ROLE OF FOSFOMYCIN TROMETHAMINE IN MODULATING NON-SPECIFIC DEFENCE MECHANISMS IN CHRONIC UREMIC PATIENTS TOWARDS ESBL-PRODUCING *ESCHERICHIA COLI*

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Antimicrobial agents and polymorphonuclear cells (PMNs) have the potential to interact in such a way that improve the therapy for infectious diseases. In immunocompromised patients highly susceptible to microbial infections with high morbidity and mortality, several metabolic and functional alterations in PMNs, mostly related to microbicidal activity, are observed. Therefore, the antibiotic of choice should have a good antimicrobial effect without impairing host defences. The aim of this study is to evaluate *in vitro* effects of sub-inhibiting fosfomycin tromethamine (FT) concentrations on the primary functions of PMNs from healthy subjects and immunocompromised patients (haemodialysed and renal transplant recipients), against an ESBL-producing *Escherichia coli*, the most common aetiological agent in urinary tract infections (UTIs). FT is considered a first line drug in the eradication of UTIs due to its appropriate antimicrobial spectrum, oral bioavailability and minimal risk of microbial resistance. Our results provide evidence that FT is able to induce enhancement of the depressed phagocytic response of PMNs from patients on chronic haemodialysis and from renal transplant recipients, restoring their primary functions *in vitro* against ESBL-producing *E. coli*. All these data permit the conclusion that uremic-infected patients might additionally benefit from the immunomodulating properties of FT.

Fosfomycin, a bactericidal cell wall synthesis-inhibiting antibiotic not structurally related to other classes of antimicrobial agents, was first described in Spain more than thirty years ago (1-3). The parenteral formulation has been widely used in Europe and Japan on account of its good tolerability and activity against aerobic bacteria, including uropathogenic species (4). However, its indications have been limited by poor bioavailability when administered orally and by the risk of microbial resistance. Persistent interest in this agent has led to elaboration of a prodrug, active against urinary pathogens, namely fosfomycin salified with trometamol (fosfomycin tromethamine; FT) that greatly increased its hydrosolubility without altering its antibacterial activity (3).

Extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* is a growing problem in many parts of the world, being the most common aetiological agent in both community-acquired or hospital-acquired urinary tract infections (UTIs) (5-7). UTIs are generally treated with β-lactams,

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co-trimoxazole and quinolones but bacterial high resistance restricts their empirical use.

FT, despite many years of use, continues to be characterized by an extremely low incidence of resistance (about 1-2%) in *E. coli* strains worldwide (4-6, 8). Microbiological, pharmacokinetic and pharmacodynamic properties of FT have been extensively studied, whereas its immunological features have only begun to attract scientific and clinical attention in recent years. The immunocompromised patients are highly susceptible to microbial infections with high morbidity and mortality related to impaired functionality of the immune system (9, 12-13). In these individuals the course of infections can be more aggressive than in normal hosts, and the antibiotic of choice should have a high antimicrobial effect without impairing host defences. Hence, the antibiotic enhancement of phagocyte functions may be of potential clinical importance in the outcome of bacterial infections (9-12). Therefore, the aim of this study is to evaluate the effects of sub-inhibiting FT concentrations on the primary functions of polymorphonuclear granulocytes (PMNs) from haemodialysed patients and renal transplant recipients, compared to that of healthy subjects against an ESBL-producing *E. coli*.

