In vitro modulatory effects of ciprofloxacin and chloramphenicol on the rabbit phagocytic cells

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Abstract

Ciprofloxacin and chloramphenicol belong to the antimicrobials used in veterinary medicine for, among others, rabbit otitis, respiratory or skin diseases treatment.

In the study ciprofloxacin (Polfa, Grodzisk) and water soluble chloramphenicol (Sigma) at clinically relevant concentrations were tested on their impact on inducible nitric oxide synthase activity, tumor necrosis factor-α production and the intracellular level of cAMP in the phagocytic cells isolated from rabbit blood. Both antibiotics exhibited some modulatory effects on the studied parameters.

Key words: *ciprofloxacin, chloramphenicol, rabbit phagocytic cells, tumor necrosis factor-α, cyclic AMP, inducible nitric oxygen synthase.*

(Centr Eur J Immunol 2009; 34 (2): 77-80)

Introduction

Ciprofloxacin (CIP) and chloramphenicol (CAP) are used in treatment of such diseases as ocular disorders, otitis, respiratory and skin diseases in rabbits.

Phagocytic cells are one of the main components of the first line defence against patogens. Both CIP and CAP accumulate inside the phagocytic cells, reaching manifold higher intra- than extracellular concentrations [1-4], which makes them able to destroy efficiently pathogens having a potency to live inside the cell after phagocytosis. Appropriate elimination of the invading microorganisms requires not only effective antimicrobial drugs, but also properly functioning defence system of the treated animal. There are some data in the literature, indicating that these two antibiotics are able to modulate immune cells functions [5, 6], so it is very important to determine the possible effects of CIP and CAP on phagocytic cells activity at clinically achievable concentrations.

In the previous studies [7] we have found that the drugs were able to modulate such rabbit cell functions as zymosan phagocytosis and respiratory burst activity. The goal of these studies was to determine the *in vitro* influence of CIP and CAP on inducible nitric oxide synthase activity, tumor necrosis factor-α production and on the intracellular level of cAMP in the phagocytic cells isolated from rabbit blood.

Material and Methods

In the study the Principles of laboratory animal care and the national laws on the protection of animals were followed (Opinion and Approval of Local Committee of Ethics Nr. 490/2004).

Antimicrobial drugs

Pure ciprofloxacin (CIP, Polfa-Grodzisk) and water soluble chloramphenicol (CAP, Sigma) at the concentrations of 5 and 30 μg/ml in RPMI 1640 (BioMed, Poland) were used.

Cell isolation

Blood of three adult, 5 kg weighing rabbits (*Oryctolagus cuniculus*), was taken into heparinised containers from the marginal ear vein. Leucocytes were isolated by density gradient centrifugation on Gradisol G (1.115 g/ml, Aqua-Medica, Łódź). The cell viability tested by the dye exclusion method (0.1 % of trypan blue) was ≥ 97%. Then the phago-

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cytic cell number was adjusted to the appropriate concentration in RPMI 1640 supplemented with 0.1% of inactivated FCS (foetal calf serum, Gibco, England) and left in 96-well microtiter plates (Nunc) for adherence. Then the supernatant with non-adherent cells was removed and the cells were used for further determinations.

Determination of inducible nitric oxide synthase (iNOS) activity

The volume of 100 µl of CIP or CAP at the appropriate concentrations or RPMI 1640 for the control wells and lipopolysaccharide (LPS from Serratia marcescens, Sigma) at 10 µg/ml were added to the cell suspension containing neutrophils at 3×10^6 cell/ml. The plate was left for 24 h, 39°C, 5% CO_2, in dark. Then the plate was centrifuged and the supernatants were collected. To the supernatant volume of 75 µl 100 µl of 1% sulphanilamide (Riedel-de Haen) in 2.5% phosphoric acid and 100 µl of 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride (Sigma) in 2.5% phosphoric acid were added. After 5 min incubation at room temperature, measurement of optic density at 540 nm with reference filter 690 nm on a microplate reader BioRad 550 was performed.

Tumor necrosis factor-α (TNF-α) assay

The volume of 100 µl of CIP or CAP at the appropriate concentrations or RPMI 1640 for the control wells and LPS at 10 µg/ml were added to the cell suspension containing monocytes at 10^6 cell/ml. The plate was left for 24 h, 39°C, 5% CO_2, in dark. Then the plate was centrifuged and the supernatants were collected. The levels of TNF-α in the supernatants were determined with the use of TNF-α Kit (Genzyme). The producer protocol was followed directly.

cAMP assay

The level of intracellular cAMP after 24 h incubation the cells (2×10^6 cell/ml) with 100 µl of CIP or CAP at the appropriate concentrations was measured with the use of bioluminescent cAMP-Glo Assay (Promega). The assay is based on the stimulation of protein kinase A activity by cAMP resulting in ATP decrease and inhibited light production in a coupled luciferase reaction. The assay was performed in a white 96-well plate (Nunc) according to producer protocol.

