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Endobronchial Inoculation with Apx Toxins of *Actinobacillus pleuropneumoniae* Leads to Pleuropneumonia in Pigs

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To establish the role of the Apx toxins in the pathogenesis of porcine pleuropneumonia, specific-pathogen-free pigs were inoculated deeply endobronchially with either culture filtrates of *Actinobacillus pleuropneumoniae* serotype 8 or 9, culture filtrates depleted of the Apx toxins by affinity chromatography, depleted culture filtrate supplemented with purified recombinant Apx toxins (rApx), or purified rApx toxins alone. Results of these experiments indicate that ApxI, ApxIII, and, to a lesser extent, ApxII are the bacterial factors that trigger the development of clinical symptoms and lung lesions typical for porcine pleuropneumonia.

Actinobacillus pleuropneumoniae causes severe hemorrhagic necrotizing pleuropneumonia in pigs. Clinical symptoms of the disease include lethargy, vomiting, fever, and labored and abdominal breathing. The lung lesions are characterized by cell infiltrate, exudation of fibrin, fibrin deposits in blood and lymph vessels, hemorrhages, and necrosis (18). The disease can be experimentally reproduced in pigs with viable *A. pleuropneumoniae* but also with sonicated *A. pleuropneumoniae* or bacterium-free culture supernatant of *A. pleuropneumoniae* (15, 19, 23).

Culture supernatant of *A. pleuropneumoniae* strains hemolyze erythrocytes and lyse alveolar macrophages and polymorphonuclear leukocytes (2, 10). These activities are due to the exotoxins of *A. pleuropneumoniae*. Three exotoxins, ApxI, ApxII, and ApxIII, have been identified. ApxI is strongly hemolytic and strongly cytotoxic, ApxII is weakly hemolytic and moderately cytotoxic, and ApxIII is not hemolytic but strongly cytotoxic (4, 9). All *A. pleuropneumoniae* strains produce one or two of these toxins (1, 11).

The Apx toxins are considered to be important virulence factors. Mutants of *A. pleuropneumoniae* serotypes 1 and 5 that lack ApxI and ApxII do not kill macrophages or neutrophils (6, 8, 21) and do not cause disease or lung lesions in pigs (6, 21). The importance of the Apx toxins in the pathogenesis of porcine pleuropneumonia is further suggested by results of experiments in which pigs were endobronchially inoculated with culture supernatant of *A. pleuropneumoniae* serotype 9. This culture supernatant induced lung lesions similar to those of a natural infection with *A. pleuropneumoniae*, but after heating at 100°C, the culture supernatant lost its hemolytic and cytotoxic activities and did not induce lung lesions (23). Whether the Apx toxins alone can induce pleuropneumonia or whether other factors of *A. pleuropneumoniae* present in the culture supernatant are also involved is not known.

This paper describes experiments in which pigs were inoculated deeply endobronchially with either culture filtrates of *A. pleuropneumoniae* serotype 8 or 9, culture filtrates depleted of the Apx toxins by affinity chromatography, depleted culture filtrate supplemented with purified recombinant Apx toxins (rApx), or purified rApx toxins alone.

For toxin production, strains 405 and 13261, reference strains of *A. pleuropneumoniae* serotypes 8 and 9, were cultured in Eagle minimal essential medium plus Earle salts (EMEM; Flow Laboratories, Irvine, England) supplemented with 10% Serum Plus (Hazelton Research Products, Lenexa, Kans.) as described earlier (10). The culture supernatants were filtered through a 0.2-μm-pore-size filter, concentrated 10-fold (Minitan system; Millipore, Bedford, Mass.), and stored at −20°C. Because the biological activity of the Apx toxins is labile, two small samples were frozen separately to enable determination of the hemolytic and cytotoxic activities on the day before preparation of the inocula. As a control, EMEM with 10% Serum Plus was concentrated 10-fold and frozen at −20°C.

For production of rApx toxins, *Escherichia coli* LE 392 containing the *hlyBD* secretion genes of *E. coli* on plasmid pLG575 (5) and either the *apxICA*, *apxIICA*, or *apxIIICA* gene on plasmid pUC19 was grown in Luria broth with 10% fetal calf serum to an optical density at 600 nm of 0.8 to 1.0 (7, 20). The cultures were centrifuged, and the supernatants were filtered through a 0.2-μm-pore-size filter.

The absence of bacteria in all preparations was checked on sheep blood agar supplemented with NAD.

