Effect of bacterial products including endotoxin on neutrophil function in infected urine

R. A. GARGAN, G. W. SMITH*, J. M. T. HAMILTON-MILLER and W. BRUMFITT

Department of Medical Microbiology, The Royal Free Hospital and School of Medicine, Pond Street, Hampstead, London NW3 2QG and *Department of Medical Microbiology, Duncan Building, Royal Liverpool Hospital, Prescott Street, Liverpool L69 3BX

Summary. Experiments were performed to determine the effects of products of bacterial growth (including endotoxin) on phagocytosis and intracellular killing by polymorphonuclear leucocytes (PMNL) in urine. Bacteriologically filtered supernates of two strains of *Escherichia coli* grown in urine were added in varying amounts to mixtures of PMNL and *E. coli*, also in urine. Phagocytosis of the two strains was reduced from > 90% in controls to 66% and 48%, respectively, in the presence of undiluted culture filtrate (containing endotoxin 2–2.5 µg/ml). Intracellular killing was also decreased and was abolished by dilutions corresponding to endotoxin concentrations of 0.6 and 0.75 µg/ml. When PMNL exposed to these inhibitory dilutions were resuspended in fresh urine, their phagocytic ability was fully restored and 13–24% of their killing activity was regained. A minimum concentration of commercially purified *E. coli* endotoxin of 200 µg/ml was required to abolish PMNL killing, with phagocytosis uninhibited. The results strongly suggest that bacterial growth metabolites, not endotoxin, are responsible for the depression of phagocytosis and intracellular killing in infected urine. A moderate dilution of the bacterial products in urine permits good PMNL function. Extrapolating this to the clinical situation, diluting the urine by water loading (as recommended for patients with urinary infections) should ensure efficient activity of PMNL under in-vivo conditions providing urinary pH and osmolality are not adversely affected.

Introduction

Among the most important factors influencing polymorphonuclear leucocyte (PMNL) function in urine are osmolality and pH.^{1–4} However, other unidentified factors have also been implicated.³ For PMNL to perform effectively, phagocytosis and killing must be able to occur in the presence of varying concentrations of bacterial products, such as endotoxin, present in infected urine.

Endotoxin affects a wide range of PMNL functions.⁵ Nanogram amounts can activate adherence and superoxide anion release,^{6,7} and microgram quantities have been reported to both enhance⁸ and depress PMNL phagocytic and bactericidal activity.^{9,10}

In one of the few reported studies on the effect of endotoxin on PMNL function in urine, Bryant *et al.*³ concluded that endotoxin was unlikely to impair PMNL function in urine, as it did not induce aggregation of these cells. Beyond this, the function of PMNL was not investigated by these authors.

As phagocytosis takes place in infected urine from patients,¹¹ it is reasonable to assume that PMNL can tolerate the relatively high levels of endotoxin that exist in infected urine.¹² The present study was

undertaken to investigate how endotoxin and metabolites produced by overnight cultures of two different *Escherichia coli* strains in urine affect both phagocytosis and intracellular killing by PMNL in urine. The effects of increasing concentrations of purified *E. coli* endotoxin (obtained commercially) on these PMNL functions in urine were also investigated.

Materials and methods

Preparation of urine

Urine was pooled from 18 healthy volunteers (equal numbers of males and females). The pH and osmolality of the pool was adjusted to pH 7.0 and 400 mOsm, which is considered favourable for phagocytosis by PMNL in urine.^{2,4} This was done with freshly voided urine samples. All urine samples were without antibacterial activity and contained < 10³ cfu/ml. Sediment and bacteria were removed by sequential filtration through a coarse, then a fine filter, and finally a 0.22-µm filter; pH was measured with a Corning 120 pH meter and osmolality with a micro-osmometer (model 3MO, Advanced Instruments Inc., Massachusetts, USA). The pool was divided into 20-ml volumes and stored at –36°C.

Isolation and preparation of PMNL

Samples of 4 ml of venous blood were taken from three healthy donors and anticoagulated with potassium EDTA. PMNL were isolated by low speed differential centrifugation in Hanks's Balanced Salts Solution (HBSS) without Ca^{2+} or Mg^{2+} (Gibco Ltd, Paisley). This method compares favourably with other standard separation techniques in its isolation of PMNL and preservation of their functions.¹³ PMNL viability, assessed with ethidium bromide and acridine orange by the method of Ford,¹⁴ was $> 95\%$. Purified PMNL were resuspended in pooled sterile urine containing serum 10% to give a concentration of $c. 4 \times 10^6$ cells/ml. Viability of the PMNL after exposure to the urine for 1 h at 37°C remained unchanged ($> 95\%$).

