INFECTION AND IMMUNITY, Sept. 1996, p. 3905–3907 Vol. 64, No. 9
0019-9567/96/$04.00+0

Influence of *Actinobacillus pleuropneumoniae* and Its Metabolites on Porcine Alveolar Epithelial Cells

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Received 18 March 1996/Returned for modification 18 April 1996/Accepted 17 June 1996

The effect of *Actinobacillus pleuropneumoniae* and its metabolites on the viability of porcine alveolar epithelial cells was studied by using a neutral-red uptake test. Alveolar epithelial cells were obtained from 5-week-old colostrum-deprived pigs. The purity of these cells as assessed by the modified Papanicolaou stain was 90 to 95%. Incubation of these cells with 10^6 CFU of a biotype 1 serotype 1 strain resulted in death of the alveolar epithelial cells within 1.5 h. A cytotoxic effect was also seen when alveolar epithelial cells were incubated with sterile culture supernatants of biotype 1 serotype 1, biotype 1 serotype 10, and biotype 2 serotype 2 strains or with ApxI, ApxII, or ApxIII produced by recombinant *Escherichia coli*. Incubation of alveolar epithelial cells with a knockout mutant of the biotype 1 serotype 1 parent strain which is unable to secrete Apx toxins or with its supernatant did not result in death of these cells. These results indicate that cytotoxicity is at least in part due to production of Apx toxins.

Actinobacillus pleuropneumoniae is the causative agent of porcine contagious pleuropneumonia. The peracute and acute forms of this disease are characterized by a hemorrhagic necrotizing pneumonia. Histologic examination of pneumonic lesions shows fibrin, neutrophils, and macrophages in the alveoli, a diffuse increase in lung interstitial tissue volume, and multiple focal areas of necrosis (1). It has been suggested that excessive oxygen radical production by Apx-stimulated lung macrophages and neutrophils contributes to the extensive lung lesions that occur in pleuropneumonia (6, 7). In the present experiments, the direct effect of *A. pleuropneumoniae* and its Apx toxins on the viability of porcine alveolar epithelial cells was studied by using a neutral-red uptake test.

First, a technique for collection and cultivation of porcine alveolar epithelial cells was developed. For collection of the cells, three balanced salt solutions (BSS) were used. BSS1, pH 7.4, contained 134 mM NaCl, 5.3 mM KCl, 2.2 mM Na_2HPO_4, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and 5.6 mM glucose. BSS2 was BSS1 with the addition of 1.9 mM $CaCl_2$ and 1.3 mM $MgSO_4$. BSS3 (elastase BSS) was BSS2 with the addition of 3 to 6 U of porcine pancreas elastase (type IV; Sigma Chemical Co, St. Louis, Mo.) per ml.

Alveolar epithelial cells were obtained from 3-week-old, pathogen-free, hysterectomy-derived pigs, using a modification of the technique of Dobbs et al. (4). They were sedated by an intramuscular injection of azaperone at 2 mg/kg of body weight and then euthanized by an intravenous injection of an overdose of sodium thiopental. The pigs were bled and the lungs were excised. Lungs were perfused with BSS2 via the truncus pulmonalis until they were free of blood. The lungs were then washed three times via the trachea with a volume equal to total lung capacity of BSS1 and twice with BSS2 to remove macrophages. Lungs were then inflated with elastase BSS, incubated for 25 min at 37°C, and minced, and the lung pieces were suspended in BSS1 containing 10% fetal calf serum (FCS) to block elastase activity. After gentle shaking of the suspension, it was filtered through two layers of 150-μm and one layer of 40-μm gauze Nitex mesh (Solana, Schoten, Belgium). Cells were centrifuged at 130 × *g* for 10 min, and the supernatant was discarded. The pellet was resuspended gently in 40 ml of Dulbecco's modified Eagle medium (MEM) with 4,500 mg of glucose per liter but without sodium pyruvate (Gibco Europe, Merelbeke, Belgium). To remove alveolar macrophages, the cell suspension was plated onto pig immunoglobulin G-coated hydrophobic surfaces as described by Dobbs et al. (4). Briefly, 4 ml of a porcine immunoglobulin G (Gibco) solution (500 μg/ml) was used to coat bacteriologic plastic dishes. After the plates were washed, 5 ml of the cell suspension was added to each plate. Plates were incubated for 1 h at 37°C in a 5% CO_2 atmosphere, and unattached cells were removed and centrifuged at 130 × *g* for 10 min. These cells were kept at 4°C, counted, and resuspended in MEM at 2×10^6/ml. This medium contained equal volumes of Dulbecco's MEM with 4,500 mg of glucose per liter but without sodium pyruvate and Dulbecco's MEM with 4,500 mg of glucose per liter and 25 mM HEPES but without sodium pyruvate (Gibco). It was supplemented with 30% FCS, 0.2 ml of L-glutamine, and 10% dimethyl sulfoxide. The cells were frozen with a controlled-rate freezer and stored in liquid nitrogen. For use in the experiments, frozen cells were thawed quickly in a water bath at 37°C. Thawed samples were washed once with phosphate-buffered saline solution (PBSS) containing 10% FCS at 4°C and twice with PBSS at 4°C. The cells were counted and their viability was determined by trypan blue exclusion. They were resuspended in MEM supplemented with 10% FCS and 10% L-glutamine (supplemented MEM) at 10^6 viable cells per ml.