Statistical analysis

Statistical analysis was performed by one-way ANOVA at significance level $p<0.05$. When significant differences were detected, Duncan's test was used to compare the experimental groups to the control.

Results

Determination of inducible nitric oxide synthase (iNOS) activity

Decreased iNOS activity was observed after cell incubation with CAP at the highest used concentration (30 µg/ml) (Fig. 1). The lower used concentration of antibiotic (5 µg/ml) had no impact on studied parameter. CIP did not change iNOS activity at any used drug concentrations.

Tumor necrosis factor-α (TNF-α) assay

At the tested concentrations of the drugs, pronounced, of similar magnitude, decrease of TNF-α levels in the LPS-stimulated cell supernatants was found (Fig. 2).

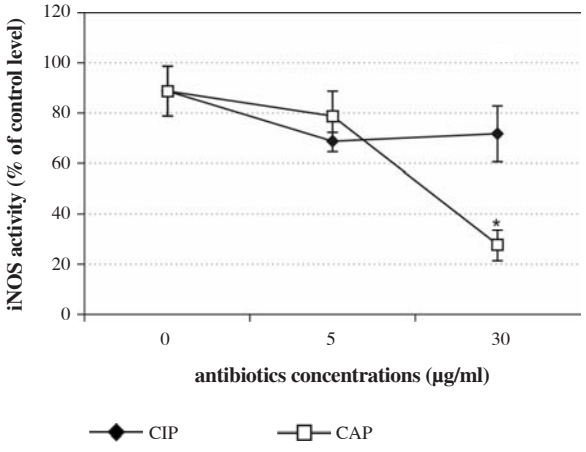

Fig. 1. The influence of ciprofloxacin and chloramphenicol on inducible nitric oxide synthase activity in rabbit blood phagocytic cells after LPS induction ($\bar{x}$ ± SD, n = 5, * $p<0.05$)

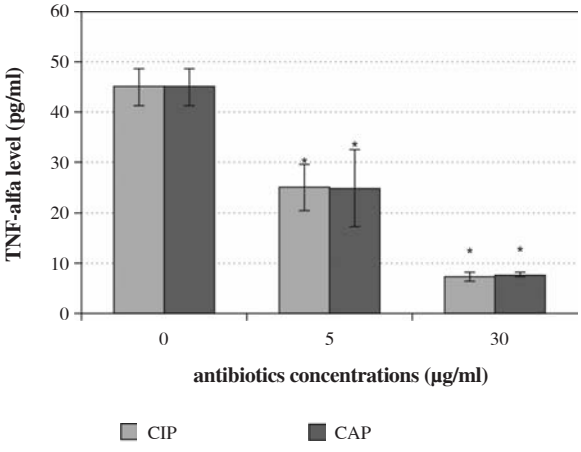

Fig. 2. The influence of ciprofloxacin and chloramphenicol on tumor necrosis factor-α level in supernatants of LPS-induced rabbit blood phagocytic cells ($\bar{x}$ ± SD, n = 5, * $p<0.05$)

cAMP assay

No statistically significant influence of the drugs was found at any used concentrations, on the intracellular cAMP levels in the phagocytic cells after 24 h incubation period, except the cells exposed on CAP at 5 μg/ml (Fig. 3) showing higher cAMP level than the control cells.

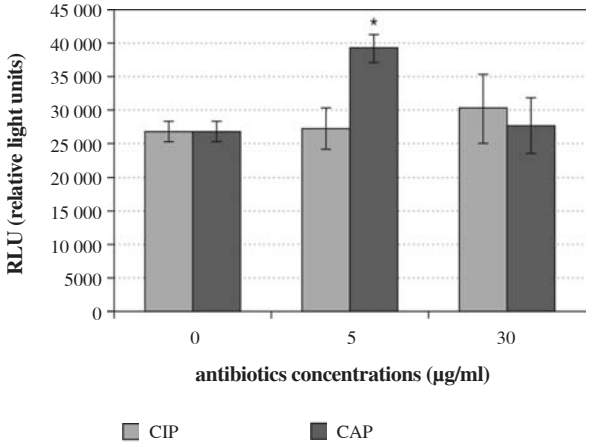

Fig. 3. The influence of ciprofloxacin and chloramphenicol on intracellular cAMP level in rabbit blood phagocytic cells ($\bar{x} \pm SD$, n = 5, * $p < 0.05$)

Discussion

The interactions with such phagocytic functions, as effective bacteria engulfing, respiratory burst activity, intracellular killing or cytokine secreting were seen in the presence of fluoroquinolones, but also chloraphenicol [5, 6, 8, 9]. Obtained results were in high degree depended on the type of the drug, used concentrations and way of testing (*in vivo*, *ex vivo*, *in vitro*). The immunomodulatory effects of antibiotics may result in disturbances of normal immune processes, however they also may have positive consequences, via the modulations of inflammatory mediators [10].

