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THE EFFECT OF ANTIBIOTICS ON LPS RELEASE FROM *SALMONELLA TYPHI* AND ITS RELATION TO TNF-α PRODUCTION BY HUMAN WHOLE BLOOD, "EX-VIVO"

GHOLAMREZA SHAJARI, Ph.D., BAHMAN TABARAEI,* Ph.D., AND ABDOLFATTAH SARRAFNEJAD,** M.D.

From the Department of Microbiology & Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, the *Department of Bacterial Vaccine and Antigen Production, Pasteur Institute of Iran, Tehran, and the **Department of Microbiology, Faculty of Hygiene, Tehran University of Medical Sciences, Tehran, Iran.

ABSTRACT

It seems that rapid destruction of gram negative bacteria by antibiotics contributes to the clinical deterioration of some patients with gram negative infections. Antibiotics increase the concentration of lipopolysaccharide (LPS) in blood and cerebrospinal fluids. Released LPS can activate blood cells to produce tumor necrosis factor-alpha (TNF-α) and other cytokines. TNF-α appears to be a major mediator in development of fever, hypotension, multi-organ failure and death.

In this research, standard *Salmonella typhi* Ty_2 -5536, a pathogenic *Salmonella* and standard *Escherichia coli* K12,QD5003 for comparing, were incubated in the presence of chloramphenicol, ampicillin and co-trimoxazole at concentrations that killed >99.9% of organisms as determined by quantitative culture techniques.

The results obtained showed that chloramphenicol produced lower LPS levels and lower TNF-α levels from whole blood cells when compared with those of ampicillin and co-trimoxazole. Therefore chloramphenicol is the preferred antibiotic against *S. typhi* because it decreases the induced-pathological effect of TNF-α in gram negative infections.

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INTRODUCTION

There has been concern almost from the beginning of the antibiotic era that rapid lysis of gram negative bacteria by antibiotics could be detrimental to the host and recent studies have provided futher support for this

Correspondence: Gholamreza Shajari , Ph.D., Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran. P.O. Box: 87155-111. Tel: 0361-550021-25(501). E-mail: Shajari@dr.com

concern.[1,2]

Rapid lysis of gram negative bacteria by antibiotics is associated with considerable release of LPS[3,4] in the blood and cerebrospinal fluid.[4,5] LPS is one of the several bacterial products that can activate whole blood cells in a host to release proinflammatory peptides such as Tumor Necrosis Factor-alpha (TNF-α).[5,6]

The importance of TNF-α as a mediator of LPS effect has been established in animal models[7,15,16] and humans.[16]

In an experimental model of lethal endotoxemia, TNF-α appears to be a major mediator of fever, hypotension, multi-organ failure and death.[8] Strategies that diminish TNF-α release may be effective in decreasing the high mortality associated with severe gram negative infections.[9,10,11]

MATERIAL AND METHODS

Cell line

The murine fibroblast cell line (L929) was obtained from American Type Culture Collection (ATCC, Rockville, MD). This tumorogenic cell line of connective tissue lineage were maintained in continuous culture using RPMI 1640 (Sigma Chemical) containing 75% (v/v) heat inactivated fetal calf serum (FCS, Gibco), Penicillin (100IU/mL) and Streptomycin (100μg/mL) in an atmosphere of 5% CO_2 at 37°C. The doubling time for these cells under these conditions was about 48 hrs.

Bacteria

Pathogenic *Salmonella typhi*, Standard *Salmonella typhi* Ty$_2$-5536 (CSBPI, B-190), and Standard *Escherichia coli K12*-QD5003 (CSBPI-D289) (Pasteur Institute of Iran) were stored until use in skimmed milk at -70°C. The stock cultures were brought to room temperature and inoculated on to trypticase soy agar containing 5% sheep blood and subcultured overnight at 37°C. Bacteria were suspended in sterile saline to a concentration of 104 cfu/mL. 0.01mL aliquots of the bacterial suspensions were transferred to 200 mL of prewarmed (35°C) RPMI 1640 (Sigma Co.) and incubated until log phase growth occurred, as determined by quantitative cultures. About 10⁶cfu/mL of organisms were added to fresh RPMI 1640 in the presence of various antibiotics as described before.

