A COMPARISON OF THE PULMONARY EFFECTS OF LIPOPOLYSACCHARIDE, LIPID A AND DETOXIFIED LIPOPOLYSACCHARIDE IN THE GUINEA PIG

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Abstract

Much evidence is available to suggest that there is a reduction in the toxicological potential of *Escherishia coli* lipopolysaccharide (LPS) after it has undergone heat and chemical detoxification[1]. The marker used to measure the change in toxic potency of the molecule was that of an alteration in the ability of LPS to induce changes in responsiveness of both isolated airway smooth muscle and that measured by plethysmography in the guinea pig. The process of detoxification resulted in the removal of the portion of LPS known as lipid A. The hypothesis was formulated that the lipid A portion of LPS was responsible for the major part of the hyperresponsive status of the airway after acute exposure.

In an effort to confirm this hypothesis samples of isolated lipid A have been obtained that bear none of the other characteristics of 'parent' LPS. Lipid A demonstrated that it is capable of causing a ten - fold leftward shift in a methacholine log dose-response curve (a similar shift is also seen after LPS treatment). Neutrophilia associated with LPS inhalation is also demonstrated after Lipid A inhalation Lipid A as well as significant decrease in sGaw at an hour post exposure and a significant increase in sGaw at twenty four hours post exposure. Lipid A is a toxicologically important component of LPS, playing a major role in mediating the damaging effects that LPS has on the airway.

Introduction

Lipopolysaccharide derived from gram-negative bacteria is firmly established as an agent capable of causing important physiological changes when it interacts with constitutive biochemical systems. The most important impact of LPS on animal systems is when the substance is introduced intravenously. Work this field has been a stimulus in the development of a fuller understanding of the action and interaction of complex biochemical systems such as the cytokines and prostanoids. In conjunction with continuing work on the toxicity of organic dusts, research into the properties of inhaled endotoxin has also has developed.

As a refinement of the studies carried out thus far; this study attempts to elucidate which portion of LPS is responsible for the mediation of its pulmonary toxicity in the guinea pig.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:208-212 (1997)
National Cotton Council, Memphis TN

The experiment is composed of four exposure types i.e. exposure to four different entities:

1. Parent LPS structure
2. Detoxified LPS (Lipid A portion removed by chemical de-esterification)
3. Lipid A alone
4. Saline (as a vehicle control)

Each of the these is examined in a well characterized animal model capable of measuring:

1. *In vitro*
2. *In vivo*
3. Inflammatory

markers in response to a challenge from the parent LPS.

Lipid A was first discovered by Boivin and co - workers in 1933[2]. The discovery occurred when a precipitate was formed on acid hydrolysis of endotoxins. This produced the first definition of lipid A. The structures of chemically isolated lipid A and of lipid A as it is present in intact LPS's are closely related in many cases e.g. *Salmonella* and *E.coli*.[3]

Lipid A was first proposed as the endotoxic component of LPS when it was recognized that this type of structure was ubiquitous for a wide range of enterobacterial lipopolysaccharides. Another piece of evidence exists in that the O - antigenic component was so varied in composition and structure that it did not seem to be a likely candidate for bearing common endotoxic properties.[4]

The chemical structure of Lipid A indicates that it as an amphipathic molecule with a hydrophobic center and hydrophilic periphery (phosphate). Furthermore, it may be an amphoteric molecule, carrying both acidic and basic functional groups.

Work carried out by Niwa[5] *et al* describes a method by which LPS can be detoxified by heating in mild alkali as assessed by biological parameters such as lethality for chick embryos and pyrogenicity in rabbits and chemical parameters such as degree of O - acetylation to characterize the degree of detoxifiaction[5].

Methods

Drugs used

The drugs used for the lavage (inflammation) study and the plethysmography (*in vivo)* were made up in non - pyrogenic, sterile saline (Baxter, Thetford, UK). The lavage vehicle used was phosphate buffered saline and all drugs used in the *in vitro* procedure were dissolved in Krebs solution (vehicle).

Methacholine chloride, detoxified LPS, Lipid A and LPS (*E. coli* serotype 026:B6 lipopolysaccharide containing 1 -

10% protein, prepared using trichloroacetic acid extraction) were obtained from Sigma Chemicals UK Ltd.

