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LIPOPOLYSACCHARIDE -BINDING PROTEIN IS PRESENT IN OVINE LUNGS LAVAGE FLUIDS

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ABSTRACT

Lipopolysaccharide Binding Protein (LBP) like activity has now been identified in ovine lung lavage fluids and in this study LBP has been purified from lung lavage using fast protein liquid chromatography (FPLC) system. On sodium dodecyl sulphate polyacrylamide electrophoresis, ovine LBP demonstrated a single band with a molecular mass of 60 kD. Ovine LBP showed a high fluorescence with FITC-LPS in human monocyte.

Key words: Lypopolysaccharide (LPS), lipopolysaccharide binding protein (LBP), FITC- LPS, acute phase protein.

INTRODUCTION

Lipopolysaccharide (LPS) is a component of the cell wall of gram-negative bacteria and the systemic release of bacterial lipopolysaccharide during infection has been well documented (Raetz *et al.*, 1991). LPS plays an important role in the pathogenesis of endotoxic diseases induced by common gram-negative bacterial pathogens, such as *Escherichia coli, Salmonella* sp. and *Pasteurella haemolytica.* LPS triggers a spectrum of pathophysiological effects in susceptible animals ranging from mild fever to fatal septic shock (Martich *et al.*, 1993; Burrel, 1994). Activated by binding endotoxin (lipopolysaccharide) via the surface glycoprotein CD 14 (Wright *et al.*, 1990), the bacterial pathogens release large amounts of inflammatory mediators including tumor necrosis factor α (TNF-α), interleukin-1 (IL- 1), interleukin-6 (IL-6), platelet activating factor (PAF), and reactive oxygen species. The interaction of LPS with monocytes also has beneficial effects to the host as monocytes contain enzymes that are capable of inactivating LPS in the circulation. Apart from the direct cellular interaction of LPS, other host mechanisms involves an acute phase serum protein, lipopolysaccharide binding protein (LBP), which forms high affinity complexes with LPS by binding to the lipid A moiety (Tobias *et al.*, 1986, Schumann *et al.*, 1990).

LBP has been purified from humans, rabbits and mice (Tobias *et al.*, 1986; Gallay *et al.*, 1993) and in bovine (Horadagoda *et al.*, 1995). There were no data available for the presence of LBP in ovine, although sheep is most popular animal throughout the world for its natural habitat, production, performance and resistance to environmental hazards. We were interested in the purification of ovine LBP from lung lavage after infection with *Pasteurella haemolytica* because ovine LBP participates in the pathogenesis of many diseases of sheep which have a gram-negative etiology. Because of the limited availability of the LBP source, we have developed an improved method for the preparation of LBP from acute phase serum using chromatographic, electrophoretic and immunological techniques. Presence of ovine LBP in the purification procedures was identified by immuno-dot blotting technique, which was a specific qualitative assay in this study.

MATERIALS AND METHODS

The study was conducted in the Department of Veterinary Clinical Studies, University of Glasgow, United Kingdom during the period from October to December 1999. The Econo-Pac Q cartridges were purchased from Bio-Rad Laboratories (Hemel Hempstead, UK). Native and fluorescein-labelled LPS (FITC-LPS) of *Escherichia coli* 0111, Histopaque were purchased from Sigma (Poole, UK). Ploylonal antisenim to bovine LBP was previously raised by Dr. Eckersall in New Zealand white rabbits by subcutaneous injection of 25 μg of purified LBP in complete Freund's adjuvant followed by two successive booster injections of 25 μg of purified LBP in incomplete Freund's adjuvant given at 4 weeks and 6 weeks. Rabbit anti-bovine IgG antibodies were purified by caprylic acid precipitation. Protein purification procedures were performed on a fast protein liquid chromatography system FPLC (Phanmcia, Milton Keynes, UK).

Collection of the ovine lungs lavage fluids

The lambs were infected with *Pasteurella haemolytica* A2 intratracheally at Moredun Research Institute, Scotland and post-mortem samples were collected (Table 1). Lambs fasted for 24 h, anaesthetised using intravenous Saffan @ 3 mg/kg body weight. The bronchoscope inserted via a principal bronchus until wedged in a small bronchiole. Segmental lavage was carried out using 2 portions of 50 ml Ca, Mg free Hank's solution, gently aspirating after each 50 ml aliquot. Recovered fluids from both aliquots pooled. Sterile heparin solution (Sigma, 1430 units/ml) added from a 1ml syringe @ 0.05 ml per 5 ml lavage fluid.

