ANTIBIOTICS AND CELL-MEDIATED IMMUNITY *In Fish* - IN VITRO STUDY

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Abstract

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The effect of chloramphenicol and florfenicol on fish T and B cell proliferation and polymorphonuclear (PMN) and mononuclear (MN) cell phagocytic ability was determined. Cells were isolated from head kidney and spleen of 20 fish *(Cyprinus carpio)*. Antibiotics were administered in vitro at concentrations of 20 and 40 μ/ml. All determinations were performed in nine repetitions. T and B cell proliferation after application of the drugs was evaluated by a spectrophotometric method with lipopolysaccharide and concanavalin A as mitogens. Under the conditions used in this study the following results were achieved: chloramphenicol and florfenicol at a concentration of 40 μ/ml reduced proliferation of T and B cells, although effect of the first one was stronger. We found that both antibiotics were more toxic for B than for T cells. Neither chloramphenicol nor florfenicol at a concentration of 20 μ/ml altered lymphocyte proliferation. The influence of the antibiotics on PMN and MN cell phagocytic ability was determined by the respiratory burst activity test based on measurement of the nitro blue tetrazolium reduction, which allowed to detect intracellular O_2^-. Chloramphenicol and florfenicol at a higher dose depressed phagocytic ability of PMN and MN cells. Moreover the cells isolated from spleen were more sensitive to suppressive activity of the drugs than those isolated from head kidney. No effects were observed for chloramphenicol and florfenicol at a concentration of 20 μ/ml. Results indicated that the lower dose of the anibiotics does not diminish lymphocyte proliferation or PMN and MN cell phagocytic ability, while the higher dose does alter both of studied parameters.

Chloramphenicol, florfenicol, immunosuppression, carp

Chloramphenicol, a wide range activity drug, was used in various infections. However, after finding its toxic activity to hematopoietic and immune system, it started to be eliminated or restricted only to cases of severe bacterial diseases. It was an urgent need to find some new analogs which would have the same effectiveness in treatment but have less toxicity.

Florfenicol is now used in veterinary practice. It possesses an antimicrobial spectrum similar to chloramphenicol, but it has not toxic influence on hematopoietic system because of lack of p-NO_2 group. That is why it seems to be the right drug to substitute chloramphenicol. Studies on pharmacokinetics and pharmacodynamics of the compound are still being carried out. Basic pharmacokinetic parameters are different in various species of animals and they depend on the way of the drug administration (Adams et al. 1987; McKellar et al. 1996; Soback et al. 1995). Generally it is well tolerated.

The purpose of our study was to determine in vitro effects of chloramphenicol and florfenicol on immunocompetence cells and nonspecific defence mechanisms in fish.

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Materials and Methods

Studies were carried out using 20 healthy carp fry *(Cyprinus carpio)* weighing 100 g. Fish were kept in containers with about 20 liters of dechlorinated, aerated and filtered water of 20-22 °C from closed circulation system with a biological filter and fed with a 40% protein diet. Fish were anasthetized using Propiscin (IFI-Poland).

Organs (head kidney and spleen) were collected for *in vitro* study. Leukocytes were isolated by pressing the tissues through a steel mesh with medium (RPMI 1640 + 10 units/ml heparin). Cells were purified by centrifuging at 2000 g for 30 min at 4 °C on Gradisol (Polfa-Kutno, Poland), washed and suspended in RPMI 1640 with 10 % FCS (Gibco, England) at a concentration of 10^5 cells/ml. Viable cells were counted with trypan blue.

The following antibiotics were used in our study:

- chloramphenicol (Detreomycine, Polfa-Kraków, Poland) at the concentrations of 20 µg/ml and 40 µg/ml
- florfenicol (Nuflor, Schering-Plough) at the concentrations of 20 µg/ml and 40 µg/ml.

Antibiotics were dissolved in ethanol and diluted to desired concentrations in RPMI 1640.