Animals and husbandry

Male Dunkin Hartley guinea pigs of weight range of 350 - 550g were employed throughout. Prior to the experiments the animals were housed in groups of up to six animals in North Kent Plastic aluminum A1 guinea pig holding units (76 x 71 x23 cm) with stainless steel grid floors. The trays beneath the cages were lined with newspaper which was changed three times per week. Tap water, oranges and pelleted food (FDP; Special Diet Services, Bicester, Oxford) were available *ad libitum*. Temperature and humidity were maintained at 21^0c and 50% respectively. A light -dark cycle of 12h was maintained throughout.

Experimental procedure

a) *In vitro* procedure.

The animals were sacrificed by cervical dislocation and exsanguination, the trachea and lungs were then removed quickly to warmed Krebs solution. (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, Na_2HPO_4 1.2, $NaHCO_3$ 25 and glucose 11.1(mmoll^{-1})). The excised respiratory organs were cleared of extraneous connective tissue and blood vessels. The lungs were cut into the left and right halves at the bifurcation and attached to a cannulae (via the bronchi) using surgical cotton. The lungs were then perfused with Krebs solution using a Watson - Marlow peristaltic pump (rate of flow: 5ml min^{-1}). Their viability was maintained by gassing with a mixture of 95% oxygen & 5% carbon dioxide and the temperature of the Krebs was maintained by using a circulator set at 35^0c. The trachea was cut into two 4cm spirals according to the method of Constantine *et al*, 1957[6] and suspended in organ baths in Krebs solution and maintained at the same conditions of temperature and oxygenation as the lungs. Both tissue types were left to equilibrate for one hour. Dose - response curves were then obtained to the bronchoconstrictors prior to adding LPS (30µg ml^{-1}), d-LPS (30µg ml^{-1}), Lipid A (30µg ml^{-1}) or vehicle alone solution to the organ baths (trachea) or perfusing LPS solution (lung). The LPS was dissolved in the vehicle (Krebs). The LPS solutions remained in contact with the tissues for one hour. One hour after replacing the bath fluid with Krebs solution, the dose - response curves to the bronchoconstrictor were repeated. The contractions in the tracheas were recorded on a Devices MX4 recorder via a Dynamometer UFI isometric transducer. The pressure changes in the lung were recorded on the same recorder via a Bell & Hartley pressure transducer. One lung half was used as a time control to monitor changes of reactivity in normal tissue. Owing to the differing responses of the left and right halves of the lung 'cross overs' were carried out i.e. the 'test' was right lung for the first experiment, left for the second experiment etc. The trachea was cut into two halves ; one 4cm spiral acting as a control and the other as the test tissue.

b) I*n vivo* procedures

i) Exposures

The guinea pigs were placed in an exposure chamber (volume 0.4m^3) for exposure to either nebulised I) 0.9% sterile saline solution, ii) lipopolysaccharide from *E. coli*, iii) detoxified lipolysaccharide from *E. coli* or iv) Lipid A (all at a concentration of 20µgml^{-1} in sterile saline) for one hour. For exposure a Hudson nebulsier was used air at a pressure of 15 p.s.i. This apparatus produced an aerosol with a greater than 60% of particles with a MMAD of 5µm or less which ensured an equilibrium concentration of 20µg m^{-1} of LPS within the exposure chamber.

ii) Procedure

All animals used in the *in vivo* procedure were trained for use in the plethysmograph according to the method of Griffiths - Johnson *et al*[7] (which also describes the determination of sGaw). Firstly, a baseline sGAW value was obtained for the response to the bronchoconstrictor methacholine (exposure to a 10µg ml^{-1} solution occurred for 1 min using a Wright nebuliser operating at 20 p.s.i.) sGaw was measured 5min prior to exposure to the bronchoconstrictor and then also measured at 2,5,10 & 15min post - exposure. The post - exposure sGaw divided by the baseline sGAW gave a percentage value for the initial reactivity (IR). Twenty four hours later, the guinea pigs were exposed to either saline, LPS, d - LPS or Lipid A as described above then at the given time points 1h,4h, 24h, 48h & 72h the bronchoconstrictor challenge was repeated and a second reactivity value was calculated (NeR) and the change in reactivity value was therefore calculated as IR - NeR. The dose of methacholine given was judged to induce an IR of 30-40% so that decreases and increases in reactivity were detectable. Groups of six to eight animals were used for each exposure.

c) Lavage and cell count procedure

Animals of the same weight range as in (a) were exposed to the test agents as described in (b) above. Each was given a lethal i.p. injection of pentobarbitone sodium (100mg/ ml). The thoracic area was cut, revealing the trachea which was incised and a Portex 6FG yellow Luer cannula inserted . The cannula was tied into place using surgical cotton and a 10ml syringe was attached to the free end. 5 x 10ml aliquots of phosphate buffered saline were introduced and removed in a gentle fashion to and from the lungs. The resulting suspension was used to perform differential and total cell counts providing that a greater than 60% of the lavage fluid was returned. This gave an indication of whether there was leakage of fluid from the lung and an assessment of the degree of physical damage suffered by the lung tissue due to experimental procedures.

