Effects of Enrofloxacin on Porcine Phagocytic Function

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The interaction between enrofloxacin and porcine phagocytes was studied with clinically relevant concentrations of enrofloxacin. Enrofloxacin accumulated in phagocytes, with cellular concentration/extracellular concentration ratios of 9 for polymorphonuclear leukocytes (PMNs) and 5 for alveolar macrophages (AMs). Cells with accumulated enrofloxacin brought into enrofloxacin-free medium released approximately 80% (AMs) to 90% (PMNs) of their enrofloxacin within the first 10 min, after which no further release was seen. Enrofloxacin affected neither the viability of PMNs and AMs nor the chemotaxis of PMNs at concentrations ranging from 0 to 10 μg/ml. Enrofloxacin (0.5 μg/ml) did not alter the capability of PMNs and AMs to phagocyte fluorescent microparticles or Actinobacillus pleuropneumoniae, Pasteurella multocida, and Staphylococcus aureus. Significant differences in intracellular killing were seen with enrofloxacin at 5× the MIC compared with that for controls not treated with enrofloxacin. PMNs killed all S. aureus isolates in 3 h with or without enrofloxacin. Intracellular S. aureus isolates in AMs were less susceptible than extracellular S. aureus isolates to the bactericidal effect of enrofloxacin. P. multocida was not phagocytosed by PMNs. AMs did not kill P. multocida, and similar intra- and extracellular reductions of P. multocida isolates by enrofloxacin were found. Intraphagocytic killing of A. pleuropneumoniae was significantly enhanced by enrofloxacin at 5× the MIC in both PMNs and AMs. AMs are very susceptible to the A. pleuropneumoniae cytotoxin. This suggests that in serologically naïve pigs the enhancing effect of enrofloxacin on the bactericidal action of PMNs may have clinical relevance.

Phagocytes are an important part of the host defense against invading microorganisms. However, some microorganisms have defense mechanisms against chemotaxis, phagocytosis, and intracellular killing by phagocytes. A number of obligate and facultative intracellular bacteria are able to survive in phagocytes, resulting in persistent infections, and antibiotic treatment is required to assist in the elimination of pathogens. Consequently, antimicrobial agents should be able to penetrate phagocytic cells and, most importantly, should maintain their activity inside the cell (34). In this context, it is important that for several antibiotics adverse effects on phagocyte function have been described (35). For this reason, it is important to study interactions between phagocytes, microorganisms, and antibiotics.

Several classes of drugs are actively accumulated in phagocytes; among these are the fluoroquinolones (10, 27, 34, 38). Fluoroquinolones used in human medicine, such as ciprofloxacin, ofloxacin, and levofloxacin, have been found to accumulate in phagocytic cells in vitro, achieving intracellular concentrations four to eight times higher than the extracellular concentration (5, 10). In vivo, the concentration of fluoroquinolones in alveolar macrophages (AMs) was 14 to 18 times higher than that in serum (38). Enrofloxacin is a fluoroquinolone exclusively developed for companion and farm animals including swine. Its potency against many bacteria (4, 14, 15, 37) and good pharmacokinetic properties (30, 31) suggest that it would be an excellent antimicrobial agent for the treatment of bacterial infections in pigs. A considerable number of clinical studies have been conducted with enrofloxacin. These studies revealed that enrofloxacin is effective in the treatment of porcine respiratory diseases (18, 22, 32). However, data from in vitro experiments that have evaluated the antimicrobial efficacy of enrofloxacin in pigs are scarce. Although much research was done to study the antimicrobial actions of fluoroquinolones in phagocytes of several species (16, 23, 26), no data on the effect of enrofloxacin on interactions between porcine phagocytes and microorganisms are available.