MATERIALS AND METHODS

Subjects

All patients participating in this study gave their informed consent. Blood samples were obtained from 60 healthy volunteers, as control, and from 91 immunocompromised patients without any evidence of active infection, treated at the Nephrology and Dialysis Unit of the Civil Hospital, Ivrea (Turin, Italy). In detail, 47 patients on haemodialysis, 26 males and 21 females (mean age 59.8 ± 12 years) and 44 patients who had undergone renal transplantation, 30 males and 14 females (mean age 50.3 ± 11 years). The mean time on dialysis was 63.2 ± 51 months and the causes of renal failure were as follows: chronic glomerulonephritis (10 cases), nephroangiosclerosis (13 cases), polycistyc kidney disease (5 cases), diabetic nephropathy (5 cases), chronic renal failure (10 cases), renal adenocarcinoma (1 case), and interstitial nephritis (3 cases). The mean normalized dose of dialysis/treatment (Kt/V single pool = 1.49 ± 0.31) and the protein catabolic rate (C-reactive protein = 1.37 ± 0.35) indicated an adequate dialysis prescription and nutrition. About 60% of the patients had secondary hyperparathyroidism (mean plasma levels of iPTH for all patients was 359.6 pg/ml, and mean calcitrol dose was 0.42 μg/d). The dialyzer membrane was modified cellulose without reuse. The mean time since transplantation was 68.3 ± 40 months and primary renal disease included chronic glomerulonephritis (14 cases), interstitial nephritis (10 cases), polycystic kidney disease (8 cases), chronic renal failure (5 cases), diabetic nephropathy (3 cases), other (4 cases). The mean serum creatinine at the time of the study was 1.5 ± 0.9 mg/dl. Post-transplant immunosuppressive treatment in 21 patients was cyclosporine A (CyA) and prednisone (P); in 18 patients CyA, P, and azathioprine (AZA); in 3 patients CyA and AZA and in 2 patients AZA and P.

Bacteria

E. coli ATCC 35218, an ESBL-producing strain, was cultured on McConkey agar (Oxoid S.p.A., Garbagnate Milanese, Milan, Italy). Young colonies (18–24 h) were picked to approximately 3–4 McFarland standard and inoculated into cryovials containing both cryopreservative fluid and porous beads to allow bacteria to adhere (Microbank, Biomérieux, Rome, Italy). After inoculation, cryovials were kept at –80°C for extended storage (13).

Antimicrobial activity of fosfomycin tromethamine against E. coli

FT was kindly supplied by Zambon Group S.r.l (Bresso, Milan, Italy). Pirogen-free aqueous solutions of the drug were freshly prepared for each experiments with the addition of 25 μg/ml of glucose 6-phosphate (Sigma-Aldrich, Milan, Italy) inductor of the enzymes responsible for the active transport of fosfomycin in the microbial cells (14). Antibiotic susceptibility testing was performed by the standardized broth microdilution method in Mueller Hinton broth (Oxoid) according to CLSI recommendations (15).

In order to evaluate the extracellular killing activity induced by FT, *E. coli* (10^7 CFU/ml) in 15 ml Brain Heart Infusion (BHI; Oxoid) broth was incubated at 37°C in duplicate with or without (control) the drug at 1/2xMIC, 1/16xMIC, 1/32xMIC and 1/64xMIC (16). 500 μl-samples were collected for colony counting at 0, 30, 60, 90, 120, 180 min and 24 h, and plated onto BHI agar plates. The mean number of CFU/ml of the duplicate samples was determined after overnight incubation at 37°C. Results were reported as log CFU/ml (16-17).

PMNs

Peripheral venous blood from haemodialysis patients and renal transplant recipients was collected into sterile evacuated blood-collecting tubes containing lithium heparin (15 USPU/10ml blood) and settled at room temperature by gravity for 30 min in 2.5% dextran

(500,000 molecular weight; Pharmacia S.p.A., Milan, Italy) in normal saline (1:1 ratio). The leucocyte-rich plasma supernatant was carefully layered on Ficoll-Paque (Pharmacia) and centrifuged twice at 1,200 *g* for 15 min; to obtain pure PMNs, residual erythrocytes were lysed by hypotonic shock for 30 sec in sterile distilled water and then centrifuged further (10, 13, 18). After being counted in Bürker cell counting chamber (Marenfield, Germany), the density of PMNs was adjusted to 10^6 cells/ml in phosphate-buffered saline (PBS) supplied with 0.1% glucose and 0.1% human albumin (Sigma, St Louis, MO). The PMNs were placed in sterile plastic capped tubes treated with RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; Gibco) and incubated at 37°C in a shaking water bath (150 rpm) before the addition of *E. coli* (10^7 CFU/ml). The viability assayed by trypan blue exclusion before and after each experiment was >95%. The time between the collection of blood and the beginning of the experiments did not exceed 3 h. The interval between PMNs harvest and the start of experiments was less than 30 min (10, 13, 18). Peripheral venous blood was pooled from sixty healthy donors, negative for the presence of microbial and viral diseases (A.S.O. San Giovanni Battista, Turin, Italy), collected and processed, as described earlier, to obtain isolated PMNs.