Monoclonal antibodies CVI-Apcly 2.2, 9.2, and 9.3 specific for ApxIII, ApxI, and ApxII, respectively (9), were coupled to CNBr-activated Sepharose 4B in accordance with the instructions of the supplier (Pharmacia, Uppsala, Sweden). Columns were used to purify the rApx toxins from the *E. coli* supernatant or to remove the Apx toxins from the 10-fold-concentrated culture filtrates of *A. pleuropneumoniae*. The columns were eluted with 0.1 M glycine-HCl (pH 2.8), and the fractions of the eluate were immediately neutralized with 1 M K_2HPO_4. Fractions of the peak were pooled and stored at −20°C until further use. A small sample of each batch was frozen separately to enable determination of the hemolytic and cytotoxic activities on the day before preparation of the inocula. The purified toxins gave single bands in Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gels.

The hemolytic and cytotoxic activities of all preparations were determined with sheep erythrocytes and porcine alveolar macrophages as described previously (10). Titers were ex-

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pressed as the reciprocal of the $\log_2$ of the highest dilution that gave at least 50% hemolysis or cell death.

To semiquantify the amount of Apx protein, twofold serial dilutions of the inocula were tested in a dot blot assay with monoclonal antibody CVI-Apcly 2.2, 9.2, or 9.3 in accordance with the recommendations of the manufacturer of the dot blot apparatus (Bio-Rad, Richmond, Calif.). The dot blot titer was expressed as the $\log_2$ of the dilution that gave approximately 50% of the maximum color intensity read by the naked eye.

Specific-pathogen-free pigs approximately 11 weeks old from our own breeding colony were deeply endobronchially inoculated (24) with 13 ml of one of the inocula. At 5, 7, 9, 11, 13, 15, and 24 h postinoculation (hpi), the body temperatures of the pigs were measured and they were inspected for the following clinical symptoms: vomiting, lethargy, refusal of food, and dyspnea. At 48 hpi, the pigs were anesthetized and exsanguinated. The lungs were examined for gross pathomorphological lesions. To avoid bias, personnel responsible for clinical inspections and pathological examinations were not informed of the groups to which the animals or tissues belonged. The bronchial lymph nodes and specimens of lung lesions were taken for bacteriological examination to verify the absence of *A. pleuropneumoniae*. Lung specimens from the center and the periphery of the lesions or, when there was no visible lesion, of the suspected inoculation site (24) were taken for histological examination.

Two experiments were conducted, each with nine groups of two animals. In the first experiment, we tested fivefold-concentrated culture filtrate of serotype 9 strain 13261 (R9s), R9s depleted of ApxI and ApxII ($R9s^-$), $R9s^-$ supplemented with purified rApxI ($R9s^-$ + rApxI), $R9s^-$ supplemented with purified rApxII ($R9s^-$ + rApxII), $R9s^-$ supplemented with purified rApxI and rApxII ($R9s^-$ + rApxI+II), purified rApxI (rApxI), purified rApxII (rApxII), purified rApxI and rApxII (rApxI+II), and a placebo that contained EMEM, Serum Plus, glycine, and K_2HPO_4.

In the second experiment, we tested fivefold-concentrated culture filtrate of serotype 8 strain 405 (R8s), R8s depleted of ApxII and ApxIII ($R8s^-$), $R8s^-$ supplemented with rApxII ($R8s^-$ + rApxII), $R8s^-$ supplemented with rApxIII ($R8s^-$ + rApxIII), $R8s^-$ supplemented with rApxII and rApxIII ($R8s^-$ + rApxII+III), purified rApxII (rApxII), purified rApxIII (rApxIII), purified rApxII plus rApxIII (rApxII+III), and a placebo that contained EMEM, Serum Plus, glycine, and K_2HPO_4.

The cytotoxin titers of the 10-fold-concentrated culture filtrate and the batches of purified rApx toxins were determined in the small samples that were frozen separately. On the next morning, concentrated culture filtrate, concentrated culture filtrates depleted of Apx toxins, and the batches of purified recombinant toxins were thawed. Batches of each rApx toxin were pooled, the cytotoxin titers of the pools were calculated, and the final toxin pools were diluted with neutralized elution buffer to the same cytotoxin titer as the corresponding 10-fold-concentrated culture filtrate.