In the study described in the present paper, the interaction between enrofloxacin and phagocytes was studied with concentrations of enrofloxacin found in vivo in clinical settings. As test organisms, we used Actinobacillus pleuropneumoniae, Pasteurella multocida, and Staphylococcus aureus for the following reasons. A. pleuropneumoniae is a pathogen that causes high rates of mortality in pigs as a result of severe infection of the respiratory tract. P. multocida type A is mainly found as a secondary respiratory infection in pigs. S. aureus is commonly used as a test organism to study interactions between phagocytes, microorganisms, and antimicrobial agents. First, the uptake and release of enrofloxacin by porcine AMs or polymorphonuclear leukocytes (PMNs) was studied. Second, the effects of enrofloxacin on chemotaxis of PMNs was measured; and third, the effect of enrofloxacin on phagocytosis and the intracellular killing of A. pleuropneumoniae, P. multocida, and S. aureus by porcine PMNs and AMs was evaluated.

MATERIALS AND METHODS

Bacterial strains. An A. pleuropneumoniae serotype 9 reference strain (25), a P. multocida type A reference strain (6), and S. aureus 42 D (20) were stored on polystyrene beads (Microbank; PCH Diagnostica, Haarlem, The Netherlands) at −70°C until they were used.

The bacteria were cultured for 18 to 24 h on sheep blood agar with 0.05% NAD (SBV) plates, passaged to fresh SBV plates, and incubated for 6 h. Then, the bacteria were rinsed off with Eagle minimal essential medium (EMEM), and the numbers of CFU were determined by plating 10-fold dilutions on SBV plates. Bacterial suspensions were stored overnight at 4°C, and on the next day the bacterial suspensions were diluted in EMEM to a concentration of 10^5 CFU/ml.
Enrofloxacin. Enrofloxacin (purity, 99.7%) was provided by Bayer AG, Leverkusen, Germany. For each experiment, a fresh stock solution of 10 mg/ml was prepared in 0.1 N sodium hydroxide. Next, the stock solution was diluted in EMEM or Mueller-Hinton bouillon (MHB) without NS.

Determination of MICs. Determination of the enrofloxacin MICs for *A. pleuropneumoniae*, *P. multocida*, and *S. aureus* was carried out by incubating bacteria with various concentrations of enrofloxacin. Bacterial suspensions in phosphate-buffered saline (PBS) with an optical density at 595 nm of 0.100 (10^3 CFU/ml) were prepared. These bacterial suspensions were diluted 1:100 in MHB with 0.05% NAD. In microtiter plates, a twofold serial dilution of enrofloxacin was made in MHB containing 0.05% NAD, bacteria were added, and the plates were incubated for 24 h at 37°C. Final concentrations of enrofloxacin in microtiter plates were prepared. These bacterial suspensions were diluted 1:100 in MHB with 0.05% NAD. Bacteria incubated without enrofloxacin served as positive controls, and MHB without bacteria and enrofloxacin served as a negative control. After incubation, microtiter plates were read turbidimetrically at 595 nm. The MIC was defined as the lowest concentration of enrofloxacin that inhibited bacterial growth. Determination of the MICs was independently carried out twice for each strain.

Isolation of porcine AMs and PMNs. Porcine PMNs were isolated from hepato-monic blood (10% (wt/vol) sodium dodecyl sulfate–50% dimethyl formamide (pH 4.7). The absorbance at 595 nm was read. Chemotaxis was expressed as a chemotactic index, which was obtained by dividing the value for chemotaxically migrated PMNs by the value for randomly migrated PMNs in the negative (NS) control. Samples of migrated PMNs were taken for determination of the enrofloxacin concentration.

Serum in phagocytosis and killing assays. In phagocytosis and killing assays, NS was used for *P. multocida* and *S. aureus*, and for *A. pleuropneumoniae*. *A. pleuropneumoniae* produces a pore-forming toxin that causes cytolysis of PMNs. Therefore, killing experiments involving AMs and *A. pleuropneumoniae* were performed with neutralizing convalescent-phase serum (CPS). With *A. pleuropneumoniae* and PMNs, NS (NS serologically naive for *A. pleuropneumoniae*) was used, but in one killing experiment CPS was also used.

