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Effect of antibiotics on the phagocytotic and respiratory burst activity of bovine granulocytes

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Abstract

The influence of antibiotics on respiratory burst (phorbol-12-myristate-13-acetate (PMA)-stimulated luminol-enhanced chemiluminescence) and phagocytosis (flow cytometry) by bovine granulocytes was studied in vitro. Phagocytosis was impaired by 1000 μg/ml of oxytetracycline, chloramphenicol, erythromycin and spiramycin. All antibiotics, except sulphadiazine, decreased chemiluminescence at 1000 μg/ml or lower concentrations. Enrofloxacin increased chemiluminescence. The inhibition by oxytetracycline and danofloxacin was due to absorption of the light emitted by luminol at 425 nm. Oxytetracycline, ceftiofur, spiramycin and erythromycin affected the myeloperoxidase–H_2O_2–halide system. Ceftiofur, penicillin and danofloxacin showed scavenging effects on H_2O_2 and OCl^-. Penicillin and ceftiofur might interfere with luminol. Chloramphenicol, penicillin and ceftiofur affected the production of superoxide radicals. In summary, the observed effects of antibiotics might be of importance during treatment of infectious diseases in normal and immunocompromised animals. However, before classifying a drug as immunosuppressive, attention has to be paid to possible interference with the chemiluminescence assay. © 1997 Elsevier Science B.V.

Keywords: Antibiotics; Polymorphonuclear leukocyte; Respiratory burst; Phagocytosis

1. Introduction

Neutrophils play a very important role in the defence against bacterial infections, such as mastitis in dairy cows (Burvenich et al., 1994). After phagocytosis of invaded bacteria, neutrophils use oxygen-dependent and oxygen-independent mechanisms to kill these pathogens. Oxygen-dependent killing is known as the respiratory burst. The respiratory burst activity of polymorphonuclear leukocytes can be measured by chemiluminescence (Allen et al., 1972). The most common bacterial infection in dairy cows is mastitis, which is economically the most costly disease in the dairy industry. Bacterial infections in general, and mastitis in particular, are generally treated with antibiotics. These antibiotics can affect the function of several immune cells directly or indirectly by changes induced in the micro-organisms (Van Den Broek, 1989). Appropriate elimination of bacteria requires both the effectiveness of the antimicrobial drug against the micro-organism and a very well functioning defence system of the animal. This is especially important for animals with an impaired immune function such as cows immediately after parturition (Burvenich et al., 1994), in which an impaired function of neutrophils has been observed (Kehrli et al., 1989; Cai et al., 1994). Indeed, in the period between partus and peak lactation, the incidence of infectious as well as metabolic diseases is very high (Burvenich et al., 1994). Severe and lethal cases of, e.g., *Escherichia coli* mastitis are only observed during early lactation. According to Cai et al. (1994), the higher incidence of these diseases is connected with the impaired function of polymorphonuclear leukocytes after parturition. The purpose of this study was to determine the effects of commonly used antimicrobial agents on the phagocytosis and respiratory burst activity of bovine polymorphonuclear leukocytes isolated from blood.

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2. Materials and methods

2.1. Animals

Seven clinically healthy high-yielding dairy cows were selected from the University of Ghent dairy herd. All cows were of the Holstein–Friesian breed, between 4 and 12 weeks after parturition and were in their 2nd to 5th lactation.

2.2. Isolation of polymorphonuclear leukocytes

Polymorphonuclear leukocytes were isolated according to the method of Mottola et al. (1980) with a few modifications. Freshly drawn peripheral blood (40 ml) was aseptically collected from each cow from the external jugular vein by venapuncture in evacuated tubes (Laboratoire EGA, Nogent-le Roi, Belgium) containing 125 I.U. heparin as anticoagulant. After initial centrifugation for 15 min at $1000 \times g$ (4°C), the plasma layer, the buffycoat and the top layer of the packed red blood cells were discarded. After washing with 0.9% NaCl and a second centrifugation (15 min; $1000 \times g$; 4°C), the supernatant, the buffycoat and the top layer of the packed red blood cells were again discarded. The red blood cells were lysed by adding 80 ml aqua bidest and gently mixing the suspension for 1 min. After restoration of the isotonicity by addition of 40 ml of 2.7% NaCl and mixing, the suspension was centrifuged again (15 min; $1000 \times g$; 4°C). The remaining cell pellet was washed with phosphate-buffered saline (PBS) and the final cell pellet was resuspended in 1 ml of Dulbecco's phosphate-buffered saline (DPBS) (Gibco BRL, Life Technologies, Gaithersburg, MD, USA). For the chemiluminescence assay, 1 mg/ml of gelatin was added to the DPBS. After isolation, the cells were counted by using an electronic cell counter (Coulter Counter ZF, Coulter Electronics, Luton, UK). The viability of polymorphonuclear leukocytes immediately after isolation was determined by trypan blue dye exclusion and differential cell counts were performed on eosin–giemsa-stained smears (Hemacolor®, E. Merck, Darmstadt, Germany). On the average, 95% of the isolated cells were polymorphonuclear leukocytes with a viability of 98%. After cell counting and differentiation, cell suspensions were adjusted to a final concentration of 4×10^5 viable polymorphonuclear leukocytes per ml.

