UNIVERSITI PUTRA MALAYSIA

IMMUNOMODULATORY ACTIVITY OF MANNHEIMIA HAEMOLYTICA A2 LIPOPOLYSACCHARIDE

AZLINA BT MOHD SALIM

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IMMUNOMODULATORY ACTIVITY OF MANNHEIMIA HAEMOLYTICA A2 LIPOPOLYSACCHARIDE

By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfillment of the Requirements for the Degree of Master of Science

September 2003
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science.

**IMMUNOMODULATORY ACTIVITY OF MANNHEIMIA HAEMOLYTICA A2 LIPOPOLYSACCHARIDE**

By

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September 2003

Chairman : Associate Professor Daud Ahmad Israf Ali, Ph.D.

Faculty : Medicine and Health Science

Bacterial lipopolysaccharides (LPS) are endotoxins. However, there ample evidence to support its role in immunomodulation. In fact LPS is commonly used as a B cell mitogen. The activity of LPS from different genera of bacteria varies considerably which makes generalisation of characteristics difficult. Despite their lethal consequences, the LPS of *Mannheimia haemolytica* A2 have not been tested for immunomodulatory activity. Therefore, the objective of this study is to investigate the adjuvant properties of LPS from *Mannheimia haemolytica* A2 upon peripheral and mucosal immunity toward protein antigen.

The experimental results indicate that, the group of mice that received 10 μg LPS in oil induced anti-BSA IgG response in serum and pulmonary antibody response when administered intraperitoneally. However, in the IgG and IgA intestinal antibody response, mice received 10 μg LPS without oil showed significantly high compared to controls.
This indicates that LPS can be used as oral adjuvant to enhance response at mucosal level. For the oral immunisation, the specific anti-OVA IgG titre in serum and IgA levels in the intestinal fluid were significantly high in group that received 500 μg LPS. The oral administration of 500 μg LPS also increased the number of anti-OVA ASC (antigen secreting cell) observed in spleen, mesentric lymph node and Peyer's patch. Hence, LPS from *Mannheimia haemolytica* A2 does stimulate peripheral and mucosal immune responses to an unrelated antigen.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

AKTIVITI IMUNOMODULATOR MEMBRAN LIPOPOLISAKARIDA DARIPADA BAKTERIA MANNHEIMIA HAEMOLYTICA A2

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Hasil kajian menunjukkan bahawa, kumpulan mencit yang diberi suntikan intraperitoneal campuran 10 µg LPS dan minyak menghasilkan antibodi anti-BSA IgG yang tinggi dalam serum dan
ekstrak pulmonari. Walau bagaimanapun, di dalam ekstrak intestin kandungan antibodi IgG dan IgA telah menunjukkan peningkatan yang baik daripada kumpulan mencit yang disuntik dengan 10 µg LPS tanpa minyak (p<0.05) berbanding kawalan. Ini menunjukkan bahawa LPS boleh diberi secara oral untuk merangsang pembentukan antibodi di dalam sistem mukosa. Keputusan eksperimen menunjukkan bahawa 500 µg LPS yang diberi secara oral kepada kumpulan mencit telah meningkatkan antibodi anti-OVA IgG dalam serum dan IgA dalam ekstrak intestin. Selain daripada meningkatkan paras antibodi, bilangan sel-sel limfoid (anti-OVA ASC) juga meningkat terutama di dalam organ limpa, sel-sel mesentrik dan Peyer's patch. Keputusan ujikaji jelas menunjukkan bahawa LPS dari bakteria jenis *Mannheimia haemolytica* A2 boleh merangsang tindakbalas imun di dalam sistem peripheral dan mukosa terhadap antigen.
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Finally, I thank my colleagues and laboratory partners, Nor Azura and Hartina for their help and great cooperation working with me in the laboratory.
I certify that Examination Committee met on 10th September 2003 to conduct that final examination of Azlina Bt Mohd Salim on her Master of Science thesis entitle “Immunomodulatory Activity of Mannheimia (Pasteurella) haemolytica A2 Lipopolysaccharide” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

AZLINA BT MOHD SALIM

Date: 4/12/2003
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LIST OF ABBREVIATIONS