Phagocytosis assay. The effect of enrofloxacin on phagocytosis of microorganisms by phagocytes was studied by flow cytometry (21) (FACS Calibur; Becton Dickinson, Franklin Lakes, N.J.) served as the upper chamber and was polyethylene terephthalate membrane with a pore size of 3 μm (Becton Dickinson Labware, Franklin Lakes, N.J.) served as the upper chamber and was placed in a well of a 24-well cell culture plate (Costar, Cambridge, Mass.), which served as the lower chamber. A total of 800 ml of EMEM or 500 ml of normal serum (NS) from conventionally housed pigs as the chemottractant was added in the lower chamber; EMEM without NS was used as the negative control. Enrofloxacin was added to the lower chambers with or without chemotactant at final concentrations of 1 or 0.1 μg of enrofloxacin per ml and, after incubation, 200 μl of the PMN suspension was added to the upper chamber and the PMNs were allowed to migrate for 1 to 2 h. After incubation in a 5% CO2 atmosphere for 90 min at 37°C, the PMNs were added to 10 μl of MTT solution and kept at 37°C. After 4 h of MTT reduction by PMNs, the cells were lysed by adding 1 ml of 20% (wt/vol) sodium dodecyl sulfate–50% dimethyl formamide (pH 4.7). The absorbance at 595 nm was read. Determination of the enrofloxacin concentration was performed essentially as described by Crujzen et al. (7) with Escherichia coli (E. coli 2.133 1099 [MIC, 0.008 μg/ml], a clinical isolate from ID-31) to test the method. Dilutions of representaives, cell homogenates, and enrofloxacin standard solutions were made in MHB incubated with E. coli 1.23 in a final volume of 100 μl in sealed microtiter plates at 37°C for 18 to 24 h. After incubation, 10 μl of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml in PBS; Sigma Chemical Company, St. Louis, Mo.) was added (24). After 5 min of incubation at room temperature the bacteria were lysed by adding 100 μl of 20% (wt/vol) sodium dodecyl sulfate–50% dimethyl formamide (pH 4.7). The contents of the plates were mixed for 5 s and the absorbance at 595 nm was read. As a negative control, MHB or enrofloxacin-free cell homogenate without bacteria was used, and as a positive control, MHB or enrofloxacin-free cell homogenate with bacteria was used. Data were fit to a sigmoid curve, and the dilution that gave 50% of the maximum signal was used for MIC determination.

Statistical analysis. Data were analyzed by ANOVA (version 7.5). C/E ratios and data concerning chemotaxis and killing experiments at the bioassays did not interpret the data. Analysis of the data was performed by means of analysis of variance (ANOVA). Data from killing experiments were natural log transformed and analyzed by ANOVA.
RESULTS AND DISCUSSION

The phagocytic part of the host defense depends on three essential steps: migration toward the invasion, ingestion, and destruction of the microorganism. Adequate antibiotic compounds should, preferably, not interfere with any of these steps. Most antibiotics accumulate in phagocytic cells, and some were described (35) to adversely affect one or more of the phagocyte functions mentioned above. Interpretation of the results described in the literature is difficult because of the wide variation in the test protocols used. Tests are performed with different microorganisms and sometimes with high concentrations of antibiotic which could never be realized in vivo. Nevertheless, for the fluoroquinolones in general, no adverse effects on phagocyte function were described. However, in contrast to other fluoroquinolones, limited in vitro data on enrofloxacin and phagocytosis are available.

The aim of the present study was to investigate the effect of enrofloxacin on porcine phagocytes by using clinically relevant enrofloxacin concentrations. With both AMs and PMNs, enrofloxacin was taken up to saturation immediately after the start of incubation. Cellular uptake of enrofloxacin is cell type dependent, and the C/E ratio is extracellular concentration independent (Table 1). This is in agreement with other studies (12, 13, 27–29) in which uptake of other quinolones in other species was studied by means of fluorometric and radioactivity assays. Enrofloxacin concentrations were twice as high in PMNs as in AMs. Similar observations were made for ciprofloxacin in mouse and human phagocytes (10, 11). A much higher value was reported for sparfloxacin by using guinea pig peritoneal macrophages (8). As was observed with other quinolones (8, 12, 13, 27–29), we also found that 80 to 90% of the intracellular enrofloxacin was released from phagocytes within 10 min when the phagocytes were placed in enrofloxacin-free medium.

