NOTES

Convalescent Pigs Are Protected Completely against Infection with a Homologous *Actinobacillus pleuropneumoniae* Strain but Incompletely against a Heterologous-Serotype Strain

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Received 9 May 1994/Returned for modification 21 January 1995/Accepted 4 March 1995

To study whether *Actinobacillus pleuropneumoniae* induces a species-specific immunity, we infected pigs in the left lung with serotype 3 or 9 and after 3 weeks we infected their right lungs with serotype 9. Convalescent pigs were protected against homologous strain reinfection, but after heterologous strain reinfection the degree of protection varied. Neutralizing antibodies were not essential for protection.

Actinobacillus pleuropneumoniae is the causative agent of an often fatal acute fibrino-hemorrhagic pleuropneumonia in pigs. To date, 12 different serotypes of *A. pleuropneumoniae* have been described, and although the prevalence of different serotypes varies widely per geographic region, different serotypes can be isolated in one area or even in one herd (11). It is generally accepted that convalescent pigs develop considerable immunity against the various serotypes of *A. pleuropneumoniae* (12). Among these serotypes heterogeneity exists in *A. pleuropneumoniae* RTX-toxins (Apx) (5). Because of this heterogeneity we expect that pigs convalescing from *A. pleuropneumoniae* infection are less protected against challenge with a heterologous-serotype strain that differs in Apx toxins than against challenge with the homologous strain. Therefore, we infected pigs in the left lung and reinfected the pigs 3 weeks later in the right lung with the homologous or with a heterologous-serotype strain.

Nineteen specific-pathogen-free pigs (8 to 12 weeks old) were anesthetized with a combination of azaperone (Stresnil; Janssen Pharmaceutica B. V., Tilburg, The Netherlands) and metomidate (Hypnodil; Janssen Pharmaceutica). A bronchoscope was advanced through the trachea into the left main bronchus. Thereafter, a small catheter was advanced through the bronchoscope deep into the bronchi, and 10 ml of bacterial suspension was slowly injected. Seven pigs were infected with 8×10^2 CFU of *A. pleuropneumoniae* 13261 (serotype 9), and six pigs were infected with 3×10^4 CFU of *A. pleuropneumoniae* 1421 (serotype 3). Details about endobronchial inoculation and preparation of bacterial suspensions are published elsewhere (17). Six control pigs were not infected in the left lung. The pigs were housed individually in separate boxes in three separate but identical climate-controlled rooms. The pigs infected with *A. pleuropneumoniae* serotype 9 were kept in one room, the pigs infected with serotype 3 were kept in another room, and the control pigs, which were not infected, were kept in the last room.

All infected pigs developed fever and dyspnea within 12 h after being infected. Signs of pleuropneumonia were so severe in the serotype 9-infected pigs that, to prevent death, we treated five of seven pigs with oxytetracycline (Engemycin 10%; Mycofarm Nederland B.V., De Bilt, The Netherlands); we administered one single injection 48 h after infection in a dose of 15 mg/kg of body weight intramuscularly. The pigs infected with serotype 9 recovered more slowly than the pigs infected with serotype 3. After 3 weeks, we infected all 13 previously infected pigs and the 6 control pigs in the right lung with 10^3 CFU of *A. pleuropneumoniae* 13261. Only one of seven homologous strain-infected pigs had fever (40.3°C), and none of these pigs had dyspnea. Of six heterologous strain-infected pigs, four had fever (40.4 to 41.5°C) and dyspnea, whereas the other two pigs stayed healthy. All control pigs had fever (39.8 to 42.5°C) and dyspnea within 24 h after infection.

