Effects of Sub-MICs of Antibiotics on Cell Surface Characteristics and Virulence of Pasteurella multocida

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Received 9 April 1992/Accepted 15 July 1992

The effects of sub-MICs of certain antibiotics, namely, penicillin G, tetracycline, and trimethoprim-sulfamethoxazole, on the cell surface characteristics and the virulences of two toxigenic isolates of Pasteurella multocida representing capsular types A and D were evaluated. Expression of proteins, in particular, outer membrane proteins and iron-regulated proteins, was not affected by exposure of bacterial cells to low concentrations of antibiotics. However, exposition of surface antigens was modified by sub-MICs of the antibiotics tested. The lipopolysaccharide profile of one isolate (capsular type D) was altered by penicillin G. Sub-MICS of penicillin G and tetracycline diminished the virulence of the capsular type A isolate and adherence to porcine tracheal rings of the capsular type D isolate. Production of dermonecrotic toxin was not affected by sub-MICS of the antibiotics tested. Our results indicate that growth of P. multocida in the presence of low concentrations of antibiotics seems to have, depending on the isolate, profound effects on cell surface characteristics, with concomitant effects on adherence or virulence. Our results also indicate that production of dermonecrotic toxin, an important virulence factor of P. multocida isolates associated with porcine atrophic rhinitis, was not affected by sub-MICS of the antibiotics studied.

Porcine atrophic rhinitis is a multifactorial disease complex that is characterized by severe necrosis of the epithelia of the upper respiratory tract and by deformity and reduction of the nasal turbinates and snout, both in volume and in size (4, 29). Bordetella bronchiseptica and Pasteurella multocida are each capable of causing atrophy of nasal turbinates in pigs, but the severity and persistence of the changes they induce are different (29). Infection with both B. bronchiseptica and P. multocida leads to more severe lesions than does infection with either microorganism alone.

Pathogenic bacteria, such as P. multocida, possess characteristics that allow them to infect and survive within the host. Porcine isolates of P. multocida usually possess a carbohydrate capsule, which may vary in size (12) and in composition (e.g., capsular types A and D) (3). Other important molecules that are also found at the cell surface of P. multocida are lipopolysaccharides (LPSs), which have endotoxic activities (8), and iron-regulated proteins (IRPs), which are usually expressed in vivo and which are probably implicated in iron transport (5, 10, 36). Although adhesion seems to be an important step in the pathogenesis of atrophic rhinitis (24), the adhesins of P. multocida are still unknown. The isolates of P. multocida implicated in atrophic rhinitis also produce a dermonecrotic toxin (DNT) of about 150 kDa (9, 22, 28) which causes characteristic lesions when injected into pigs (30).

Among the variety of antimicrobial agents used in swine, feed medication containing tetracycline alone or in combination with penicillin and sulfonamides can reduce the clinical signs of atrophic rhinitis (25, 29). However, it is not very clear whether bactericidal drug concentrations are achieved on the mucosal of the upper respiratory tracts of pigs. The level of antibiotics in certain areas, such as secretions in the nasal cavity or bronchial tree, may not reach adequate concentrations to prevent multiplication of organisms. Also, levels of antibiotics decrease with time following administration. For example, levels of penicillin G in nasal washings of rabbits at 4 h after intramuscular injection were 0.063 μg/ml, or one-fourth the MIC for 80% of the rabbit P. multocida isolates tested (41).

An increasing number of reports indicate that antibiotics at levels below the MIC (sub-MIC) can affect bacteria in ways other than the expected bactericidal or bacteriostatic action. Sub-MIC antibiotic effects include morphological changes (1, 6, 13, 19), reduction of growth (1, 13, 39), inhibition of enzyme or toxin production (33), and loss of adhesive properties (31, 34).