No significant differences (P = 0.426) in chemotactic indices were found between control PMNs and enrofloxacin-containing PMNs (Fig. 1), showing that chemotaxis of PMNs remained unaffected by enrofloxacin, similar to other fluoroquinolones and human PMNs in the leading-front technique (23) and the under-agarose assay (1). The Boyden chamber technique enabled us to show that migrated cells indeed contained high levels of enrofloxacin, with a C/E ratio within the range described for the uptake of enrofloxacin.

Phagocytosis was performed by flow cytometry with three different fluorescein-labelled microorganisms and with inert particles (fluospheres) as controls. After the FITC labelling procedure the viabilities of A. pleuropneumoniae, P. multocida, and S. aureus were 80% greater than those of the unlabelled controls. Phagocytosis assays were performed uniformly with various serum concentrations, one concentration of enrofloxacin (0.5 μg/ml), and PMNs and AMs isolated from the same animal. Significant differences (P < 0.001) between PMN- and AM-phagocytizing inert particles or FITC-labelled bacteria were found. Serum increased the uptake of bacteria and particles by AMs or PMNs in a dose-dependent manner (P < 0.001) for all bacteria and particles except P. multocida (P = 0.371). In the latter case, phagocytosis by AMs and PMNs was independent of the serum concentration. Under all variations of serum concentration and particle type, no significant differences in phagocytosis by PMNs or AMs were observed in the presence or absence of enrofloxacin (P > 0.570), as was described by others (8, 12, 23) for other quinolones. As is evident from Fig. 2, different mechanisms are involved in the phagocytosis of the microorganisms; e.g., S. aureus is easily taken up in the presence of NS and no serum is needed to phagocytize P. multocida. It is also clear that enrofloxacin does not interfere with either mechanism.

Reduction of bacterial numbers by enrofloxacin alone was dose dependent. By using 1× the MIC of enrofloxacin and phagocytes, the reduction of bacterial numbers in the presence of enrofloxacin was not significantly different compared with the reduction for controls without enrofloxacin. The following results were obtained with 5× the MIC of enrofloxacin. The MIC of enrofloxacin for A. pleuropneumoniae serotype 9 and P. multocida type A was 0.015 μg/ml, and that for S. aureus 42 D was 0.125 μg/ml. No significant differences in phagocyte viability (established by nigrosine dye exclusion) between treated and control preparations were found when phagocytes were incubated with bacteria at any time except at 3 h when phagocytes were incubated with A. pleuropneumoniae. As a result of A. pleuropneumoniae toxin (7), PMN viability decreased to 60% and AM viability decreased to 30%. In controls without bacteria or with P. multocida and S. aureus, phagocyte viability decreased significantly only for AMs (to an average of 80% at 3 h). The effect of phagocyte death is compensated for by expression of the results as the ratio of the number of CFU of enrofloxacin-treated bacteria to the number of CFU of the non-enrofloxacin-treated counterpart separately for incubation with and without phagocytes (Fig. 3) and is given below as a percentage at 3 h.

S. aureus was reduced to 4.1% ± 4.3% of the original number of bacteria in the presence of enrofloxacin alone. No S. aureus cells were recovered from PMNs (NS) because this bacterium was killed very rapidly by these phagocytes. This shows that porcine PMNs are very efficient in killing S. aureus.

<table>
<thead>
<tr>
<th>Phagocyte</th>
<th>Enrofloxacin concn (μg/ml of medium)</th>
<th>C/E ratio (mean ± SD) [no. of exps]</th>
<th>% Release after 10 min (mean ± SD) [no. of exps]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMs</td>
<td>10 1</td>
<td>4.03 ± 1.71 (5)</td>
<td>81.1 ± 9.0 (3)</td>
</tr>
<tr>
<td>PMNs</td>
<td>10 1</td>
<td>7.84 ± 2.20 (5)</td>
<td>88.5 ± 2.1 (3)</td>
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FIG. 1. PMN chemotaxis (Boydren chamber technique) in the presence of different concentrations of enrofloxacin (EFL) and chemoattractant. Values are means ± standard deviations.
unlike human PMNs, which in the presence of ciprofloxacin (11, 13, 26) or sparfloxacin (12) are bacteriostatic rather than bactericidal (3). Porcine AMs are not capable of intracellular antimicrobial activity against _S. aureus_, with enrofloxacin (at 5× the MIC, NS), AMs reduced the proportion of _S. aureus_ cells to 44.8% ± 15.7% of the original number. Similar results were described for _S. aureus_ and mouse and guinea pig peritoneal macrophages both without fluoroquinolones and with ciprofloxacin (10) and sparfloxacin (8).

