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CYTOPROTECTION AGAINST NEUTROPHIL-DELIVERED OXIDANT ATTACK BY ANTIBIOTICS

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Abstract—In the present study we have investigated the effect of six antibiotics (penicillin G, ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin) on the neutrophil cytolytic activity by using a system constituted of phorbol-12-myristate-13-acetate-triggered neutrophils and ^{51}Cr-labelled lymphoblastoid Daudi target cells. The results demonstrate that five of these drugs (ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin) are capable of inhibiting the neutrophil cytolytic activity by inactivating the hypochlorous acid (HOCl) generated extracellularly by the myeloperoxidase pathway and crucial to the target cell lysis. Penicillin G had no effect on neutrophil-mediated cytolysis. Thus, these data demonstrate that ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin lower the neutrophil-mediated target cell damage by a HOCl-scavenging mechanism, suggesting a possible cytoprotective role for these drugs during infections.

Neutrophils are the front-line agents in the immune defence against microorganisms. They exert their bactericidal activity after being chemotactically recruited to sites of infection [1, 2]. Moreover, it is well known that neutrophils may release extracellularly both the products of the oxidative burst and the granule contents, resulting in the injury of tissues surrounding the infected area [3–6]. In recent years, neutrophils have also been implicated as mediators of tissue damage in different non-infectious diseases such as adult respiratory distress syndrome [7], myocardial infarction [8], ulcerative colitis [9], rheumatoid arthritis [10], glomerulonephritis [11], emphysema [12] and chronic obstructive pulmonary disease [13].

Several studies have been reported regarding possible interactions between phagocytes and antimicrobial agents [14–23]. Some of these investigations indicate a synergistic activity of antibiotics with neutrophils in their antimicrobial action [15, 16, 20–22]. Other reports suggest a down-regulation in the phagocyte function by some of these drugs [14, 17–19].

In this paper we report evidence that five antibiotics, ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin, all widely used in the therapy of bacterial infections, are endowed with the capacity of limiting the hypochlorous acid (HOCl†)-dependent tissue damage induced by neutrophils at the sites of infection.

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† Abbreviations: HOCl, hypochlorous acid; HBSS, Hank's balanced solution without phenol red; FCS; fetal calf serum; PMA, phorbol-12-myristate-13-acetate; Tau, taurine; MPO, myeloperoxidase.

MATERIALS AND METHODS

Media and reagents. HBSS, RPMI 1640 and FCS were from Flow Labs (Irvine, U.K.). Catalase (bovine liver), superoxide dismutase (type I, bovine blood), taurine (Tau-NH_2), L-methionine, benzoate, mannitol, ferricythocrome *c* and Triton X-100 were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Sodium azide was obtained from Merck (Darmstadt, Germany). Heparin (Liquemin) was purchased from Roche (Milan, Italy). Ficoll-Hypaque was from Nyegaard & Co. (Oslo, Norway). PMA (Sigma), stored at −20° as a stock solution of 2 mg/mL in dimethylsulfoxide (C. Erba, Milan, Italy) was diluted and used at the final concentration of 10 ng/mL. 5-Thio-2-nitro benzoic acid was prepared by reducing 5,5′-dithio-bis(2-nitrobenzoic)acid (Sigma), as described by Aune and Thomas [24]. HOCl was generated by adding sodium hypochlorite (B.D.H. Ltd, Poole, U.K.) into solution buffered at pH 7.4 [24]. $Na_2(^{51}Cr)O_4$ was purchased from the Radiochemical Centre (Amersham, U.K.). Ceftazidime (Sigma Tau, Rome, Italy), Cephotaxime (Hoechst Italia, L'Aquila, Italy), Cephoperazon (Pfizer Italiana, Latina, Italy), Ampicillin (Squibb S.p.A., Rome, Italy), Piperacillin (Cyanamid Italia S.p.A., Catania, Italy) and Penicillin G (Squibb S.p.A., Rome, Italy) were dissolved in HBSS adjusting the solutions osmolarity (290 mOsm/L) and pH (7.4) before using.

