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THE ROLE OF LIPOPOLYSACCHARIDE OF *PASTEURELLA HAEMOLYTICA* IN THE DEVELOPMENT OF EXPERIMENTAL PASTEURELLOSIS IN RABBITS

H.G. Heng, M. Zamri-Saad, A. Nor'Izah, A.R. Sheikh-Omar and A. Rasedee

Faculty of Veterinary Medicine and Animal Science,
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

SUMMARY

Twelve adult New Zealand white rabbits were divided into four groups before the first three groups were inoculated intravenously with 0.7 µg/kg body weight, 0.4 µg/kg body weight and 0.2 µg/kg body weight of lipopolysaccharide (LPS) isolated from *Pasteurella haemolytica* A2 respectively. Rabbits in group 4 were the unexposed control. All rabbits were observed for signs of pasteurellosis while post mortem examinations were carried out on all dead rabbits. All rabbits in groups 1, 2 and 3 were found dead within 24 h post inoculation with lesions of peracute pasteurellosis which were mainly severe congestion of internal organs and haemorrhages in the tracheal mucosa and lungs. The control rabbits survived without any lesions. This report confirmed the ability of LPS isolated from *P. haemolytica* A2 to induce clinical signs and lesions similar to those of pasteurellosis in rabbits.

Keywords: Lipopolysaccharide, *Pasteurella haemolytica*, rabbits.

INTRODUCTION

Pasteurellosis is a disease of many species of animals caused by either *Pasteurella multocida* or *Pasteurella haemolytica*. The infection may develop to either the septicaemic or the pneumonic form, leading to deaths and great economic losses in cattle, sheep and goats worldwide (DeAlwis, 1993; Gilmour, 1993). Several virulence factors of the organisms have been recognised and associated with the development of this disease (Gonzalez and Maheswaran, 1993) including the lipopolysaccharide (LPS) which is one of the most important components of the outer membrane of all gram negative bacteria (Rietschel *et al.*, 1985). It is generally agreed that the clinical signs associated with gram negative bacterial infection such as *P. multocida* and *P. haemolytica* occur as result of the host response to endotoxin or lipopolysaccharide (Ulevitch, 1993). The typical LPS molecule is composed of two chemically dissimilar domains; the hydrophilic, polysaccharide core and O-antigen structure and the hydrophobic region known as lipid A. The core-oligosaccharide is structurally similar among many gram negative bacteria while the O-antigen is more chemically diverse (Morrison and Ulevitch, 1978), but the LPS-induced clinical responses are lipid A dependent (Rietschel *et al.*, 1982; Kotani *et al.*, 1983). Although the lipopolysaccharide appears to be the major surface antigen on formalin killed *P. haemolytica*, the high serum antibody responses to the polysaccharide chain of LPS do not correlate with resistance to experimental pneumonic pasteurellosis

This study was designed to determine the role of the lipopolysaccharide (LPS) particularly the lipid A of *P. haemolytica* A2 which causes pneumonic pasteurellosis in goats and sheep, in the development of experimental pasteurellosis in rabbits.

MATERIALS AND METHODS

Bacterial strain

Pasteurella haemolytica serotype A2 isolated from a caprine nasal swab (CNSA2) taken from a goat with pneumonic pasteurellosis was used in this study. The bacteria were grown in brain heart infusion broth (BHIB; Oxoid) overnight at 37°C to the stationary phase.

Lipopolysaccharide preparation

Following overnight growth of bacteria in BHIB, the LPS was extracted and purified by aqueous-phenol extraction (Ali *et al.*, 1992). Briefly, 1% phenol (w/v) was added into the BHIB before it was centrifuged at 5000 *g* for 30 min. The bacterial sediment was washed once with distilled water and with same volume of 70% ethanol followed by twice with acetone. The final wash was carried out using diethyl ether. The bacteria were air-dried before two grams of the dried bacteria was added into 10 mL aqueous phenol, warmed to 65°C, homogenised for 5 min with high speed stirring and sonicated for 5 min. Eighty millilitres of petroleum ether and 50 mL chloroform were then added, warmed to 65°C, homogenised for 5 min and stirred at room

for 30 min. The supernatant was filtered and the sediment was extracted again as described above. Chloroform and petroleum ether were removed from the supernatant by using the rotary evaporator. Water was added to the solution until the LPS precipitated followed by sedimentation of the LPS by centrifugation at 100,000 *g* for 1 h. The final pellet was washed twice in ether and dried under vacuum. The dried material added into 3-5 mL of water, with warming if necessary, using a syringe and 23-gauge needle and the LPS was sedimented by centrifugation at 100,000 *g* for 4 h.