2.3. Drugs

Na^+-ceftiofur (The Upjohn, Kalamazoo, MI, USA) and sulphadiazine (Sigma, St. Louis, MO, USA) were dissolved in DPBS. Chloramphenicol (Sigma), danofloxacin (Pfizer, NY, USA), erythromycin (Sigma), oxytetracycline HCl (Sigma) and spiramycin (Sigma) were dissolved in dimethyl sulphoxide (DMSO). Enrofloxacin (Bayer, Leverkusen, Germany) and benzathin benzylpenicillin (Sigma) were dissolved in HCl/NaOH. The 200 mg/ml stock solutions of all antibiotics were further diluted in DPBS. Six concentrations of antibiotics were tested: 0.01 μg/ml, 0.1 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml and finally 1000 μg/ml.

2.4. Effects of antibiotics on phagocytosis

E. coli P4 was cultured on brain heart infusion broth (BHI, Oxoid) and labeled with fluorescein isothiocyanate (FITC isomer I, Sigma) according to the method of Gelfand et al. (1976). Phagocytosis was tested according to a method described by Saad and Hageltorn (1985) with a few modifications. Polymorphonuclear leukocytes (2.10^5/ml, final concentration), *E. coli* (10 bacteria/polymorphonuclear leukocyte) and 5% (v/v) pooled adult bovine serum were incubated in DPBS without and with 6 concentrations of antibiotics for 30 min at 37°C. Phagocytosis was stopped by addition of 250 μl ice-cold formaldehyde in PBS and by putting the tubes on ice. Trypan blue was used for quenching extracellular fluorescence according to the method of Hed et al. (1987). The addition of 800 μl of a 0.3% (w/v) trypan blue solution in PBS resulted in a final concentration of 1.2 mg/ml, which caused complete quenching of all extracellular fluorescence. FITC fluorescence was measured by flow cytometry (FACScan, Becton Dickinson European HQ, Erembodegem-Aalst, Belgium) at 530–560 nm and recorded on a logarithmic scale from 1 to 10 000. Percentage fluorescence and mean fluorescence intensity before and after quenching were measured. Percent fluorescence indicates the percentage of the neutrophil population that is fluorescent, with at least one FITC-labeled *E. coli* attached or ingested per neutrophil. Mean fluorescence intensity represents the mean number of fluorescent bacteria per cell (Saad and Hageltorn, 1985).

2.5. Effect of antibiotics on viability of polymorphonuclear leukocytes

Percent viability was estimated by addition of 10 μl of a 0.002% (w/v) propidium iodide solution to polymorphonuclear leukocytes (2×10^5 final concentration) in DPBS with adult bovine serum before and after sham and antibiotic incubation for 30 min at 37°C. The red fluorescence of dead cells was registered by flow cytometry with a 630 nm long pass filter. The viability of the polymorphonuclear leukocytes incubated with antibiotics was compared with the viability of polymorphonuclear leukocytes after sham treatment to study the effect of antibiotics on viability.

2.6. Effect of antibiotics on cell size

Cell size was measured after a 30 min incubation of polymorphonuclear leukocytes in the presence of 0–1000 μg/ml of antibiotics and DPBS with adult bovine serum

at 37°C by flow cytometric measurement of the forward scatter in a forward scatter–side scatter-histogram on a linear scale from 0 to 1000.

2.7. Chemiluminescence assay

Luminol-enhanced phorbol-12-myristate-13-acetate (PMA)-stimulated chemiluminescence was used to measure the respiratory burst activity of the isolated polymorphonuclear leukocytes. The chemiluminescence assay was performed at 26°C with a liquid scintillation counter (Rackbeta Spectral 1219, LKB Wallac Oy, Turku, Finland), using the tritium channel and the out-of-coincidence mode. Six concentrations of antibiotics and two blanks with the same composition as the drug solution, with the exception of the drug itself, were monitored. The cell suspension (2.10^5 cells/ml, final concentration) was incubated with the medium and the antibiotics for 30 min at 26°C. DMSO was added to get the same % of DMSO in all the tubes. After this incubation, luminol (0.1 mM, final concentration) and PMA (10 ng/ml, final concentration) were added and chemiluminescence was registered immediately for 30 min at 26°C. After measurement, the area under the curve over the 30 min period was calculated. A chemiluminescence index was calculated by dividing the area under the curve (AUC) of the antibiotic-incubated polymorphonuclear leukocytes by the AUC of the control polymorphonuclear leukocytes and multiplying by 100.

