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Circulating immune complexes of calves with bronchopneumonia modulate the function of peripheral blood leukocytes: *In vitro* evaluation

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ABSTRACT

In this work we studied if circulating immune complexes (CIC) of calves with bronchopneumonia have the capacity to modulate function of peripheral blood leukocytes of healthy cattle.
CIC of three month old calves (6 healthy and 6 diseased) were isolated by PEG precipitation. Peripheral blood mononuclear cells (MNCs) and granulocytes from healthy calves and cows were the CIC responder cells in *in vitro* tests.
The most remarkable increase of adhesiveness to polystyrene and ROS synthesis (assessed by NBT test) was detected in cows' granulocytes stimulated with CIC of diseased calves. Results of MTT test showed that CIC of both healthy and diseased calves reduced granulocytes' viability. The strongest effect of inhibition of cows' granulocytes resulted from CIC of diseased calves. CIC only moderately reduced spontaneous viability of calves' MNCs. Again, the strongest effect of CIC isolated from diseased calves was observed. In contrast to the low impact of CIC on non-stimulated cells, their inhibitory effect on viability of mitogen stimulated MNCs was very strong. With CFSE assay we showed that both types of CIC stimulated spontaneous, but inhibited mitogen induced proliferation of calves' MNCs. Propidium iodide staining reviled that CIC increased apoptosis/necrosis of both non-stimulated and mitogen stimulated MNCs.
CIC of both healthy and diseased calves modulated the function of peripheral blood MNCs and granulocytes, but a stronger effect of CIC of diseased calves was shown. The age of the donors (calves or cows) of the responder cells, and the activation state of these cells, were also of influence.

1. Introduction

Calf bronchopneumonia is a complex disease caused by viruses (BHV-1, PI-3V, BVDV, BRSV) or/and bacteria (*Mannheimia haemolytica*, *Pasteurella multocida*, *Haemophilus somnus*, *Mycoplasma bovis*). These infectious agents are ubiquitous in cattle populations, and the disease occurs when the calves' immune response is diminished by environmental stress factors (Mosier, 2014). The important role of humoral immunity in the defense was confirmed by data showing that the highest incidence of pneumonia occurs in calves aged two to four months. At this age the concentration of total serum immunoglobulins (Corbeil et al., 1984), and IgG specific for some of the pathogens associated with bronchopneumonia (Prado et al., 2006) are at their lowest level, due to an almost complete degradation of passively acquired maternal IgG and insufficient synthesis of their own antibodies. Numerous studies confirmed, directly or indirectly, that complexes of causative antigens and specific antibodies (*i.e.* immune complexes) are of importance for the pathogenesis of bronchopneumonia in humans (Mizutani and Mizutani, 1986; Mellencamp et al., 1987; Monsalvo et al., 2011). Deposits of immune-complexes in the lung tissue, together with histological finding of massive inflammation (McBride et al., 1999; Mulongo et al., 2015) indicate that immune-complexes play a role in the pathogenesis of calf bronchopneumonia. Studies conducted on rodent models (reviewed in Gao et al., 2006; Ward, 2010) showed that intra-alveolar deposition of IgG immune complexes activate complement system and lung resident macrophages and, recruit and activate blood neutrophils. The activated cells secreted pro-inflammatory cytokines, reactive oxygen species (ROS) and proteinases which trigger acute lung injury. The immune-complexes mediated lung inflammation is normally terminated endogenously by anti-inflammatory cytokines, and inhibitors of leukocyte proteinase and matrix metalloproteases which mediate the repair of injured lung tissue. However, an imprecise regulation of pro-inflammatory and anti-inflammatory components in the injured lung tissue are critical for the pathogenesis of pneumonia in humans

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and experimental animals (Quinton and Mizgerd, 2015), but also in calves (Mosier, 2014).

In our previous work we showed that calf bronchopneumonia is accompanied with an increased level of PEG precipitable circulating immune complexes (CIC) (Fratrić et al., 2012). We have also showed that IgG from CIC of diseased calves express more galactose and sialic acid than IgG from CIC of healthy calves. Such IgG glycosylation pattern could influence IgG-FcγR binding (Kapur et al., 2014) and we have assumed that it could also influence effector functions of these IgG containing CIC. Therefore in this study, with *in vitro* tests, we analyzed if CIC of three month old calves with bronchopneumonia were able to modulate function (adhesiveness, ROS and NO generation, viability and proliferation) of quiescent peripheral blood mononuclear cells (MNCs) and granulocytes of healthy calves. Given that age influences the effectiveness of the immune response in cattle (Chase et al., 2008), we also analyzed if CIC have a different impact on functional responses of blood leukocytes of young, three month old calves, and adult cows, at 30 days after parturition.