To identify type II alveolar epithelial cells in cell suspensions, a modified Papanicolaou stain (3) was used. This stain

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allows visualization of lamellar bodies, which are characteristic for type II cells.

In this study *A. pleuropneumoniae* biotype 1 serotype 1 reference strain S4047 (which produces ApxI and ApxII) (9), its knockout mutant (which is unable to secrete toxins) (9), biotype 1 serotype 10 strain 13039 (which produces ApxI only), and biotype 2 serotype 2 strain 2168/1 (which produces ApxII only) were used. The biotype 1 serotype 10 and biotype 2 serotype 2 strains were isolated from pigs in Belgium that had died from pleuropneumonia. The generation of the knockout mutant has been described previously (9).

The effect of crude toxin produced by these strains and Apx toxin produced by recombinant *Escherichia coli* on alveolar epithelial cells was studied. For production of crude toxin, 10^4 CFU of each *A. pleuropneumoniae* strain was inoculated in RPMI 1640 supplemented with 10% nonessential amino acids, 10% L-glutamine, 10% FCS, 1% sodium pyruvate, 10^{-2} mol of $CaCl_2$ per liter, 10% yeast extract, and 0.03% NAD. Bacteria were grown for 12 h at 37°C on a rocking platform. The suspensions were then centrifuged at 10,000 × *g* for 3 min and the resulting supernatant was filtered through a 0.2-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.). The production and collection of sterile ApxI-, ApxII-, and ApxIII-containing supernatants of logarithmically growing cultures of recombinant *E. coli* cells has been described elsewhere (8, 10, 12). Alveolar epithelial cells, 10^5 viable cells in 100 μl of supplemented MEM, were added to wells of a 96-well microtiter plate and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. Subsequently, 100 μl of a twofold dilution (in supplemented MEM) of crude toxin preparation or supernatant of recombinant *E. coli* was added. Controls consisted of alveolar epithelial cells in 200 μl of supplemented MEM. The plates were incubated for 2 h at 37°C. Then, 25 μl of a 0.1% neutral red solution was added to the cups and neutral-red uptake and cytotoxic titers were determined as described previously (6).

To study the effect of *A. pleuropneumoniae* serotype 1 on alveolar cells, these cells were cultured for 2 h in 96-well microtiter plates as described above. Then, 10^6 CFU of biotype 1 serotype 1 strain S4047 or its knockout mutant in 100 μl of supplemented MEM was added. To study the effect of inactivated bacteria on alveolar epithelial cells, 10^6 CFU of strain S4047 per 100 μl was inactivated (20 min at 56°C). The inactivated bacteria were washed once in PBSS and resuspended in the same volume of supplemented MEM. Then, 100 μl of the inactivated strain S4047 was added to the alveolar epithelial cells. Controls consisted of alveolar epithelial cells in 200 μl of supplemented MEM. After 1, 1.5, 2, and 3 h of incubation at 37°C in a 5% CO_2 atmosphere, neutral red uptake was assessed as described above.

Neutralization tests were carried out with inactivated monospecific sera of rabbits immunized against ApxI or ApxII produced by recombinant *E. coli*. Preimmune sera obtained from these rabbits before immunization were used as negative control sera. Twofold dilutions in supplemented MEM of crude toxin preparations were incubated for 2 h at 4°C with equal volumes of 1/4-diluted negative control serum, 1/4-diluted serum against ApxI, 1/4-diluted serum against ApxII, or a 1/1 mixture of 1/2-diluted serum against ApxI and ApxII. Then, 200 μl was added to alveolar epithelial cells that had been incubated for 2 h in microtiter plates as described above. Controls consisted of alveolar epithelial cells in 200 μl of supplemented MEM and alveolar epithelial cells incubated with 100 μl of supplemented MEM and 100 μl of sera only. The plates were incubated for 2 h at 37°C, and neutral-red uptake was assessed as described above.

