxithromycin, or trimethoprim. EHNA, which inhibits adenosine deaminase and thereby increases the available adenosine, blocked the generation of superoxide in response to FMLP and zymosan stimulation. The effect of EHNA was additive to that of adenosine but was not altered in the presence of clindamycin.

Hydrogen peroxide production. Clindamycin and adenosine modulated PMN hydrogen peroxide production in a manner similar to that seen with superoxide generation. Thus, clindamycin (>10⁻⁵ M) and adenosine (≥10⁻⁵ M) suppressed hydrogen peroxide release after ingestion of opsonized *S. aureus* (Fig. 2) or opsonized zymosan (Fig. 3). FMLP-stimulated hydrogen peroxide production was decreased in the presence of adenosine but not clindamycin (Table 4). Penicillin G had no effect on hydrogen peroxide generation.

Nucleosides or antibiotics theoretically might have a direct effect on the extinction of scopoletin fluorescence by hydrogen peroxide. In a cell-free system, hydrogen peroxide was generated by the reaction between glucose and glucose

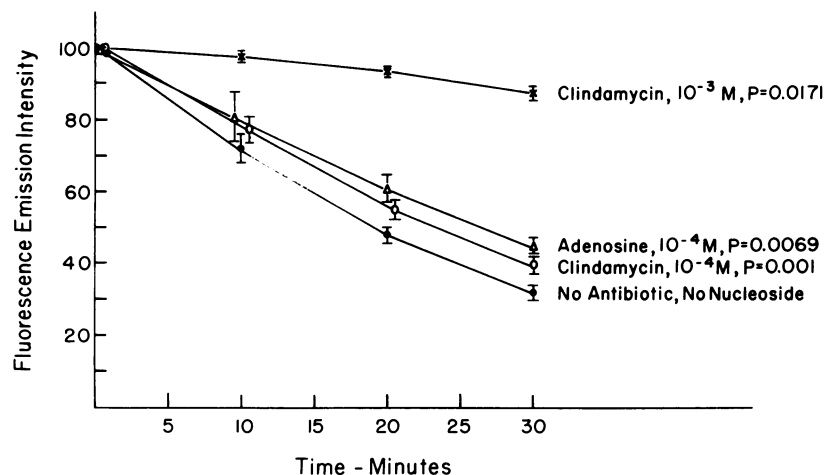


FIG. 2. The influence of clindamycin and adenosine on hydrogen peroxide release by human PMN after ingestion of *S. aureus*.

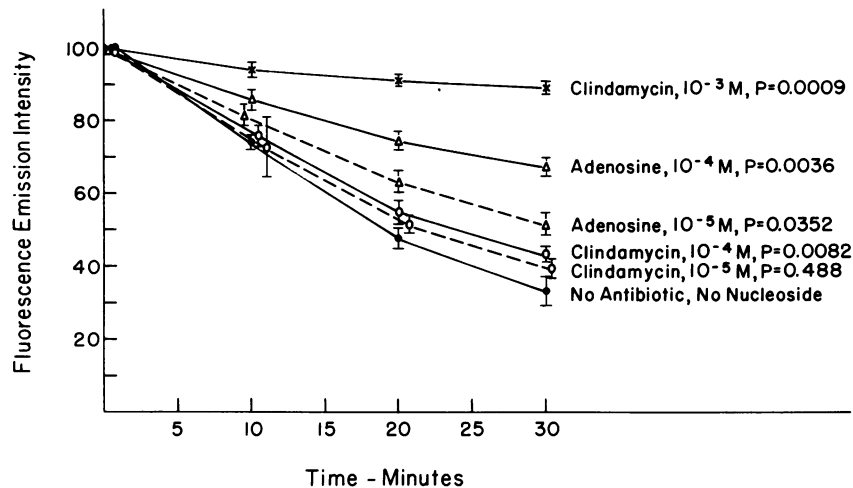


FIG. 3. Release of hydrogen peroxide by zymosan-stimulated human PMN exposed to clindamycin or adenosine.

oxidase. The tested antibiotics and nucleosides, at concentrations identical to those used in experiments with PMN, had no influence on the production or measurement of H_2O_2 in this system.