To determine the purity, the LPS was separated by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% acrylamide stacking gel and 12% acrylamide resolving gel. Electrophoresis was performed in a vertical slab gel apparatus containing 25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS (pH 8.3) buffer at a constant current of 50 mA until the dye reached the end of the gel. The LPS was then visualised with silver stain (Davies *et al.*, 1991).

Experimental procedure

Four groups of rabbits consisted of three animals per group were used in this experiment. Group 1 was inoculated intravenously with 0.7 µg/kg body weight, group 2 with 0.4 µg/kg body weight and group 3 with 0.2 µg/kg body weight of the LPS isolated from *P. haemolytica* A2. Rabbits in group 4 represented the unexposed control. The rabbits were observed for clinical signs of pasteurellosis, and post mortem examinations were carried out on all dead rabbits. Lung samples were fixed in 10% buffered formalin, embedded in paraffin and prepared in 4 µm sections for histological examinations.

RESULTS

SDS-PAGE

The SDS-PAGE profile of the LPS from *P. haemolytica* A2 following silver staining revealed a rough LPS consisting of only one band. The LPS had the appearance of lipid A core oligosaccharide as there was no ladder-like pattern representation of O-antigen.

Clinical observations

All rabbits that were inoculated with 0.7, 0.4 and 0.2 µg/kg body weight of LPS isolated from *P. haemolytica* A2 were found dead on the second day post inoculation (p.i.) except one rabbit that was inoculated with 0.2 µg/kg body weight of the *P. haemolytica* A2 LPS that was found dead on the third day. The affected rabbits were found recumbent with congested mucous membranes and high body temperatures, and showing heavy breathing as early as 8 h p.i. The rabbits that were untreated control did not show any abnormal clinical signs (described earlier) and none of those rabbits died during the 14-day trial

Pathological observations

Grossly, all dead rabbits showed cyanotic skin particularly of the muzzle, lips, ears and thigh. The muscles and most internal organs showed severe and generalised congestion. The lungs were severely congested and moderately oedematous with patches of red haemorrhagic lesions. Blood-tinged fluid oozed out from cut surfaces of the lungs. The entire tracheal mucosa was severely congested and haemorrhagic with oedema fluid found in the lumen of trachea.

Histologically, the alveoli in the entire lung sections were filled with pink homogeneous oedema fluid. Thrombus was observed in all major blood vessels of the lungs while the interalveolar septa were thickened due to the dilatation of the congested capillaries. Inflammatory cells, however, were not observed in any of the lung sections. Similarly the blood vessels in tracheal mucosa were dilated as a result of congestion. Haemorrhages were observed in the lumen of trachea.

DISCUSSION

The SDS-PAGE analysis of the LPS isolated from both *P. multocida* type B and *P. haemolytica* A2 used in this study showed that the gel contained only a single band. This means that the LPS used in this study consisted of only one type known as the lipid A core oligosaccharide as observed earlier by Donachie and Gilmour (1988). The ladder-like pattern to signify the presence of other components of LPS particularly the O-antigen observed earlier by Ali *et al.* (1992) was not observed in this study. Similarities between the LPS of different serotypes of *P. haemolytica* have been observed earlier (Durham *et al.*, 1988).

