A COMPARISON OF THE EFFECTS OF LPS AND DETOXIFIED LPS ON REACTIVITY CHANGES INDUCED IN THE GUINEA PIG AIRWAY.

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Abstract

Lipopolysaccharide (LPS) obtained from the bacterial species *Escherchia coli* has previously been shown to induce reactivity changes in guinea pig airway smooth muscle[1]. As part of a continuing investigation into the mechanisms involved in the induction of hyperresponsiveness, a study has been carried out to determine if one constituent of the LPS unit is responsible for the changes involved. *In vitro* and *in vivo* experiments were carried out to compare changes between normal 'untreated' LPS and that obtained from a preparation of LPS subject to treatment by mild alkali[2] to remove the lipid A (d - LPS).

In vitro, 'normal' LPS induced a phase of smooth muscle hyperreactivity 1h post LPS exposure. However, the detoxified LPS showed no deviation from the control dose - response relationship to an applied spasmogen and hence does not cause any change in reactivity.

In vivo, a similar pattern was observed 1h post -LPS exposure. 24h after exposure to 'normal' LPS the airway was hyporeactive to an inhaled bronchconstrictor and this persisted for a further 24h after which a return to baseline values occurred. The detoxified LPS caused no significant change compared to that seen in the saline exposed group, *in vivo*.

The detoxification of LPS (the removal of lipid A) is an important aspect in prevention of induction of airway hyperreactivity changes in the guinea pig.

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 significant impact that LPS has on animal systems occurs 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 cytokine network. In conjunction with continuing work on the toxicity of organic dusts, research into the properties of inhaled LPS has also has developed. As part of the work into developing further understanding of the mechanism by which lipopolysaccharides cause airway hyperresponsiveness, an *in vitro* and *in vivo* investigation was undertaken to establish whether any particular component of the LPS molecule was important in the development of reactivity changes of the guinea pig airway to a bronchoconstrictor agent (methacholine).

This experiment involved the use of detoxified LPS (removal of the lipid A component) as a comparison to the complete LPS . Lipid A was first found by Boivin and co - workers in 1933[3], discovery occurring when a precipitate was formed on acid hydrolysis of LPSs. The hydrolytic process undertaken gave rise to the first true definition of lipid A as a component of gram negative LPS. The chemical 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*[4] and can also be widely different e.g the comparison between lipid A from *Salmonella* and *Chromobacterium*[4].

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[5]. Further support for the toxicological importance of Lipid A comes indirectly from the observation that the composition and structure of the o - antigenic fragment of the molecule varied greatly among different LPSs, the latter all exhibiting common endotoxic properties[4].

The chemical structure of lipid A indicates that it as an amphipathic molecule with a hydrophobic center (acylated disaccharide) and hydrophilic periphery (phosphate). Furthermore, it can be an amphoteric molecule, carrying both acidic and basic functional groups. This provides further evidence that it is capable of interaction both in a physical and chemical manner with biological organelles such as membranes and other large protein molecules such as receptors.

Work carried out by Niwa[2] *et al* describes a method by which LPS can be detoxified by heating in mild alkali using 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 detoxification[6].

Detoxification has proven to be important in the control of the effects of inhalation of organic dusts[7] . The same may true in the case of LPS and this preliminary investigation looks at the effect of lipid A removal.

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Methods

Materials used

Methacholine chloride, LPS (*E. coli* serotype 026:B6 lipopolysaccharide containing 1 - 10% protein, prepared using trichloroacetic acid extraction) and detoxified LPS were obtained from Sigma Chemicals UK Ltd.

For use in the plethysmography work, all compounds were dissolved in non - pyrogenic sterile saline (Baxter, Thetford, UK). All drugs used in the *in vitro* procedure were dissolved in Krebs solution.

