Interactions of the oxygen-dependent antimicrobial system of the human neutrophil with difloxacin, ciprofloxacin, pefloxacin and fleroxacin in the intraphagocytic eradication of *Staphylococcus aureus*

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Summary. The effect of the O₂-dependent antimicrobial systems of the human neutrophil on the intraphagocytic activity of difloxacin, ciprofloxacin, pefloxacin and fleroxacin was determined by use of a radioassay with *Staphylococcus aureus* as the test organism. The fluoroquinolones exhibited good intraphagocytic activity with normal neutrophils. However the intracellular bioactivities of the four antimicrobial agents were substantially less in tests with neutrophils from two patients with chronic granulomatous disease. These observations suggest a synergic interaction between fluoroquinolones and the O₂-dependent antimicrobial systems of phagocytes in the eradication of intracellular microbial pathogens.

Introduction

Immunosuppression may contribute to unsuccessful antimicrobial chemotherapy. The use of a chemotherapeutic agent with intraphagocytic activity in the treatment of immunocompromised individuals infected with intracellular microbial pathogens may be important. We have previously found that the fluorinated piperazinyl quinolones, difloxacin, ciprofloxacin, pefloxacin and fleroxacin, possess intracellular bioactivity (van Rensburg *et al.*, 1989). In the present study we have compared the ability of these fluoroquinolones to eradicate *Staphylococcus aureus* in neutrophils with intact O₂-dependent antimicrobial systems and neutrophils from two children with chronic granulomatous disease (CGD). CGD is associated with failure of phagocytes to activate O₂-dependent antimicrobial systems (Curnutte *et al.*, 1974).

Materials and methods

Antimicrobial agents

Difloxacin, ciprofloxacin, pefloxacin and fleroxacin were obtained from the South African affiliates of Abbott Laboratories, Bayer-Miles Ltd, May and Baker Ltd and Roche Ltd respectively. The agents were dissolved in Hanks's Balanced Salts Solution (HBSS; Grand Island Biological Co., NY, USA) buffered to pH 7.2 with

HEPES (Sigma Chemical Co., St Louis; MO, USA) 1 g/L to achieve final concentrations of 0.5 and 2.5 mg/L.

Bacteria

A 24-h culture of *S. aureus* ATCC 25923 in Nutrient Broth (Difco) was washed three times in phosphate-buffered saline and diluted to 2×10^8 cfu/ml.

Neutrophils

Venous blood from two healthy volunteers and two patients with CGD was collected into preservative-free heparin (5 units/ml of blood). The heparinised blood was fractionated by centrifugation (400 *g* for 15 min) on Ficoll (Pharmacia, Uppsala, Sweden) metrizoate cushions. The resultant fraction was sedimented with gelatin 3% for 30 min at 37°C to remove most of the erythrocytes. The neutrophil-rich supernate was centrifuged at 250 *g* for 10 min and the residual erythrocytes in the cell pellet were lysed by exposure to ammonium chloride 0.85%. The neutrophils were centrifuged, washed once and resuspended in HBSS to a concentration of 1×10^7 /ml.

Radioassay

Intracellular bioactivity of the antimicrobial agents was measured as described previously (Anderson *et al.*, 1986). Human neutrophils were pre-incubated with *S. aureus* at a ratio of 1 neutrophil: 20 bacterial cfu for 30 min at 37°C in a final volume of 1 ml of HBSS containing autologous serum 10% and 5×10^6 neutrophils. The mixtures of neutrophils and bacteria were rotated on

a turntable during the 30-min incubation period; lyso-staphin 10 mg/L was added and the tubes were incubated for a further 15 min at 37°C. The neutrophils and phagocytosed bacteria were then centrifuged (250 *g* for 10 min) and washed to remove the extracellular bacteria. Antimicrobial agents were added to the neutrophils and intracellular bacteria after phagocytosis to achieve a final concentration of 0.5 or 2.5 mg/L. These mixtures were incubated for 4 h at 37°C after which the neutrophils were washed three times in phosphate-buffered saline (0.15 M, pH 7.2) to remove the antibiotics and the intracellular bacteria were released by lysis of the neutrophils with distilled water. Nutrient broth containing 5 μ Ci of a radiolabelled amino acid mixture [L-amino acid mixture, L-¹⁴C (U), specific activity 55 mCi/milligram carbon, New England Nuclear Corp.] was inoculated with 100 μ l of the lysate and incubated at 37°C for 5 h, after which the bacteria were centrifuged and washed three times in PBS. The extent of incorporation of radiolabelled amino acids into bacterial cells was measured in a liquid scintillation counter (Isocap/300, Searle, Chicago, IL, USA) spectrophotometer. The results were expressed as uptake of radiolabelled amino acids as radioactive counts/min (cpm). Neutrophils were omitted from identically processed control systems containing bacteria and antibiotics only.