Intravenous administration of LPS isolated from *P. haemolytica* A2 has successfully re-produced the clinical and pathological features of acute pasteurellosis in rabbits. This is due to the presence of a specific membrane receptor that has a lipid A-binding site of *P. haemolytica* A2 on the lipid A-dependent cells (Ulevitch, 1993). The clinical manifestation of sudden deaths within 24 h p.i., the gross lesions of generalised congestion and peracute pneumonia, and the histopathological lesions of pulmonary oedema and thrombosis observed in the dead rabbits in this study were very much similar to those observed in cases of peracute infection of goats by *Pasteurella multocida* type A and D (Maswati *et al.*, 1995) as well as those cases of peracute *P. multocida* type B infection (haemorrhagic septicaemia) in cattle and buffaloes (Graydon *et al.*, 1993).

Intravenous administration of LPS of *P. haemolytica* has been known to induce the release of mediators that act as autocrine and paracrine signals (Nathan, 1987). These mediators include thromboxane A_2, prostaglandins, serotonin, cAMP and cGMP that mediate endotoxin effects (Emau *et al.*, 1987), the

factor, lipid mediators from arachidonic acid, and toxic oxygen radicals (Ulevitch, 1993). The LPS is absorbed to the cell membrane and activates the complement system as well as binding to the lung surfactant (Brogden *et al.*, 1986) to disrupt the alveolar stability leading to oedema, haemorrhage and atelectasis in the lungs. The *P. haemolytica* LPS is also able to cause direct cell membrane damage to the pulmonary endothelial cells (Paulsen *et al.*, 1989) leading to generalised congestion, pulmonary oedema and disseminated intravascular coagulation observed grossly and histologically in all dead rabbits in this study.

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REFERENCES

Ali, Q., Davies, R.L., Parton, R., Coote, J.G. and Gibbs, H.A. (1992). Lipopolysaccharide heterogeneity in *P. haemolytica* isolates from cattle and sheep. *J. Gen. Microbiol.*, **138**: 2185-2195.

Brogden, K.A., Rimler, R.B., Cutlip, R.C. and Lehmkuhl, H.D. (1986). Incubation of *Pasteurella haemolytica* lipopolysaccharide with sheep lung surfactant. *Am. J. Vet. Res.*, **47**: 727-729.

Confer, A.W. and Simons, K.R. (1986). Effects of *Pasteurella haemolytica* lipopolysaccharide on selected functions of bovine leukocytes. *Am. J. Vet. Res.*, **47**: 154-157.

Confer, A.W., Panciera, R.J. and Mosier, D.A. (1986). Serum antibodies to *Pasteurella haemolytica* lipopoly-saccharide: Relationship to experimental bovine pneumonic pasteurellosis. *Am. J. Vet. Res.*, **47**: 1134-1138.

Davies, R.L., Ali, Q., Parton, R., Coote, J.G., Gibbs, A. and Freer, J.H. (1991). Optimal condition for the analysis of *P. haemolytica* lipopolysaccharide by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *FEMS Microbiology Letters*, **90**: 21-28.

DeAlwis, M.C.L. (1993). Pasteurellosis in production animals: a review. *In*: Pasteurellosis in Production Animals. ACIAR Proceedings No43, 11-22

Donachie, W. and Gilmour, N.J.L. (1988). Sheep antibodies respond to cell wall antigens antigens expressed *in vivo* by *P. haemolytica* serotype A2. *FEMS Microbiology Letters*, **56**: 271-276.

Durham, J.A., Antone, S.M., Cunningham, M.W. and Confer, A.W. (1988). Monoclonal antibodies to *Pasteurella haemolytica* serotype 1 lipopolysaccharide: Demonstration of antigenic similarities among several serotypes. *J. Clin. Microbiol.*, **26**: 885-889.

Emau, P., Giri, S.N. and Bruss, M.L. (1987). Effects of smooth and rough *Pasteurella haemolytica* lipopolysaccharide on plasma cyclicnucleotides and free fatty acids in calves. *Vet. Microbiol.*, **15**: 279-292.