2.8. Effects of antibiotics on chemiluminescence in a cell-free system

Chemiluminescence in a cell-free system was measured in duplicate according to the method of Briheim and Dahlgren (1987) with a few modifications. Reaction mixtures with a total volume of 200 μl included DPBS, 0.1 mM of luminol (final concentration), 20 μl of the antibiotic or medium and 0.1 mM of H_2O_2 (final concentration). Chemiluminescence was measured for 30 min at 26°C with a microtiter plate luminometer (LB 96P, EG and G Berthold, Bad Wildbad, Germany) after addition of 25 μl of the supernatant of sonicated polymorphonuclear leukocytes. Polymorphonuclear leukocytes (8×10^5/ml) from one cow were sonicated for 3×10 s with an Ultrasonic Disintegrator (MSE, Manor Royal, Crawley, UK) and centrifuged for 10 min at $400 \times g$. Thereafter, the supernatant was stored at −20°C and immediately before use thawed and again sonicated for 3×10 s.

2.9. Effect of antibiotics on myeloperoxidase (EC 1.11.1.7) activity

The effect of the antibiotics on myeloperoxidase activity was measured in duplicate, according to the method of Somersalo et al. (1990), as the oxidation of *ortho*-dianisidine (Sigma) by neutrophil extract containing H_2O_2 in the presence or absence of the drugs. Supernatant of sonicated cells (25 μl) from one cow (see Section 2.8), medium and antibiotics (20 μl) were incubated at 26°C for 5 min. After incubation, H_2O_2 (0.1 mM, final concentration) and *ortho*-dianisidine (0.8 mM, final concentration) were added and absorption was measured in a spectrophotometer (Multiskan Plus Type 314, Labsystems Oy, Helsinki, Finland) at 450 nm.

2.10. Chemiluminescence from added OCl^-

Chemiluminescence from added OCl^- was measured in duplicate according to the method of Gunther et al. (1993). Reaction mixtures including DPBS, 0.04 mM of luminol (final concentration) and antibiotics or medium were placed in the sample chamber of a luminometer and chemiluminescence was measured after addition of Na^+OCl^- (100 μl from a stock prepared by diluting Na^+OCl^- 1:1000 in DPBS) (Sigma-Aldrich, Deisenhofen, Germany).

2.11. Effect of antibiotics on generation of superoxide anions by stimulated polymorphonuclear leukocytes

Freshly isolated polymorphonuclear leukocytes (1×10^7/ml) from one cow were incubated with medium or antibiotics for 15 min at 37°C. After incubation, cytochrome *c* (Sigma) (0.2 mM, final concentration) was added to all the wells and superoxide dismutase (EC 1.15.1.1) (25 μg/ml, final concentration) was added to the blanks. After incubation for 5 min at 37°C, polymorphonuclear leukocytes were stimulated with PMA (200 ng/ml, final concentration). Absorption was measured in duplicate in a microtiter plate spectrophotometer (Multiskan Plus Type 314, Labsystems Oy) at 550 nm after 5 min and 35 min of incubation.

2.12. Absorption of antibiotics at 405–630 nm

Because luminol emits light at 425 nm, absorption at 405 to 630 nm in the presence or absence of antibiotics was monitored, using a spectrophotometer (Multiskan Plus Type 314, Labsystems Oy). The effect of the coloured antibiotics danofloxacin and oxytetracycline on chemiluminescence was studied as follows: a vial with a control was inserted into a larger vial containing the antibiotics at the highest concentration. Chemiluminescence of this sample was compared with that of another control sample without antibiotics.

2.13. Statistical analyses

Statistical analysis of the results of the chemiluminescence assay (Section 2.7) was performed for each antibiotic by a two-way analysis of variance with the concentration of the antibiotics as a fixed factor, the cows ($n = 7$) as a randomized factor and their interaction term. Comparison

of means was performed by way of the method of least significant differences. Logarithmic transformation of the absolute values of the chemiluminescence data of the calculated area under the curve was used for further statistical analyses. Bartlett's test of equal variances was used to study the equality of variances.

Statistical analyses of the results of the cell-free assay, the myeloperoxidase assay, the assay with luminol and Na^+OCl^-, and the cytochrome *c* reduction test were performed for each antibiotic by a one-way analysis of variance ($n = 2$). Comparison of means was performed by way of the method of least significant differences. Bartlett's test of equal variances was used to study the equality of variances. The Statistix program package (v. 4.0, 1992, Analytical Software, Tallahassee, FL, USA) was used. Significant differences were determined at $P < 0.05^*$, $P < 0.01^{**}$ and $P < 0.001^{***}$.

3. Results

3.1. Effects of antibiotics on viability of polymorphonuclear leukocytes

The effects of antibiotics might be due to effects on the viability and integrity of cells. Therefore, the effects on viability were studied by trypan blue or propidium iodide dye exclusion. Viability immediately after isolation of polymorphonuclear leukocytes was ±98%. Sham treatment of the polymorphonuclear leukocytes for 30 min at 37°C did not alter the viability. No important significant decreases in viability were found between control polymorphonuclear leukocytes and polymorphonuclear leukocytes incubated with antibiotics, except for 1000 μg/ml of oxytetracycline, which decreased the viability by 29% ($P < 0.001$) as compared to the control polymorphonuclear leukocytes. A dose of 1000 μg/ml of ceftiofur caused a decrease in viability of 2% ($P < 0.01$).