- Total cell count.

A coverslip was placed on an improved Neubauer Haemocytometer. To obtain a total cell count a small amount of the suspension was drawn into a capillary tube an

a drop placed at the edge of the coverslip. Capillary action drew the cell suspension into a 'monolayer' under the coverslip. The cells were allowed to settle for 1 min. Under the microscope it can be seen that the counting chamber consists of 25 squares. For each cell suspension four counts were performed and the mean was calculated.

- Differential cell counts.

The cell suspension (0.1 ml) was transferred to chambers prepared for use in a Shandon Cytospin III. The cells were spun at 1000rpm for 6 min at the low acceleration setting. On removal they were dried in air by a fan. They were than fixed by placing in 95% ethanol for 5 mins. The slides were than redried and placed in Giemsa stain for 5 min. Following this they were washed with distilled water and placed in May - Grünwald stain for 2 min. The rinsing procedure was then repeated and left to dry. Differential cell counts of monocytes (mainly macrophages), eosinophils and neutrophils were performed counting at least three hundred cells per slide. The counting procedure entailed starting at the left edge of the circle of cell produced by the cytospin on the slide. All cells in the field of vision were counted and the slide was adjusted so that the next field of vision immediately to the right was obtained. This ensured the sample of cells counted was representative and reduces possible errors that may occur due to an uneven distribution of cells by the cytospin caused by the different masses of the cells.

Results

In figure 1 it can be observed that after an hours' exposure to LPS the response of the lung tissue to the bronchoconstrictor methacholine has shifted significantly to the left ($p<0.05$). The shift is approximately one logarithmic cycle. This indicated an increase in the sensitivity of the tissue to methacholine. The maximum response is unaltered. Similar results are also observed in the trachea[1]. Figure 2 highlights the fact that no significant shift is seen in the tracheal preparation after to a similar exposure to d-LPS, the same lack of shift is observed with the perfused lung preparation. However exposure to Lipid A causes a similar shift to that caused by the parent LPS (figure 3). Once again the shift is to the left suggesting an increase in the sensitivity of the tissue on exposure to the agent. The same phenomenon is seen in the tracheal preparation treated to the same exposure protocol. The shift appears to be approximately one log - cycle. Time matched controls suggest that there is no significant, spontaneous change in reactivity over the time span of the experiment.

Figure 4 illustrates the *in vivo* responses to the agents employed. As previously illustrated[1] a phase of hyperresponsiveness at one hour post exposure LPS exposure, followed by a return to baseline values and a phase of hyporesponsiveness at 24h to 72h when values return to baseline values. Lipid A causes a similar pattern in change of reactivity. The d - LPS causes no significant change in reactivity, at the salient time points illustrated above, when compared to the effects of saline.

Both LPS and Lipid A caused an increase in total cell numbers, numbers of macrophages and neutrophils in the broncho- alveolar lavage fluid but no significant change in the number of eosinophils (Figure 5 - 8). d -LPS caused no increase in the total numbers of cells in the airway or any significant increase in each individual cell type that is increased by the inhalation of LPS or Lipid A.

Discussion

The results suggest that the Lipid A portion of the molecule is the most important in terms of inhaled toxicological potential. Most importantly the results illustrate the role of Lipid A in the development of the changes in responsiveness (both *in vitro* and *in vivo*) and in the development of airway inflammation. The induction of these changes illustrates the potential damage that LPS and Lipid A can cause both in the long and short term. Confirmation of the importance of Lipid A is made by the fact that the d - LPS is ineffective in causing any of the changes that are induced by the isolated Lipid A or the parent LPS.

Although Lipid A is not statistically different from the intact LPS structure, there is a trend suggesting that the intact LPS is a more potent agent. This would indicate that wherever LPS binds a complete structure with o - specific side chain may be more important than isolated Lipid A.

