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Escherichia coli Administered into Pig Amniotic Cavity Appear in Fetal Airways and Attract Macrophages into Fetal Lungs

I. ŠPLÍCHAL, I. TREBICHAVSKÝ, A. ŠPLÍCHALOVÁ[1], L. DÍTĚTOVÁ, M. ZAHRADNÍČKOVÁ

Division of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Nový Hrádek, [1]PRO.MED.CS and Institute of Experimental Biopharmacy, Academy of Sciences of the Czech Republic, Hradec Králové, Czech Republic

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Summary

Escherichia coli ($2x10^4$ bacteria) of the non-pathogenic O86 strain or enteropathogenic O55 strain were administered into the pig amniotic cavity at 79 to 86 days of gestation for six or ten hours. Translocation of bacteria into fetal lungs was confirmed by cultivation as well as by light and electron microscopy. Infection caused an influx of macrophages that were immunostained in cryostat sections by monoclonal antibody recognizing calprotectin.

Key words

Intraamniotic infection • *Escherichia coli* • Pig • Fetal lungs • Macrophages

Introduction

Intraamniotic infections are a serious cause of preterm labor. Accumulating evidence indicates an association between intraamniotic infection and rising concentrations of amniotic inflammatory cytokines that are the leading cause of perinatal morbidity and mortality (reviewed by Šplíchal and Trebichavský 2001). *Escherichia coli* is a frequent pathological agent of intraamniotic infections both in humans and pigs. Bacteria can be translocated from amniotic liquid, into the fetal organism directly by swallowing and by respiratory movements. The fetal lungs begin a pattern of respiratory activity early in intrauterine life (Laudy and Wladimiroff 2000). In humans, intrauterine aspiration of thorotrast was confirmed as early as at four months of gestation (Maximow and Bloom 1952).

Defense mechanisms of fetal lungs are immature. Macrophages and other immune cells occur in porcine bronchoalveolar lavages after birth (Trebichavský *et al.* 1986). In the pig, bacteria which appear in the circulation are trapped mostly by pulmonary intravascular macrophages and not by splenic or liver phagocytes as in humans. Porcine lung capillaries are colonized perinatally by monocytes that replicate and differentiate into pulmonary intravascular macrophages. These phagocytes represent only 6 % of capillary volume in newborn pigs but 25 % in 30-day-old pigs (Winkler and Cheville 1987). Their activity also increases. Whereas only 12 % of *Salmonella typhimurium* were recovered from the

Fax+4202 24920590
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

circulation in the lungs of newborn piglets (Mouton et al. 1963), 60 % of *Pseudomonas aeruginosa* and 75 % of *Staphylococcus aureus* were cleared by the lungs in two-month-old pigs (Dehring *et al.* 1983). Phagocytosis and oxidative burst of macrophages from pig fetuses, newborn and germ-free piglets are decreased. They are upregulated, however, by the presence of gut microflora (Řeháková *et al*. 1998). The pulmonary mucociliar apparatus that plays an important role in defense against respiratory infections differentiates in the last third of gestation. Pig bronchial cells are ciliated at 80 days of gestation, and mucosal glands and bronchiolar cilia does not appear until 92 days of gestation when definitive alveoli also differentiate (Baskerville 1976). Intraamniotic infections therefore seriously endanger the fetal respiratory tract. The aim of this work was to study the translocation of common bacterial pathogen from the amnion into fetal lungs.