2. Material and methods

2.1. Animals

In this study, 12 three months old Holstein-Friesian calves were donors of CIC. Calves were from a commercial farm owned by PKB Corporation (Padinska Skela, Belgrade, Serbia). The use of animals was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade in accordance with the National Regulation on Animal Welfare. All calves were clinically examined by a veterinarian. The calves were classified according to signs of respiratory disease as healthy ($n = 6$) or diseased ($n = 6$). Healthy animals had body temperature < 39.5 °C, respiratory rate < 40 min^{-1}, no nasal discharge, no coughing and normal respiratory sounds. The overall clinical score for diseased calves was calculated according to McGuirk (2005). The scoring system is expressed in numbers from 0 to 3 (0: normal, 1: mild, 2: moderate, 3: severe). Total respiratory score < 4 is normal; = 4 is to be monitored; and >4 indicates the need for treatment. Calves were considered diseased when they scored 6 or more and presented two or more clinical signs of respiratory disease. Diseased animals had at least three of the following clinical signs: body temperature ≥ 39.5 °C, respiratory rate ≥ 45 min^{-1}, nasal discharge, coughing or increased respiratory sounds. Calves' nasal swab cultures are representative of their lung isolates (DeRosa et al., 2000) and in order to identify the bacteria and ensure adequate antibiotic therapy, deep nasal swabs were taken. Tests were done by applying conventional bacteriological methods and an automatic identification system, BBL Crystal 134 Enteric/nonfermenter ID kit (Becton Dickinson GmbH, Heidelberg, Germany), at the Department of Microbiology and Immunology, Faculty of Veterinary Medicine, Belgrade. All samples from diseased calves were positive for *P. multocida*, while other pathogenic bacteria and fungi were not isolated. In nasal swab specimens of healthy calves no pathogenic bacteria or fungi were isolated. The analysis of basic hematological parameters of peripheral blood of these calves was done at the Faculty of Veterinary Medicine, Belgrade using the Hematology Analyzer 901,062 (Diatron, Arcus, GmbH, Wien, Austria). The obtained results are shown in Table 1.

As donors of peripheral blood leukocytes, used as responders in the below listed *in vitro* tests, 5 three months old healthy calves and 5 healthy cows at 30 days after parturition, were included in this study. These calves and cows were also from the same above-mentioned commercial farm.

2.2. Polyethylene glycol (PEG) precipitation assay

CIC were isolated by PEG precipitation assay (Fratrić et al., 2006). Blood samples from diseased calves, prior to treatment with antibiotics, and healthy calves were collected *via* jugular vein puncture. Blood serum was separated after spontaneous coagulation and centrifugation. In 2 ml of fresh serum, 6 ml of 4.5% (*w*/*v*) PEG (MW 6000) in 100 mM sodium borate buffer pH 8.3–8.5 (all from Sigma Taufkirchen, Germany) was added. After 2 h of incubation at 4 °C, samples were centrifuged for 30 min at 2060 × *g*, at 4 °C. The precipitated proteins were redissolved in 2 ml of sterile PBS (0.8% NaCl, 10 mM sodium-phosphate, pH 7.2–7.4). Optical densities at 350 nm (OD_{350}) of the redissolved PEG precipitates were measured on Ultrospec 3300pro spectrophotometer (Amersham Bioscience, Uppsala Sweden). Aliquots of the PEG precipitates were stored at −20 °C pending analysis.

Table 1
Basic hematological indices of peripheral blood of healthy calves and calves with bronchopneumonia.

	Healthy calves ($n = 6$)	Calves with bronchopneumonia ($n = 6$)
Erythrocytes (10^{12}/l)	9.9 ± 0.4	11.1 ± 0.3**
Hemoglobin (g/l)	87 ± 6	102 ± 3**
Hematocrit (%)	24.8 ± 1.8	28.5 ± 1.3**
Leukocytes (10^9/l)	7.0 ± 1.2	14.8 ± 3.7**
Lymphocytes (10^9/l)	6.2 ± 0.8	6.9 ± 1.0
Monocytes (10^9/l)	0.3 ± 0.5	0.4 ± 0.5
Granulocytes (10^9/l)	2.2 ± 1.6	7.6 ± 3.8*
Lymphocytes (%)	81.6 ± 3.9	49.8 ± 15.7**
Monocytes (%)	0.8 ± 0.2	2.3 ± 3.2
Granulocytes (%)	17.7 ± 3.9	47.9 ± 15.7**
Gr/Ly ratio[a]	0.3 ± 0.2	1.0 ± 0.6**
Platelets (10^9/l)	420 ± 96	390 ± 103

Data are mean ± SD.
The significant difference between healthy and diseased calves: *$P < 0.05$; **$P < 0.01$.
[a] Gr/Ly — granulocytes/lymphocytes.

2.3. Concentration of γ globulin in sera and CIC

Agarose gel electrophoresis of total serum proteins and PEG precipitated serum proteins was performed according to the procedure of Johansson (1972). The relative content of γ globulins (percentage) was quantified by densitometry using ImageMaster Total- Lab v1.11 software (Amersham Pharmacia Biotech, Uppsala Sweden). Concentration of γ globulins was calculated based on total protein concentration determined by the BCA protein assay kit (Pierce, Rockford IL, USA).

2.4. Isolation of peripheral blood leukocytes

Blood of healthy calves and cows was drawn from the jugular vein into 50 ml sterile tubes containing 5 ml 3.8% sodium-citrate. Separation of MNCs was performed by whole blood centrifugation over Ficoll-Hypaque (1.077) density gradient (PAA Laboratories, Pasching, Austria), for 35 min, at 400 × *g* at 4 °C. The interface MNCs were collected, washed with PBS and resuspended in a complete cell culture medium (CM), RPMI 1640 (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (PAA Laboratories), and penicillin/streptomycin (PAA Laboratories). Granulocytes from the lower layer were purified by lysis of erythrocytes in isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4; all from AppliChem GmbH, Darmstadt, Germany). Granulocytes were then washed in PBS and resuspended in the CM.

2.5. Measurement of cell adhesion to polystyrene

Adhesion of MNCs and granulocytes to plastic (polystyrene) was assessed with the assay described by Vlaški et al. (2004). Peripheral blood granulocytes or MNCs (5×10^5 cells in 100 μl CM /well) were plated in 96-well flat bottom plates with 10 μL of CIC and incubated for 60 min. Spontaneous adhesion was determined in control cultures of cells incubated in CM only. Cultures of cells stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/ml) (Sigma-Aldrich, St. Louis, MO) were the assay positive control. After incubation, non-