Radioactive labelling protocol

A total of 200 µl of the frozen culture was placed into fresh BHI broth containing 150 µCi of ^{3}H-uracil (specific activity, 1.27TBg/mmol; NEN Products, Milan, Italy) at 37°C. The radiolabelled bacteria were centrifuged several times with BHI broth and re-suspended in fresh medium and adjusted to yield 10^7 CFU/ml, as confirmed by colony counts in triplicate.

Phagocytosis assay

In all experiments, the bacterium:PMN ratio was 10:1. Aliquots of 1 ml of *E. coli* in RPMI 1640 with 10% FCS were added to PMNs in sterile plastic tubes (10^6 cells) and the tubes were then incubated at 37°C in a shaking water bath. After incubation for a period of 30, 60 or 90 min, the tubes were centrifuged at 1,200 *g* for 5 min. The pellet was then suspended in phosphate saline, and the mixture was centrifuged at 1,200 *g* for 5 min to remove free bacteria. The cells were then suspended in 1 ml of sterile distilled water for 5 min, and 100 µl samples of this suspension were placed in scintillation fluid (Atomlight, NEN) and counted by liquid scintillation spectrophotometry. Radioactivity was expressed as the counts/min/sample. The percentage of phagocytosis at a given sampling time was calculated as follows: phagocytosis (%) = (cpm in PMN pellet/cpm in total bacterial pellet)x100 (12-13).

Measurement of antimicrobial activity of PMNs

In all the experiments, the bacterium:PMN ratio was 10:1. Aliquots of 1 ml of *E. coli* (10^7 CFU) and PMNs in sterile plastic tubes (10^6 cells) were incubated in RPMI 1640 at 37°C in a shaking water bath for 30 min to allow phagocytosis to proceed. The PMN-bacterium mixtures were centrifuged at 1,200 *g* for 5 min and washed with phosphate saline to remove the free extracellular bacteria. An aliquot of the cells containing bacteria was taken, and lysed by adding sterile water, and a viable count of intracellular *E. coli* was performed (time zero). The cells were then incubated further, and at intervals (time x) the viable counts of the surviving intracellular bacteria were measured in the same way. The PMN killing values were expressed as the survival index (SI), which was calculated by adding the number of surviving microorganisms at time zero to the number of survivors at time x and dividing by the number of survivors at time zero (10, 12, 18). According to this formula, if bacterial killing was 100% effective, the SI would be 1.

Influence of FT on phagocytosis and intracellular killing

The effects of FT on either the phagocytosis of *E. coli* or intracellular bacterial killing by PMNs were investigated by incubating the bacteria and the phagocytes (bacteria:PMN ratio 10:1) at 37°C in a shaking water bath for periods of 30, 60, or 90 min in the presence of sub-inhibiting concentrations of the drug. To differentiate between any separate effect of FT on the bacteria and the PMNs, the experiments were conducted after exposure of each of them to sub-inhibiting antibiotic concentrations for 1 h, before they were incubated together (17). After the withdrawal of the drug, pre-exposed bacteria were added to PMNs and bacteria to pre-exposed PMNs. A control system was assayed in parallel with no antibiotic. The phagocytic and bactericidal activities of PMNs were determined as described above.

Statistical analysis

Each test was performed in triplicate, and the results compared with those obtained with the control systems and expressed as the means and standard errors of the means (S.E.M.s) for 10 separate experiments. Statistical evaluation of the differences between test and control results was performed by an analysis of variance by Tukey's test. Extracellular killing was compared using Student's unpaired *T*-test (16).