Methods

Animals and intramniotic infection

Four healthy pregnant (79-86 days of gestation) primiparae (aged 9-12 months) of Minnesota-derived miniature pigs had free access to water but fasted before the first surgery. They were pre-medicated with atropine sulphate (i.m. 0.5 mg per 30 kg of body weight, Hoechst-Biotika, Martin, Slovakia) and they were anesthetized with 1.5-2.5 % halothane (Léčiva, Prague, Czech Rep.) mixed with O_2 and N_2O. After laparotomy of the gilts, $2x10^4$ *Escherichia coli* bacteria in 3 ml of PBS (pyrogen-free phosphate buffered saline, PAA, Austria) were injected through uterine wall into the amniotic cavity. Non-pathogenic *E. coli* O86 strain and virulent *E. coli* O55 strain (enteropathogenic for both humans and pigs) were freshly prepared on agar (Immuna, Šarišské Michaľany, Slovakia), diluted in PBS, measured at 550 nm and their dose was calculated from a calibration curve. The viability of bacteria was confirmed by cultivation on bovine blood agar and MacConkey agar (Merck, Darmstadt, Germany) for 24 h at 37 °C. Sham-infected controls in the second uterine horn were treated by PBS only. The incisions were then sutured and the gilts placed in a post-surgical care unit. They had free access to water but a limited amount of food.

The second laparotomy was performed six or ten hours later. The total number of 28 fetuses in four independent experiments was obtained by uterotomy. Fetuses were exsanguinated under the above-mentioned halothane anesthesia of the gilts. Lung samples were homogenized in PBS and cultivated in different dilutions 24 hours on MacConkey's agar at 37 °C.

All experiments were approved by The Ethical Committee of the Institute.

Mouse monoclonal antibodies

Three mAbs recognized surface markers on pig leukocytes: the K252.1E4 (IgG1) directed against CD45 – leukocyte common antigen and MIL2 (IgG2b) recognizing a marker of pig myeloid cells (Haverson *et al.* 1994) were kindly donated by K. Haverson (Langford, Bristol, U.K.), 74-22-15 (IgG2b) recognizing the SWC3a antigen restricted to the myelomonocytic hematopoietic lineage in pigs (Lunney 1993, Saalmüller 1996) was kindly donated by J. Lunney (Beltsville, MD). The MAC387 mAb (IgG1) recognizing calprotectin (in the pig and some other mammalian species) was purchased from Serotec (Oxford, U.K.).

Immunofluorescence

Rabbit anti-O55 antiserum (V. Dlabač, Inst. Microbiol. AS CR, Prague) was prepared by immunizing rabbits with bacteria killed by phosphate buffered saline saturated with chloroform. The agglutination titer of the antiserum was 1:400, ELISA titer with *E. coli* O55 lipopolysaccharide was more than 1:10 000.

Cryostat sections through lungs of *E. coli* O55-infected and control saline-treated fetuses were incubated with 10 % pig serum in 37 ^{o}C for 10 min, rabbit anti-O55 antiserum and F(ab)2 swine anti-rabbit Ig labeled with FITC (DAKO, Glostrup, Denmark) in optimal dilutions. They were thoroughly washed after each incubation and observed using an Orthoplan fluorescence microscope (Leitz, Wetzlar, Germany). The fading of fluorescence was limited with Vectashield (Vector Laboratories, Burlingame, CA).