The inocula were prepared in such a way that all contained similar amounts of EMEM, Serum Plus, glycine, and K_2HPO_4. The composition of R9s, R8s, $R9s^-$, and $R8s^-$ was 1 volume of 10-fold-concentrated culture filtrate or 10-fold-concentrated culture filtrate depleted of Apx toxins plus 1 volume of neutralized elution buffer. The composition of $R9s^-$ + rApxI, $R9s^-$ + rApxII, $R8s^-$ + rApxII, and $R8s^-$ + rApxIII was 1 volume of depleted 10-fold-concentrated culture filtrate plus 1 volume of the purified recombinant toxin. The composition of $R9s^-$ + rApxI+II and $R8s^-$ + rApxII+III was 1 volume of depleted 10-fold-concentrated culture filtrate and 0.5 volume of each purified recombinant toxin. The composition of rApxI, rApxII, and rApxIII was 1 volume of 10-fold-concentrated EMEM + Serum Plus and 1 volume of the purified recombinant toxin, and that of rApxI+II and rApxII+III was 1 volume of 10-fold-concentrated EMEM + Serum Plus and 0.5 volume of each of the purified recombinant toxins.

TABLE 1. Apx protein contents and hemolytic and cytotoxic activities of inocula used in this study

Bacterium and inoculum	Dot blot titer			Hemolytic activity[d]	Cytotoxic activity[d]
	ApxI	ApxII	ApxIII		
Serotype 9					
R9s[a]	6	4	NT[b]	7	9
$R9s^-$[a]	<1[c]	<1	NT	<1	<1
$R9s^-$ + rApxI	11	<1	NT	8	9
$R9s^-$ + rApxII	<1	8	NT	1	8
$R9s^-$ + rApxI+II	10	8	NT	8	8
rApxI	11	<1	NT	8	9
rApxII	<1	8	NT	1	7–8
rApxI+II	10	8	NT	8	8
Placebo	0	0	NT	0	0
Serotype 8					
R8s[a]	NT	6	4	<1	18
$R8s^-$[a]	NT	<1	<1	0	<1
$R8s^-$ + rApxII	NT	7	<1	1	10
$R8s^-$ + rApxIII	NT	<1	9	0	14
$R8s^-$ + rApxII+III	NT	6	9	<1	14
rApxII	NT	7	<1	1	10
rApxIII	NT	<1	9	0	14
rApxII+III	NT	6	9	<1	14
Placebo	NT	0	0	0	0

[a] R9s or R8s, fivefold-concentrated culture filtrate of serotype 9 or 8, respectively, that contains Apx toxins. $R9s^-$ or $R8s^-$, R9s or R8s depleted of Apx toxins.

[b] NT, not tested.

[c] A titer of <1 means that an effect is visible but there is less than 50% of the maximum color intensity, hemorrhage, or cell death.

[d] Hemolytic and cytotoxic activities are expressed as the reciprocals of the $\log_2$ of the highest effective dilutions.

Specimens of the inocula were kept on ice. Directly after the pigs were inoculated, these specimens were tested in the hemolysin, cytotoxin, and dot blot assays.

The experiments were approved by the ethical committee of the Institute for Animal Science and Health in accordance with Dutch law on animal experiments.

Experiment with serotype 9. The R9s inoculum contained ApxI and ApxII and was strongly hemolytic and cytotoxic (Table 1). Affinity chromatography removed nearly all of the ApxI and ApxII proteins from R9s; thus, $R9s^-$ was only very weakly hemolytic and cytotoxic (Table 1). Our aim was to prepare inocula of rApx toxins that had biological activities similar to those of R9s. This was, of course, not possible for the hemolytic activity of those inocula that contained rApxII only because ApxII is a much weaker hemolysin than ApxI. Still, all of the inocula that contained rApxI had a hemolysin titer similar to that of R9 and all of the inocula containing rApx toxins had cytotoxin titers similar to those of R9s (Table 1). The dot blot titers of the inocula containing rApx were, however, higher than those of R9s (Table 1).

Both pigs inoculated with R9s became severely ill; they had fever, vomited, refused to eat, were lethargic, and had dyspnea. Clinical symptoms were first observed at 5 hpi. At 15 hpi, both pigs were still ill but on the next morning, at 24 hpi, they had largely recovered, their temperatures were normal, and they had eaten but their breathing was still labored. In contrast, of

the pigs inoculated with R9S$^-$, one appeared clinically normal whereas the other became slightly depressed and refused to eat from 5 to 11 hpi but was recovered at 13 hpi.