Assays of phagocyte granular and cytoplasmic enzymes. Exposure of PMN to FMLP or microbial particles causes degranulation, with release of granular enzymes to the external environment. Neither antibiotics nor adenosine in concentrations of 10^{-4} or 10^{-5} M had any effect on FMLP (or zymosan)-stimulated release of lysozyme and β -glucuronidase (Table 5).

Since the influence of antibiotics and nucleosides on PMN oxidative metabolism might be due to the toxic effects of these substances, cell viability (trypan blue exclusion) and release of the cytoplasmic enzyme LDH were monitored as indicators of cellular injury. Release of cytoplasmic LDH to the external environment by control or stimulated PMN was not influenced by adenosine or tested antibiotics at 10^{-4} or 10^{-5} M. At very high concentrations (10^{-3} M) of clindamycin and adenosine, there was a marked increase in the quantity of LDH released by the phagocytes (Table 5), although the ability of the cells to exclude trypan blue was not altered.

Antibiotic and nucleoside uptake. Exposure of human PMN to microbial particles (zymosan and *S. aureus*), but not PMA and FMLP, stimulated the uptake of clindamycin by these cells. Entry of adenosine into neutrophils was increased by PMA, zymosan, and *S. aureus*, but FMLP had only a minor stimulatory effect on adenosine uptake (Table 6). In previous studies we found that uptake of erythromycin

and roxithromycin by PMN was slightly decreased after phagocytosis, although cellular levels of these drugs were still quite high (18, 20, 36).

NBTI, which binds to membrane nucleoside transport receptors, inhibited the entry of adenosine into control and FMLP-stimulated PMN. To a minor extent, this agent also decreased the entry of adenosine into cells which ingested zymosan or *S. aureus* (adenosine uptake was still greater than in control cells). However, the uptake of clindamycin by neutrophils was not influenced by NBTI.

Adenosine deaminase is present in large quantities and rapidly metabolizes adenosine in some cells (25). To obtain an accurate determination of adenosine uptake (transport) in such cells, it is necessary to use an inhibitor (such as EHNA) of adenosine deaminase. We found that incubation with EHNA led to only a minor increase in apparent adenosine uptake by PMN.

DISCUSSION

In a recent study we showed that clindamycin and erythromycin, which are highly concentrated within human PMN, failed to kill intraphagocytic *S. aureus*, even though the intracellular level of clindamycin, at least, exceeded the MBC for the test organism (18). Therefore, the possibility that certain antibiotics might inhibit neutrophil antibacterial activity was considered. Since clindamycin enters phagocytes by the cell membrane nucleoside (adenosine) transport system (16, 36) and since adenosine modulates PMN superoxide generation via regulatory cell membrane receptors (9,

TABLE 4. Influence of clindamycin and adenosine on FMLP-stimulated hydrogen peroxide production by PMN

Exptl group	Hydrogen peroxide production—nmol/min—at the following drug concn ^a :			
	Control	10^{-5} M	10^{-4} M	10^{-3} M
No additions	38.6 ± 2.3 (16)			
Clindamycin		38.7 ± 5.3 (4) <i>P</i> = 0.98 ^b	38.5 ± 1.7 (11) <i>P</i> = 0.99	51.6 ± 2.1 (6) <i>P</i> = 0.006
Adenosine		24.6 ± 3.0 (9) <i>P</i> = 0.002	19.5 ± 3.0 (9) <i>P</i> = 0.00008	

^a Data are means ± standard errors of the mean of observations (number of experiments) for each group.

^b *P* values reflect differences between control and experimental groups.

TABLE 5. Influence of adenosine and antibiotics on FMLP-stimulated release of granular and cytoplasmic enzymes by human PMN