Table 1. Collection of ovine lungs lavage

Treatment	Sample collected
Control	6 day post-infection
P. haemolytica	2 hours post-infection
P. haemolytica	1 day post- infection

Purification of ovine lungs lavage fluid

Collected lavage fluids were dialysed separately against distilled water overnight at 4^0C and then centrifuged 1800-x g for 15 min. Discarded the precipitate the supernatant again dialysed against 2 mM diethanolamine buffer pH 8.3 overnight at 4^0C and then concentrated 10 times by dialysing against polyethylene glycol. The chromatographic step was performed on an anion exchanger with a volume of 5 ml (Econo-Pac Q cartidge; Bio-Rad) equilibrated with 2 mM diethanolamine buffer, pH 8.3. Aliquots (5 ml) of the elute froui Bio-Rex column, were applied to the Econo-Pac Q cartridge which was then washed with 20 ml of equilibrating buffer. Proteins adsorbed to the cartridge were eluted with increasing concentrations of saline in 2 mM diethanolamine, pH 8.3. Fractions were collected and, on the basis of the absorbance at 280 run of the elute, immuno-dot blotting and protein concentration of the fractions was carried out. The protein concentration of the fractions was measured by the bicinchonic acid protein assay using bovine serum albumin (BSA) as standard (Sigma).

Electrophoresis and immunoblotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10- 15% acrylamide gels (Laemmli, 1970). Novex pre-cast SDS gels were also used. Acute phase bovine serum and fractions from the different steps of the purification procedure were applied onto the acrylarylamide gels under reducing conditions in dithioerythritol and after migration the gels were stained with Coomassie Blue. Immunoblotting was performed onto nitrocellulose sheets in a Trans blot cell. Immnostaining of the nitrocellolose sheets were performed according to the procedure described by Eckersall and Conner (1990) using appropriate antibody.

Test for LBP activity

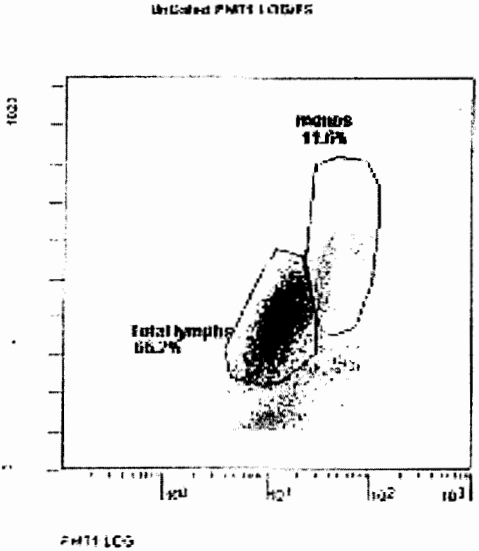
Human peripheral blood mononuclear cells (PBMC) were prepared from blood collected from healthy donors. Blood samples, collected by venepuncture, were mixed equal volume of Alsever's solution and 30 ml aliquots were layered onto 20 ml Histopaque in 50 ml conical polypropylene tubes. The tubes were centrifuged at 1600 x g for 40 min at 23^0C; mononuclear cells at the interphase were collected and suspended in an equal volume of Alsever's solution and then pelleted by centrifugation (5 min, 1800 x g, 23^0C). To remove platelets, the cells were further washed (x 3) in Alsever's solution by repeated suspension and centrifugation (1800 x g, 23^0 C) and the final pellet was suspended in RPMI 1640 medium. Cells were counted. Samples (0.1 ml) to be tested for LBP activity were added to PBMC (106) suspended in 1 ml of RPMI medium (final volume) and then incubated 1 h at 37^0C with FITC-LPS (μg/ml). At the end of the incubation period the cells were washed in cold phosphate buffered saline and analyzed with a EPICS ELITE flow cytometer (Coulter) fitted with an air cooled argon laser operating at 488 nm and 19 mW power. The fluorescence signal was recorded on a logarithmic scale and expressed as fluorescence units (FU).