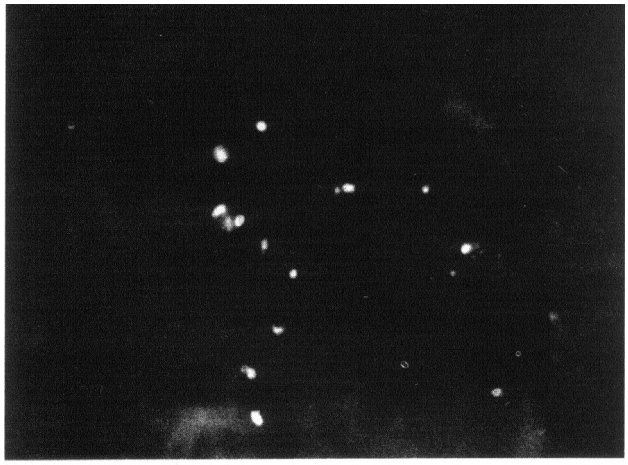
APAAP immunohistochemistry

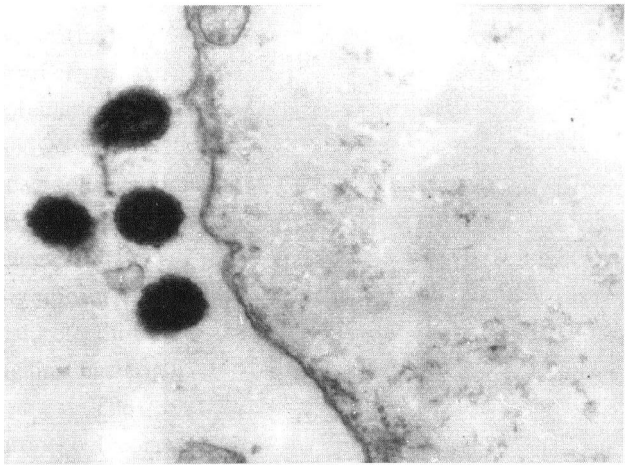
Lung samples were snap-frozen in cold isopentane and liquid nitrogen and kept in a deep freezer until sectioning. Cryostat sections were air dried and fixed for 5 min in cold acetone. Dry sections were washed and incubated with optimally diluted mouse monoclonal antibody (Tris buffer containing 0.1 % saponin Sigma). Sections were thoroughly washed and treated with an alkaline phosphatase-antiphosphatase (APAAP) kit (DAKO). A Fast Red system with levamisole inhibition was used for detection of the enzyme marker. Sections were stained with McGill's hematoxylin and mounted in Glycergel medium (DAKO).

Positive cells that were binding specific antibodies were stained red on a blue background. Negative controls consisted of sections treated without a specific antibody. Furthermore, mouse IgG1 negative control (X0931, DAKO) was used as a control of specific binding of the MAC387 mAb .

Electron microscopy

Samples of fetal lungs were fixed in fresh 2.5 % glutaraldehyde (grade I, Sigma) in 0.2 M cacodylate buffer pH 7.2 for one hour and postfixed in 2 % buffered OsO_4. Dehydrated samples were embedded in Vestopal W polyester resin (Serva, Heidelberg, Germany), and ultrathin sections were observed in a Tesla BS500 (Brno, Czech Republic) transmission electron microscope.





Bacteria translocate into fetal lung after intraamniotic infection. A porcine fetus on the 85th day of gestation.

Fig. 1. (Upper) *Immunofluorescence of E.coli O55 in the alveolar duct (1000x).*

Fig. 2. (Lower) *Electron micrograph of E.coli O55 in the alveolar duct near the epithelium (10 000x).*

Results

E. coli are cultivated from fetal lungs after intraamniotic infection

Bacteria were cultivated from the lungs of all *E. coli*-treated fetuses. The range of *E. coli* CFU is shown in Table 1. No bacteria were found in sham-infected control fetuses.

Bacteria are found in fetal airways

Using the immunofluorescence technique on cryostat sections, E. coli O55 were found mostly in alveolar ducts of fetal lungs (Fig. 1). Electron microscopy confirmed that the bacteria were distributed only in the respiratory space near the epithelium (Fig. 2). Macrophages that were found in pulmonary tissue by electron microscopy contained occasionally phagocytozed red blood cells but no bacteria.

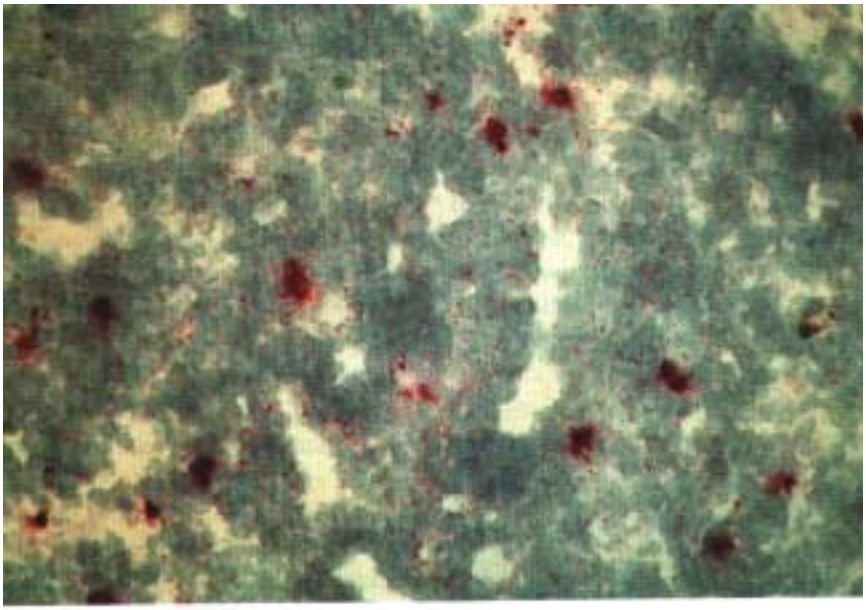
Macrophages appear in infected fetal lungs