Lymphocyte proliferation

Lymphocyte proliferation assay is based on a spectrophotometric method – the MTT test (Mossman 1983).

Lymphocytes were distributed to microplate wells (100 µl) with each antibiotic (100 µl) at a concentration of 20 and 40 µg/ml (nine wells for each antibiotic at each concentration) with 10 µg/ml of LPS (Lipopolysaccharide, Sigma-Aldrich) as a B cell mitogen or 10 µg/ml of Con A (Concanavalin A, Sigma-Aldrich) as a T cell mitogen. After 3 days of incubation at 22 °C (5 % of CO_2), MTT (thiazolyl blue, Sigma-Aldrich) solution at a concentration of 10 mg/ml was added and the plate was left in an incubator for the next 4 hours. Then medium was removed and 100 µl of DMSO (Dimethyl sulphoxide, PSPark, U.K.) were added to each well. The optical density was measured at 620 nm on a plate reader Stat Fax 2600 (Awareness Technology, FI).

Phagocytic ability

Phagocytic ability was measured by the respiratory burst activity (RBA) test, using PMA (Phorbol 12-Myristate Acetate, Sigma-Aldrich) as a stimulator (Siwicki et al. 1993).

PMN and MN cells were distributed to microplate wells (100 µl) with each antibiotic (100 µl) (nine wells for each test) and left for incubation (22 °C, 5 % CO_2). Then medium was removed and PMA in 0,1 % NBT(Nitro blue tetrazolium, Sigma) / RPMI 1640 solution was added. After half an hour medium was removed and the remaining cells were fixed with ethanol. When the plate was dry, each well was filled with 120 µl of 2N KOH and 140 µl of DMSO in order to dissolve the produced formazan inside the cells. The content of the wells was mixed. The optical density was measured at 620 nm.

The same procedures were carried out on the antibiotics-free control samples. Medium used in these samples was the same as used with antibiotics. Used in the control samples cells originated from the same fish material as used in the test samples.

Statistical analysis of the results was performed by the Student's *t*-test. Differences on means are considered statistically significant at $p < 0.05$.

Results

T lymphocyte proliferation

Chloramphenicol, at a concentration of 40 µg/ml, induced statistically significant ($p < 0.05$) reduction of T cell proliferation while at a concentration of 20 µg/ml that compound did not affect the process.

After application of chloramphenicol at 40 µg/ml, MTT reduction for T lymphocytes isolated from head kidney and spleen was diminished about 40% and 30%, respectively, compared to the control. Florfenicol, at the same concentration, reduced T cell proliferation about 30 % and 15% respectively. At a concentration of 20 µg/ml of that compound reduction of T cell proliferation was not observed (Fig.1).

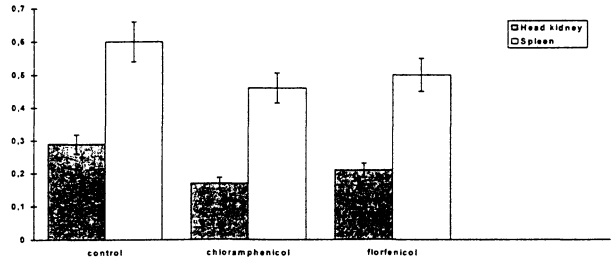

Fig. 1.

In vitro effects of chloramphenicol and florfenicol on T lymphocyte proliferation from head kidney and spleen in fish (concentration 40 µg/ml). (Mean ± SD less than 10 %, n=9)

B lymphocyte proliferation

Similar results were obtained for B lymphocyte proliferation. Both antibiotics at a concentration of 20 µg/ml did not give any statistically significant ($p > 0.05$) changes in MTT reduction values in comparison to the control. Chloramphenicol application at a concentration of 40 µg/ml suppressed B cell proliferation about 50% from head kidney and 30% from spleen and florfenicol applied at the same concentration reduced the reaction about 45% and 20%, respectively (Fig. 2).