FCA  Freund’s complete adjuvant
FIA  Freund’s incomplete adjuvant
LPS  Lipopolysaccharides
DAG  2,3 -dideoxy-D-glucose
OMPs  Outer membrane proteins
BSA  Bovine serum albumin
OVA  Ovalbumin
MPL  Monophosphoryl lipid A
MDP  Muramyl dipeptide
CWS  Cell wall skeleton
EAU  Experimental autoimmune uveoretinitis
CTL  Cytotoxic T lymphocyte
TNF  Tumour necrosis factor
IL  Interleukin
IFN  Interferon
APC  Antigen presenting cell
CT  Cholera toxin
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
BHI  Brain heart infusion
TEMED  N-N’-N’-tetramethylethylenediamine
TNP  Trinitropenyl hepten
PBS  Phosphate buffered saline
PMSF  Phenylmethylsulfonyl fluoride
ELISA  Enzyme linked immunosorbent assay
TMB  Tetramethylbenzidine
LT  Heat-labile enterotoxin
GALT  Gut-associate lymphoid tissues
UPM  Universiti Putra Malaysia
IP  Intraperitoneal
HRPO  Horseradish peroxidase
ELISPOT  Enzyme linked immunospot assay
AEC  Amino ethyl carbazole
Immunomodulators are compounds that can cause either suppression or potentiation of the immune system. Immunomodulators are used to stimulate immune responses to vaccines. Adjuvants are generally considered to be materials that are added to vaccines with the intent of potentiating the immune response so that a greater amount of antibody is produced. The most common adjuvants that are safe for human use are aluminium hydroxide, aluminium phosphate and calcium phosphate (Edelman, 1980). Adjuvants that have been used for research purposes are oil emulsions which were first used by Le Moignic and Pinoy (1916), who found that a suspension of killed Salmonella typhimurium in mineral oil increased the immune response. Freund’s complete adjuvant (FCA), a water mineral oil emulsion with killed mycobacterium is used extensively to augment the immune response of laboratory animals for experimental purposes. Freund’s incomplete adjuvant (FIA) which is water in oil emulsion without mycobacterium is used successfully in veterinary rabies, parainfluenza and Newcastle disease. There are also other adjuvants derived from other bacteria and their products.
They play an important role in the immune response. The common bacterial used as adjuvants mainly for experimental purposes include mycobacterium, *Corynebacterium parvum* or *Corynebacterium granulosum*, *Bordetella pertussis* and lipopolysacharides (Rajesh et al., 1993).

Lipopolysacharides (LPS) are common bacterial products that can be used as adjuvants. It is a constituent of the outer membrane of gram-negative bacteria that contains 2,3-dideoxy-D-glucose (DAG), an amino sugar attached to a lipid A backbone. LPS has long been known to be a potent modulator of immune reactions such as B cell mitogenicity, polyclonal antibody synthesis, immunogenicity, adjuvanticity, macrophage activation, monokine secretion and regulation of IgA responses (Qureshi et al., 1990). LPS also induces regression of spontaneous, induced and transplanted tumours in laboratory animals. Its toxicity which involves pyrogenicity, tumour necrosis and lethality has limited exploration of its therapeutic potential in man. However, studies have shown that the toxic compound of the LPS molecule can be cleaved chemically (Ribi et al., 1986). The dose of LPS and the time of exposure will determine the resultant effects that will be seen in the host. It has been shown to effect on B cell but not on T lymphocytes. LPS has been suggested to bind non-specifically to the cell surface followed by intercalation of its hydrophobic lipid A moiety into the plasma membrane (Jacobs, 1992). Others have implicated the involvement of specific receptors on the surface (Tahiri & Chaby, 1990). Recently, it has
been reported that the existence of membrane-associated LPS and lipid A-specific binding proteins on responsive cells, including macrophages, lymphocytes and B cells (Hara Kuge et al., 1990).