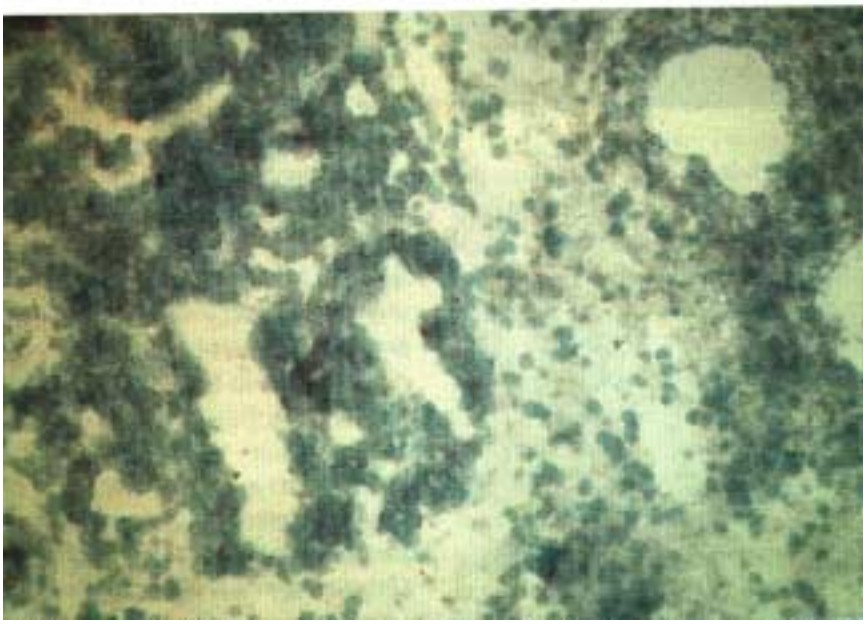
All four monoclonal antibodies recognizing pig leukocytes and myeloid cells stained many more cells in the lungs infected with *E. coli* bacteria than in the lungs of saline-treated control fetuses. The most significant difference between the two groups of fetuses was found in the population of cells stained with the MAC387 mAb directed against calprotectin (Table 1, Figs 3 and 4). MAC387+ cells in fetal lungs were tissue macrophages that were localized outside the vessels and airways. They were large (15-20 μm) rounded or oval non-granular mononuclear cells (neutrophils were found rarely and only as intravascular cells) that sometimes had processes. They had extensive cytoplasm that was stained by the APAAP technique dark red and therefore contrasted well with the blue background of negative cells. Negative controls (isotype controls or controls where specific antibody was omitted) never showed any positivity.

Discussion

The lungs act as the principal site of blood clearance in several mammalian species, particularly in pigs, ruminants and cats. Macrophages occur in pig lungs (Pabst and Binns 1994) in different compartments: in the interstitium, as tingible-body macrophages in bronchiole mucosa-associated lymphoid tissue (Huang *et al.* 1990), in bronchoalveolar space or as pulmonary intravascular macrophages that play an important role in the removal of blood-borne (Winkler 1988, 1989) and also intratracheally administered bacteria (Bertram 1986).