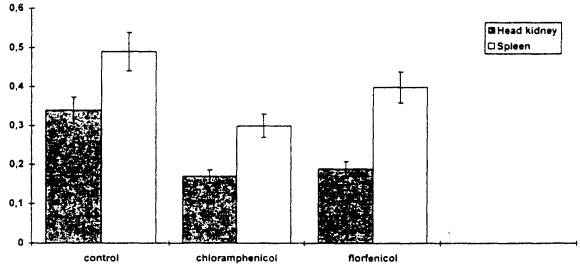

Fig. 2.

In vitro effects of chloramphenicol and florfenicol on B lymphocyte proliferation from head kidney and spleen in fish (concentration 40 µg/ml). (Mean ± SD less than 10 %, n=9)

Phagocytic ability of PMN and MN cells

At a concentration of 20 µg/ml of the drugs, phagocytic ability of PMN and MN cells was not suppressed as compared to the control.

Respiratory burst activity values for PMN and MN cells isolated from head kidney and spleen were decreased at a concentration of 40 µg/ml of both drugs. After application of 40 µg/ml of chloramphenicol, RBA values of cells isolated from head kidney were suppressed about 23% and of cells isolated from spleen about 34% compared to the control. After application of 40 µg/ml of florfenicol, RBA values of cells isolated from head kidney were suppressed about 15% and of cells isolated from spleen about 30% compared to the control.

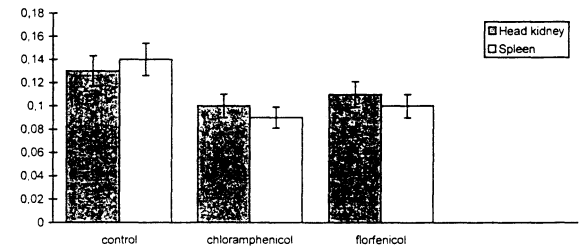

Fig. 3.

In vitro effects of chloramphenicol and florfenicol on phagocytic ability of PMN and MN cells from head kidney and spleen in fish (concentration 40 µg/ml). (Mean ± SD less than 10 %, n=9)

Discussion

The immunosuppressive properties of chloramphenicol are well known (Banack et al. 1979; DaMert et al. 1979). It was found that chloramphenicol has a negative influence on antibody production in man (Hauser et al. 1982). It also affects the cellular immunity. As a result of T cell deficiency after prolonged use of the antibiotic, chronic mucocutaneous candidosis and viral superinfections were observed. T cell suppression affects production of interferon, leading to spread of viral infections (Munmster et al. 1977). Another effect of the activity of

chloramphenicol is its toxic influence on neutrophil metabolism. At higher concentrations it inhibits their migration and chemotaxis (Forsgren et al. 1977; Hauser et al. 1982).

Neu et al. (1980) noticed a negative influence of chloramphenicol on phagocytosis of PMN cells isolated from peripherial blood in cattle. It was also shown that despite of being able to take up microorganisms, neutrophils have completely inhibited mechanism of intracellular killing.

On the contrary, Paape et al. (1990) did not observe any negative effects of florfenicol on phagocytosis and chemiluminiscence of PMN cells. However, the antibiotic altered morfology of those cells, e.g. caused a loss of pseudopodia.

The results obtained in our laboratory showed, that both chloramphenicol and its derivative - florfenicol, at a concentration of 40 μg/ml suppress humoral and cellular immunity. This concentration inhibits T and B cell proliferation. Chloramphenicol strongly suppresses lymphocyte proliferation in comparison to the control and is more toxic for B than T cells. Florfenicol at a concentration of 40 μg/ml acts similarly, inhibiting more strongly B than T cell proliferation. In addition at a concentration of 40 μg/ml of the two compounds, chloramphenicol is more toxic for lymphocytes than florfenicol. Very few data is available on this subject in literature, especially about the influence of florfenicol on lymphocyte proliferation. There is a need for further study on the diferences in sensitivity of T and B cells to the same amount of the antibiotic.