Antibiotics

Stock solutions of ampicillin (Jaber ebne Hyyan, Iran-Tehran), chloramphenicol (Al-havi Co. Iran-Tehran), and co-trimoxazole (Tehran Daru Co. Iran-Tehran) were prepared according to methods supplied by the manufacturer. These solutions were filtered (0.22 μm filter) and stored at -70°C until use. Appropriate dilutions of the antibiotics were made using RPMI 1640. The MIC and MBC of antibiotics were determined by using standard serial dilution method.[12] After thawing, aliquots from the various antibiotic stock solutions were added to sterile test tubes containing 10 mL of RPMI 1640 to give a final Minimal Bactericidal Concentration of each antibiotic.

Bacterial killing

1 mL of log-phase organisms (~10⁶ cfu/mL) were added to prewarmed sterile tubes containing 9 mL of RPMI 1640 with or without antibiotics and incubated at 35°C for 4 hrs. For all antibiotics, these conditions produced >99.9% killing of bacteria as determined by quantitative culture techniques. After incubation, the bacterial suspensions were filtered (0.22μm filter) and the filtrates were collected for further studies.

Pyrogenic test

Aliquots of each filtrate were dialyzed against free-endotoxin distilled water containing merthiolate (1:10,000) and then condensed to 1: 20 volume of primary concentration in a dessicator containing phosphorous pentaoxide (P_2O_5). Pyrogenic test in rabbits were done according to UK Pharmacoepia 1998.[13]

Quantitative assay for LPS

Released LPS was measured using a new commercially available chromogenic L.A.L. microtiter plate method (Chromogenics, Sweden).[14] Aliquots (50μL) of bacterial filtrates were added in triplicate in a 96-well microplate. After mixing vigorously, the plates were incubated at 37°C for exactly 5 minutes. Standard endotoxin *E. coli* O111:B4 at concentration up to 150 pg/mL were used as positive control and sterile, free - endotoxin distilled water was added to some wells as negative control. After incubation, 50μL Limulus Amebocyte Lysate (L.A.L.) was added to each well and after mixing the plates were incubated at 37°C for exactly 5 minutes, followed by addition of 100μL substrate-buffer solution (37°C) to the well, then incubated at 37°C for 5 minutes. By adding 100μL acetic acid 20% to each wells, the photometric reaction was stopped and absorbance of the samples was read in a photometer at 405 nm (Table II).

TNF-α Production by human whole blood "ex-vivo"

We used a slightly modified version of a recently described method for stimulating TNF-α production by heparinized human whole blood cells.[6,16] Briefly, 15 mL of blood from one of the investigators (the same donor for all experiments) was drawn in to a heparinized syringe (10 U/mL). 225μL of heparinized whole blood was placed into each well of a 24-well tissue culture plate (Costar, Cambridge, MA) followed by addition of 750μL RPMI 1640. Finally 25μL of test samples or standard endotoxin were

added to each well and mixed by gentle agitation of plates, making the final volume of each well 1 mL. Tissue culture plates were then incubated at 37°C in 5% CO_2. Each experiment was run in triplicate. After incubation for 6 hours, the suspensions were transferred to 1.5 mL conical polypropylene tubes and centrifuged at 800 g for 15 minutes and the supernatants were saved (-70°C) for TNF-α assay. Control experiments to evaluate the direct or indirect effect of each antibiotic to stimulate whole blood cells to release TNF-α were also done.

TNF-α bioassay using L929 cells

Initially, an assay previously described by Wang et al.[16] was used. Briefly, 5×10^4 TNF-α sensitive L929 cells were added to wells of a 96-well flat-bottom microtiter plate in 50μL of medium consisting of RPMI 1640. containing 10% (V/V) FCS plus 2μg/ mL Actinomycin-D (Boehringer Mannheim, Laral, Quebec). The microtiter plates were incubated at 37°C in 5% CO_2 in air for 2 hours. 50 mL of test samples that have been determined to have released LPS in them, or recombinant human TNF-α, were added and the plates were incubated for an additional 16 hours prior to addition of 50 μL of a filtered neutral red solution (Sigma, 0.05% (w/v) in

normal saline, stored in the dark at 4°C but warmed to room temperature prior to use) were added to each well of the microtiter plates. Plates were incubated for 2 hours at 37°C in an atmosphere of 5% CO_2 in air, emptied by inversion and rinsed once with 0.2 mL of room temperature Phosphate - Buffered Saline (PBS) using a multichannel pipettor. In order to avoid variable cell loss, the rinsing was carried out quickly but carefully and the emptying by inversion of the plate as smoothly and uniformly as possible. 100μL of a sodium phosphate-alcohol solution (0.05 M NaH_2PO_4 in 5% (V/V) ethanol) was added to each well and the plates gently mixed on a shaker for at least 20 minutes. Absorbance (optical density) at 570 nm was determined using an automated microplate spectrophotometer with a reference wavelength of 360 nm as previously described (Branch & Guilbert, 1986) (Table III).