All of the pigs inoculated with R9S$^-$ supplemented with rApxI or rApxI + rApxII or with rApxI or rApxI + rApxII alone also became severely ill. Some had fever, but all were lethargic, refused to eat, and had dyspnea. The clinical symptoms were observed from 5 to 15 hpi. On the next morning, at 24 hpi, all of the pigs appeared clinically normal. Both of the pigs inoculated with R9S$^-$ supplemented with rApxII showed milder disease symptoms. They refused to eat and were lethargic, and their breathing was a little faster than normal. These clinical symptoms were recorded from 5 to 11 hpi, but from 13 hpi on, both pigs appeared clinically normal. Both pigs inoculated with rApxII alone showed even less disease symptoms; one appeared clinically normal, and the other was slightly depressed and refused to eat from 7 to 9 hpi. The pigs inoculated with the placebo were active but refused to eat from 7 to 9 hpi.

At autopsy, both pigs inoculated with R9s had severe fibrinohemorrhagic consolidation of the lung parenchyma and one had fibrinous pleuritis. Histologically, in the lungs of both pigs, we observed multifocal confluent necrosis of the alveolar parenchyma and hemorrhages distending the interlobular septa and into the alveolar space in the center of the lesions. In the periphery of the lesions, alveoli were filled by a severe fibrinopurulent exudation. In contrast to bacterially induced lesions, a demarcation zone of streaming cells around areas of necrosis was absent.

Macroscopically, the lung parenchyma of both pigs inoculated with R9s$^-$ had a grey, firm appearance at the site of inoculation. Histologically, the lungs of both pigs showed mild catarrhal pneumonia and areas of atelectasis. In some small vessels, thrombi were observed.

All of the pigs inoculated with R9s$^-$ supplemented with rApxI or rApxI + rApxII or with rApxI or rApxI + rApxII alone had lung lesions similar to those of the pigs inoculated with R9s (Fig. 1 and Table 2). Both pigs inoculated with R9s$^-$ + rApxII only had moderate catarrhal-purulent pneumonia with few foci of fibrin formation or interstitial bleeding, whereas the pigs inoculated with rApxII alone had very mild lesions only. One had mild catarrhal purulent pneumonia with few hemorrhages in the interlobular septa, and the other had thickened interalveolar septa, similar to the pigs inoculated with R9s$^-$ (Table 2).

The lungs of the two control pigs appeared normal. In one of these pigs, mild exudation of macrophages and neutrophils into alveoli was seen histologically.

Experiment with serotype 8. The culture filtrate of serotype 8 contained ApxII and ApxIII and was weakly hemolytic and strongly cytotoxic (Table 1). Affinity chromatography removed nearly all of the ApxII and ApxIII from R8s; thus, R8s$^-$ was not hemolytic and only very weakly cytotoxic (Table 1). The cytotoxin titers of the inocula that contained rApx toxins were lower than the titer of R8s, whereas their dot blot titers were higher than the titers of R8s (Table 1).

Both pigs inoculated with R8s became severely ill. They had fever, refused to eat, were lethargic, and had dyspnea. Clinical symptoms were observed from 5 to 15 hpi, but on the next morning, at 24 hpi, both pigs appeared clinically normal. In contrast, both pigs inoculated with R8s$^-$ appeared clinically normal except that one had a temperature of 40°C at 15 hpi and the other had not eaten and breathed a little faster than normal at 24 hpi.

Both pigs inoculated with R8s$^-$ supplemented with rApxIII or rApxII + rApxIII or with rApxIII or rApxII + rApxIII alone also became severely ill. They had fever, refused to eat, were lethargic, and had dyspnea. Clinical symptoms were observed from 7 to 15 hpi, but on the next morning, the pigs had largely recovered except that some pigs still showed labored breathing. Both pigs inoculated with R8$^-$ supplemented with rApxII showed milder disease symptoms. One had a temperature of 40.3°C at 9 hpi and breathed faster than normal from 5 to 15 hpi, whereas the other had a temperature of ≥40°C at 7 and 9 hpi (maximum temperature, 40.4°C) and breathed faster than normal at 13 and 15 hpi, but they were active and ate. Both pigs inoculated with rApxII alone showed even less disease symptoms; they breathed a little faster than normal at 11 hpi but otherwise seemed clinically normal.

