**ORIGINAL ARTICLES**

# The Immunomodulatory Effect of Antibiotics on the Secretion of Tumour Necrosis Factor Alpha by Peripheral Blood Mononuclear Cells in Response to *Stenotrophomonas maltophilia* Stimulation

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## *ABSTRACT*

*Some antibiotics have been shown to modify the host immune response. Infection with* Stenotrophomonas maltophilia*, is often difficult to treat due to multiresistance to antibiotics. The authors examined the effect of four commonly used antimicrobial agents (ciprofloxacin, ceftazidime, cotrimoxazole and piperacillin-tazobactam) on tumour necrosis factor alpha (TNFα) production by human peripheral blood mononuclear cells (PBMC) stimulated with heat-killed* S maltophilia. *Cotrimoxazole was the only antibiotic that suppressed TNFα secretion at clinically achievable concentrations. This may explain its use with good effect in the treatment of* S maltophilia *infections. However at supratherapeutic concentrations, ceftazidime and ciprofloxacin, but not piperacillin-tazobactam, also inhibited significantly the production of TNFα. Cotrimoxazole, in addition to its antimicrobial effect against* S maltophilia, *has an immunomodulatory effect on peripheral blood mononuclear cells stimulated by* S maltophilia.


# Efecto Inmunomodulatorio de los Antibióticos Sobre la Secreción del Factor de Necrosis Tumoral Alfa por Células Sanguíneas Mononucleares Periféricas en Respuesta a la Estimulación de *Stenotrophomonas Maltophilia*

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## *RESUMEN*

*Algunos antibióticos han mostrado ser capaces de modificar la respuesta inmune del huésped. Las infecciones con* Stenotrophomonas maltophilia *– un patógeno emergente – son difíciles de tratar debido a su multiresistencia a los antibióticos. Examinamos el efecto de cuatro agentes antimicrobianos comúnmente usados (ciprofloxacina, ceftazidima, cotrimoxazol, y piperacilina-tazobactam) sobre la producción del factor de necrosis tumoral alfa (FNTα) por las células sanguíneas mononucleares periféricas humanas (PBMC) estimuladas con* S maltophilia *inactivadas mediante calor. El cotrimoxazol – en concentraciones clínicamente posibles – fue el único antibiótico que eliminó la secreción FNTα. Esto puede explicar su uso efectivo en el tratamiento de las infecciones por* S maltophilia. *Sin embargo, en concentraciones supraterapéuticas, la ceftazidima y la cipro-floxacina – pero no la piperacilina-tazobactam – también inhibieron significativamente la producción de FNTα. El cotrimoxazol, además de su efecto antimicrobiano contra* S maltophilia, *tiene un efecto inmuno-modulatorio sobre las células sanguíneas mononucleares periféricas estimuladas por* S maltophilia.


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## INTRODUCTION

Antibiotics that are given to patients in order to treat infection may have dual actions. These are an antimicrobial action on the pathogenic bacteria and interference with the host's immune cells such as lymphocytes and phagocytes. The latter effect, called immunomodulation, is believed to occur via alterations in chemotaxis, phagocytosis, oxidative burst, complement activation and cytokine secretion (1, 2).

Sepsis may represent an exaggerated systemic inflammatory response involving cytokines with harmful effects on the host system. Suppression of cytokine production by antibiotics may therefore be of great clinical benefit especially to patients who are immunocompromised or who have severe infection (3).

*Stenotrophomonas maltophilia* is a nonfermentative Gram-negative bacterium which is considered an emerging pathogen (4). Over the last decade, there has been an increased frequency of isolation of this organism from patients with nosocomial infections including pneumonia, bacteraemia and wound infection (5, 6). Important risk factors for infection include immunosuppression, prolonged hospitalization and prolonged broad spectrum antibiotic therapy (5–7). Infections with *S maltophilia* are usually difficult to treat due to its resistance to multiple antibiotics. Mortality may therefore be high. Cotrimoxazole is the drug of choice but therapy should be guided by routine antimicrobial susceptibility testing (8–9). The effect of cotrimoxazole and other commonly used antibiotics on the secretion of TNFα from PBMC stimulated with *S maltophilia in vitro* was investigated in this study.