Our results are similar to those of other authors' findings about the toxic influence of chloramphenicol on the cellular immunity. Watson (1980), Grądzki (1991, 1995) proved that chloramphenicol affects the total number of lymphocytes in cats, chickens and guinea-pigs. DaMert et al. (1979) and Hauser et al. (1982) showed that chloramphenicol is toxic for human B lymphocytes, and Munmster et al. (1977) found that chloramphenicol induces toxic effects in T lymphocyte proliferation.

Phagocytosis is an important immunological response as far as bacterial and other pathogens diseases are concerned. Activated MN and PMN phagocytes have increased bactericidal capacity by upregulating production of reactive oxygen species. We noticed reducted phagocytic ability of those cells after application of the higher concentration (40 μg/ml) of chloramphenicol and florfenicol. However chloramphenicol at this concentration possesses stronger inhibiting properties on respiratory burst activity (RBA) than florfenicol. Another finding is that both drugs suppress the phagocytic activity of the cells isolated from spleen more than those from head kidney.

Under the experimental conditions used in our study the results were similar to those found by Forsgren et al. (1977), Hauser et al. (1982), Ziv et al. (1983), and Paape et al. (1990) that chloramphenicol at therapeutic doses has an immunosuppressive effect on phagocytic ability of PMN cells. Paape et al. (1990) did not notice any toxic influence of florfenicol administered at therapeutic doses on PMN cells in cattle, however in our studies we have observed a distinct immunosuppressive effect of florfenicol at 40 μg/ml on phagocytic ability of PMN and MN cells and also on carp B and T lymphocyte proliferation. The same concentration of both antibiotics decreases the metabolic activity of PMN and MN cells, and leukocyte proliferation, although florfenicol acts to a less extent than chloramphenicol does. Considering common use of various types of antibiotics at different doses and their multiplicity of effects on animals we see a need to continue that kind of sudies on the influene of drugs on the immune system.

Antibiotika a buněčná imunita u ryb - studie *in vitro*

V práci byl sledován vliv chloramfenikolu a florfenikolu na proliferaci T a B buněk a fagocytární schopnost polymorfonukleárních (PMN) a mononukleárních (MN) buněk.

Buňky byly izolovány z hlavové části ledvin a ze sleziny u 20 ryb (*Cyprinus carpio*). Antibiotika byla podána in vitro v koncentraci 20 a 40 μg-ml. Všechna stanovení byla provedena v devíti opakováních. Proliferace T a B buněk po aplikaci léčiva byla zjišťována spektrofotometrickou metodou při použití lipopolysacharidu a conkanavalinu jako mitogenů. V podmínkách naší studie bylo zjištěno, že chloramfenikol a florfenikol v koncentraci 40 μg/ml redukují proliferaci T a B buněk, ačkoliv vliv chloramfenikolu byl silnější. Zjistili jsme, že obě antibiotika jsou toxičtější pro B buňky. Chloramfenikol ani florfenikol v koncentraci 20 μg/ml nemění proliferaci lymfocytů. Působení antibiotik na fagocytární schopnost PMN a MN buněk bylo stanoveno pomocí testu aktivity respiračního vzplanutí založeném na měření redukce nitro blue tetrazolium, která umožňuje detekovat intracelulární O_2. Ve vyšší dávce chloramfenikol i florfenikol snižují fagocytární schopnost PMN a MN buněk. Přitom buňky izolované ze sleziny jsou citlivější k supresivnímu působení antibiotik než buňky izolované z hlavové části ledvin. V koncentraci 20 μg/ml chloramfenikolu a florfenikolu nebyly pozorovány žádné změny. Výsledky ukazují, že nízké dávky antibiotik nesnižují proliferaci lymfocytů nebo fagocytární schopnost PMN a MN buněk. Vyšší dávky pak snižují hodnoty obou sledovaných parametrů.

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