3.2. Effect of antibiotics on cell size

Because forward scatter is a measure of cell size, the influence of antibiotics on forward scatter was measured by flow cytometry (Fig. 1). At a dose of 1000 μg/ml oxytetracycline, chloramphenicol, erythromycin and sulphadiazine reduced forward scatter of polymorphonuclear leukocytes very significantly ($P < 0.001$) from 525 (blank) to 362, from 544 (blank) to 359, from 527 (blank) to 440, and from 526 (blank) to 437, respectively. Spiramycin and penicillin reduced forward scatter at the same dose from 528 (blank) to 456 ($P < 0.01$) and from 516 (blank) to 433 ($P < 0.05$).

3.3. Effects of antibiotics on phagocytosis

To study phagocytosis, mean fluorescence intensity before and after quenching and % fluorescence were estimated by flow cytometry. Quenching was used to distinguish between internalised and adhered bacteria. Effects on phagocytosis (Fig. 2) were observed only at doses of 1000 μg/ml. Oxytetracycline decreased phagocytosis very significantly: mean fluorescence intensity before and after quenching was reduced by 19 and 9%, respectively. The decrease in % fluorescence before and after quenching amounted to 54 and 56%. Chloramphenicol caused a decrease in fluorescence before and after quenching of 34 and 49%, respectively. A smaller decrease of mean fluorescence intensity was also observed. Erythromycin reduced fluorescence before and after quenching by 33 and 29% ($P < 0.05$) respectively, but no marked effect on mean fluorescence intensity was observed. After quench-

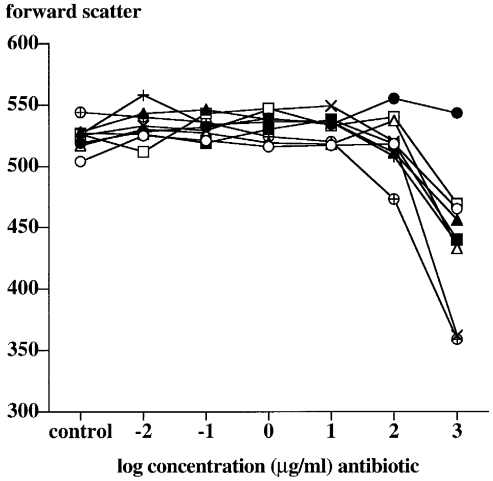

Fig. 1. Influence of different doses of antibiotics on cell size measured as forward scatter by flow cytometry. ○, danofloxacin; ●, Na^+-ceftiofur; △, penicillin; ▲, spiramycin; □, enrofloxacin; ■, erythromycin; X, oxytetracycline; +, sulphadiazine; ⊕, chloramphenicol.

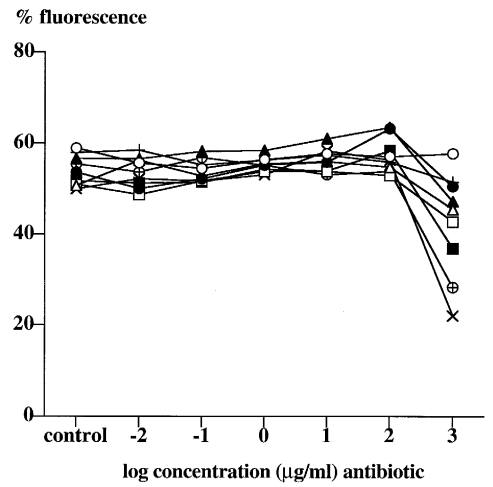

Fig. 2. Effect of several antibiotics on neutrophil phagocytosis of FITC-labeled *E. coli*. Percent fluorescence was measured after quenching with Trypan blue. ○, danofloxacin; ●, Na^+-ceftiofur; △, penicillin; ▲, spiramycin; □, enrofloxacin; ■, erythromycin; X, oxytetracycline; +, sulphadiazine; ⊕, chloramphenicol.

ing, spiramycin reduced fluorescence by 17% ($P < 0.05$). The remaining antibiotics had no marked effect on phagocytosis.

