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Original article

Effect of porcine respiratory coronavirus infection on lipopolysaccharide recognition proteins and haptoglobin levels in the lungs

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Abstract

Porcine respiratory coronavirus (PRCV) potentiates respiratory disease and proinflammatory cytokine production in the lungs upon intratracheal inoculation with lipopolysaccharide (LPS) at 1 day of infection. This study aimed to quantify LPS-binding protein (LBP), CD14 and haptoglobin in the lungs throughout a PRCV infection. LBP and CD14 recognize LPS and enhance its endotoxic activity, whereas haptoglobin dampens it. Gnotobiotic pigs were inoculated intratracheally with PRCV ($n = 34$) or saline ($n = 5$) and euthanized 1–15 days post inoculation (DPI). Virus was detected in the lungs from 1 to 9 DPI. Cell-associated CD14 in lung tissue increased up to 15 times throughout the infection, due to an increase in highly $CD14^+$ monocyte-macrophages from 1 to 12 DPI and $CD14^+$ type 2 pneumocytes from 7 to 9 DPI. LBP and soluble CD14 levels in bronchoalveolar lavage fluids were elevated from 1–12 DPI, with up to 35- and 4-fold increases, respectively. Haptoglobin levels increased significantly (×4.5) at 7 DPI. In addition, we found that PRCV could sensitize the lungs to LPS throughout the infection, but the response to LPS appeared less enhanced at the end of infection (7 DPI). The marked increases in LBP, CD14 and haptoglobin were not correlated with the extent of the LPS response.

Keywords: Porcine respiratory coronavirus; Coronavirus; Lipopolysaccharide; LPS-binding protein; CD14; Haptoglobin; Lungs

1. Introduction

The porcine respiratory coronavirus (PRCV) is highly prevalent in swine populations all over the world. PRCV shares several pathogenetic characteristics with the ''severe acute respiratory syndrome'' coronavirus (SARS CoV) that recently emerged in humans. Both viruses have a tropism for lung epithelial cells and cause bronchointerstitial pneumonia and necrotizing alveolitis [1,2]. Although PRCV infections often remain mild under experimental conditions, it is generally accepted that PRCV can be involved in respiratory disease in the field, when complicated with secondary (unknown) agents [3].

We have previously shown that PRCV synergizes with lipopolysaccharide (LPS) in the induction of severe respiratory disease [4]. LPS, also called endotoxin, is a major component of the outer membrane of Gram-negative bacteria and a potent inducer of proinflammatory cytokines. In the above-mentioned study, pigs were inoculated intratracheally with PRCV followed by LPS (20 μg/kg) at a time interval of 12–24 h. This led to excessive production of proinflammatory cytokines in the lungs and the simultaneous appearance of high fever, depression and breathing difficulties, which did not occur after inoculation with PRCV or LPS alone.

LPS induces proinflammatory cytokines after binding to its specific receptor complex [5]. LPS-binding protein (LBP) and CD14 are major components of this complex and mediate the early recognition of LPS. LBP is produced by hepatocytes and

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is constitutively present in plasma [6]. Because plasma LBP levels rise several times during systemic acute-phase responses, LBP is considered an acute-phase protein. Recently, type 2 pneumocytes were also shown to produce LBP after in vitro stimulation with different proinflammatory cytokines [7]. LBP acts as a lipid transfer molecule, extracting single LPS molecules from bacterial membranes or LPS aggregates and transferring them to CD14. CD14 is a pattern-recognition receptor, which is expressed by monocytes and macrophages and to a lesser extent by neutrophils [8]. CD14 also exists in a soluble form, which is enzymatically cleaved from the membrane or directly secreted from the cytoplasm [9]. Like LBP, soluble CD14 is constitutively produced by hepatocytes, and plasma concentrations can rise significantly during acute-phase responses. Importantly, soluble CD14 facilitates LPS-dependent activation of CD14-negative cells, such as epithelial and endothelial cells [10]. CD14 presents LPS to Toll-like receptor 4, which leads to the activation of different proinflammatory genes. Depending on the amount of inflammatory mediators that are induced, the endotoxic effect of LPS varies from mild inflammation to live-threatening functional disorders.

In contrast to the previous proteins, haptoglobin dampens the endotoxic activity of LPS [11]. This acute-phase protein is produced by hepatocytes and, according to some reports, also by lung epithelial cells [12]. Interestingly, haptoglobin desensitizes monocytes for the effects of LPS, protects mice from lethal endotoxic shock and reduces LPS-induced bronchial hyperreactivity [11].