Preparation of neutrophils. Heparinized (heparin 10 units/mL) venous blood was obtained from healthy male volunteers. Neutrophils were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll-Hypaque density gradient, as described previously [25]. Contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were then washed three times with HBSS and resuspended in HBSS. Final cell suspensions contained 97% or more neutrophils and more than 97% viable cells as

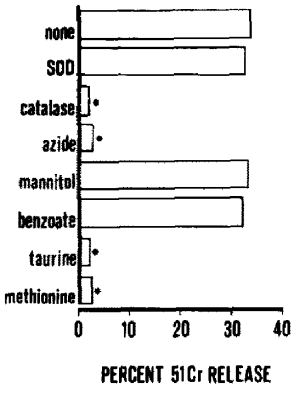

Fig. 1. Effects of oxidant scavengers and inhibitors on the neutrophil-mediated Daudi cell lysis. Superoxide dismutase (SOD) = 300 units/mL; catalase = 4000 units/mL; azide = 1 mmol/L; mannitol, benzoate, taurine and methionine = 20 mmol/L. The results are expressed as means of at least three experiments (SD ≤ 13% of the means). * $P < 0.001$.

detected by the ethidium bromide fluorescein diacetate test [26].

Preparation of Daudi cells. The Daudi cell line (B lymphoblasts) supplied by Prof. G. Damiani (Department of Biochemistry, University of Genoa, Italy) was grown in suspension (RPMI–FCS) and subcultured every 4–5 days. The cells were labelled with 100–200 μCi $Na_2(^{51}Cr)O_4$ by incubating for 1 hr at 37° [25] and resuspended in HBSS.

Cytolytic assay. The Daudi cell lysis by PMA-triggered neutrophils was measured by using a ^{51}Cr release method [25]. The experiments were carried out in duplicate using 2×10^6 neutrophils, 5×10^5 ^{51}Cr-labelled Daudi cells and 10 ng/mL PMA in a final volume of 0.5 mL. The tests were performed in Falcon plastic tubes (12 × 100 mm, Falcon Plastic, Oxnard, CA, U.S.A.) in a shaking water bath (100 rpm) at 37°. After incubation for 2 hr, the ^{51}Cr release from labelled target cells was determined in the cell-free supernatants. The percentage of cytolysis (% ^{51}Cr release) was calculated according to the formula $(E-S)/(T-S) \times 100$, where E is the counts per minute released in the presence of effectors, T is the counts per minute released after lysing target cells with 5% Triton X-100 and S is the counts per minute spontaneously released by target cells in the absence of effectors (in each case ≤ 10%).

HOCl assay. The generation of HOCl by neutrophils was measured by the Tau-NH_2-trapping technique [27], as described previously [24]. The reactions were carried out with 5×10^5 neutrophils, 20 mmol/L Tau-NH_2 and 10 ng/mL PMA in a final volume of 0.5 mL. After 60 min of incubation, the amount of HOCl trapped by Tau-NH_2 (yielding taurine-monochloramine, Tau-NHCl) in the cell-free supernatants was determined by measuring spectrophotometrically ($\varepsilon = 1.36 \times 10^4$ mol/L/cm at 412 nm) the oxidation of 5-thio-2-nitro benzoic acid [25]. A series of experiments was carried out by using reagent HOCl (40 μmol/L) and Tau-NH_2 (20 mmol/L) in the presence of each antibiotic in a final volume of 1 mL. After incubation (15 min at 37°) the amount of the Tau-NHCl generated from reagent HOCl and Tau-NH_2 was measured as described earlier.

Other assays. The generation of superoxide anion (O_2^-) by neutrophils, incubated (20 min) in the presence or absence of antibiotics, was studied by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* [28]. The effects of antibiotics on the neutrophil viability was studied by the ethidium bromide fluorescein diacetate test [26]. The effects of the antibiotics on the viability of Daudi target cells was studied by using the ^{51}Cr release method described above.