We have recently shown that the sub-MICS of some of the antibiotics commonly used in the swine industry, namely, penicillin G, tetracycline, and trimethoprim-sulfamethoxazole, can alter the morphology and the growth of P. multocida (13). Sub-MICS of penicillin G, but not sub-MICS of tetracycline or trimethoprim-sulfamethoxazole, markedly reduced the growth of P. multocida. In addition, cells treated with sub-MICS of penicillin G were totally devoid of capsule and filamentation occurred. Treatment with tetracycline or trimethoprim-sulfamethoxazole led to filamentous cells with intact capsules. The purpose of the study described here was to evaluate the effects of sub-MICS of these antibiotics on cell surface characteristics and on the virulences of P. multocida strains of porcine origin.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. Toxigenic P. multocida isolates 1703 (capsular type D) and 23-4 (capsular type A) retrieved from the nasal cavities of pigs with atrophic rhinitis were used in the present study. The MICs of penicillin G, tetracycline, and trimethoprim-sulfamethoxazole (1:5) were determined by the agar dilution method (13, 40). Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bacterial isolates were grown on Mueller-Hinton agar plates containing sub-MICS (i.e., one-half, one-
fourth, or one-eighth the MIC of the antibiotics tested for 18 h at 37°C. Control media without antibiotics were always used in parallel. Bacterial isolates were also grown on Mueller-Hinton agar plates containing 160 μM 2,2'-dipyridyl (iron-restricted conditions) (36), with or without sub-MICs of the antibiotics tested, for 18 h at 37°C.

**Protein profiles.** Bacteria were suspended in solubilization buffer containing 10% glycerol, 5% β-mercaptoethanol, 3% sodium dodecyl sulfate (SDS), 0.0625 M Tris hydrochloride (pH 6.8), and 0.01% bromophenol blue. Samples were heated for 20 min at 100°C. Samples were separated by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) by using stacking gels of 4.5% (wt/vol) polyacrylamide and separating gels of 12.5% (wt/vol) polyacrylamide (16). Samples were electrophoresed at 100 V (stacking gel) and 200 V (separating gel) by using a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, Calif.). Gels were stained with Coomassie brilliant blue R-250 or were stained with silver by the procedure of Morrissey (21) with a 2% glutaraldehyde fixation step added (32).

**OMPs.** Outer membrane proteins (OMPs) were prepared by using sarcosine (5, 36). Briefly, bacteria were harvested in 0.85% (wt/vol) NaCl, washed twice, and resuspended in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid) buffer (pH 7.4). The suspension of cells was passed twice through a French pressure cell at 1,534 kg/cm². Cell debris was removed by centrifugation (1,700 × g for 20 min). The supernatant was centrifuged at 100,000 × g for 60 min at 4°C. The pellet, which contained the crude membrane extract, was resuspended in 2% sodium lauryl sarcosinate in HEPES buffer, and the solution was incubated at 22°C for 60 min. The detergent-insoluble OMP-enriched extracts, which were obtained by centrifugation at 100,000 × g for 60 min at 4°C, were then washed twice in distilled water, resuspended in solubilization buffer, and stored at −20°C.

**Immunoblotting.** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed as described by Towbin et al. (37). Rabbit antiserum against *P. multocida* whole cells (17) was used as the first antibody, and goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate (Bio-Rad) was used as the second antibody. The reaction was revealed by the addition of 4-chloro-1-naphthol. Prestained low-range molecular weight markers (Bio-Rad) were used.

**LPS profiles.** Boiled bacterial cells in solubilization buffer were treated with proteinase K (1 mg/ml; Sigma). Samples were then applied onto a stacking gel of 4.5% (wt/vol) polyacrylamide and a separating gel of 15% (wt/vol) polyacrylamide. Running conditions were as described above. The silver-staining procedure of Tsai and Frasch (38) was used.

**Immunoelectron microscopy.** Bacteria were harvested in phosphate-buffered saline (PBS; 0.01 M, pH 7.2). Single drops of bacterial suspension were placed on Formvar-coated grids and were allowed to partially air dry. The grids were then placed sequentially on drops of PBS containing 1% (wt/vol) egg albumen for 5 min and rabbit antiserum raised against *P. multocida* for 30 min. They were then washed in distilled water and placed on drops of colloidal gold particles (10 nm) conjugated to goat anti-rabbit IgG (Sigma) for 30 min. After a final wash in distilled water, they were stained with 0.2% (wt/vol) phosphotungstic acid (pH 7.1) and were examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV. The number of labeled cells, out of 100 cells per grid, was determined; results were compared for statistical significance by Student's t test.

**Surface-exposed antigens.** The surface-exposed antigens of *P. multocida* were examined by the method of Kadurugamuwa et al. (14). Briefly, cells were exposed to *P. multocida* antiserum, disrupted with Zwittergent 3-14 (Calbiochem, San Diego, Calif.), and then transferred to a protein A-Supersol column (Pharmacia, Uppsala, Sweden). The antigen-antibody complexes were eluted by using citric acid (0.1 M, pH 3). The eluate was dialyzed for 48 h against distilled water, lyophilized, and resuspended in solubilization buffer.