RESULTS

Antimicrobial activity of FT against E. coli

The MICs of FT for ESBL-producing E. coli

Table I. *Functional activity of PMNs from healthy subjects, haemodialysed patients and renal transplant recipients: phagocytosis and intracellular killing against ESBL-producing E. coli in the absence and in presence of 1/16xMIC of fosfomycin tromethamine (FT).*

Time (min)	Mean % phagocytosis ± SEM		Survival Index ± SEM	
	Controls	FT	Controls	FT
Healthy subjects				
30	14.44 ± 1.9	24.6[a] ± 2.3	1.52 ± 0.08 (48 %*)	1.04[a] ± 0.08 (96 %)
60	10.81 ± 0.95	4.5 ± 0.8	>2	1.03[a] ± 0.04 (97 %)
90	9.1 ± 0.78	2.9 ± 0.2	>2	1.01[a] ± 0.02 (99 %)
Haemodialysed patients				
30	10.2 ± 1.2	14[b] ± 1.47	>2	1.07[a] ± 0.01 (93 %)
60	7.85 ± 0.34	2.8 ± 0.01	>2	1.04[a] ± 0.02 (96 %)
90	6.94 ± 0.19	2.55 ± 0.35	>2	1.04[a] ± 0.01 (96 %)
Renal transplant recipients				
30	12.5 ± 1.6	18.15[b] ± 1.9	>2	1.08[a] ± 0.03 (92 %)
60	10.3 ± 0.9	3.8 ± 0.85	>2	1.02[a] ± 0.01 (98 %)
90	10.1 ± 0.5	3.5 ± 1.4	>2	1.04[a] ± 0.05 (96 %)

[a] *Significantly different from the controls (P<0.01);* [b] *Significantly different from the controls (P<0.05);* * *% Percentage of initial bacterial population killed by PMNs*

Table II. *Functional activity of PMNs from healthy subjects: intracellular killing against ESBL-producing E. coli following 1h pre-exposure of bacteria (A) or PMNs (B) to 1/16xMIC of fosfomycin tromethamine (FT).*

	Survival Index ± S.E.M		
Time (min)	Controls	A	B
30	1.52 ± 0.08 (48 %)	1.41[b] ± 0.2 (59%)	1.26[a] ± 0.05 (74%)
60	>2	1.6[a] ± 0.2 (40 %)	1.48[a] ± 0.08 (52 %)
90	>2	>2	>2

[a]*Significantly different from the controls (P<0.01);* [b]*Significantly different from the controls (P<0.05); % Percentage of initial bacterial population killed by PMNs*

were 4 and 8 μg/l with inocula of 10^5 and 10^7 CFU/ml, respectively. The extracellular killing of *E. coli* in BHI broth with the drug is shown in Fig. 1. At all concentrations tested the activity of FT was bactericidal as indicated by a significant decrease in live bacteria after 3 h incubation: 4.3, 3.5, 3.2 and 2.1 log in the presence of 1/2xMIC, 1/16xMIC, 1/32xMIC and 1/64xMIC, respectively, indicating that bactericidal activity of FT was proportional to its concentration.

Functional activity of PMNs

In all the experiments, the viability of PMNs remained unchanged throughout the experiments. The pattern of phagocytosis and intracellular killing against *E. coli* by PMNs harvested from healthy subjects, haemodialysed patients and renal transplant recipients is shown in Table I. A slight reduced phagocytic efficiency in PMNs from hemodialysis and renal transplant recipients was detected when compared with that of PMNs from healthy subjects. In fact, PMNs harvested from healthy subjects were able to engulf *E. coli* in a range percentage between 9.1% and 14.44% during 90 min of incubation, while PMNs from haemodialysed patients and renal transplant patients were able to phagocytose 6.94-10.2% and 10.1-12.5 %, respectively. This reduced phagocytic activity in the immunocompromised patients was accompanied by the total inability of PMNs to kill ingested bacteria at any time of observation (SI >2, indicating absence of killing), if compared to 48% of intracellular killing by phagocytes from healthy subjects until 30 min (SI =1.52; Table I).

Effect of FT on functional activity of PMNs

The direct exposure of PMNs from healthy subjects and bacteria to sub-inhibiting concentration (1/16xMIC) of FT significantly enhanced the phagocytosis until the first 30 min of incubation (24.6 versus 14.44; $P<0.01$; Table I), whereas the subsequent phagocytosis was not even half that of the antibiotic-free controls (Table I). Conversely, in the same experimental conditions, FT had a marked effect on intracellular killing, resulting in increased numbers of killed *E. coli* for all three incubation times compared with those of the controls: during the 90-min period, the intracellular bacterial load was reduced by 99% (SI = 1.01; $P<0.01$; Table I). PMNs from haemodialysed patients and renal transplant recipients displayed a similar pattern in functionality (Table I): FT at sub-inhibiting concentration significantly enhanced phagocytosis within 30 min ($P<0.05$), whereas its subsequent percentages were similar to those observed with PMNs from healthy subjects. PMNs from the immunocompromised patients were totally unable to kill ingested cells at any time (SI>2), while in the presence of a sub-inhibiting drug, the killing values ranged from 93 to 96% and 92 to 98%, for haemodialysed patients and renal transplant recipients, respectively ($P<0.01$; Table I).