The main objective of this study was to determine the kinetic profiles of LBP, CD14 and haptoglobin levels in the lungs throughout the PRCV infection. Both cell-associated and soluble CD14 were quantified and CD14-positive cells were identified using different markers. In a previous study, we demonstrated that PRCV potently enhances the response to LPS at 1 day of infection. Additional aims of the present study were therefore to verify whether this also occurs at later time points of infection (namely at 3 and 7 days post inoculation [DPI]) and whether virus-induced sensitization to LPS can be correlated with changes in the bronchoalveolar lavage (BAL) fluid levels of the above-mentioned proteins.

2. Materials and methods

2.1. Virus and LPS preparations

The Belgian 91V44 isolate of PRCV was used at the second passage in swine testis cells. The inoculation dose was 10^7 50% tissue culture infective doses ($TCID_{50}$) per pig.

LPS of *Escherichia coli* (serotype 0111:B4, Sigma–Aldrich, St. Louis, USA) was used at a dose of 20 μg/kg. This dose has been used in previous experiments and causes no respiratory disease signs and minimal production of proinflammatory cytokines in the lungs upon intratracheal inoculation [4,13]. Virus and LPS were diluted in sterile pyrogen-free phosphate-buffered saline (PBS; Gibco, Merelbeke, Belgium) to obtain a 3 ml inoculum.

2.2. Pigs, experimental design and sampling

Forty-eight caesarean-derived colostrum-deprived pigs were used at the age of 3.5 weeks. The pigs originated from six sows and were housed in Horsefall-type isolation units with positive-pressure ventilation and fed with ultrahigh-temperature-treated cow's milk. All inoculations were performed intratracheally with a needle that was inserted through the skin cranial to the sternum.

Thirty-four pigs were inoculated exclusively with PRCV and euthanized at 1 ($n = 5$), 2 ($n = 3$), 3 ($n = 5$), 4 ($n = 3$), 5 ($n = 3$), 7 ($n = 6$), 9 ($n = 4$), 12 ($n = 3$) or 15 ($n = 2$) DPI. Five pigs were mock-inoculated with PBS and euthanized at 1 ($n = 1$), 7 ($n = 2$) or 15 ($n = 2$) DPI. Additionally, six pigs were inoculated with PRCV and 3 ($n = 2$) or 7 ($n = 4$) days later with LPS. They were euthanized 4 h after the LPS inoculation. Previous experiments showed that this time point is optimal for detection of de novo synthesis of cytokines upon LPS inoculation [4]. Three control pigs were inoculated exclusively with LPS and euthanized 4 h later.

The right lung was used for lung lavage. The main right bronchus was clamped and a needle was inserted distally. Cold PBS (3 × 20 ml) was injected, followed by massage of the lung tissue and aspiration. About 75% of the BAL fluid could be aspirated and was kept on ice. BAL fluids were separated into cells and cell-free fluids by centrifugation. Cell-free BAL fluids were concentrated 20 times by dialysis. Tissue samples from the apical, cardiac and diaphragmatic lung lobes of the left lung were collected for virological and bacteriological examinations and immunofluorescence staining. Serum from all pigs was collected at euthanasia.

2.3. Virological and bacteriological examinations

Infectious PRCV in lung tissue homogenates of pooled samples of apical, cardiac and diaphragmatic lung lobes was quantified by virus titration in swine testis cells [4]. Routine bacteriological examination of lung tissue was performed.

2.4. Antibody titration

PRCV-neutralizing antibodies in sera were titrated using a virus-neutralization assay [14].

2.5. BAL cell analysis

Total cell numbers in BAL fluids were counted in a Türk chamber. The percentage of neutrophils was determined using Diff-Quik® (Baxter, Düdingen, Switzerland) staining of cytocentrifuge preparations. The percentage of sialoadhesin-, SWC3a-, CD3- and IgM-positive cells was determined using flow cytometric analysis (Becton Dickinson FACSCalibur™, BD Cellquest software) [15]. Sialoadhesin (mAb 41D3) is expressed exclusively on the membrane of macrophages and SWC3a (mAb 74-22-15) is expressed on monocytes, macrophages and neutrophils [16,17]. Resident macrophages of uninfected lungs are sialoadhesin-positive, whereas newly