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Research paper

Erysipelothrix rhusiopathiae exploits cytokeratin 18-positive epithelial cells of porcine tonsillar crypts as an invasion gateway

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ABSTRACT

Tonsils are important organs for mucosal immunity and are gateways for various pathogens, including bacteria and viruses. The purpose of the present study was to reveal how *Erysipelothrix rhusiopathiae*, the causative agent of swine erysipelas, invades the mucosal epithelium of the tonsils of pigs. Two germ-free piglets were orally infected with *E. rhusiopathiae* Koganei 65-0.15, an attenuated vaccine strain in Japan, and their tonsils of the soft palate were histologically examined four weeks after infection. Bacterial organisms were observed in dilated crypt lumens and a few epithelial cells of the crypt. Immunohistochemical examination revealed that some epithelial cells of the crypt were positive for cytokeratin (CK) 18, a specific marker for M cells in the Peyer's patches of pigs. Confocal laser scanning microscopy showed that bacterial antigens were present in the cytoplasm of CK 18-positive epithelial cells. Furthermore, an ultramicroscopic examination revealed that the bacteria-containing epithelial cells did not have microfolds or microvilli, both of which are characteristic of membranous epithelial cells (M cells), and that they were in close contact with intraepithelial phagocytes. Thus, the present observations suggest that the tonsillar crypt epithelium is a site of persistent infection for orally administered *E. rhusiopathiae*, and the bacteria exploit cytokeratin 18-positive epithelial cells of the crypts as portals of entry into the body.

1. Introduction

Erysipelothrix rhusiopathiae, a Gram-positive, non-acid-fast, non-spore-forming bacterium, is a pathogen of pigs and many other mammals including humans as well as birds (Wood, 1992). Swine erysipelas, a systemic infectious disease of the pig caused by *E. rhusiopathiae*, is clinically divided into several forms. Acute swine erysipelas presents as sudden death following septicemia, while the subacute form shows the characteristic erythematous cutaneous lesions. The chronic form shows polyarthritis and/or proliferative endocarditis (Wood, 1992). *E. rhusiopathiae* is widely distributed in soil, water and marine environments, and 30–50% of healthy pigs carry the organism in their lymphoid organs, especially in the tonsils (Conklin and Steele, 1979; Stephenson and Berman, 1978; Wood, 1992).

The tonsil is a secondary lymphoid organ located at the oropharyngeal mucosa. As the front line of defense against infections of the throat and upper airways, tonsils capture foreign bodies and microorganisms entering through the mouth or nares and initiate the immune response to antigens. Five tonsils are present in the pig, and the tonsils of the soft palate are especially well-developed (Casteleyn et al., 2011). The tonsils of the soft palate have

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many crypt structures. The tonsillar crypts are covered with non-keratinized stratified squamous epithelium containing many intraepithelial lymphocytes and dendritic cells (Casteleyn et al., 2011). The tonsils of the pig are often colonized by many resident and pathogenic swine bacteria, including *Salmonella enterica* spp., *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Haemophilus parasuis* and *Streptococcus suis*, and are used as a site of primary replication and/or persistent chronic infection (Fedorka-Cray et al., 1995; Chiers et al., 2002; Arends et al., 1984; MacInnes et al., 2008; Horter et al., 2003).

The mucosal epithelium generally acts as a barrier to pathogens, but some microorganisms can invade from the mucosal surface. Many enteric bacteria, such as *Salmonella* Typhi, *Salmonella* Typhimurium, *Vibrio cholerae*, *Yersinia enterocolitica* and *Campylobacter jejuni*, utilize membranous epithelial cells (M cells) in the Peyer's patches as a portal of entry for infection (Kohbata et al., 1986; Jones et al., 1994; Owen et al., 1986; Grutzkau et al., 1990; Walker et al., 1988). However, little is known about how bacterial pathogens invade into the mucosa of the tonsils. In the present study, to understand the interactions between mucosal bacterial pathogens and host mucosal immunity, we histologically investigated how *E. rhusiopathiae* colonize and break the mucosal epithelial barrier of tonsils of pigs.