Approximately 5×10^6 to 6×10^7 cells were obtained from each lung, and 90 to 95% were type II alveolar epithelial cells, as determined by the modified Papanicolaou stain. After storage in liquid nitrogen and thawing, viability of the alveolar epithelial cells was 50 to 60% as determined by trypan blue exclusion.

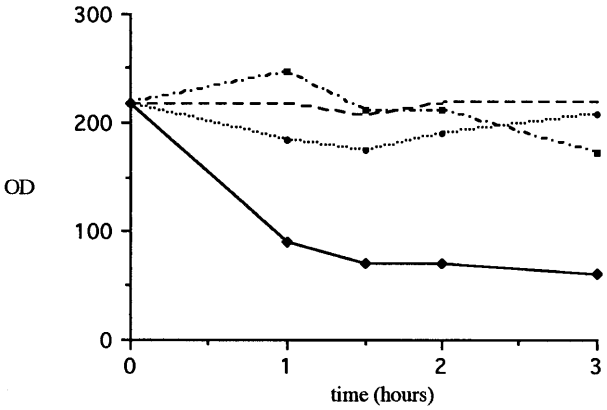

FIG. 1. Neutral-red uptake of alveolar epithelial cells after incubation with viable biotype 1 serotype 1 strain S4047 (◆), viable knockout mutant (●), heat-inactivated biotype 1 serotype 1 strain S4047 (■) or supplemented MEM (– – – – –). OD, optical density (10^{-3}).

Cytotoxic titers of the supernatant of the biotype 1 serotype 1 strain S4047, the biotype 1 serotype 10 strain, and the biotype 2 serotype 2 strain were 32 to 128, 128 to 256, and 8, respectively. Cytotoxicity was not observed with supernatant of the knockout mutant. Cytotoxic titers of the supernatants of recombinant *E. coli* containing ApxI, ApxII, and ApxIII were 8-16, 4, and 32, respectively.

The viability of alveolar epithelial cells at various times during incubation with biotype 1 serotype 1 strain S4047, its knockout mutant, and heat-inactivated biotype 1 serotype 1 strain S4047 or supplemented MEM is shown in Fig. 1. Most alveolar epithelial cells were killed within 1.5 h after incubation with viable biotype 1 serotype 1 strain S4047 but not after incubation with the knockout mutant or inactivated biotype 1 serotype 1 strain S4047.

When crude toxin preparations of the biotype 1 serotype 1 strain S4047, the biotype 1 serotype 10 strain, and the biotype 2 serotype 2 strain were preincubated with the mixture of serum against ApxI and ApxII, cytotoxic titers declined by a factor of at least four. The cytotoxicities of the toxin preparations of the biotype 1 serotype 10 strain and the biotype 2 serotype 2 strain were also neutralized by sera against ApxI and ApxII, respectively.

The results of the present studies demonstrate that *A. pleuropneumoniae* and its metabolites are able to kill type II alveolar epithelial cells. This toxic effect on alveolar epithelial cells together with toxic and other effects already described for endothelial cells and phagocytes (6, 7, 11) are most likely responsible for the extensive lung lesions that occur in pleuropneumonia.

Several findings in these experiments show that toxicity for alveolar epithelial cells is at least in part due to production of Apx toxins. Viable *A. pleuropneumoniae* serotype 1 strain S4047 killed these cells, whereas inactivated bacteria and a knockout mutant which did not secrete Apx toxins did not. Furthermore, cytotoxic factors could be filtered from the culture medium, and their effects were reproduced by ApxI, ApxII, and ApxIII produced by recombinant *E. coli*. Finally, rabbit sera against ApxI and ApxII were able to neutralize the cytotoxicity of crude toxin preparations.

In the present studies the effect of *A. pleuropneumoniae* and its metabolites was tested only for type II alveolar epithelial cells. These cells make up 15% of all lung cells, although they cover less than 5% of the alveolar surface. They perform many important functions within the lung, including regulation of surfactant metabolism, ion transport, and alveolar repair. For the latter function, they transform into type I alveolar epithelial cells. In the present studies, cells were cultivated for only a short period before being used. It is possible that longer cultivation periods would result in in vitro differentiation of type II cells to type I cells, as has already been described for alveolar epithelial cells obtained from rabbits (2) and rats (5). Further studies are necessary to determine if type I cells are equally susceptible.

The technical assistence of N. Van Loocke, N. Van Rysselberghe, S. Mekeirle, J. Vekeman, and J. Vermeulen is gratefully appreciated.

This work was supported by the Institute for the Encouragement of Research in Industry and Agriculture, Ministry of Agriculture, Brussels, Belgium.

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Editor: P. E. Orndorff