There are many gram-negative bacteria that have been used as a source of LPS, particularly members of the enterobacteriaceae {Salmonella, Shigella, Escherichia, Proteus, Pseudomonas, Klebsiella, Mannheimia (Pasteurella)}. Mannheimia haemolytica serotype A2 is the most commonly occurring serotype associated with pneumonic pasteurellosis in cattle and sheep. Immunological studies have been done to determine responses in the respiratory tract using killed M. haemolytica A2 (Zamri et al., 1999). Although experimental vaccines against pneumonic pasteurellosis have been done including both live and killed bacteria (Purdy et al., 1986), research has attempted to identify important immunogenic components of M. haemolytica such as iron-regulated outer-membrane proteins (OMPs) (Gilmor et al., 1991), carbohydrate-protein subunits (Lesley et al., 1985) and LPS (Confer et al., 1986). The immunomodulatory properties of LPS from M. haemolytica A2 have not been studied despite being an important pathogen of livestock. Therefore, the objectives of the study are:-

1. To extract and purify LPS from Mannheimia haemolytica A2.

2. To determine the immunostimulatory effect of M. haemolytica A2 LPS upon peripheral and mucosal immunity toward model protein antigens such as bovine serum albumin (BSA) and ovalbumin (OVA).
CHAPTER 2

LITERATURE REVIEW

2.1 Adjuvants

The response to an immunogen can be enhanced by the use of adjuvants. Adjuvants are compound that potentiate the immune response when mixed and administered with antigen. They enhance and prolong antibody production and increase effector cell counts (Coleman et al., 1992). From this definition, it is obvious that adjuvants are useful and necessary components of the development of vaccines. An adjuvant must be able to increase both amount and duration of antibody and T helper cell response. The mode of action of adjuvants was summarised by Chedid et al., (1975). First, the formation of a depot of antigen at the site of inoculation which is slowly released. Secondly, the presentation of antigen to immunocompetent cells and the production of different lymphokines such as various interleukins and tumour necrosis factor.

It has long been known that many adjuvants are surface-active agents (Allison, 1979). The importance of surface activity in adjuvanticity has been confirmed with modern adjuvants. Saponins, lipid A and muramyl dipeptide (MDP) derivatives are all surface active
in that they consist of discrete hydrophilic and hydrophobic domains. With the use of adjuvants, less antigen is required, thus reducing vaccine production cost (Rajesh et al., 1993). For many years there has been a search to find adjuvants with the ability to potentiate the immune response but with minimal side effects. Freund’s complete adjuvant (FCA) is one of the most potent oil adjuvants described so far and used extensively to augment the immune response of laboratory animals for experimental purposes. FCA as originally formulated was too toxic to be used in human. The water in mineral oil emulsion without Mycobacteria is known as Freund’s incomplete adjuvant (FIA). FIA was used successfully in a number of veterinary vaccines including foot-and-mouth disease, rabies, parainfluenza and Newcastle disease. FIA has been used in human, particularly with influenza and killed poliomyelitis vaccines by enhancing the immunogenicity of the vaccines (Salk, 1977). However, FIA is unsuitable for routine human application because of previously reported side effects (Table 2.1). From these classical adjuvants, there are many alternative adjuvant that have been used for many years such as monophosphoryl lipid A (MPL), Titermax®, saponins, liposome, muramyl dipeptide (MDP), lipopolysaccharides (LPS), cholera toxin and heat labile enterotoxin.
Table 2.1: Side effect of FIA (Rajesh et al., 1993)

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2.1.1 Monophosphoryl lipid A (MPL)

MPL is a non-toxic lipid A with the lack of phosphate on the reducing end of the disaccharide backbone which has 1000-fold less toxic than endotoxin (Ribi, 1984). MPL has immunostimulatory activities such as B cell mitogenicity, activation of macrophages, colony-stimulating activity and the induction of interleukin-1 (IL-1) production by human monocytes (Rudbach & Cantrell, 1990). Tomai et al., (1987) showed that MPL has an adjuvant activity with poly-L-lysine antigen in LPS-hyporesponsive C3H/HeJ and C57BL/10ScN mice and in aging BALB/c mice, which have a low capacity to produce antibody, when examined by a modified haemolytic plaque assay. They also reported an adjuvant effect to sheep red blood cell (SRBC) antigen in young and ageing mice (Johnson et al., 1987). Schneerson et al., (1991) also reported that both the primary and secondary serum antibody responses to the capsular polysaccharides (CP) antigens in mice were stimulated