Exptl group	Release of enzyme—% of total (supernatant/whole cell) ^a		
	Lysozyme	β -Glucuronidase	LDH
Control (PMN only)	10.6 \pm 1.4 (10)	4.2 \pm 0.6 (11)	14.3 \pm 1.5 (12)
FMLP-stimulated, no addition	25.3 \pm 2.2 (10) <i>P</i> = 0.0004 ^b	11.8 \pm 1.6 (12) <i>P</i> = 0.0004 ^b	18.4 \pm 2.1 (12) <i>P</i> = 0.14 ^b
Adenosine			
10 ⁻⁴ M	21.6 \pm 2.1 (6) <i>P</i> = 0.29 ^c	9.1 \pm 1.2 (6) <i>P</i> = 0.29	17.5 \pm 2.5 (6) <i>P</i> = 0.81
10 ⁻⁵ M	22.9 \pm 1.9 (6) <i>P</i> = 0.49	9.0 \pm 1.0 (6) <i>P</i> = 0.26	16.9 \pm 1.6 (6) <i>P</i> = 0.66
Clindamycin			
10 ⁻³ M		15.4 \pm 3.6 (3) <i>P</i> = 0.36	46.2 \pm 4.3 (3) <i>P</i> = 0.0001
10 ⁻⁴ M	30.3 \pm 2.3 (6) <i>P</i> = 0.18	13.4 \pm 2.3 (7) <i>P</i> = 0.58	18.0 \pm 2.4 (6) <i>P</i> = 0.92
10 ⁻⁵ M	24.3 \pm 1.7 (5) <i>P</i> = 0.77	8.9 \pm 2.3 (5) <i>P</i> = 0.33	16.7 \pm 2.9 (5) <i>P</i> = 0.68
Erythromycin			
10 ⁻⁴ M	26.4 \pm 3.2 (5) <i>P</i> = 0.79	11.9 \pm 3.3 (5) <i>P</i> = 0.97	22.8 \pm 2.5 (5) <i>P</i> = 0.26
10 ⁻⁵ M	26.1 \pm 1.7 (4) <i>P</i> = 0.85	11.2 \pm 2.4 (4) <i>P</i> = 0.84	19.9 \pm 2.6 (4) <i>P</i> = 0.71
Chloramphenicol			
10 ⁻⁴ M	28.8 (1)	11.0 (1)	20.6 (1)
10 ⁻⁵ M	28.6 (1)	13.5 (1)	20.7 (1)
Gentamicin, 10 ⁻⁵ M	28.3 \pm 7.6 (2) <i>P</i> = 0.64	11.1 \pm 3.1 (2) <i>P</i> = 0.86	18.5 \pm 2.7 (2) <i>P</i> = 0.98

^a Data are means \pm standard errors of the mean of observations (number of experiments) for each experimental group.

^b *P* values reflect differences between control (PMN only) and experimental (FMLP-stimulated) groups.

^c Remaining *P* values reflect differences between FMLP-stimulated (no addition) and FMLP-stimulated (plus adenosine or antibiotic) groups.

11), we postulated that clindamycin might also alter phagocyte oxidative antibacterial function.

With this background, we investigated the influence of antibiotics on human PMN oxidative metabolism. Those antibiotics which achieve the highest intraphagocytic concentrations modulated the respiratory burst response in neutrophils. Thus, clindamycin, macrolide antibiotics (roxithromycin and erythromycin), and trimethoprim, which are avidly concentrated by human phagocytic cells (15, 18–20, 27, 36), inhibited the production of superoxide by stimulated PMN. Antibiotics, including beta-lactam (penicillin G), aminoglycoside (gentamicin), and lincosamine (lincosamin) agents, which enter phagocytes less readily, had little

effect on PMN superoxide production. Rifampin, a lipid-soluble drug concentrated two- to threefold by phagocytic cells, was also inactive in this system.

Adenosine was a better inhibitor of superoxide production stimulated by FMLP than of microbial particle-induced activity. In contrast, clindamycin had a modest effect on FMLP-induced superoxide production but was an efficient inhibitor of microbial particle-stimulated respiratory burst activity. Roxithromycin and trimethoprim were more effective modulators of soluble stimulus-induced superoxide production than of the zymosan-stimulated respiratory burst response. Erythromycin inhibited ConA-mediated superoxide generation. A recent report indicated that erythromycin,

TABLE 6. Influence of FMLP, zymosan, and NBTI on the uptake of clindamycin and adenosine by human PMN