3.4. Effects of antibiotics on chemiluminescence of isolated polymorphonuclear leukocytes

Chemiluminescence was quantified by the area under the curve of the chemiluminescence dose–response curve. Most antibiotics decreased chemiluminescence at a concentration of 1000 μg/ml (Fig. 3). Sulphadiazine had no effect on cellular chemiluminescence. Therefore, the influence of this antibiotic was not further investigated in the other experiments. Increasing doses of enrofloxacin enhanced chemiluminescence. Doses of 10 to 100 μg/ml of enrofloxacin induced a significant ($P < 0.001$) increase in chemiluminescence of 23 and 40%, respectively. However, 1000 μg/ml of this antibiotic caused a drop to control values. Danofloxacin, Na^+-ceftiofur and chloramphenicol caused a significant inhibition of chemiluminescence at doses of 100 μg/ml ($P < 0.01$) and higher ($P < 0.001$). Spiramycin ($P < 0.001$), erythromycin ($P < 0.01$) and penicillin ($P < 0.001$) significantly decreased chemiluminescence at 1000 μg/ml by 48, 17 and 66%, respectively. Oxytetracycline at doses ranging from 10–1000 μg/ml significantly ($P < 0.001$) decreased chemiluminescence by 70, 93 and 100%, respectively.

3.5. Effect of antibiotics on chemiluminescence in a cell-free system

To know whether the drugs act on the NADPH-oxidase in the cell membrane, chemiluminescence was studied in a cell-free system. Danofloxacin stimulated cell-free chemiluminescence at doses of 0.01 to 1 μg/ml by about 7% (Fig. 4). Higher doses inhibited cell-free chemiluminescence significantly ($P < 0.001$) by 97 and 99%, respectively. Na^+-ceftiofur inhibited this reaction significantly ($P < 0.001$) at doses of 10, 100 and 1000 μg/ml by 67, 99 and 100%, respectively. Penicillin severely ($P < 0.001$) decreased cell-free chemiluminescence at concentrations of 1 to 1000 μg/ml by 54, 92, 97 and 98%, respectively. Spiramycin had an inhibitory effect at 100 μg/ml ($P < 0.01$) and 1000 μg/ml ($P < 0.001$), causing 28 and 87% inhibition. Enrofloxacin had no influence on cell-free chemiluminescence except at 100 and 1000 μg/ml, when it inhibited the reaction by about 19%, which was not significant. Erythromycin stimulated cell-free chemiluminescence already at a dose of 0.01 μg/ml ($P < 0.05$) and at higher doses ($P < 0.01$) by about 20%, but decreased cell-free chemiluminescence at a dose of 1000 μg/ml ($P < 0.01$). Oxytetracycline inhibited cell-free chemiluminescence at concentrations of 10–1000 μg/ml by 72, 99 and 99%, respectively ($P < 0.001$). Chloramphenicol had no important influence on cell-free chemiluminescence.

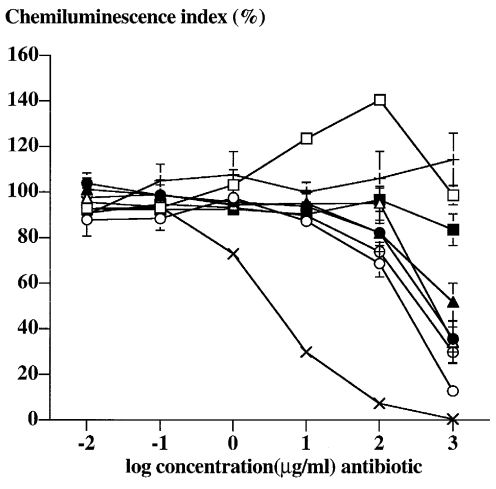

Fig. 3. Influence of different doses of antibiotics on PMA-induced chemiluminescence of polymorphonuclear leukocytes. Data are means for seven cows ± S.E.M. Chemiluminescence index = (AUC of the antibiotic-incubated cells/AUC of the vehicle-treated cells)∗100. ○, danofloxacin; ●, Na^+-ceftiofur; △, penicillin; ▲, spiramycin; □, enrofloxacin; ■, erythromycin; X, oxytetracycline; +, sulphadiazine; ⊕, chloramphenicol.

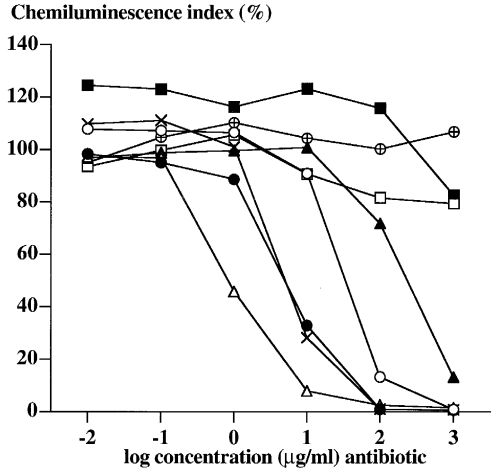

Fig. 4. Influence of different doses of antibiotics on chemiluminescence in a cell-free system. Chemiluminescence index = (AUC of the antibiotic-incubated cells)/(AUC of the vehicle treated cells)∗100. ○, danofloxacin; ●, Na^+-ceftiofur; △, penicillin; ▲, spiramycin; □, enrofloxacin; ■, erythromycin; X, oxytetracycline; ⊕, chloramphenicol.