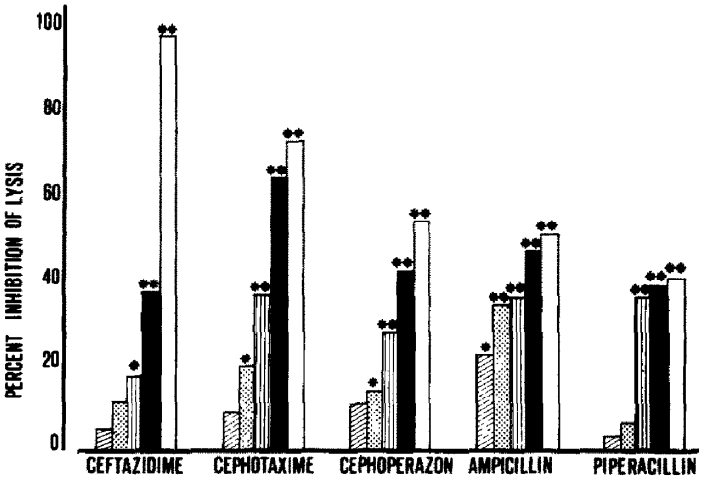

Fig. 2. Inhibition of the neutrophil cytolytic activity by various doses of ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin. The results are expressed as means of at least three experiments with SD ≤ 15% of the means. The % ^{51}Cr release from labelled Daudi cells incubated with PMA-triggered neutrophils in the absence of antibiotics was 30.3 ± 5.6 (mean ± SD). (▨) 0.1 mg/mL; (▩) 0.5 mg/mL; (▥) 1.0 mg/mL; (■) 2.5 mg/mL; (□) 5.0 mg/mL. * $P < 0.05$; ** $P < 0.001$.

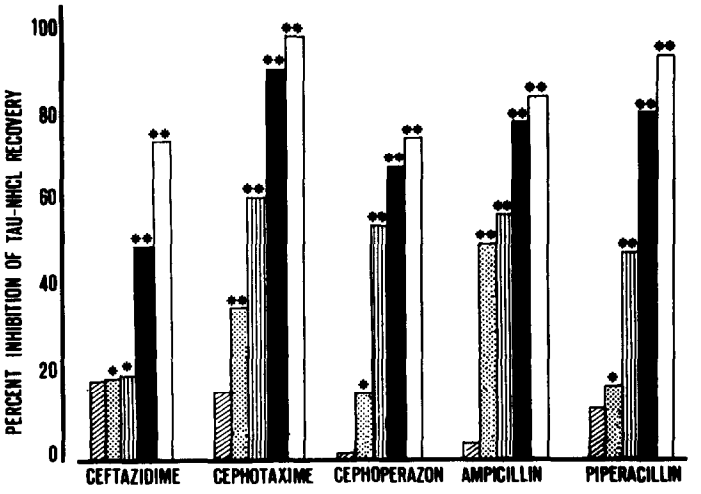

Fig. 3. Per cent inhibition of the recovery of Tau-NHCl from PMA-triggered neutrophils by ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin. The results are expressed as means of at least three experiments (SD ≤ 12% of the means). The Tau-NHCl recovery from PMA-triggered neutrophils in the absence of antibiotics was 56.54 ± 4.7 nmol/10^6 neutrophils/60 min (mean ± SD). (▨) 0.1 mg/mL; (▩) 0.5 mg/mL; (▥) 1.0 mg/mL; (■) 2.5 mg/mL; (□) 5.0 mg/mL. * $P < 0.05$; ** $P < 0.001$.

Statistical analysis. Data were evaluated by variance analysis.

RESULTS

As depicted in Fig. 1, the cytolytic activity of PMA-triggered neutrophils against Daudi target cells was inhibited by catalase (which degrades H_2O_2), azide (inhibitor of MPO), Tau-NH_2 and methionine (HOCl scavengers): these findings suggest that the target cell lysis depends on the HOCl production via the MPO-catalysed transformation of the generated H_2O_2. Finally, the lysis was unaffected by SOD (superoxide anion, O_2^- scavenger), benzoate and mannitol (hydroxyl radical, $OH^\cdot$ scavengers) (Fig. 1), ruling out the involvement of O_2^- and $OH^\cdot$ in the lytic event.

As shown in Fig. 2, five of six antibiotics investigated (ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin) inhibited the neutrophil-mediated lysis of Daudi cells in a dose-dependent manner. No inhibition of the cytolytic activity was observed when penicillin G was added (per cent inhibition < 10%). None of the antibiotic inhibited the superoxide production by neutrophils. Finally, antibiotics did not affect neutrophil or Daudi cell viability.