Bacteria and culture methods

Two strains of serum-resistant *E. coli*, nos. 398 (serotype O6) and 441 (O18:K5), from urinary tract infections were supplied by Dr A. Roberts, Department of Microbiology, West London Hospital, London. These were stored on nutrient-agar slopes. Cultures were grown for 18 h at 37°C in 5 ml of the pooled urine, to give viable counts of $(2-3) \times 10^8$ cfu/ml, washed twice in HBSS, suspended in 0.5 ml of HBSS, and kept at 4°C until they were resuspended in urine for use the same day. The organisms were kept in HBSS and not urine until used, because *E. coli* in the stationary phase, if exposed before phagocytosis to a nutrient source such as serum or urine, can develop resistance within 20 min to subsequent intracellular killing.¹⁵

Urine culture filtrates and endotoxin

After the *E. coli* strains had been removed from the two urine cultures by centrifugation, the supernates were passed through a $0.22\text{-}\mu\text{m}$ filter. The pH and osmolalities of the sterile filtrates were unchanged, despite bacterial growth. Dilutions of these culture filtrates were made in fresh sterile urine from the pool to give concentrations ranging from 1% to 40% v/v.

E. coli endotoxin O55:B5 (prepared by phenol extraction; Sigma Chemical Co.) was dissolved in HBSS and diluted in pooled urine to give concentrations from 10 to $200\text{ }\mu\text{g/ml}$.

The endotoxin concentration of the two neat culture filtrates was measured spectrophotometrically at 405 nm with a *Limulus* amoebocyte lysate (LAL) chromogenic substrate assay method (Kabi Coatest endotoxin kit, Kabi Diagnostica, Sweden). The pooled sterile urine in which experiments were conducted contained $< 5\text{ ng}$ of endotoxin/ml. This represented the lower limit set for the test.

Killing and phagocytosis

Suspensions of bacteria (viable count 2×10^7 cfu/ml) and PMNL (4×10^6 PMNL/ml) were

made up in various dilutions (expressed as percentage v/v) of urine culture filtrate in sterile urine, in a final volume of $400\text{ }\mu\text{l}$. Serum (from the same donor as the PMNL) was added to give a 10% final concentration (without serum opsonins phagocytosis did not occur in the urine). The bacteria:PMNL ratio was $c. 5:1$. The same procedure was also followed with increasing concentrations of commercial endotoxin in sterile urine instead of dilute culture filtrate. Controls without urine culture filtrate or endotoxin were also set up to assess maximal phagocytosis and killing in the sterile urine.

Immediately after adding the organisms to the PMNL, $40\text{ }\mu\text{l}$ of reaction mixture was removed and added to 4.96 ml of endotoxin-free distilled water (Travenol Ltd), adjusted to pH 11, and held for 5 min at 37°C . This completely lysed the PMNL without harming the micro-organisms.¹⁶ The sample was shaken vigorously, diluted in distilled water (pH 5-6) and $100\text{-}\mu\text{l}$ samples were plated out on MacConkey Agar (CM7b; Oxoid) for duplicate viable counts after incubation for 18 h at 37°C . The mixture was rotated for 1 h at 37°C , after which it was sampled as before. Bacterial killing was expressed as the percentage reduction of the initial viable count. All experiments were done in triplicate. Controls were set up with bacteria in urine with serum 10% and the highest concentration of urine culture filtrate (40% v/v) or *E. coli* commercial endotoxin ($200\text{ }\mu\text{g/ml}$), but without PMNL. This was to eliminate the possibility of bactericidal activity of serum, endotoxin, urine or water at pH 11. Bacterial counts in the controls after 1 h were always in excess of the starting population.