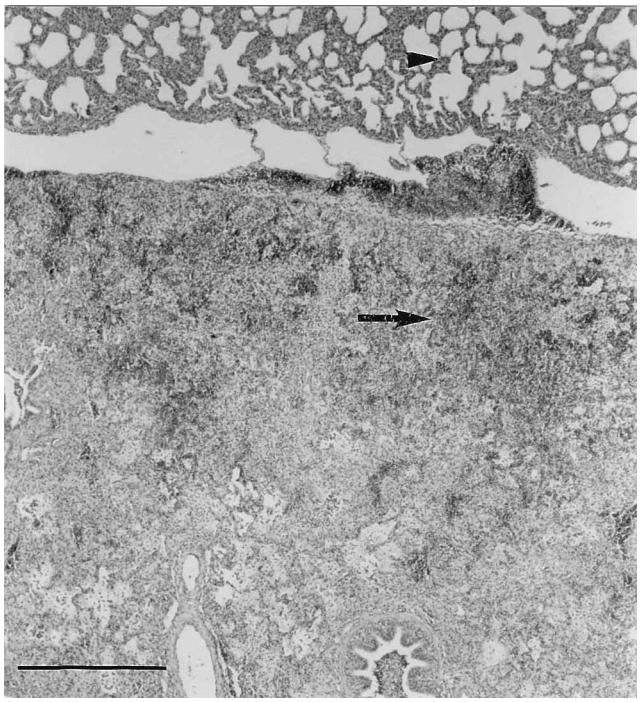

FIG. 1. Lung tissue of a pig inoculated with culture filtrate of serotype 9 depleted of Apx toxins and supplemented with recombinant ApxI (R9s$^-$ + rApxI). It shows lobuli without alterations (arrowhead) adjacent to tissue with severe hemorrhagic necrosis (arrow) and fibrinous exudate in the alveoli. These lesions are similar to the lesions of pigs inoculated with complete culture filtrate of serotype 9 (R9s). Note the absence of the cellular demarcation seen after infection with viable bacteria (12). Bar, 100 μm. Staining was done with hematoxylin and eosin.

At autopsy, both pigs inoculated with R8s had severe fibrinopurulent pneumonia with multiple foci of hemorrhages in the areas of inflammation. Interlobular septa were severely distended by fibrin and serosanguineous fluid. A demarcation zone of streaming cells was absent. Macroscopically, the lungs of the pigs inoculated with R8s$^-$ showed no coherent lesions but multiple small grayish foci in the parenchyma. Histologically, thickening of interalveolar septa, activation of septal cells, and atelectasis were seen.

All pigs inoculated with R8s$^-$ supplemented with rApxIII or with rApxIII or rApxII + rApxIII alone had severe lesions similar to those of the pigs inoculated with R8s, whereas the pigs inoculated with R8s$^-$ supplemented with rApxII + rApxIII or rApxII or with rApxII alone had somewhat milder lesions with fewer hemorrhages (Table 2). The lungs of the control pigs were normal.

Pigs inoculated with bacterium-free culture supernatant of

TABLE 2. Major morphological changes in the lungs

Bacterium and inoculum	Severity[b] of changes in alveolar and interlobular parenchyma					
	Necrosis		Fibrino-hemorrhagic pneumonia		Purulent pneumonia	
	Pig 1	Pig 2	Pig 1	Pig 2	Pig 1	Pig 2
Serotype 9						
R9s[a]	4	4	4	4	4	4
R9s^{-a}	0	0	0	0	2	3
R9s$^-$ + rApxI	3	3	4	3	4	4
R9s$^-$ + rApxII	0	0	1	2	3	3
R9s$^-$ + rApxI+II	3	4	3	3	4	4
rApxI	3	4	3	4	4	4
rApxII	0	0	0	2	0	3
rApxI+II	2	4	4	4	3	3
Placebo	0	0	0	0	0	1
Serotype 8						
R8s[a]	0	0	4	4	3	3
R8s^{-a}	0	0	0	0	0	1
R8s$^-$ + rApxII	0	0	1	2	3	4
R8s$^-$ + rApxIII	0	0	4	4	4	4
R8s$^-$ + rApxII+III	0	0	2	2	4	3
rApxII	0	0	2	1	4	4
rApxIII	0	0	4	4	4	4
rApxII+III	0	0	4	4	4	4
Placebo	0	0	0	0	0	0

[a] R9s or R8s, fivefold-concentrated culture filtrate of serotype 9 or 8, respectively, that contains Apx toxins. R9s$^-$ or R8s$^-$, R9s or R8s depleted of Apx toxins.

[b] The severity of each pathological feature is expressed on an arbitrary scale of 0 to 4. A 0 means no changes, and a 4 means the most severe changes.

serotype 9 or 8 became severely ill, and their clinical symptoms were similar to those caused by experimental infection with viable bacteria (24) except that the onset of the disease was earlier and the duration was shorter. At autopsy, the pigs had pneumonic lesions that were macroscopically and microscopically similar to those of pigs infected with viable *A. pleuropneumoniae* except that the demarcation zone of streaming cells was absent (12). Probably, the absence of bacterial multiplication and constant toxin formation can account for these differences in clinical disease and lung lesions.