In the previous studies we have found that CIP and CAP at the concentrations of 5, 10, 30 μg/ml stimulated zymozan phagocytosis and reactive oxygen intermediates production of rabbit peripheral blood phagocytic cells with no influence on the cell viability [7]. The aim of that study was to assess the drugs impact on reactive nitrogen intermediates production tested as iNOS activity, on TNF-α release and on intracellular cAMP level with the use of the same experimental model.

It is known that endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) types of nitric oxide (NO) synthases are involved in the production of NO in mammals and take part in the intracellular killing of the pathogens. As both tested antibiotics reach high concentrations inside the phagocytic cells, it is essential to elucidate their possible impact on the process of intracellular killing.

Wong et al. (2000) found that in mice treated with CIP at 40 mg/kg b.w. given intraperitoneally, both phagocytic activity and levels of nitrite significantly increased [11]. On the other hand, Szczypka and Obmińska-Domoradzka (2002) found, that CIP given to mice at the therapeutic doses, caused decrease of phagocytic and killing activity of peritoneal macrophages, however NO production was stimulated [12]. Moreover, in the study by Kolios et al. (2006) on human colonic adenocarcinoma cells (HT-29) and colonic biopsies from patients with ulcerative colitis, CIP at 10-100 μg/ml was found to significantly inhibit NO production induced by the mixture of proinflammatory cytokines: IL-1α, TNF-α and IFN-γ, in a concentration-dependent manner. The observed lower NO level was the result of the inhibition of the cytokine-induced iNOS mRNA expression [10].

While in the previous study we saw the increase of phagocytic activity [7], now we did not observe any changes in the level of nitric oxide induced by LPS after cell incubation with the tested CIP concentrations. However the cells in the presence of CAP at 30 μg/ml produced significantly lower amounts of NO compared to the control. In the literature less data are available for CAP impact on iNOS activity. Similarly to our findings, it was reported [13], that incubation of RAW 264.7 murine macrophages with pneumococci and CAP at 10 μg/ml induced less detectable iNOS protein accumulation. It is difficult to speculate on the implications of our observation in the process of pathogen destruction, as in the former study stimulation of phagocytic ability and respiratory burst activity was seen [7].

TNF-α is a highly conserved protein with the specific cell receptors present on essentially all cells of the body [14]. That potent pleiotropic cytokine exerts critical functions in the activation and regulation of immune and inflammatory responses. It regulates among others such cell functions as proliferation, apoptosis, phagocytosis, it also increases oxidative metabolism and aggregation of neutrophils [15]. Some antibiotics, particularly fluoroquinolones, are known to modulate that cytokine production [6, 16]. Increase or decrease of TNF-α level is connected with changing cAMP amount, as amplification or attenuation cycles are centered around that cyclic nucleotide [14].

In the present study, we examined the effects of CIP and CAP on TNF-α synthesis by LPS-stimulated rabbit phagocytic cells, as well as on intracellular cAMP level in the cells. Both antibiotics reduced in similar degree TNF-α release in dose dependent manner. At the same time higher cAMP level was seen, however only at the lowest tested concentration of CAP.

Ciprofloxacin was shown to decrease TNF-α level in *in vivo*, *ex vivo* and *in vitro* studies [6]. Similar to our findings effects were observed in the *in vitro* studies by Nwariaku

et al. (1997), where CIP at 4 μg/ml strongly suppressed TNF release from the alveolar rabbit macrophages. The observed suppression was more pronounced for CIP that for β-lactam antibiotics [17]. In the other study filtrates of bacteria killed by CIP affected release of TNF from monocyte THP-1 cell line, and the authors suggested that the drug may modulate the inflammatory response in addition to its antibacterial activity [18]. Moreover in mice after *in vivo* administration at the therapeutic doses [6 and references therein], as well as in the *in vitro* studies on human cells at concentrations higher that 25 μg/ml [19] similar inhibitory effects were seen. On the other hand, it was reported [20] that application of CAP caused the increase of TNF-α level, however as the result of rapid bacteria destruction and the increase of the concentration of LPS in blood and cerebrospinal fluids. Moreover, CAP produced lower LPS levels and lower TNF-α levels from whole human blood cells when compared with other tested drugs, ampicillin and cotrimoxazole.

As the level of TNF-α is associated with the intracellular cAMP concentration [14], we also determined amounts of that nucleotide in the cells incubated with the antibiotics. Only CAP at the lowest used concentration caused increase of cAMP. That effect was not seen at the higher tested drug concentration, although TNF-α production was even more diminished. On the contrary, CIP at any tested concentrations did not change cAMP levels in the cells compared to the control. It was found [16] that CIP at higher concentration (100 μg/ml) was responsible for elevation of cAMP production in human monocytes. Surprisingly, in that study only in the presence of IL-18 CIP prevented the production of IL-12, INF-γ and TNF-α, while in the absence of the cytokine it had no effects.

In summary, CIP and CAP may have the modulatory effects on rabbit phagocytic cells, depending on the drug concentration. Further studies are needed on that field, as the obtained and some available in the literature data are in conflict.

Acknowledgments

Authors thank to K. Kaca for her technical assistance.

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