After 48 h, the pigs were killed and submitted for necropsy. Lungs were examined macroscopically, and tissue samples were plate cultured. The left lungs of all homologous strain-infected pigs had necro-purulent lesions in the diaphragmatic lobe with chronic adhesive pleuritis; the right lungs had no lung lesions in six of seven pigs. The pig that developed fever had a catarrhal pneumonia in the right lung (Table 1). The left lungs of four heterologous strain-infected pigs had necro-purulent lesions, whereas those of the other two pigs had small lesions with connective tissue. All six heterologous strain-infected pigs had chronic adhesive pleuritis in the left thorax. The right lungs of four of six pigs had an acute, fibrinous, hemorrhagic pneumonia, whereas the other two pigs that developed no fever had no lesions in the right lungs (Table 1). All control pigs ($n = 6$) had an acute, fibrinous, hemorrhagic pneumonia of the right diaphragmatic lobe. Pathomorphological and bacteriological examination confirmed that the first infection was in the left lung and the second infection was in the right lung. From all acute and chronic lesions the causative *A. pleuropneumoniae* strain was isolated, except from two serotype 3-induced chronic lesions with connective tissue that were sterile. The overall differences between the three groups (homologous strain infection, heterologous strain infection, and control) were tested with the Kruskal-Wallis ranking order test, and the

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TABLE 1. Experimental reinfection of pigs in the right lung with *A. pleuropneumoniae* serotype 9, strain 13261

Convalescent group ^a and pig no. ^b	ApxI-ApxII titer ^c		ApxII-ApxIII ^d CTN ^e titer	Clinical sign		Necropsy result ^f			
	HTN ^g	CTN		Temp (°C) ^h	Dyspnea	Left lung		Right lung	
						Type of lesion	Vol ⁱ	Type of pneumonia	Vol ⁱ
Infected with strain 13261 (serotype 9)									
4890	80	20	<5	39.1	No	Necrosis	324	None	
4891	80	40	<5	39.3	No	Necrosis, sequestered	160	None	
4893	80	40	<5	39.4	No	Abscess	48	None	
4894	80	40	<5	39.2	No	Abscess	672	None	
4895	80	20	<5	39.3	No	Abscess	385	None	
4896	40	20	<5	39.5	No	Abscess	60	None	
4897	20	5	<5	40.3	No	Necrosis	24	Catarrhal	8
Infected with strain 1421 (serotype 3)									
4898	10	<5	20	41.3	Yes	Necrosis, sequestered	30	Hemorrhagic	108
4900	<5	<5	20	41.5	Yes	Necrosis, sequestered	84	Hemorrhagic	648
4902	<5	<5	5	39.5	No	Connective tissue	4	None	
4903	10	<5	5	40.5	Yes	Connective tissue	4	Hemorrhagic	32
4904	5	<5	<5	39.4	No	Necrosis	8	None	
4905	<5	<5	20	40.4	Yes	Necrosis, sequestered	31	Hemorrhagic	126
Control (not previously infected)									
4884	<5	<5	<5	39.8	Yes			Hemorrhagic	14
4885	5	<5	<5	41.5	Yes			Hemorrhagic	63
4886	<5	<5	<5	40.1	Yes			Hemorrhagic	40
4887	<5	<5	<5	40.7	Yes			Hemorrhagic	105
4888	<5	<5	<5	41.3	Yes			Hemorrhagic	32
4889	<5	<5	<5	40.7	Yes			Hemorrhagic	180

^a Six-week-old specific-pathogen-free pigs were infected in the left lung with *A. pleuropneumoniae* 13261 or *A. pleuropneumoniae* 1421 and 3 weeks later were subsequently reinfected in the right lung with strain 13261.

^b Pig no. 4892 died 24 h after infection with strain 13261 in the left lung, and pigs no. 4899 and 4901 were killed 48 h after infection to confirm the pneumonia in the left lungs induced by strain 1421 (positive controls).

^c Three weeks after the pigs were infected in the left lung, serum samples were collected and tested for their ability to neutralize the toxic effect of a sterile culture filtrate of strain 13261 (ApxI and ApxII) on sheep erythrocytes and porcine alveolar macrophages.

^d As for footnote c, except that serum samples were tested for their ability to neutralize a sterile culture filtrate of strain 405 (ApxII and ApxIII) towards porcine alveolar macrophages.

^e CTN, cytotoxin neutralization assay with porcine alveolar macrophages.