3.6. Effects of antibiotics on myeloperoxidase activity

The myeloperoxidase in azurophilic granules reacts with H_2O_2 and Cl^- to generate OCl^-. This reaction is necessary to generate chemiluminescence. Na^+-ceftiofur and oxytetracycline caused a significant inhibition at 100 μg/ml of 29 ($P < 0.01$) and 20% ($P < 0.05$) and at 1000 μg/ml caused an inhibition of 76 ($P < 0.001$) and 37% ($P < 0.001$), respectively (Fig. 5). Erythromycin and chloramphenicol ($P < 0.05$) slightly reduced myeloperoxidase activity at 1000 μg/ml. The reduction by erythromycin was not significant. Danofloxacin ($P < 0.001$), spi-

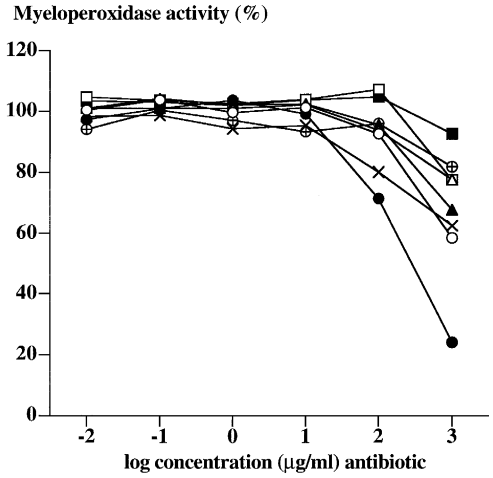

Fig. 5. Influence of different doses of antibiotics on myeloperoxidase activity measured in terms of oxidation of *ortho*-dianisidine. ○, danofloxacin; ●, Na^+-ceftiofur; △, penicillin; ▲, spiramycin; □, enrofloxacin; ■, erythromycin; X, oxytetracycline; ⊕, chloramphenicol.

ramycin ($P < 0.01$), penicillin and enrofloxacin reduced myeloperoxidase activity only at 1000 μg/ml by 41, 32, 12 and 12%, respectively. The decreases elicited by penicillin and enrofloxacin were not significant.

3.7. Scavenging effects of antibiotics on OCl^-

Addition of Na^+OCl^- to luminol resulted in an immediate chemiluminescence response. Danofloxacin, ceftiofur, penicillin and oxytetracycline significantly ($P < 0.001$) decreased the reaction at a dose of 1000 μg/ml by 97, 99, 100 and 100%, respectively, and at a dose of 100 μg/ml by 34, 34, 99 and 76%, respectively ($P < 0.001$) (Fig. 6). Na^+-ceftiofur induced a significant inhibitory effect (20%)

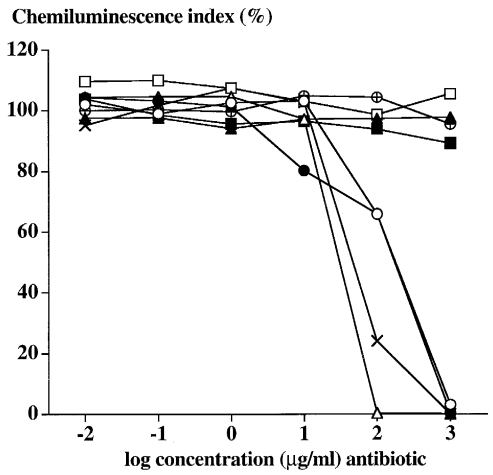

Fig. 6. Influence of different doses of antibiotics on chemiluminescence after incubation of luminol and Na^+OCl^- with the antibiotics in relation to blanks without antibiotics. Chemiluminescence index = (RLU of the antibiotic-incubated reaction)/(RLU of the blanks) * 100. ○, danofloxacin; ●, Na^+-ceftiofur; △, penicillin; ▲, spiramycin; □, enrofloxacin; ■, erythromycin; X, oxytetracycline; ⊕, chloramphenicol.

at a concentration of 10 μg/ml ($P < 0.001$). Enrofloxacin significantly ($P < 0.01$) stimulated the reaction by 10% at a dose of 0.1 μg/ml. Chloramphenicol induced a small but significant ($P < 0.05$) inhibitory effect (5%) at the highest concentration. Spiramycin and erythromycin had no significant effects in this assay.

3.8. Absorption by antibiotics at 405–630 nm

Because luminol emits blue light at a wavelength of 450 nm, the possible absorption of this light by the antibiotics was studied. This is primarily important for the coloured drugs oxytetracycline and danofloxacin. Highest absorptions were noticed at 405 nm and lowest at 630 nm. At 405 nm, danofloxacin (0.060 versus 0.015), oxytetracycline (1.330 versus 0.015) and ceftiofur (0.053 versus 0.015) showed a clearly increased absorption at 1000 μg/ml. Oxytetracycline also showed an increased absorption at 100 μg/ml (0.250 versus 0.015) and at 10 μg/ml (0.050 versus 0.015). When a control vial, which did not contain oxytetracycline, was inserted into a vial containing 1000 μg/ml of oxytetracycline, the chemiluminescence response was much lower than that of a normal control sample, but it was higher than that of a sample in which the cells were directly incubated with oxytetracycline. The same was observed with danofloxacin.