**Virulence in mice.** Tests for virulence were performed with 19- to 21-g CD1 male mice by using the intraperitoneal route (2). Bacteria were grown overnight, harvested, and suspended in PBS to a concentration of approximately 10⁷ CFU/ml. Virulence was estimated by determining the 50% lethal dose with groups of five mice (20).

**DNT production.** DNT production was evaluated by an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies M815 and E816 (Dakopatts, Glostrup, Denmark), as described by Foged et al. (9).

**Adherence to porcine tracheal rings.** Adherence of *P. multocida* to porcine tracheal rings maintained in culture was measured as described previously (2, 7). Adherence was measured after 1 h of incubation with an inoculum of approximately 10⁸ CFU/ml. Results were compared for statistical significance by Student's t test.

**RESULTS**

No significant changes were observed in the protein profiles of lysates of cells exposed to sub-MICs of the antibiotics tested (Fig. 1A). When isolate 1703 was grown under iron-restricted conditions, IRPs were apparent (Fig. 1A, lane 2). These IRPs were not affected by sub-MICs of antibiotics (Fig. 1A, lanes 3 to 5). The protein profiles of cells grown on iron-repleted media (Fig. 1A, lane 6) were similar to the profiles of control cells grown under iron-sufficient conditions. Similar results were obtained for isolate 23-4.

OMPs were extracted from untreated cells and cells exposed to sub-MICs of antibiotics under iron-restricted conditions (Fig. 1B). The OMP profiles were not significantly affected by the antibiotics tested. Four high-molecular-mass IRPs (102, 91, 87, and 81 kDa) present in the OMP extracts were not affected by the antibodies tested. Furthermore, immunoblotting experiments performed with these OMP extracts revealed that sub-MICs of antibiotics did not seem to modify the reactivities of major OMPs with antibodies (Fig. 2).

LPS profiles of cells exposed to sub-MICs of antibiotics were similar to the profiles of control cells (Fig. 3) except for that of isolate 1703 grown in the presence of penicillin G, from which some bands corresponding to the longest O chains were missing (Fig. 3, lane 6).

Surface antigens were investigated by immunogold labeling and electron microscopy (Fig. 4). The frequency of labeled cells decreased significantly (from 76 to 50%; P < 0.05) when isolate 1703 was exposed to trimethoprim-sulfafoxazole, but increased (to 100%; P < 0.05) after growth in the presence of penicillin G. Growth of isolate 23-4 in the presence of sub-MICs of the antibiotics tested did not affect the percentage of labeled cells.

In order to study the accessibility of surface-exposed antigens to antibodies, bacterial cells were incubated with antiserum against *P. multocida*, and the surface antigen-
antibody complexes were purified by affinity chromatography. Results showed that both isolates had one or two major surface-exposed antigens (Fig. 5). For isolate 1703, an antigen of 40 kDa was less accessible to antibodies at the surface of cells grown in the presence of penicillin G (Fig. 5A, lane 4), and another one (42 kDa) was more accessible for antibodies at the surfaces of cells grown in the presence of tetracycline (Fig. 5A, lane 5). An antigen of 34 kDa was more accessible on cells of isolate 23-4 grown in the presence of the three antibiotics used in the study (Fig. 5B, lanes 4 to 6).

The influence of the antibiotics tested on adherence to porcine respiratory tract cells was evaluated by using tra-
DISCUSSION

Until now, little was known about the influence of sub-MICs of antibiotics on *P. multocida*, an important swine pathogen. We previously reported that the growth and the morphology of *P. multocida* can be affected by sub-MICs of antibiotics (13). We found that *P. multocida* exposed to penicillin G was totally devoid of capsular material and that treatment with penicillin G, tetracycline, or trimethoprim-sulfamethoxazole induced filamentation. The aim of the present work was to study the cell surface characteristics and the virulence of *P. multocida* after treatment with low concentrations of antibiotics commonly used in the swine industry, namely, penicillin G, tetracycline, and trimethoprim-sulfamethoxazole.

The first part of the study was designed to compare the cell surface components of treated and untreated cells. We did not see many significant changes in whole-cell protein profiles. In order to ascertain whether there were some modifications in the outer membranes of the bacterial cells, we extracted OMPs. Other investigators (18) have reported that *Escherichia coli* OMPs can be affected after treatment with sub-MICs of antibiotics. Our results showed that the major OMPs of *P. multocida* did not seem to be modified after growth in the presence of antibiotics. We also noted the presence of IRPs when the isolates were grown under iron-restricted conditions. These IRPs, similar to IRPs described by other investigators (5, 36), were not affected by sub-MICs of the antibiotics tested.