## MATERIALS AND METHODS

### Preparation of heat-killed *S maltophilia*

*S maltophilia* strains (SM 7805, SM 6887) were kindly provided by Tyrone Pitt (Health Protection Agency, Colindale, London, UK). Strains were stored in 30% glycerol at -80°C. To prepare cultures for experiment, isolated single colonies incubated overnight on blood agar were inoculated into 10 mls of Luria-Bertani broth (Oxoid, UK) and grown at 37°C with shaking until the log phase was reached (3–4 hours). Aliquots were taken based on the spectrophotometer reading of an optical density at 600 nm wavelength established by a standard curve. Samples were washed thrice with RPMI (Rosswell Park Memorial Institute) 1640 culture medium (Sigma, Poole, UK) and concentration confirmed by viable counts of colony forming units on bacterial plates. Heat-killed preparations were made by incubating at 65°C for 30 minutes in a water bath and confirmed by negative viable counts.

### Peripheral blood mononuclear cell separation

Samples of human venous blood (10 ml) were obtained from consented healthy volunteers in heparinised tubes and diluted with an equal volume of RPMI 1640 medium supplemented with 10 mM HEPES and 2 mM L-glutamine (herein referred to as RPMI). Peripheral blood mononuclear cells were recovered from the leukocyte-rich buffy coat after standard density gradient centrifugation with histopaque 1077 (Sigma, UK) for 20 mins at x 600 g. Peripheral blood mononuclear cells were then washed thrice with RPMI supplemented with 2% Fetal Calf Serum (FCS) (Harlan, Loughborough, UK). Cells were resuspended in RPMI with 10% FCS for enumeration using a haemocytometer and trypan blue dye. This final cell suspension was adjusted to 1 x $10^6$ cells/ml and used for culture.

### *In vitro* stimulation

Aliquots of cell suspensions (1 ml) were dispensed into sterile polypropylene tubes (Elkay, UK). The cell suspensions were pre-incubated with heat-killed *S maltophilia* (1x$10^6$ CFU/ml) for one hour followed by addition of the antibiotics at therapeutic and supratherapeutic concentrations (10). The antibiotics used included cotrimoxazole (as Bactrim, La Roche, Switzerland); ceftazidime (as Fortum, Glaxo, England); ciprofloxacin (as Ciproxina, Bayer, El Salvador) and piperacillin-tazobactam (as Zosyn, Lederle, Puerto Rico). Cell suspensions with *S maltophilia* but no antibiotics were used as controls. Unstimulated cell suspensions were without *S maltophilia* and antibiotics. All cell suspensions were further incubated at 37°C in a 5% $CO_2$ incubator for five hours then stored at 4°C overnight.

### Intracellular and cell surface staining

Twenty microlitre of 100mM EDTA (Sigma, UK) was added to each tube which was then vortexed and incubated at room temperature for 15 minutes. Cells were next fixed with 4% formaldehyde in phosphate buffered saline PBS for five minutes before being washed with PBA (PBS/0.1% bovine serum albumin/ 0.1% sodium azide) at x 200 g for five minutes. Cell surface staining was performed using the following anti-human monoclonal antibodies: anti-CD 14 phycoerythrin/ (PE)/texas red (ECD). For intracellular cytokine staining, the monoclonal antibodies used were: anti-TNFα PE and mouse $IgG_2$ PE as an isotope control. This was followed by two further washings, first with saponin buffer (PBA/0.1% saponin) then saponin buffer with 10% FCS. Tubes were blot dried and aliquots of the appropriate fluorochrome-labelled antibodies were added as a cocktail and incubated on ice for two hours with half hourly shaking. Finally cells were washed three times with saponin buffer and then resuspended in 1 ml of 0.5% formaldehyde/PBS.

### Flow cytometric analysis

An Epics Altra flow cytometer (Beckman coulter) was used to acquire 100 000 cells from each tube. WinMDI software (Joe Trotter, California, USA) was used for detailed analyses. Dot plots of forward scatter versus side scatter were displayed from which gates were created around viable

mononuclear cells. The region containing monocytes was defined on dot plots of CD14 versus side scatter. This region was used as a second gate for a third dot plot of TNFα versus forward scatter linear to which quadrants were applied.

**Statistical analysis**

The computer software package SPSS version 11 (SPSS Inc., Chicago, USA) was used to perform statistical analyses while charts were done using Microsoft Excel (Microsoft, Washington, USA). Data were first assessed for normality and then the appropriate parametric or nonparametric statistical analysis was used. A $p$ value of less than or equal to 0.05 was used as being statistically significant.

## RESULTS

Peripheral blood mononuclear cells stimulated by *S maltophilia* induced significant production of TNFα (83.6% ± 1.13) compared to unstimulated control cell cultures (12.6% ± 0.57 ) ($p$ = 0.000 ).