Macrophages comprise up to 98 % cells in bronchoalveolar lavages from specific-pathogen-free pigs but only about two thirds in conventionally bred pigs (van Leengoed and Kamp 1989).





Influx of macrophages into fetal lung after intraamniotic infection. Macrophages are stained red with the MAC387 mAb and a Fast Red system of APAAP. A porcine fetus on the 83th day of gestation. (400x).

Fig. 3. (Upper) *Numerous macrophages appear after infection with E. coli O86.*

Fig. 4. (Lower) *Macrophages are missing in saline control.*

Table 1.
Frequency of MAC387$^+$ cells in fetal pig lungs increases after intraamniotic infection.

	Control (3)	**Control (4)**	***E. coli* (2)**	***E. coli* (4)**	***E. coli* CFU**
Exp. 1. *E. coli* O86 for 10 h		1.0±0.8		45.5±15.5	3.0-23.5 x 10^7.g $^{-1}$
Exp. 2. *E. coli* O55 for 6 h		3.0 ± 1.9	14.0 ± 1.0		1.1-100.0 x 10^3.g $^{-1}$
Exp. 3. *E. coli* O55 for 10 h	3.7 ± 3.2			26.0±14.1	0.03-18.0 x 10^7.g $^{-1}$
Exp. 4. *E. coli* O55 for 10 h	0.7 ± 0.6			16.8±1.5	1.0-6.0 x 10^7.g $^{-1}$

Counts of positive cells/mm^2 of cryostat section (mean ± S.D., numbers of fetuses in four experiments are in parentheses). Bacterial CFU in lung tissue (range of values in E. coli-treated fetuses) are presented in each experiment.

We have shown that fetal pig lungs are rapidly colonized by bacteria after intraamniotic infection. Bacterial CFU in fetal blood, spleen and liver were much lower than CFU in the gut and lungs (not published). Intraamniotic infection with *Escherichia coli* caused a significant increase of tissue MAC387+ macrophages in fetal lungs. The MAC387 mAb recognizes intracytoplasmic calprotectin – an antimicrobial protein of the S100 family. Although the definite function of calprotectin is not completely understood, this protein might be involved in some calcium-mediated stages of cell function, differentiation and nonspecific defense mechanisms (Clohessy and Golden 1995). Calprotectin is found in neutrophils, reactive tissue macrophages, non-keratizing squamous epithelia and reactive epidermal cells (Lehrer 1998). Expression of calprotectin is a marker of cells that are newly recruited from the circulation (Rugtveit *et al.* 1996). Calprotectin was shown to be a highly specific marker of monocyte-like macrophages in the acute phase of inflammation and was associated with the influx of inflammatory cells into the lungs (Střiž *et al.* 2001). In this study, we have observed significant immigration of macrophages containing calprotectin during the acute response of fetal pig lungs against bacterial infection.

The absence of macrophages in the bronchoalveolar space of infected lungs could be explained by compartment immaturity. Macrophages observed by electron microscopy in fetal lungs occasionally contained phagocytozed erythrocytes but no bacteria. Erythrophagocytosis occurs physiologically in neonatal pig lungs (Winkler and Cheville 1984).

Bacteria were found only in the lungs of infected fetuses. Both strains of *Escherichia coli* did not translocate into the control horn during the experimental periods examined. They were never cultivated from sham-infected fetuses located in the other uterine horn. The bacteria did not penetrate the respiratory epithelium ten hours after infection. At the same time after intraamniotic infection, enteropathogenic *E. coli* O55 caused, however, the effacement and necrosis of amniotic and intestinal epithelia, and the bacteria penetrated into the underlying tissues. The influx of macrophages expressing calprotectin was also observed in the infected amniotic mesenchyme (unpublished observation).

In the present study, pig fetuses were shown to be a convenient animal model for studies of intraamniotic infections and prenatal antibacterial responses.

Acknowledgements

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Reprint requests

Igor Šplíchal, Institute of Microbiology AS CR, 549 22 Nový Hrádek, Fax +420 441-478264, e-mail splichal@biomed.cas.cz