In *E. coli*, it has been shown that some antigens can be affected by antibiotics (15). We looked at *P. multocida* OMPs to see whether sub-MICs of antibiotics might modify OMP conformation and antigen presentation. Using immuno-blot, we noted that the reactivities of the major OMPs with antibodies did not seem to be modified after growth in the presence of sub-MICs of the antibiotics tested. To examine further the cell surface antigens, we performed immunogold labeling. Exposure to low concentrations of antibiotics seemed to affect the labeling of cells for isolate 1703, although not for 23-4. To get a more precise idea of which surface-exposed antigens were affected, we used an electrophoretic method described by Kadurugamuwa et al. (14), who have shown that, in *Klebsiella pneumoniae*, some antigens become more accessible to antibodies when the bacteria are treated with cephalosporins. Our results suggest that some surface-exposed antigens of *P. multocida* are indeed affected by low concentrations of antibiotics. Depending on the isolate, antibodies can increase or decrease the accessibilities of antigens to antibodies.

We also looked at the LPS profiles of control cells as well as those of cells exposed to sub-MICs of antibiotics. As described previously (20, 27), LPSs of *P. multocida* were rough (isolate 23-4) or semirough (isolate 1703) and migrated in the same region as LPSs of the *Salmonella minnesota* mutant Ra. There were no differences in the LPS profiles of treated and untreated cells of isolate 23-4. With isolate 1703 exposed to penicillin G, the LPS molecules were truncated, and bands in the ladderlike pattern of LPS were missing. Sub-MICs of penicillin G may interfere with the biosynthesis or assembly of LPS of *P. multocida*, as was observed with *E. coli* (23).

Adherence of *P. multocida* to porcine respiratory tract cells is reportedly an important step in the pathogenesis of atrophic rhinitis (24). Adherence of *P. multocida* 1703 was significantly diminished by penicillin G and, to a lesser
TABLE 1. Adherence to porcine tracheal rings, virulence in mice, and DNT production of P. multocida 1703 and 23-4 grown in the presence of one-half the MIC of penicillin G, tetracycline, or trimethoprim-sulfamethoxazole

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>P. multocida 23-4</th>
<th>P. multocida 1703</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adherence&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>83 ± 17</td>
<td>1,000</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>79 ± 5</td>
<td>23,000</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>100 ± 6</td>
<td>&lt;10</td>
</tr>
</tbody>
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<sup>a</sup> Adherence to porcine tracheal rings after incubation for 1 h. The results are expressed as a mean percentage of the control ± standard deviation.
<sup>b</sup> Virulence in mice (50% lethal dose [LD<sub>50</sub>]) expressed as the number of CFU.
<sup>c</sup> DNT production was determined by ELISA.
<sup>d</sup> Significant differences (P < 0.01) were observed between the adherence of bacterial cells exposed to an antibiotic and the adherence of control, untreated bacterial cells.

material, an important virulence factor of P. multocida (4, 11). For isolate 1703, perhaps the capsular material is not as important as it is with the capsular type A isolate, and other factors contribute to the virulence of this isolate. It is known that P. multocida capsular type A isolates are covered by a much thicker capsular material layer than that covering capsular type D isolates (12). Previous studies have shown that sub-MICs of antibiotics can affect the production of toxins (35). We found that DNT production, one of the most important virulence factors of P. multocida (4, 30), was not affected by growth in the presence of sub-MICs of antibiotics.

This is the first study which dealt with the effects of sub-MICs of commonly used antibiotics on the cell surface characteristics and virulence of P. multocida. Our results indicate that although expression of proteins, in particular, OMPs and IRPs, was not significantly affected by sub-MICs of three important antibacterial agents, the LPS of one isolate was altered and exposition of surface antigens of both isolates seemed to be modified. Furthermore, our results indicate that sub-MICs of some antibiotics can affect the virulence or adherence of P. multocida to respiratory tract cells, although the responses differ for different isolates. Finally, the production of DNT, an important virulence factor associated with atrophic rhinitis, was not modified by growth in the presence of low concentrations of antibiotics.

ACKNOWLEDGMENTS

This work was supported by a grant (2375) from Conseil de Recherche en Pêche et Agro-alimentaire du Québec. A. Lebrun is the recipient of a studentship from Conseil de Recherche en Pêche et Agro-alimentaire du Québec.

We thank Bernadette Foiry for excellent technical assistance, Daniel Dubreuil for helpful discussions, and Charles Dozois for reviewing the manuscript.

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