Statistical analysis

Data are expressed as mean±SD. All analyses were done with statistical software. LPS and the TNF-α concentrations produced by each antibiotic were compared using one-way analysis of variance. Post hoc multiple comparisons were tested using the Bone-ferroni test at $p = 0.01$ (significant).

Table I. MIC and MBC determinations of antibiotics on bacteria.

Organisms / Antibiotics	S. typhi Ty2-5536 (CSBPI,B-190)		Pathogenic S. typhi		E. coli K12,QD5003 (CSBPI,D-289)	
	MIC	MBC	MIC	MBC	MIC	MBC
Ampicillin	1.25 µg/mL	1.25 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL
Chloramphenicol	2.5 µg/mL	160 µg/mL	5 µg/mL	160 µg/mL	2.5 µg/mL	160 µg/mL
Co-trimoxazole	1.25 µg/mL	5 µg/mL	2 µg/mL	10 µg/mL	1.25 µg/mL	10 µg/mL

Table II. LPS levels released by S. typhi Ty$_2$-5536 exposed to antibiotics (using chromogenic L.A.L. microtiter plates).

Sample	RPMI$_{1640}$solution, PBS, D.W, Each antibiotic (alone) and other solutions	Filtrate of viable standard S. typhi	Filtrate of standard S. typhi killed by:		
			Chloramphenicol	Ampicillin	Co-trimoxazole
Endotoxin Level (Pg/mL) (Mean ± SD)	0	18.26 ± 1.13	86. 68 ± 2.53	113.33 ± 8.0	134.18 ± 11.59

RESULTS

The MIC and MBC of ampicillin, chloramphenicol and co-trimoxazole were 1.25, 1.25µg/mL (MIC= MBC), 2.5, 160µg/mL and 1.25, 5µg/mL respectively (Table I).

Administration of 1 mg/kg of dialyzed filtrates of antibiotic killed *S. typhi* in rabbits, raised normal temperature of the animals by 1.2°C compared with control groups that showed the presence of free -LPS in the filtrates.

Table II shows the amount of released LPS of *S. typhi* Ty_2-5536 exposed to antibiotics. Co-trimoxazole released greater quantities of LPS than did chloramphenicol and ampicillin. Ampicillin induced an intermediate amount of LPS. Low levels of LPS were detected in the filtrates of *S. typhi* not exposed to antibiotics. Each antibiotic alone, RPMI 1640. solution, PBS and distilled water were assayed for LPS, and had no detectable levels of LPS (Table II).

Using quantitative culture techniques at the end of the incubation period (4-6 hours) did not show any growth of bacteria on the media. Also gram staining after incubation period didn't show any viable bacteria on microscopic examination that indicated the complete lysis of bacteria by each antibiotic at the end of the incubation period.

Table III summarizes the TNF-α production by filtrates of antibiotic - killed *S.typhi* from human whole blood cells. Unstimulated blood released only small amounts of TNF-α (75 pg/mL). Whole blood cells generated TNF-α in response to standard endotoxin *E. coli*. O111: B4 in a dose related fashion. Standard endotoxin at concentrations of 50, 100 and 150 pg/mL stimulated human whole blood cells to release 135±9.12; 250±18.60 and 360±26.63 pg/mL of TNF-α respectively (values are respective mean±SD). The rate of TNF-α production by human whole blood cells was greatest 4-6 hours after endotoxin was added.

As is shown in Table III, exposure of *S. typhi* to each antibiotic produced a significant increase in TNF-α levels compared with viable *S. typhi* ($p<0.05$). Low levels of TNF-α were produced in response to filtrates of viable *S. typhi* in the absence of antibiotics (115±7.89 pg/mL) compared with *S. typhi* killed by antibiotics ($p<0.05$). The addition of each antibiotic to human whole blood cells failed to directly stimulate TNF-α production. Thus, antibiotics alone had no effect on TNF-α production from human whole blood cells (Table III).