In the presence of exogenous Tau-NH_2, the neutrophil-derived HOCl reacts with this amino acid (HOCl + Tau-NH_2 → Tau-NHCl + H_2O) to yield long-lived chloramine Tau-NHCl, which is measurable [27]. The addition of ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin to PMA-triggered neutrophils, incubated with Tau-NH_2, resulted in a dose-dependent reduction of the Tau-NHCl recovery (Fig. 3). This suggests that the five antibiotics are likely to compete with Tau-NH_2 for neutrophil-derived HOCl. Consistent with such a possibility, the aforementioned drugs also inhibited the recovery of Tau-NHCl when added to a mixture of reagent HOCl and Tau-NH_2 (Table 1). On the contrary and according to the results from the cytolytic assays, penicillin G was ineffective in limiting the Tau-NHCl recovery from both PMA-triggered neutrophils and the aforementioned HOCl/Tau-NH_2 cell-free system (data not shown).

Table 1. Antibiotic-induced inhibition of Tau-NHCl recovery from the mixture HOCl –Tau-NH_2

Antibiotic	Dose (mg/mL)	% Inhibition Expt. 1	% Inhibition Expt. 2
Ceftazidime	10	92.7	98.9
Ceftazidime	1	60.3	48.4
Cephotaxime	10	95.5	99.9
Cephotaxime	1	59.1	62.1
Cephoperazon	10	90.1	92.7
Cephoperazon	1	81.5	88.7
Ampicillin	10	99.8	97.7
Ampicillin	1	72.7	55.0
Piperacillin	10	87.7	80.1
Piperacillin	1	45.4	47.8

The experiments were carried out using 40 nmol HOCl and 20 μmol Tau-NH_2 in a final volume of 1 mL.

DISCUSSION

The present data suggest that five antibiotics, ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin, are capable of inhibiting the neutrophil-mediated lysis of target cells, while penicillin G, the sixth antibiotic investigated, is ineffective. As demonstrated by previous data

[5, 29, 30] and confirmed here, the neutrophil-mediated cell lysis is strictly dependent on the HOCl production by the MPO pathway ($H_2O_2 + Cl^- \xrightarrow[H^+]{MPO} HOCl + H_2O$). In fact, when the main steps of the MPO pathway were inhibited by catalase, azide, Tau-NH_2 or methionine, the neutrophil-mediated lysis of Daudi cells was impaired. As the five antibiotics were found to compete with Tau-NH_2 for reagent- or neutrophil-derived HOCl, they are likely to inhibit the neutrophil cytolytic activity by inactivating the generated HOCl. It is suggestive that the observed cytoprotective ability of these antibiotics reflects the presence of $-NH_2$ groups in the molecule, prone to be chlorinated by HOCl ($A\text{-}NH_2 + HOCl \rightarrow A\text{-}NHCl + H_2O$, where A- represents antibiotic molecule). Similar reactions have been reported for other drugs containing $-SH$ groups [31]. Finally, it is noteworthy that the antibiotics studied cannot traverse across neutrophil membranes [14, 15, 32]. Consequently, their HOCl-scavenging action can be expected to be limited to the extracellular surroundings without affecting the intracellular bactericidal activity of neutrophils.

The lowest drug concentration able to exert the described inhibitory effects generally was 0.5 mg/mL. The concentration is higher than that detectable in serum after 1 g intravenous administration of these antibiotics (0.1–0.2 mg/mL) [33, 34]. Nevertheless, high doses of these drugs (up to 4–6 g every 6–8 hr for at least piperacillin and cephoperazon) can be used in severe conditions, such as septicemia, genitourinary and respiratory tract, intraabdominal and soft tissue infections [33, 35]. Also, these antibiotics can reach biliary and/or urinary concentrations several times higher than those in the serum [33–35]. Therefore, the present data suggests a possible role of these antibiotics in moderating the neutrophil cytolytic potential at tissue sites of infection. Moreover, they might also limit the HOCl-dependent activation of latent collagenase [36] and gelatinase [37], as well as the inactivation of α-1-proteinase inhibitor (inactivator of elastase) [38]. Thus, it is possible that the HOCl-scavenging properties of the five antibiotics may also limit the degradation of connective matrix components by neutrophils recruited at infection sites. Although speculative, the cytoprotective activities of ceftazidime, cephotaxime, cephoperazon, ampicillin and piperacillin demonstrated here may have a role especially in the treatment of severe pulmonary infections, considering the involvement of neutrophils in the pathogenesis of the lung tissue damage, for example during chronic obstructive pulmonary disease and the adult respiratory distress syndrome.

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