Phagocytosis was assessed by microscopical examination of a cytocentrifuged preparation of the mixture, after incubation for 1 h, prepared with a Cytospin 2 (Shandon Southern Products) as described by Horwitz and Silverstein.¹⁷ The slides were fixed and stained with Prodiff 1 and 2 (Braidwood Labs). There was little difficulty in distinguishing organisms which had been ingested from those which had not. Cytocentrifuged slides of PMNL-bacteria suspensions in which phagocytosis had not taken place showed no bacteria within the boundaries of the PMNL. These large clear areas of PMNL were in stark contrast to the distribution of bacteria over the rest of the surface of the slide. Thus bacteria were not centrifuged either into the PMNL or on to the cell surface, but were dispersed to the periphery of the cell during cytocentrifugation. This is an extremely useful artefact of preparations made with the Cytospin. When phagocytosis had occurred, the majority of bacteria were clearly within the boundary of the PMNL membrane, and there were often organisms within vacuoles. We have previously shown that this method compares well with fluorescent extracellular quenching,⁴ in which crystal violet is used to quench the fluorescence of extracellular and cell-associated organisms, but not ingested bacteria.

PMNL (100) were examined microscopically to

determine the percentage of PMNL that had phagocytosed bacteria (percentage phagocytosis) and the mean number of bacteria phagocytosed by each PMNL.

Phagocytosis and killing after exposure to urine culture filtrate

PMNL were incubated in urine in the presence of the culture filtrate 30% v/v at 37°C for 30 min with rotation. The PMNL were then centrifuged once and resuspended in fresh sterile urine together with serum and bacteria and determination of phagocytosis and killing were performed as described above.

Results

Phagocytosis in the presence of urine culture filtrate

PMNL were able to phagocytose both strains of *E. coli* in the presence of increasing concentrations of

Table. Phagocytosis of two strains of *E. coli* in urine in the presence of increasing concentrations of urine culture filtrate over 1 h at 37°C, pH 7.0

Culture filtrate (% v/v)	Percentage phagocytosis*		Mean number of bacteria ingested/PMNL	
	strain 398	strain 441	strain 398	strain 441
0†	98 (2.5)	93 (3)	8.3	4.1
10	96 (1)	86 (6)	5.3	3.1
20	80 (12)	80 (16)	4.6	3.7
30	72 (23)	80 (16)	5.2	3.0
50	75 (8)	53 (28)	2.1	1.4
100 (undiluted)	66 (6)	48 (26)	1.6	1.5

* Percentage of PMNL that had phagocytosed *E. coli*, mean (SD) of a minimum of three experiments.

† Sterile pooled urine.

respective urine culture filtrates. Undiluted culture filtrates of strains 398 and 441 contained endotoxin 2 and 2.5 µg/ml, respectively, after overnight growth in the urine. Both strains were still phagocytosed (66 and 48%) at this concentration, but to a lesser extent than in the controls (table). The average number of bacteria ingested by each PMNL decreased from means of 5.9 and 3.5 for strains 398 and 441, respectively, with ≤ 30% v/v of culture filtrate to 1.9 and 1.5 in the presence of 50% v/v and undiluted urine culture filtrate.

Killing by PMNL in the presence of urine culture filtrate

Increasing the concentrations of urine culture filtrates from both of the *E. coli* strains from 1% to 20% v/v in the reaction mixture caused a proportionate decline in PMNL killing ability. However, abrupt abolition of PMNL killing of both strains occurred at 30% v/v (i.e., 0.6 and 0.75 µg of endotoxin/ml; fig. 1).

Killing by PMNL after prior exposure to urine culture filtrate

PMNL that had been exposed to urine culture filtrates 30% v/v for 30 min, and had thus lost all their killing ability, recovered some of their bactericidal activity when transferred to fresh urine. Killing of strain 398 was 13 SD 12% and of 441 was 24 SD 7% (n = 3), compared to control figures of 52 SD 9% and 46 SD 5%, respectively. Phagocytosis by PMNL was fully restored in these experiments to > 90% (p > 0.3 for both strains, Student's *t* test; n = 3).

*Phagocytosis and killing in the presence of *E. coli* O55:B5 endotoxin*

Intracellular killing of strain 398 by PMNL was not affected by endotoxin 10 µg/ml, but higher levels

Fig. 1. Effect of PMNL killing in urine, after exposure for 1 h at 37°C, of increasing concentrations of urine culture filtrate from 1% to 40% v/v. Filtrates were from 18-h cultures (in urine) of *E. coli* strains 398 (—) and 441 (---). Bar is SEM of a minimum of three experiments. The mean viable count at time 0 was 1.6×10^7 cfu/ml for strain 398 and 1.7×10^7 cfu/ml for strain 441. When PMNL were omitted from experiments containing urine culture filtrate 40% v/v, the mean viable counts rose to 2×10^7 and 2.1×10^7 cfu/ml for the two strains, respectively.