Exptl group	Antibiotic uptake (C/E) ^a			
	5 min	15 min	30 min	60 min
Clindamycin				
Control	8.07 \pm 1.76 (7)	10.09 \pm 1.09 (20)	10.17 \pm 1.21 (17)	9.46 \pm 1.83 (15)
FMLP	7.22 \pm 1.54 (7) <i>P</i> = 0.009 ^b	8.99 \pm 0.84 (20) <i>P</i> = 0.008 ^b	9.78 \pm 1.17 (17) <i>P</i> = 0.19 ^b	
Zymosan	9.58 \pm 1.79 (4) <i>P</i> = 0.46 ^b	13.61 \pm 1.61 (7) <i>P</i> = 0.003 ^b	15.06 \pm 1.73 (7) <i>P</i> = 0.002 ^b	16.57 \pm 2.79 (5) <i>P</i> = 0.01 ^b
Adenosine				
Control	0.78 \pm 0.54 (7)	0.67 \pm 0.12 (21)	1.06 \pm 0.18 (20)	1.88 \pm 0.36 (14)
Control plus NBTI		0.26 \pm 0.10 (15) <i>P</i> = 0.0001 ^b	0.40 \pm 0.14 (14) <i>P</i> = 0.002 ^b	0.84 \pm 0.25 (11) <i>P</i> = 0.001 ^b
FMLP	0.35 \pm 0.14 (7) <i>P</i> = 0.25 ^b	0.82 \pm 0.11 (21) <i>P</i> = 0.01 ^b	1.62 \pm 0.21 (20) <i>P</i> = 0.002 ^b	2.34 \pm 0.32 (14) <i>P</i> = 0.008 ^b
FMLP plus NBTI	0.21 \pm 0.25 (3) <i>P</i> = 0.004 ^c	0.44 \pm 0.14 (9) <i>P</i> = 0.02 ^c	0.60 \pm 0.13 (8) <i>P</i> = 0.0003 ^c	1.08 \pm 0.32 (5) <i>P</i> = 0.04 ^c
Zymosan		1.82 \pm 0.21 (10) <i>P</i> = 0.006 ^b	2.39 \pm 0.28 (10) <i>P</i> = 0.0003 ^b	3.10 \pm 0.63 (10) <i>P</i> = 0.06 ^b
Zymosan plus NBTI		1.54 \pm 0.24 (9) <i>P</i> = 0.20 ^c	2.56 \pm 0.36 (9) <i>P</i> = 0.19 ^c	2.63 \pm 0.40 (9) <i>P</i> = 0.66 ^c

^a Data are means \pm standard errors of the mean of observations (number of experiments) for each group.

^b *P* values reflect differences between control and experimental groups.

^c *P* values reflect differences between stimulated (FMLP or zymosan) group and stimulated group plus NBTI.

and especially roxithromycin, inhibited lucigenin-enhanced chemiluminescence in human PMN stimulated by FMLP and the calcium ionophore A23187 (but not zymosan or PMA) (2). The means by which macrolides interfere with this assay was not identified.

Adenosine failed to alter superoxide production stimulated by PMA, which bypasses membrane receptor-related mechanisms of activation and directly stimulates protein kinase C (6). Clindamycin, roxithromycin, and trimethoprim (mostly at high concentrations) exerted only a small inhibitory effect on PMA-mediated superoxide generation.

Another parameter of oxidative metabolism, the production of hydrogen peroxide, was also inhibited by clindamycin and adenosine in phagocytes ingesting microbial particles. However, only adenosine inhibited FMLP-induced H_2O_2 production in human PMN.

The cellular regulatory activity of nucleosides is mediated through external cell membrane receptors which can be either stimulatory (A_2) or inhibitory (A_1) (12, 24) for guanine nucleotide regulatory (G or N) protein-adenylate cyclase function (30, 35). Specific receptor-G protein complexes may also regulate phospholipase C activity, with an effect on polyphosphoinositide breakdown and intracellular Ca^{2+} mobilization, thereby influencing the function of the respiratory burst enzyme (34). Adenosine and certain other nucleosides inhibit FMLP and other stimulus-induced superoxide and hydrogen peroxide production by PMN, a phenomenon due to binding of adenosine A_2 cell membrane receptors (9-11).