3.9. Effect of antibiotics on the generation of superoxide anions

Penicillin significantly ($P < 0.01$) reduced the production of superoxide radicals at a concentration of 1000 μg/ml by 60%. Chloramphenicol induced significant inhibitory effects at concentrations of 100 and 1000 μg/ml, with 14 ($P < 0.05$) and 40% ($P < 0.001$) inhibition, respectively. Na^+-ceftiofur significantly stimulated the production of superoxide radicals by 20 ($P < 0.01$) and 37% ($P < 0.001$) at doses of 100 and 1000 μg/ml, respectively.

4. Discussion

Phagocytosis was estimated by flow cytometry by using FITC-labeled *E. coli*, whereas respiratory burst activity was studied by using PMA-induced chemiluminescence. The soluble stimulant PMA directly activates the respiratory burst without being phagocytised, which is in contrast with opsonised particles. Therefore, PMA made it possible to study the direct effects of the antimicrobials on the respiratory burst, independent of the effects on phagocytosis.

The therapeutic concentrations of most antibiotics are between 10–20 μg/ml (Ziv et al., 1973). Since after local and repeated treatment (e.g., intramammary) much higher

concentrations can be achieved, concentrations up to 1000 μg/ml were studied. The antibiotics used in this study are commonly used to treat bacterial infections in dairy cattle.

The decrease in polymorphonuclear leukocyte viability and cell size elicited by high concentrations (1000 μg/ml) of oxytetracycline might indicate a certain toxicity of this molecule towards these cells or their cell membranes in particular. This has also been suggested by others (Nickerson et al., 1985; Paape et al., 1990). Chloramphenicol, erythromycin, spiramycin, sulphadiazine and penicillin G also reduced cell size at the highest concentration. These decreases in cell size were probably due to the significant toxicity of the highest concentrations of these antibiotics.

Only oxytetracycline, chloramphenicol, erythromycin and spiramycin decreased phagocytosis at the highest concentration. The percentage of phagocytic polymorphonuclear leukocytes was much more decreased than was the number of bacteria per polymorphonuclear leukocyte. The mean number of bacteria per neutrophil seemed to be less sensitive to antibiotics than the percentage phagocytic neutrophils. Apart from oxytetracycline, the effects of antibiotics on phagocytosis were not due to a decrease in viability. The reduction in phagocytosis occurred mainly in the 1 mg/ml doses, where forward scatter of the polymorphonuclear leukocytes was also reduced. A reduction in forward scatter could indicate shrinking of the cells with a possible loss of membrane flexibility and consequently phagocytic capacity (sublethal effects). The severe reduction of the forward scatter by oxytetracycline may indicate a loss of pseudopodia. Impairment of phagocytosis by tetracyclines has also been observed by others (Forsgren et al., 1974; Gnarpe and Belsheim, 1981). Tetracyclines inhibit the ingestion phase of phagocytosis (Forsgren et al., 1974). They may affect the assembly of F-actin by chelating Ca^{2+} and consequently interfere with Ca^{2+}-dependent mechanisms involved in the formation of actin dimers and trimers (Vandekerckhove, 1990). Chloramphenicol has been reported to decrease human (Kaplan et al., 1969) and bovine polymorphonuclear leukocyte phagocytosis (Nickerson et al., 1985; Paape et al., 1990). The loss of pseudopodia and inhibition of the assembly of actin monomers into filaments, which is important for degranulation and maintenance of pseudopodia (Paape et al., 1990), may account for the decreased phagocytosis at high concentrations. In general, β-lactam antibiotics, macrolides, sulphonamides and quinolones do not significantly affect phagocytosis by human (Van Den Broek, 1989; Akamatsu et al., 1995) and bovine granulocytes (Nickerson et al., 1985; Paape et al., 1991), as confirmed in this study.

Apart from sulphadiazine, all antibiotics had inhibitory effects on chemiluminescence at 1000 μg/ml. Lower concentrations had no significant effect, with the exception of enrofloxacin, which significantly stimulated chemiluminescence, and oxytetracycline, which significantly reduced chemiluminescence. Because chemiluminescence is a very sensitive method which can be easily disturbed by factors other than functional alterations of the cells, additional experiments were performed in order to study subcellular interactions. Chemiluminescence in a cell-free system was studied to see whether the effects observed in the cellular assay were due to effects on the cell membrane (NADPH-oxidase). Because the myeloperoxidase–H_2O_2–halide system is necessary to yield luminol chemiluminescence, the myeloperoxidase assay and the chemiluminescence assay were performed in the presence of hypochlorite, and because the first step of the burst is the formation of O_2^-, the cytochrome *c* reduction test was performed.