A benefit would be obtained if these results were repeated using a different strain of the *E coli* bacterium, repeated using an entirely different gram negative organism and also carried out in a different species other than the guinea pig. To appreciate the value of these experiments in terms of the potential toxicity of cotton dust or its' various extracts it would be worth assessing the content of the dusts and extracts to quantify the presence of LPS of various types and the presence of Lipid A from those wild types that do not possess o - specific side chains.

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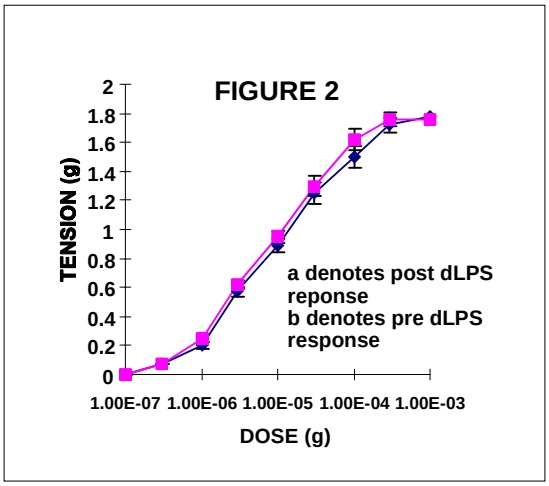
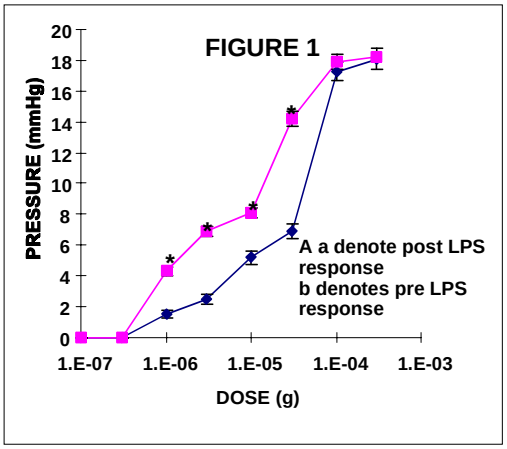
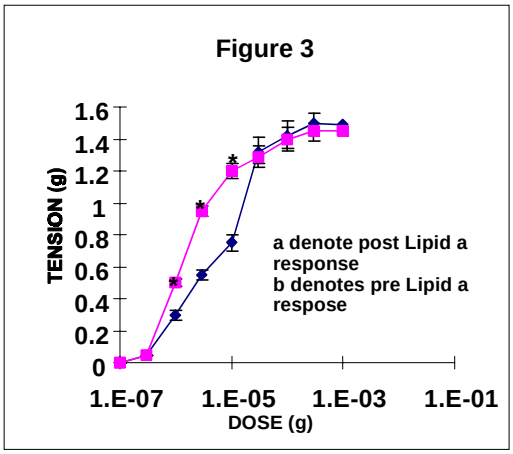


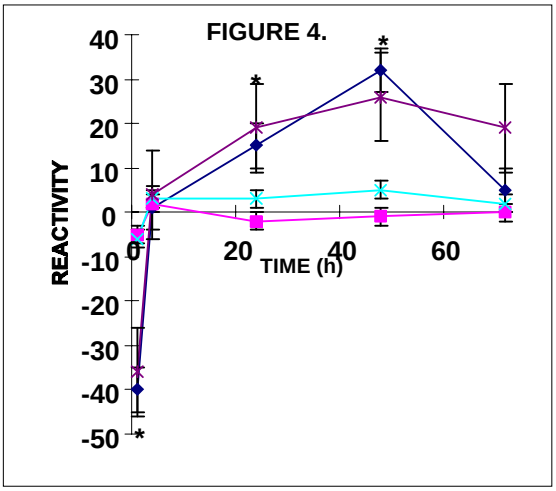
Change in reactivty of tracheal spirals to methacholine one hour post - dLPS exposure.
Values are means+/- S.E.M. n= 6. Paired Students t -test.