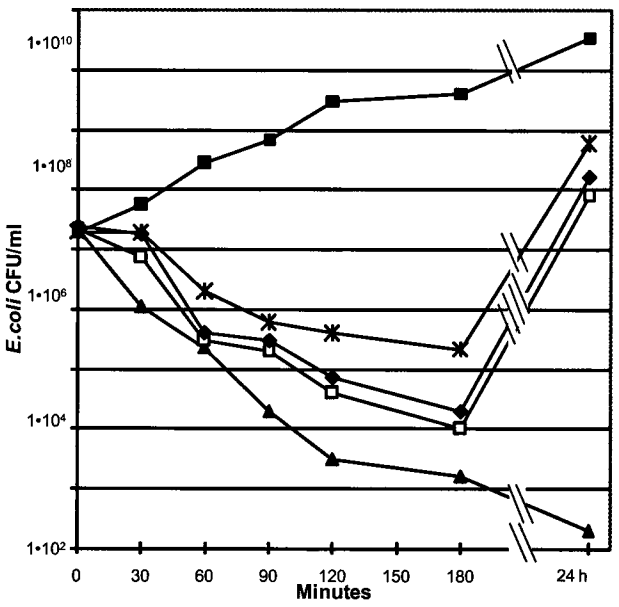

Fig. 1. *Effect of fosfomycin tromethamine (FT) on the extracellular killing of ESBL-producing E. coli.*
E. coli (10^7 CFU/ml) was incubated with the drug at 1/2xMIC (▲), 1/16xMIC (□), 1/32xMIC (♦) and 1/64xMIC (*). A control (■) without FT was set up. The mean number of CFU/ml of the samples was determined after overnight incubation at 37°C. Results were reported as log CFU/ml.

To investigate the direct effect of FT on the phagocyte functions, bacteria and PMNs from healthy subjects were pre-incubated for 1 h with sub-inhibiting drug. After withdrawal of the antibiotic, bacterial uptake and microbicidal activity were determined. Drug pre-treatment of the bacteria during their growth phase had no effect on phagocytosis itself. In fact, PMNs engulfed the bacteria at the same rate as the untreated ones (data not shown). However, drug-pre-treated *E. coli* were killed far more efficiently by PMNs during the 60 min of incubation compared with untreated bacteria: 59-40% versus 48% and >2 (Table II; Column A). Pre-exposure of the PMNs to sub-inhibiting FT for 60 min had no effect on phagocytosis: *E. coli* were phagocytosed at the same rate as controls (data not shown). In contrast, there was enhanced intracellular killing, leading to a significant decrease in SIs for 60 min, compared with the controls ($P<0.01$; Table II; Column B).

DISCUSSION

It is known that PMNs play an important role in host defence mechanisms against the invading microorganisms, especially in the cell-mediated defence system (9-10, 17). Phagocyte-dependent host defences are frequently impaired in patients with chronic renal failure who are particularly exposed to serious infections by Gram-negative and Gram-positive bacteria, characterized by high morbidity and mortality. Therefore, the therapy protocol for these individuals should take into consideration the immunomodulatory properties of antibiotic and its antimicrobial effects (1, 9, 13, 19).

Since FT is a first line drug in the eradication of UTIs, including the appropriate antimicrobial spectrum, oral bioavailability and minimal risk of microbial resistance (4), in the present study its effect on the primary functions of PMNs from haemodialysed patients and renal transplant recipients, compared to that of healthy subjects, against an ESBL-producing *E. coli* was evaluated.