Erythromycin had an inhibitory effect on the myeloperoxidase–H_2O_2–halide system, which is in agreement with the findings of Van Rensburg et al. (1981). Spiramycin had a similar effect.

The present results and the fact that penicillin G poorly penetrates into polymorphonuclear leukocytes (Van Den Broek, 1989) suggest that penicillin G acts as a scavenger of OCl^-, rather than an inhibitor of myeloperoxidase. This is in agreement with the findings of Briheim and Dahlgren (1987) and others (Siegel and Remington, 1982; Soltisova and Lokaj, 1989). Because the greatest inhibition was observed when luminol was present in the incubation mixture, interference with this compound may play a role. This was also suggested by Briheim and Dahlgren (1987). Oxidative cleavage of the β-lactam ring of ampicillin by H_2O_2, resulting in the formation of a nitrone species, has been reported (Lagercrantz, 1992). The same effect may also be involved in the reaction with penicillin. The reduced production of O_2^- is also an important reason for the decreased chemiluminescence response.

The reduction of chemiluminescence at the highest concentration of Na^+-ceftiofur was partially due to the absorption of the light emitted by luminol (425 nm). The present results and the poor intracellular penetration of Na^+-ceftiofur suggest that Na^+-ceftiofur, like cefdinir (Labro et al., 1994), impairs myeloperoxidase activity, has scavenging effects on reactive oxygen species and possibly interferes with luminol. The stimulatory effect of ceftiofur on the production of O_2^- was completely abolished by these negative effects.

The reduction of chemiluminescence by danofloxacin was mainly due to absorption of the blue light emitted by luminol, which was demonstrated in the absorption assays. Indeed, danofloxacin dissolved in DMSO has an orange colour at the highest concentration. An inhibitory influence on myeloperoxidase activity and a scavenging effect on H_2O_2, but possibly not on OCl^-, may also be responsible for the decreased chemiluminescence.

The significant stimulatory effect of enrofloxacin on cellular chemiluminescence could not be confirmed in the other assays. This indicated a possible stimulatory effect on the production of O_2^- by stimulation of the membrane-bound NADPH-oxidase or of hexose monophosphate shunt activity. However, no effect on the production of O_2^- could be observed. The stimulatory effect of enrofloxacin

in the cellular system could not be confirmed in the other assays, because in these assays, H_2O_2 was added externally, which indicates that enrofloxacin has a stimulatory effect on the production of H_2O_2. The production of O_2^- might be unchanged, but that of H_2O_2 may be increased due to an increased activity of superoxide dismutase in the cytosol or to a decreased activity of catalase (EC 1.11.1.6). Stimulatory effects on the penetrability of luminol into the cells may also play a role (Hoeben et al., 1997), since intracellular generation of chemiluminescence is very much dependent on the diffusion of luminol into the cells. Enrofloxacin may also potentiate the action of PMA by increasing the concentration of free Ca^{2+} in the cytosol (Hoeben et al., 1997).

Oxytetracycline may have an inhibitory effect on myeloperoxidase or a scavenging effect on reactive oxygen species, but it is clear that the decrease in chemiluminescence is mainly due to absorption of the blue light emitted by luminol (Siegel and Remington, 1982), rather than a direct effect on the respiratory burst. Indeed, we observed that the yellow colour partially decreases chemiluminescence, but this colour is not the only factor responsible. The decreased viability of the cells also plays a role. The Ca^{2+}-chelating effect may play a minor role since the extracellular Ca^{2+} concentration is not important for the generation of PMA-induced chemiluminescence (Dechatelet and Shirley, 1982), although, the intracellular Ca^{2+} concentration is very important.

Chloramphenicol may inhibit myeloperoxidase, but inhibition of NADPH-oxidase is more likely. This is in agreement with other findings (Kaplan et al., 1969; Briheim and Dahlgren, 1987) that chloramphenicol does not affect directly the production of reactive oxygen species, but partially inhibits the oxidation of NADH, associated with impaired activation of the hexose monophosphate shunt (Kaplan and Finch, 1970). We observed an inhibitory effect on the production of O_2^-. However, there is no effect on hexose monophosphate shunt activity at low concentrations (20 μg/ml) (Pickering et al., 1978).

In summary, respiratory burst activity (chemiluminescence) seems to be more sensitive to antibiotics than is phagocytosis. These effects are mainly due to effects on subcellular metabolism rather than to effects on viability. One exception has to be made for oxytetracycline. Apart from sulphadiazine, which had no effect, and enrofloxacin, which stimulated chemiluminescence, chemiluminescence was decreased at very high concentrations of most antibiotics. Attention has to be paid to possible interference with the assay, especially at the highest antibiotic concentrations. Because the respiratory burst plays a very important role in the killing of *E. coli*, research into this function of polymorphonuclear leukocytes seems to be very interesting. Further research into the immunostimulating effects of drugs on polymorphonuclear leukocytes would be very useful in order to achieve a better clearance of bacteria in the udder.

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