DISCUSSION

Although outcome from gram negative septicemia depends on host factors, the specific infecting organism and site of infection, antibiotic selection has received little attention.

Recent studies demonstrate that rapid lysis of bacteria by antibiotics during treatment of gram negative sepsis or meningitis is associated with brisk release of susbstantial amounts of LPS in to blood or cerebrospinal fluid in animals[15,16] and humans.[6] In a mouse model of inoculated *E. coli*, antibiotics stimulated LPS release that followed a marked increase in whole blood TNF-α levels.[15]

We used a recently reported and physiologically relevant *"ex-vivo"* whole blood system to measure TNF-α production.

In contrast to methods using isolated monocytes, this method avoids monocyte activation related to an adherence step and reduces the risk of LPS contamination during isolation.[5] More importantly, it examines periph-

Table III. Tumor necrosis factor- alpha (TNF-α) generated by human whole blood cells stimulated by *S. typhi* Ty_2-5536 exposed to antibiotics (using bioassay method).

Groups	n	TNF-α (pg/mL)
Controls:	(4)	
Unstimulated blood (negative control)	(4)	75 ± 5
Standard Endo. (150 pg/ml) (positive control)	(4)	360 ± 26.63
Viable *S. typhi*		115 ± 7.89
S. typhi exposed to:		
- Ampicillin (1.25 µg/mL)	(8)	
- Chloramphenicol (160 µg/mL)	(8)	283 ± 20.77
- Co-trimoxazole (5 µg/mL)	(8)	232 ± 16.84
		340 ± 25.00

Values are mean±SD of 'n' experiments. Compared with viable *S. typhi*, unstimulated whole blood and standard endotoxin ($p<0.05$).

eral blood cell TNF-α release in response to different stimuli in their relevant physiologic environment.[14] Although the major cells producing TNF-α in human peripheral blood are thought to be monocytes, circulating mature granulocytes, T and B lymphocytes and NK cells are also capable of synthesizing and secreting TNF-α.[5,8,9]

Several variables could potentially affect TNF-α production in this "ex-vivo" whole blood system. Monocytopenia may decrease and monocytosis may increase TNF-α production from whole blood. However we did not attempt to count or adjust for monocyte numbers.[14,16] We used human whole blood from the same normal adult for all experiments.[6,16]

In our studies bacterial filtrates of Salmonella typhi Ty_2-5536 exposed to antibiotics provoked human whole blood cells to TNF-α production. Co-trimoxazole and ampicillin-induced killing of *S. typhi* were associated with susbstantial release of LPS and thus TNF-α production from human whole blood, whereas chloramphenicol was associated with minimal LPS release from *S. typhi* and thus minimal TNF-α production from human whole blood cells "ex-vivo" (Table III).

Unstimulated blood released only small amounts of TNF-α (75pg/mL). TNF-α release indiuced by viable *S. typhi* alone, despite the lack of direct contact between bacteria and antibiotics, was probably due to cell wall fragments, including endotoxin shed from multiplying bacteria into the medium.

The differences in human whole blood cells TNF-α production by the action of antibiotics may be related to differences in the pharmacodynamic properties of these antibiotics. On the other hand, bactericidal antibiotics are responsible for the release of large amounts of LPS, whereas bacteriostatic antibiotics do not appear to liberate significant quantities of LPS. Other investigators[8] confirmed these *in-vitro* studies and documented *in-vivo* that the release of free LPS is dependent on the class of antibiotics used, not necessarily on the rate of bacterial killing. Despite similar rates of killing, antibacterials that lyse the cell wall released considerably more free LPS than did those that inhibit protein synthesis.[8]

The importance of TNF-α as a mediator of LPS effect has been established in animal models[7,15] and humans.[6,7,16] Overproduction of TNF-α in response to LPS is believed to be one of the major contributing factors to mortality and morbidity in gram negative sepsis. Strategies designed to neutralize circulating LPS or to block the production or biologic effects of LPS-induced cytokines, appear to improve survival in gram - negative sepsis and shock and are under intense investigation.

Our data, together with other recent experimental findings,[6,7,15,16] suggest that initial antibiotic choice may influence the degree of LPS release and resultant in-duction of TNF-α production.

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