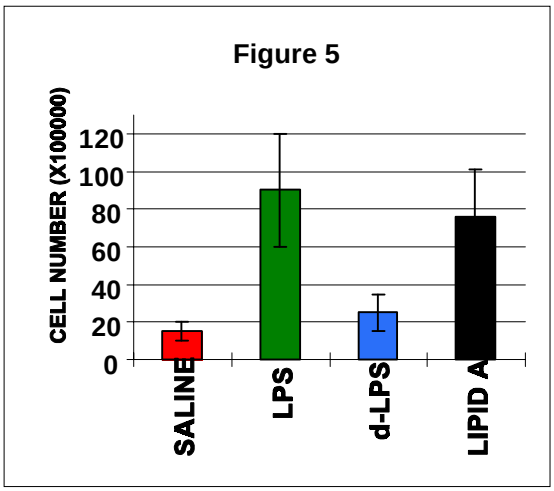
Change in reactivty of perfused half lung to methacholine one hour post - LPS exposure.
(* indicates p<0.05). Values are means+/- S.E.M.
n= 6. Paired Students t - test.



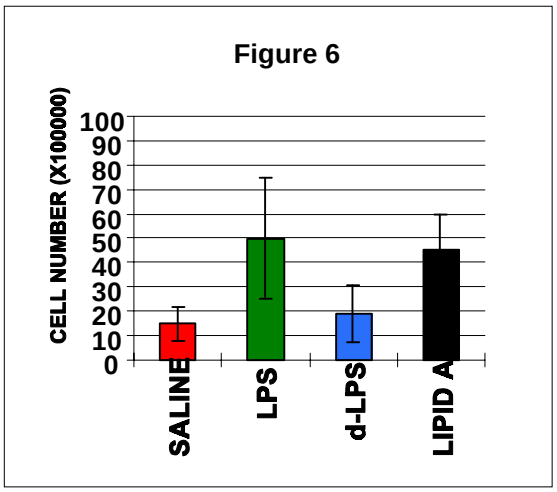
Change in reactivty of perfused half lung to methacholine one hour post - Lipid A exposure.
(* indicates p<0.05). Values are means+/- S.E.M.
n= 6. Paired Students t - test.



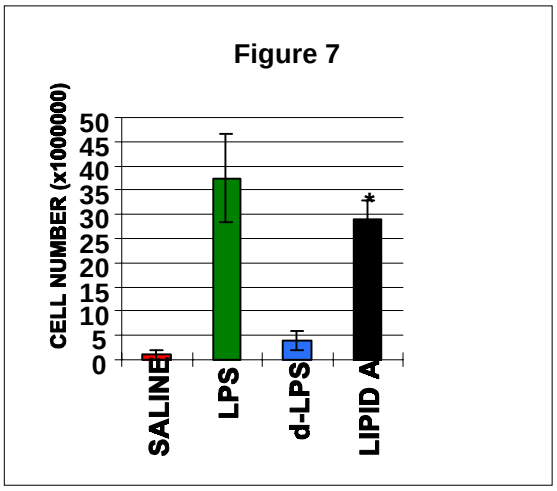
The effect of a single exposure of A) LPS, B) d - LPS, C) Lipid A or D) saline on the reactivity of guinea pig airways to methacholine at various time points post - exposure.



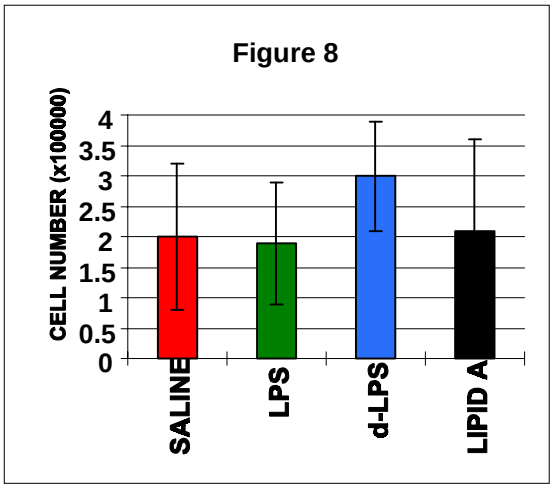
The effects of saline, LPs, d-LPS and Lipid A twenty four hours post exposure on total cell number recovered from lavage fluid. n=6
***p<0.05. Errors are +/- S.E.M.**



The effects of saline, LPs, d-LPS and Lipid A twenty four hours post exposure on macrophage cell number recovered from lavage fluid. n=6
*p<0.05. Errors are +/- S.E.M.



The effects of saline, LPs, d-LPS and Lipid A twenty four hours post exposure on neutrophil cell number recovered from lavage fluid. n=6
***p<0.05. Errors are +/- S.E.M.**



The effects of saline, LPs, d-LPS and Lipid A twenty four hours post exposure on eosinophil cell count recovered from lavage fluid. n=6*p<0.05. Errors are +/- S.E.M.