Surprisingly, intracellular _S. aureus_ in AMs appeared to be less susceptible to the bactericidal effect of enrofloxacin than extracellular _S. aureus_. In previous studies (8, 10) with _S. aureus_, peritoneal macrophages from different species, and different fluoroquinolones, no direct comparison between intra- and extracellular bactericidal effects was made. How intracellular _S. aureus_ is able to attenuate the intracellular activity of enrofloxacin is not clear since fluoroquinolones are suggested to have an even distribution in the cytoplasm (27).

In contrast to _S. aureus_, porcine PMNs are not able to phagocytize _P. multocida_ (with NS). Porcine AMs do phagocytize _P. multocida_, but intracellular bacterial numbers remained constant. In the presence of enrofloxacin, intracellular bacteria were reduced to 3.4% ± 1.7% of their original number. _P. multocida_ was reduced to 2.5% ± 2.2% of the original number by enrofloxacin alone (without AM), confirming the inability of AMs to kill _P. multocida_ and the excellent intracellular penetration of enrofloxacin activity.

AMs with CPS did phagocytize _A. pleuropneumoniae_, reducing its intracellular numbers to 51.1% ± 17.1% of the original number. The addition of enrofloxacin further reduced the number of _A. pleuropneumoniae_ to 14.7% ± 0.1% of the original number. Since in the absence of AMs enrofloxacin reduced the number of _A. pleuropneumoniae_ to 57% ± 41.3% of the original number, this shows that enrofloxacin has a potentiating effect on AM-associated killing of _A. pleuropneumoniae_ in the presence of CPS. PMNs with NS phagocytized _A. pleuropneumoniae_ but acted bacteriostatically (120.0% ± 58.1% of the original number). Enrofloxacin-laden PMNs, however, reduced the number of _A. pleuropneumoniae_ to 8.7% ± 9.6% of the original number, and in the presence of CPS the number was reduced to 0.02% (data not shown). As we found for AMs with CPS, in PMNs with NS, enrofloxacin had a large additional effect on intracellular killing of _A. pleuropneumoniae_. By using PMNs with CPS, this effect of enrofloxacin became even more pronounced. Thus, enrofloxacin showed excellent intracellular activity against _A. pleuropneumoniae_ in both AMs and PMNs. However, AMs are bactericidal for _A. pleuropneumoniae_ only in the presence of neutralizing antibodies. Clinically, this means that in serologically naive pigs the enhancing effect of enrofloxacin on the bactericidal action of PMNs will be the most relevant.

In conclusion, this study showed that enrofloxacin accumulated in porcine PMNs and AMs and had no effect on chemo-taxic action of porcine PMNs. Furthermore, enrofloxacin did not inhibit the phagocytosis of _A. pleuropneumoniae_, _P. multocida_, _S. aureus_, or fluorospheres by porcine PMNs or AMs. It was also observed that this antimicrobial agent is active intracellularly against _A. pleuropneumoniae_ and _P. multocida_. Intracellular _S. aureus_ in AMs was less susceptible than extracellular _S. aureus_ to the bactericidal effect of enrofloxacin. Enrofloxacin did not interfere with the intracellular killing of _A. pleuropneumoniae_ by PMNs and AMs; moreover, an additive effect of enrofloxacin was seen. _A. pleuropneumoniae_ is an important pathogen of swine and is not killed efficiently by phagocytic action alone. The present results suggest that enrofloxacin is particularly well suited for use in the treatment of _A. pleuropneumoniae_ infections in pigs.

**FIG. 2.** Effects of different serum concentrations on the phagocytosis of _A. pleuropneumoniae_, _P. multocida_, _S. aureus_, and fluorospheres by porcine phagocytes. Values are means ± standard deviations. △, AMs; ■, PMNs.
**ACKNOWLEDGMENT**

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**REFERENCES**