The modulatory effect of clindamycin, and probably the other inhibitory antibiotics, is by a different pathway. We found major differences between adenosine and antibiotics in their influence on respiratory burst activity induced by specific stimuli. The effects of both antimicrobial agents and adenosine are strongly related to their extracellular and intracellular concentrations. There is a direct association between inhibition of the respiratory burst by antibiotics and uptake of the drugs in stimulated human PMN. Clindamycin had more effect on the PMN respiratory burst induced by stimuli (i.e., zymosan and *S. aureus*, but not FMLP and PMA) which increase membrane transport (uptake) and intracellular concentration of the drug. Conversely, roxithromycin modulation of PMN superoxide production stimulated by ConA (which does not alter drug uptake) was greater than its effect on the respiratory burst induced by phagocytic particles (which decrease macrolide uptake) (20). Nucleoside regulation of the respiratory burst, due to binding of regulatory external membrane receptors, was less pronounced with stimulating agents (zymosan and *S. aureus*) which markedly increase adenosine uptake (with a reduction in extracellular adenosine) than with a stimulus (FMLP) which only slightly alters nucleoside transport.

Adenosine and clindamycin were additive in their modulation of superoxide production stimulated by zymosan (which greatly increases PMN clindamycin uptake). This also suggests that these agents have different mechanisms of action. There was no additive inhibitory effect of these combined agents in studies with FMLP, which does not stimulate clindamycin uptake.

Studies with NBTI and dipyrindamole provided useful information. These substances inhibited adenosine uptake in unstimulated and FMLP-stimulated PMN. Superoxide production in FMLP-activated neutrophils was inhibited by NBTI and dipyrindamole, because the diminished nucleoside transport resulted in more extracellular adenosine to bind cell membrane regulatory receptors. NBTI had little effect on the augmented adenosine uptake observed in microbial

particle-activated PMN. As a result, NBTI and dipyrindamole failed to inhibit zymosan-stimulated generation of superoxide. This is probably due to the increased adenosine uptake, decreased extracellular adenosine concentration, and perhaps the subsequent displacement of adenosine at membrane receptor sites by NBTI or dipyrindamole. The uptake of clindamycin was not affected by NBTI in either stimulated (FMLP or zymosan) or unstimulated PMN.

We do not know as yet how the avid accumulation of clindamycin, roxithromycin, and trimethoprim, resulting in very high intracellular drug concentrations, alters activation of the neutrophil respiratory burst. NADPH oxidase, the cell membrane-associated respiratory burst enzyme, is composed of a cytochrome *b*, a flavoprotein, and possibly a quinone (3, 8, 14, 31, 32). A portion of the cytochrome is translocated from specific granules to the cell membrane when stimulation of PMN leads to degranulation (4). Clindamycin, at very high concentrations ($\sim 10^{-3}$ M), has been reported to inhibit degranulation of PMN lysosomes (22, 23). However, we found that at nontoxic concentrations, clindamycin (and other antibiotics) had no effect on zymosan- or FMLP-induced degranulation in PMN. Furthermore, production of superoxide by stimulated PMN cytoplasts, which contain few granules, is inhibited by clindamycin and adenosine (W. L. Hand and D. L. Hand, unpublished observations). Thus, the influence of clindamycin on PMN oxidative metabolism is not mediated through an effect on granule function.

Other pathways by which high levels of intracellular antibiotics might alter activation of the oxidase have been evaluated. The modulatory effects of clindamycin are not mediated by external cell membrane receptors. We have excluded a role for nucleoside receptors. The modulatory activity of clindamycin is related to high intraphagocytic concentrations and is observed with a variety of stimuli. Certainly, no specific receptor or class of receptor could account for these observations. Thus, the antibiotic affects the activation signal chain at a point(s) beyond these membrane receptors. Furthermore, the terminal activation sequence, mediated through protein kinase C and the oxidase, is apparently intact, since clindamycin and other modulatory antibiotics have little effect on PMA-stimulated superoxide production. Potential sites at which clindamycin might alter the oxidase activation pathways include G protein function, phospholipase (C, D, or A_2) activation and phospholipid metabolism, and cytosolic Ca^{2+} levels.

Whatever the mechanism, the possibility that this inhibition of oxidative antimicrobial function might interfere with the ability of neutrophils to kill ingested bacteria is of concern. On the other hand, the control by specific antibiotics and their derivatives of toxic oxidative radical production in phagocytes has potential beneficial implications.

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