^f Pigs were killed and necropsied 48 h after the reinfection in the right lung.

^g HTN, hemolysin neutralization assay with sheep erythrocytes.

^h Maximum measured rectal temperature after the reinfection in the right lung.

ⁱ Volume of the lung lesion (in cm³).

pairwise differences between groups were tested with the Wilcoxon-Mann-Whitney two-sample ranking order test (16). The sizes of acute lung lesions differed significantly between the three groups ($P = 0.015$). The ranking of the sizes of acute lung lesions differed significantly ($P = 0.0027$) between homologous strain-infected pigs and control pigs and did not significantly differ between homologous strain- and heterologous strain-infected pigs ($P = 0.063$) or between heterologous strain-infected and control pigs ($P = 0.92$). Pre- and postinfection serum samples were tested for neutralization of the hemolytic and cytotoxic activity of the culture filtrates of *A. pleuropneumoniae* 13261 and of strain 1421 towards sheep erythrocytes and porcine alveolar macrophages (10). The titers for individual pigs are given in Table 1.

In this study we found a strong strain-specific immunity with complete protection, and we found no protection but typical pleuropneumonia in all control pigs. The severe lesions in four heterologous strain-infected pigs indicate that no strong species-specific immunity against other serotypes was developed. In contrast, two other heterologous strain-infected pigs were completely protected, which indicates a cross immunity between serotypes after infection, as found by Nielsen (12). The variation in results for the heterologous strain-infected pigs is

difficult to explain, but it suggests that there is either complete protection or no protection, but nothing in between.

Apx toxins are thought to be important for the induction of protective immunity in pigs (3). In this study we infected pigs with virulent strain 13261, which produces ApxI and ApxII, or with virulent strain 1421, which produces ApxII and ApxIII. All pigs developed pleuropneumonia after the initial infection. Three weeks later, but before the reinfection with strain 13261, the ApxI-ApxII-neutralizing antibody titers were low in pigs initially infected with strain 13261 and below the detection level in pigs 3 weeks after initial infection with strain 1421. Nevertheless, two heterologous strain-infected pigs and all homologous strain-infected pigs were completely protected.

Antibodies to virulence factors other than Apx toxins, like capsular antigens (7, 14), lipopolysaccharides (4), iron-regulated outer membrane lipoproteins (6, 15), and other outer membrane proteins (13) may also contribute to protection. Although antigenic heterogeneity of these factors exists between serotypes, common antigens might play a role in the complete protection of the two heterologous strain-infected pigs. Another common antigen of strains 13261 and 1421 is ApxII (8), a protein with a weak hemolytic effect and a moderate cytotoxic effect (9). Neutralizing antibodies directed

against ApxII went almost undetected by our assays, because ApxI is a strong hemolysin and cytotoxin. Nevertheless, antibodies directed to ApxII might contribute to cross-protection.

Oral infection of pigs with *A. pleuropneumoniae* results in a transient increase of polymorphonuclear leucocytes (PMN) in the bronchoalveolar lavage fluid (2). Accumulation of PMN in alveolar spaces is a common histological finding in acute and chronic pleuropneumonia. Recently, we demonstrated that PMN can rapidly phagocytize and kill *A. pleuropneumoniae* in vitro, whereas alveolar macrophages cannot (1). In this study it is likely that PMN were abundant in lungs before reinfection, and this might have influenced our results.

Infected herds can harbor different serotypes (11). Until now it has been thought that a serotype that causes pleuropneumonia leads to lasting immunity against reinfection from all other serotypes (12). However, our results indicate that pigs can repeatedly suffer from pleuropneumonia. In conclusion, we have shown that convalescent pigs surviving pleuropneumonia are protected only against homologous strain reinfection and that the protective immunity of convalescent pigs subsequently reinfected with heterologous serotypes varies. Apx-neutralizing antibodies were not essential for protection.

We gratefully thank Elbarte Kamp and Norbert Stockhofe-Zurwieden for their assistance.

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