The Apx toxins were essential for the development of clinical disease and typical lesions, because pigs inoculated with culture supernatant specifically depleted of Apx toxins were clinically normal or showed only a few mild disease symptoms. At autopsy, these pigs had no lesions or very mild catarrhal bronchopneumonia. Moreover, it could be concluded that rApxI and rApxIII were able to induce clinical disease and pneumonic lesions similar to those of the pigs inoculated with complete culture filtrate, even in the absence of other components in the culture supernatant of *A. pleuropneumoniae*. Clearly, the combination of depleted culture filtrate and/or rApxII with rApxI or rApxIII did not induce more severe disease or more severe pneumonic lesions than rApxI or rApxIII alone. This indicates that ApxI and ApxIII are the most important factors in the culture filtrates that trigger the development of severe clinical pleuropneumonia and typical pneumonic lesions.

The role of ApxII in the induction of clinical disease and typical pneumonic lesions was much less clear, since pigs inoculated with rApxII appeared clinically normal or showed a few mild disease symptoms and only mild lung lesions. When depleted culture filtrate was added, the clinical disease appeared somewhat more severe but the lung lesions were not more severe, suggesting that ApxII is not as important as ApxI and ApxIII. This agrees with the observations of Rycroft et al., who showed that a chemically induced mutant of serotype 2 that produced ApxIII but not ApxII had virulence for pigs that was similar to that of the parent strain (17). However, we did expect to find clinical disease and typical lung lesions after inoculation with rApxII. After all, ApxII is present in almost all serotypes; strains of serotype 7, which produce ApxII but no other Apx toxins, cause clinical disease and typical lung lesions and even mortality after experimental infection (16), and strains of serotype 7 are commonly isolated from natural cases of pleuropneumonia in the United States and Canada (13, 14). Furthermore, a transposon mutant of serotype 1 and a chemically induced mutant of serotype 5 which both produce only ApxII appeared to cause clinical disease and typical lung lesions in pigs. Both mutants were, however, substantially less virulent than their parent strains (6, 21). Finally, although it is the weakest of the three toxins, ApxII has in vitro biological activities similar to those of ApxI and ApxIII. For instance, they lyse alveolar macrophages, are toxic for alveolar epithelial cells, and stimulate polymorphonuclear neutrophils at low concentrations but kill these cells at high concentrations (3, 4, 10, 22).

We aimed at a similar cytotoxic activity for all toxin-containing inocula, but we did not always succeed. In the experiment with serotype 9, the inocula containing rApxII were a little less cytotoxic in vitro than the inocula containing rApxI. In the experiment with serotype 8, differences in cytotoxicity of the inocula were even bigger and the inocula containing ApxII as the sole toxin had the lowest titer. Furthermore, the inocula containing rApx toxins had higher dot blot assay titers than the inocula containing nonrecombinant toxins (Table 1). This indicates that they contained inactive protein. The lower cytotoxic activity of the rApxII inocula and the presence of inactive protein, which may compete with active protein for binding to receptors, for instance, may explain why we found mild disease symptoms and mild lung lesions after inoculation of pigs with inocula that contained rApxII as the sole Apx toxin.

Culture filtrate of serotype 9 and the inocula containing rApxI induced hemorrhagic and necrotic lung lesions, whereas culture filtrate of serotype 8 and the inocula containing rApxIII induced hemorrhagic lesions without necrosis, which are less severe than the former. This suggests that ApxI causes more severe lesions than ApxIII. However, the absence of necrosis is not a consistent finding. We conducted the experiment with serotype 8 twice. The first experiment was not included in this paper because the positive control R8s had a very low titer and induced only minor lesions. In general, the results of this first experiment were very similar to the results of the experiment described in this paper, except that the lesions induced by the inocula that contained rApxIII were hemorrhagic and necrotic. This is in full agreement with results of experiments in which we inoculated pigs with 1,000 viable bacteria of either serotype 8 or 9. Both serotypes induced similar lung lesions with hemorrhages and necrosis.

In conclusion, the results of this study clearly show that ApxI, ApxIII, and, to a much lesser extent, ApxII are the bacterial factors that trigger the development of clinical symptoms and lung lesions typical of porcine pleuropneumonia.

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