2. Materials and methods

2.1. Animal experiments

Two Large White piglets obtained by caesarian section were used in this study. They were kept in sterile positive-pressure isolators and fed with a sterile milk replacer (SPF-LAC; Weyerhaeuse, Eaton, OH). *E. rhusiopathiae* strain Koganei 65-0.15, a Japanese live vaccine strain, was used in the experiments. *E. rhusiopathiae* strains were grown in brain heart infusion medium (BHI; Becton, Dickinson and Company, MD) containing 0.1% Tween 80, pH 8.0 (BHI-T80). Ten-day-old piglets were inoculated orally with 4.2×10^7 cfu of *E. rhusiopathiae* mixed in the sterile milk replacer. Four weeks after inoculation, the piglets were euthanized and necropsied. Tonsils of the soft palate and other major organs were collected for histopathology, and the number of bacteria in tonsils, blood and knee and elbow joint fluids was determined on BHI-T80 agar plates.

Animal experiments were carried out according to the regulations and guidelines approved by the Animal Ethics Committee of the National Institute of Animal Health.

2.2. Histopathology, immunohistochemistry and immunofluorescence

For histopathology, collected tissues were fixed in 10% neutral buffered formalin and embedded in paraffin by a routine method. Paraffin sections (2 μm thick) were stained with either hematoxylin and eosin (HE) or Gram stain (Hucker-Conn method). For immunohistochemistry, the tissue sections were autoclaved at 120 °C for 10 min in 10 mM Tris–EDTA buffer pH 9.0 for antigen retrieval, treated with 1% hydrogen peroxide in methanol and incubated in 5% skim milk in Tris-buffered saline (TBS) to block non-specific reactions. Sections were incubated at 37 °C for 1 h with primary antibodies. Monoclonal antibodies against cytokeratin (CK) 18 (clone CY-90, Sigma, ST. Louis, MO), CK 5/6 (clone D5/16 B4, DAKO-Japan, Kyoto, Japan) and rabbit polyclonal antibodies raised against formalin-killed whole *E. rhusiopathiae* cells were used. Thereafter, the sections were incubated with the Envision polymer reagent (DAKO-Japan) at 37 °C for 1 h. Visualization was performed with 3,3′-diaminobenzidine tetrahydrochloride, and sections were counterstained with hematoxylin. For immunofluorescent dual staining, tonsil tissues were rapidly frozen and cryosectioned at 15 μm thickness. The sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C for 20 min and treated in 5% skim milk in TBS with 0.05% Tween 20. The sections were incubated with an anti-CK 18 monoclonal antibody and an anti-*E. rhusiopathiae* polyclonal antibody at 37 °C for 1 h and then incubated with secondary antibodies (fluorescein-conjugated goat anti-mouse IgG and Alexa Fluor 546-labeled goat anti-rabbit IgG, Molecular Probes®) at 37 °C for 1 h. The sections were examined with a Leica TCS-SP5 for confocal scanning laser microscopy.

Table 1
Number of *E. rhusiopathiae* bacteria isolated from the organs of germ-free pigs after oral inoculation.

Samples from	The number of isolated bacteria (log_{10} CFU)	
	Pig no. 1	Pig no. 2
Tonsils of soft palate	7.69	7.82
Blood	2.04	1.60
Elbow synovial fluid	Not detected	1.00
Knee synovial fluid	1.60	Not detected

2.3. Electron microscopy

Each tonsil tissue sample was cut to a 2 mm^3 size and placed for 2 h in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) at 4 °C. Samples were washed 3 times with PBS and post-fixed for 2 h in 1% OsO2 at 4 °C. They were then washed three times with distilled water, dehydrated in increasing grades of ethanol, replaced with QY-1 (Nisshin EM Corporation, Tokyo, Japan) and embedded in Quetol651 resin (Nisshin EM). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7500 transmission electron microscope.

3. Results

3.1. In vivo colonization of E. rhusiopathiae

In the infection experiment, there were no clinical signs and no visible gross pathology at necropsy in the *E. rhusiopathiae* strain Koganei 65-0.15 orally-infected piglets. To confirm *in vivo* colonization and the peripheral distribution of *E. rhusiopathiae*, the number of bacteria was determined in the tonsil of the soft palate, blood and synovial fluid. As shown in Table 1, a large number of bacteria were isolated