Fig. 2. Effect of increasing concentrations of purified endotoxin from *E. coli* O55: B5 on PMNL killing of *E. coli* strain 398 after 1 h at 37°C in urine. Bar is SEM of a minimum of three experiments. The mean viable count at time 0 was 1.1×10^7 cfu/ml. When PMNL were omitted from experiments containing the highest concentration of endotoxin the mean viable count rose to 1.7×10^7 cfu/ml.

caused a steady decline in PMNL killing ability, until at 200 µg/ml there was no killing (fig. 2). Phagocytosis was unaffected at this concentration of endotoxin.

Discussion

In urinary infections there are usually large numbers of both gram-negative bacteria and PMNL present in the urine, hence there is ample opportunity for endotoxin to affect PMNL function. We investigated the effect of the products of bacterial growth, including endotoxin, on phagocytosis and intracellular killing by PMNL in pooled sterile urine by adding increasing amounts of a urine culture filtrate produced by a maximal population of the same *E. coli* strain under test. This simulated the effects of an increasing bacterial metabolite concentration in urine as would occur during urinary infection. Intracellular killing was abolished by the presence of urine culture filtrate 30% v/v; phagocytosis proved far more resistant to the deleterious effects of a filtrate of infected urine. An average of 57% of the PMNL ingested bacteria of either strain, even in the presence of undiluted culture filtrate.

PMNL exposed to urine culture filtrate recovered all of their phagocytic and some of their killing ability when transferred to fresh urine. This suggests that in a patient with urinary tract infection, PMNL phagocytic activity can be regained if the urine is diluted by increased water intake. Providing the organisms are efficiently phagocytosed and the PMNL are shed or migrate into the urine, clearance will occur at the time of voiding. Although intracellular killing would hasten the eradication of bacteria, in this situation it might not be essential.

PMNL killing was not significantly affected by

endotoxin 10 µg/ml added at the same time as bacteria to PMNL, but was steadily inhibited by higher concentrations—200 µg/ml completely prevented killing after 1 h. Phagocytosis was unaffected even at these high levels of endotoxin. Proctor,¹⁰ in experiments with HBSS, not urine, similarly found that endotoxin concentrations in excess of 10 µg/ml inhibited PMNL killing, but not phagocytosis. The work of Creamer *et al.*¹⁸ further indicated that PMNL may be suited to function in the presence of large amounts of endotoxin (up to 1 mg/ml).

The endotoxin concentrations in the two urine culture filtrates were 2 and 2.5 µg/ml. This corresponds with the study of Jorgensen and Jones¹² who, with the LAL assay, set a minimum endotoxin level of 0.5 µg/ml to indicate a significant bacteriuria ($\geq 10^5$ cfu of *E. coli*/ml). The concentrations of endotoxin in the undiluted urine culture filtrates (2 and 2.5 µg/ml), were well below those that inhibited PMNL killing. Although purified endotoxin from a different strain of *E. coli* to that of the cultures was used, it appears most likely that bacterial growth metabolites, and not endotoxin alone, were responsible for the observed inhibition of phagocytosis and killing by the urine culture filtrate. It is interesting that PMNL killing was abolished by the same concentration of urine culture filtrate (30% v/v) from both *E. coli* strains.

The nature of the bacterial products responsible for the inhibition of PMNL activity was not investigated in this study. However, it is known that several different bacterial species can produce substances that inhibit PMNL function. These include a lethal cytotoxin for PMNL from *Pseudomonas aeruginosa*,¹⁹ succinic acid from *Bacteroides fragilis* that inhibits neutrophil migration,²⁰ and catalase from *Listeria*

monocytogenes that inactivates the oxidative anti-bacterial agents of PMNL.²¹

It might be considered that bacterial consumption of factors required for killing had occurred and were restored by dilution with pooled urine. However, this is unlikely as the experiments were controlled in HBSS. This showed that killing was not dependent on other unknown factors that might have been utilised by the bacteria.

This study showed that the growth metabolites from

a large population of *E. coli* in urine—like pH and osmolality—are factors that can affect PMNL phagocytosis and killing. Phagocytosis takes place, but at a reduced rate in the neat culture filtrate from 10⁸ cfu of *E. coli*/ml, and adequate killing occurs in a 10% v/v dilution of this filtrate. Hence, in infected urine of suitable pH and osmolality, it is possible that when lower bacterial counts occur or when the patient dilutes the urine by water intake, near normal PMNL function could take place.

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