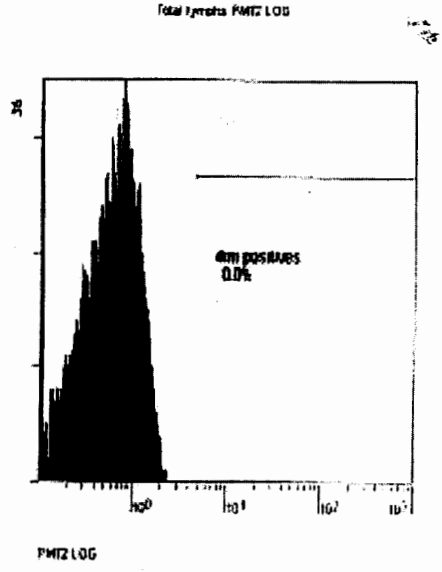
An ELISA was carried out by coating the plate overnight at 4^0C with different fractions of purified acute phage bovine serum (APBS) containing 5 μg protein / ml in 10 mM sodium bicarbonate buffer pH 9.4. Then was washed 3 times with saline, blocked with 250μl 1% BSA/saline for 1 h at 37 ^{0}C, and washed 4 times with saline. Hundred microliter 1st anti-LBP diluted 500 times with CRP buffer pH 7.4 (50 mM Tris, 0.9% NaCl, 10 mM CaC12 and 0. 1 % Tween-20) were added in each well and the plate was incubated for 2 h at room temperature. With the 2nd antibody 100 μl HRP- anti rabbit IgG 1: 1000 the plate was again incubated for 1 h at room temperature, washed 3 times with

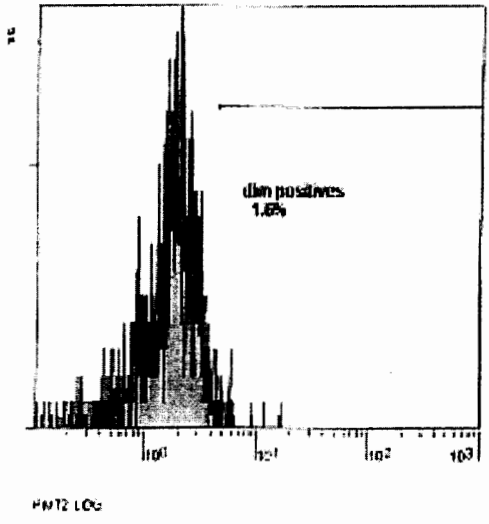
CRP assay buffer. Hundred microliter TMB was added and the plate was incubated 30 min at room temperature. The reaction was then stopped by addition of 50 µl 2M H_2SO_4 and the plate was read at 450 nm.

RESULTS AND DISCUSSION

Chromatography of ovine lungs lavage fluids effectively concentrated the LBP activity. Purification of ovine LBP was achieved by applying to Econo-Pac Q, a strongly anionic exchange column and LBP peak was eluted with increasing concentration of saline. The FPLC peak was assayed for protein. Immuno-dot blotting confirmed that it contained LBP and dialysed finally the peak was further purified by gel filtration column.







Mean Calculation Method:	LOG-LOG			
Statistic	monos >dim positives	Total lymphs >dim positives	Ungated >monos	Ungated >Total lymphs
Number	19	1	1158	6624
% Total 66.24	0.19	0.01	11.58	
% Gated 66.24	1.64	0.02	11.58	
X-Mean	6.4	4.6	45.2	13.7
X-Mode	5.8	4.6	37.1	12.7

When samples from purification procedure were analysed by SDS-PAGE, both FPLC and Gel filtration showed a single band and with comparison to standard the molecular mass was 60 kDa and on western blot the band was clearly shown. ELISA detected the LBP in presence of negative control and was very effective to determine quantitative value, whereas immuno-dot blotting was a very efficient method to determine LBP qualitatively. Flow cytometric analysis in the presence of FITC-LPS and monocyte showed high level of fluorescence but in absence of FITC-LPS or monocyte showed very weak activity (Table 2).

Table 2. Flow cytometric analysis in the presence of FITC-LPS and monocytes

Samples	Activity (FU)
Only cells	1.61%
0.1 ml LBP + FITC-LPS + Cells	27.7%
(T1) Sample 25 + FITC-LPS + Cells	35.3%
(T2) Sample 28 + FITC-LPS + Cells	20.6%
(Control) Sample 14 + FITC-LPS + Cells	12.6%

This is our first report about presence of LBP in ovine lungs lavage fluids. Previous studies in man and laboratory animals (Tobias *et al.*, 1986) and bovine (Horadagoda *et al.*, 1995) demonstrated that the presence of LBP activity in serum which is involved in initiating cytokine mediated host response to LPS. All the previous authors found a similarity among the LBP from different mammals even the mass within a range 50-60 kD. Purification of LBP is important for the pathogenesis of bacterial infection as well as to develop various immunological tests. LBP has been considered primarily as a protein able to allow the host to detect low concentrations of circulatory LPS, turning on the synthesis and release of mediators and cytokines. LBP was a mediator which exacerbated the lethal effects of endotoxaemia. Immuno-dot blotting and ELISA was used in this purification procedure and these were very reliable and less time consuming. The vast difference between FU activities suggests *Pasteurella haemolytica* A2 infection in lambs increased LBP activity, and there was a direct correlation between infection and LBP activity.

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