Neutrophil viability

Neutrophil viability was determined before phagocytosis and after the 4-h incubation period of the neutrophils and intracellular bacteria with and without antibiotics by standard dye exclusion methods.

Results

In tests of neutrophils from healthy volunteers with intact O₂-dependent antimicrobial systems, the antimicrobial agents at concentrations of 0.5 and 2.5 mg/L decreased the survival of intracellular bacteria. There was evidence of a fall in survival of bacteria in all cases except with feroxacin tested at 0.5 mg/L (figure). Considerably less antimicrobial activity was obtained in similar experiments with neutrophils from patients with CGD. Evidence of decreases in bacterial growth at an antimicrobial concentration of 2.5 mg/L (figure) was obtained only with ciprofloxacin and difloxacin.

Exposure of *S. aureus* to pefloxacin and feroxacin at a concentration of 2.5 mg/L in HBSS for 4 h at 37°C in the absence of neutrophils did not significantly affect bacterial growth. However, difloxacin and ciprofloxacin, at 2.5 mg/L in the absence of neutrophils, caused 76.0 (SE 2.8) % and 57.5 (SE 9.2) % reduction in bacterial growth respectively.

No detectable loss of viability of neutrophils was demonstrated when the cells, containing intracell-

Figure. Measurement of the intracellular bioactivity of (a) difloxacin, (b) ciprofloxacin, (c) pefloxacin and (d) feroxacin with the radioassay and *S. aureus* as the target intraphagocytic pathogen. Normal neutrophils (●---●) as well as CGD neutrophils (●—●) were used in these experiments. Data are mean values from two different experiments; bars are $\pm$ SEM.

ular bacteria, were incubated with and without antibiotic for 4 h at 37°C.

Discussion

Investigations of the intracellular bioactivity of antimicrobial agents have mainly been done on neutrophils with an intact O₂-dependent antimicrobial system (Vosbeck *et al.*, 1984) without taking into account possible interactions between this system and the drug. Use of antimicrobial agents which need an intact intraphagocytic O₂-dependent antimicrobial system for optimal intracellular effectiveness may lead to less efficient eradication of

intracellular pathogens from patients such as those with CGD, in whom the oxidative system of neutrophils is defective. In previous studies we have found that the intracellular bioactivity of amoxycillin, clindamycin, erythromycin and roxithromycin is optimal with normal neutrophils and much less efficient with CGD neutrophils (Anderson *et al.*, 1986, 1987) indicating that the co-operative interactions between the O₂-dependent antimicrobial systems of the neutrophil and the intracellular antibiotic should not be under-rated.

We have previously found the radioassay used in the present study to be more sensitive than colony counting procedures (Anderson *et al.*, 1986). All four fluoroquinolones tested, at concentrations of 0.5 and 2.5 mg/L, were found to possess intracellular activity against *S. aureus* in phagocytes with an intact O₂-dependent antimicrobial system. However, when CGD-neutrophils were used, only difloxacin and ciprofloxacin exhibited intraphagocytic activity against the test organism and only at a concentration of 2.5 mg/L. This suggests that the

therapeutic efficacy of pefloxacin and fleroxacin may be decreased in individuals with impaired phagocyte functions. The MICs of the fluoroquinolones for *S. aureus* ATCC 25923 were 0.25 mg/L (difloxacin and ciprofloxacin) and 0.5 mg/L (pefloxacin and fleroxacin) (van Rensburg *et al.*, 1989). In a neutrophil-free system only ciprofloxacin and difloxacin, but not pefloxacin and fleroxacin, effectively decreased bacterial growth at a concentration of 2.5 mg/L. Therefore, the differences in intracellular activity between the drugs could be ascribed to differences in activity against *S. aureus*. Although a concentration of 2.5 mg/L is achievable in serum with normal dosages of most of the fluoroquinolones (Eggleston and Park, 1987) these findings stress the importance of obtaining high enough serum levels to optimise interactions with phagocytes.

These results suggest the existence of beneficial interactions between fluoroquinolones and the O₂-dependent antimicrobial systems of phagocytes in the eradication of intracellular pathogens.

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