PMNs from haemodialysed patients and renal transplant recipients showed a significant diminished efficiency with reduced phagocytosis and bactericidal activity compared with that seen in PMNs from healthy subjects (Table I), confirming the literature data (9, 12-13): in uremic patients, the number of PMNs is normal, but several metabolic and functional alterations are observed, namely those related to phagocytic and bactericidal activity (12-13, 20-21). These results may, in part, explain the high incidence of infection among these patients.

The addition of FT (1/16xMIC) to PMNs and *E. coli* enhanced the phagocytic activity by PMNs from healthy subjects within the first 30 min of incubation, compared with controls (Table I). Reduction of the phagocytosis percentage after 60 and 90 min could depend on extracellular killing exerted by the drug rather than inhibition of phagocytosis itself. Since FT, in our *in vitro* conditions, was in direct contact with the bacteria, such extracellular killing would reduce the number of bacteria exposed to further PMNs ingestion. This hypothesis is also confirmed by the results of extracellular killing assays. The number of bacteria still alive at 60 min in the presence of 1/16xMIC FT fell by about 2 log from 10^7 to 10^5 (Fig. 1). Data on the influence of FT on non- specific immune defences are scant and fragmentary, sometimes contradictory and hard to compare because of the variations in methods used for assessing phagocyte functions and the differences in experimental design. Our results regarding the phagocytosis disagree with those of Amurrio et al. (22) and Krause et al. (23), who found that fosfomycin did not alter the ability of neutrophils to ingest *Candida guilliermondii* and *E. coli*, respectively. It should, however, be noted that Krause et al. incubated heat-killed *E. coli* but for only 30 min.

On the other hand, the addition of FT (1/16xMIC) to PMNs and ESBL-producing *E. coli* significantly stimulated intracellular killing with percentage values up to 99% ($P<0.01$). This result may be in part related to FT low molecular weight and hence to its active transport allowing it to penetrate PMNs and cooperate with them in eradication of their ingested bacteria (1, 8, 24). Moreover, this high PMN killing could be related to the inclusion of glucose-6-phosphate in our experimental conditions since this substance facilitates the bactericidal action of FT by inducing the enzymes responsible for its active transport into the bacteria (5, 14, 25).

The addition of FT to PMNs from immunocompromised patients resulted in a similar pattern of functionality in comparison with that observed in healthy subject PMNs (Table I): FT significantly enhanced the bacterial uptake, within the first 30 min of incubation, whereas during the entire period of observation exerted a marked intracellular microbicidal activity with values ranging from 92% to 98% similar to those observed for healthy subjects ($P<0.01$; Table I), indicating that *E. coli* was eradicated from PMNs only in the presence of antibiotic. This result indicates that FT synergized for bacterial killing with PMNs, being able to kill *E. coli* when the killing mechanisms of the phagocytes failed because they were not sufficient: PMNs from uremic patients showed a total incapability of stopping the growth of intracellular bacteria (SI>2; Table I).

To determine whether the increase in microbicidal activity induced by FT was due to its direct action on the bacteria or on the PMNs, both types of cells were separately exposed to the drug for 1 h prior to phagocytosis and killing tests. The results showed that FT did not depress bacterial uptake, since the

percentage of intracellular drug-pre-treated bacteria was similar to that observed in the controls with untreated bacteria (data not shown). There was more intracellular killing of *E. coli* that had been pre-exposed to FT (Table II): the direct action of FT makes the bacteria more susceptible to PMN lytic mechanisms, probably related to phenotypic and biochemical changes in *E. coli* induced by FT. Pre-treatment of PMNs with the drug also significantly increased intracellular microbicidal activity over the course of 1 h (Table II). This may provide indirect evidence of FT penetration of PMNs. Once penetrated, the drug probably remains available in a biologically active form and damages ingested bacteria, which become more susceptible to PMNs killing.

Taken together these results provide evidence that FT, even at sub-inhibiting concentration, is able to induce stimulation of the depressed phagocytic response of PMNs from patients on chronic haemodialysis and from renal transplant recipients, restoring their primary functions *in vitro*. It has to be underlined that these beneficial immunological properties of FT are observed at FT concentration much lower than those usually detected after FT administration in patients for treatment of uncomplicated UTIs (6). All of these data permit the conclusion that both uremic-infected patients and immunocompromised patients might additionally benefit from the immunomodulating properties of FT.

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