Of the four antibiotics tested, the inhibition of TNFα production was only significant for cotrimoxazole at clinically achievable serum concentrations ($p$ = 0.0277) (Fig). The

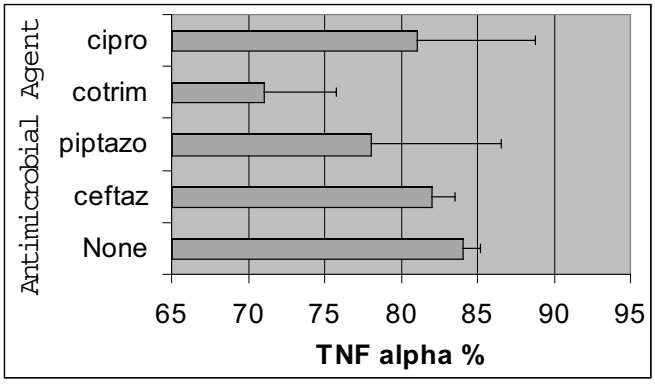


Figure: Effect of therapeutic concentrations of antibiotics on the production of TNFα

Results are given as mean ± SEM for at least four experiments

Ceftaz = ceftazidime (5 µg/ml); piptazo = piperacillin-tazobactam (30 µg/ml piperacillin); Cotrim = cotrimoxazole (48 µg/ml sulfamethoxazole) and Cipro = ciprofloxacin (5 µg/ml)

other antibiotics showed a decreasing trend in TNFα production by PBMC.

At supratherapeutic concentrations, the other antibiotics except piperacillin-tazobactam, as shown in Table, demonstrated a significant concentration-dependent suppression of TNFα production.

## DISCUSSION

The finding that cotrimoxazole significantly suppressed the synthesis/production of TNFα at therapeutic concentrations endorses its current use as the drug of choice in the treatment of *S maltophilia* infections. There are no published data of the effect of cotrimoxazole on cytokine production.

Table: Effect of supratherapeutic concentrations of antibiotics on TNFα secretion by PBMC stimulated with heat-killed *S maltophilia*

| Antimicrobial Agent | TNFα (%) | Inhibition (% of control) | $p$ value |
|---|---|---|---|
| None | 84 ± 1.2 | | |
| Ceftazidime (µg/ml) | | | |
| 20 | 83 ± 3.1 | 1.2 | NS |
| 100 | 77 ± 5.7 | 8.3 | NS |
| 200 | 72 ± 6.6 | 14.3 | 0.0405241 |
| Ciprofloxacin (µg/ml) | | | |
| 25 | 75 ± 7.8 | 10.4 | NS |
| 50 | 67 ± 8.8 | 20.2 | 0.0051904 |
| 80 | 50 ± 7.8 | 40.5 | 0.0000007 |
| Piperacillin-tazobactam (µg/ml)* | | | |
| 67.5 | 76 ± 8.6 | 9.5 | NS |
| 135 | 74 ± 8.6 | 11.9 | NS |
| 270 | 74 ± 6.6 | 11.9 | NS |

Results are given as mean ± SEM for at least four experiments
NS = not significant. * expressed as piperacillin component

Agents that accumulate intracellularly within lymphoid cells or those that interfere with protein or DNA synthesis are believed to have the prerequisites to modify the host cell responses (2). As such, macrolides (10–200 fold accumulation) and quinolones (3–20 fold accumulation) have been widely studied using various stimulatory agents (2, 11). In this study, ciprofloxacin showed no inhibitory effect on TNFα production at therapeutic levels and this is in agreement with a previous report by Schultz *et al* who used heat-killed *Pseudomonas aeruginosa* as the stimulus (12). Ciprofloxacin at supratherapeutic concentrations as demonstrated in this study suppressed TNFα synthesis and is also known to potentiate interleukin-1 and interleukin-2 release (13–14).

As reported in a previous study, ceftazidime was not observed to have an inhibitory effect on TNFα synthesis (15). Therefore it was of interest that ceftadazime inhibited TNFα synthesis by *S maltophilia* stimulated PBMC. In the previous study, a Gram-positive organism was used as the stimulus while in this study a Gram-negative organism was used which may explain the different findings. Piperacillin-tazobactam did not demonstrate any significant inhibitory effect on TNFα synthesis in this study even when supratherapeutic concentrations of this drug were used. Piperacillin, however, was shown in another study to enhance the production of interleukin-1 (13).

Studies are continuing to identify the molecular target of cotrimoxazole in the signal transduction pathway of TNFα synthesis in peripheral blood mononuclear cells. In conclusion, the results of this study demonstrated that cotrimoxazole, in addition to its antimicrobial effect against *S maltophilia*, has an immunomodulatory effect on peripheral blood mononuclear cells stimulated by *S maltophilia.*

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