Gilmour, N.J.L. (1993). Pasteurellosis. *In*: Pasteurella in Production Animals. ACIAR Proceedings No. 43, pp 79-82

Gonzalez, C.T. and Maheswaran, S.K. (1993). The role of induced virulence factors produced by *Pasteurella haemolytica* in the pathogenesis of bovine pneumonic pasteurellosis: Review and hypothesis. *Brit. Vet. J.*, **149**: 183-193

Graydon, R.J., Patten, B.E. and Hamid, H. (1993). The pathology of experimental haemorrhagic septicaemia in cattle and buffalo. *In:* Pasteurellosis in Production Animals. ACIAR Proceedings No. 43, pp. 105-107.

Kotani, S., Takada, H., Tsujimoto, M., Ogawa, T., Mori, Y., Sakuta, M., Kawasaki, A., Nage, M., Kusumoto, S., Shiba, T. and Kasai, M. (1983). Immunological activities of synthetic lipid A analogs and related compounds as compared with those of bacterial lipopolysaccharide, re-glycolipid, lipid A, and muramyl dipeptide. *Infect. Immun.*, **41**: 758

Maswati, M.A., Zamri-Saad, M., Effendy, A.W.M. and Sheikh-Omar, A.R. (1995). Ultrastructural changes in the lungs of goats died of peracute infection by *Pasteurella multocida*. *In*: Proc. 5th Scientific Conference of the Electron Microscopy Society Malaysia, pp. 52-54.

Morrison, D.C. and Ulevitch, R.J. (1978). The effects of bacterial endotoxins on host mediation system. *Am. J. Pathol.*, **93**: 527-617.

Nathan, C.F. (1987). Secretory products of macrophages. *J. Clin. Invest.*, **79**: 319

Paulsen, D.B., Monsier, D.A., Clinkenbeard, K.D. and Confer, A.W. (1989). The direct effects of *Pasteurella haemolytica* lipopolysaccharide on bovine pulmonary endothelial cells *in vitro*. *Am. J. Vet. Res.*, **50**: 1633-1637.

Rietschel, E.T., Galanos, C., Luderitz, O. and Westphal, O. (1982). *In*: Immunopharmacology and the Regulation of Leukocyte Function. Webb, D.R. (Ed.), Dekker, New York. pp. 183-229.

Rietschel, E.T., Brade, H., Brade, L., Kaca, W., Kawahara, K., Lindner, B., Luderitz, T., Tomita, T., Schade, U., Seydel, U. and Zahringer, U. (1985). *In* Bacterial Endotoxins: Structure, Biomedical Significance and Detection with the Limulus Amebocyte Lysate Test. Alan R. Liss, New York. pp. 31-50.

Ulevitch, R.J. (1993). Recognition of bacterial endotoxins by receptor-dependent mechanisms. *In*: Advances in Immunology Dixon, F.J. (Ed.), Academic Press Inc. San Diego, **53**: 267-289

RINGKASAN

PERANAN LIPOPOLISAKARIDA PASTEURELLA HAEMOLYTICA *DALAM PERKEMBANGAN PASTEURELOSIS UJIKAJI DALAM ARNAB*

Dua belas ekor arnab New Zealand White telah dibahagikan kepada empat kumpulan sebelum 3 kumpulan pertama diinokulatkan secara intravena masing-masing dengan 0.7 µg/kg berat badan, 0.4 µg/kg berat badan dan 0.2 µg/kg berat badan lipopolisakarida (LPS) yang dipencil daripada Pasteurella haemolytica *A2. Arnab daripada kumpulan 4 merupakan kawalan bukan terdedah. Kesemua arnab dicerap untuk petanda pasteurelosis sambil pemeriksaan post mortem dijalankan terhadap semua arnab mati. Kesemua arnab dalam kumpulan 1, 2 dan 3 telah didapati mati dalam tempoh 24 j pascapenginokulatan dengan lesi pasteurelosis perakut terdiri terutama sekali daripada kesebakan teruk organ dalaman dan hemoraj dalam mukosa trakea dan peparu. Arnab kawalan terselamat tanpa sebarang lesi. Laporan ini mengesahkan keupayaan LPS dipencil daripada* P. haemolytica *A2 untuk mengaruh penyakit yang menyerupai pasteurelosis dalam arnab.*