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 aluminium 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 procedures

a) *In vitro* procedures

The animals were sacrificed by cervical dislocation and exsaguination, 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(mmol l^{-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 each was attached to a cannula (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}). Viability was maintained by gassing with a mixture of 95% oxygen & 5% carbon dioxide and the temperature of the Krebs was maintained at 35^0c. The trachea was cut into two 4cm spirals according to the method of Constantine[8] 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 bronchoconstrictor prior to adding LPS / d - LPS solution to the organ bath (trachea) or perfusing LPS / d - LPS solution (lung). The LPS's was dissolved in the 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 over experiments were carried out i.e. the 'test' was right lung for the first experiment, left for the second experiment etc. In the case of the trachea it was cut into two halves ; one 4cm spiral acted as a control and the other as the test tissue. Time matched controls were undertaken to ensure that time of perfusion alone had no effect on reactivity change.

b) I*n vivo* procedure

Exposure

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) lipolysaccharide from *E. coli* or ii) detoxified lipolysaccharide from *E. coli* (both at a concentration of 20µg/ml in sterile saline) for one hour. For exposure a Hudson nebuliser was used with air at a pressure of 15 p.s.i. This apparatus produced an aerosol with greater than 60% of particles having a MMAD of 5µm or less which ensured an equilibrium concentration of $20\mu g/m^3$ of LPS within the exposure chamber.

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*[9] (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 & 15 min 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 or d - LPS 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.

Results

a) *In vitro*

In figure 1 it may be observed that 1h after exposure to perfused LPS the lung had become significantly ($p<0.05$) more sensitive to the constrictor agent, methacholine, as detected by the leftward shift of the dose - response curve. However it should also be observed that the maximum response of the lung was unaltered. A similar pattern may be observed in figure 3 for the tracheal preparation. Figures 2 and 4 illustrate that no significant change of reactivity was observed post d - LPS exposure i.e. there was no

significant leftward or rightward shift compared to control values indicating the was no change in reactivity status of the tissues and no change in maximum response occurred. Time matched control suggested that no significant ($p < 0.05$) change in reactivity occurred in control experiments over the time span of the experiments.

b) I*n vivo* procedure

Figure 5 illustrates the change in bronchial reactivity to inhaled methacholine caused by exposure to normal LPS *in vivo*. Hyperreactivity is witnessed 1h post exposure, followed by a recovery to baseline values at about four hours post - exposure. At a point 24h post -exposure a phase of hyporeactivity begins until a recovery to baseline values at 72h. The detoxified LPS has caused no significant change compared to that seen in the saline exposed group, at any of the key time points. It may be clearly observed that exposure to d -LPS was unable to affect the bronchoconstrictor action of inhaled methacholine when examine at several time points up to 72h after LPS inhalation.

Discussion

The results obtained suggest that the lipid A portion of LPS is an important factor in the production of changes in reactivity status of the airways. In both models used the removal of the lipid A from the LPS results in a significant detoxification and virtual elimination of changes in reactivity status. Further investigation would now be worthwhile in other models to discover if results are comparable.

These results may have a significant bearing on results obtained from work carried out on aqueous cotton dust extracts that whose original cotton had been heat - treated in order to 'detoxify' it. The heat - treatment has been shown to significantly reduce endotoxin content[10]. The heat - treated cotton has been shown to have a reduced propensity to induce reactivity changes in the guinea pig airway[7] . It would be prudent to investigate the structure of the LPS present in the untreated and heat -treated cotton dusts and comparing their structures to elucidate whether any significant change has taken place.

The current results will be further expanded by an investigation into the effects of d - LPS on the neutrophilia witnessed at 24 & 48h post - exposure to untreated LPS. A sample of isolated lipid A from *E. coli* has also been obtained and a study paralleling the d - LPS undertaken her using the lipid A alone is being undertaken.

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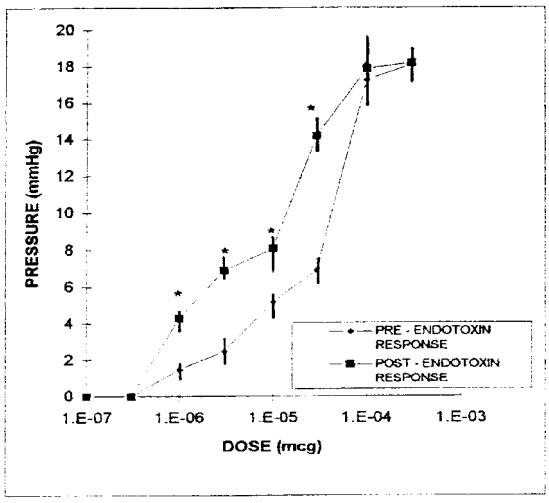

Figure 1. Change in reactivity of perfused half lung to methacholine one hour post- LPS exposure. (*indicates $p<0.05$). Values are means +/- S.E.M. n=6. Paired students t-test.

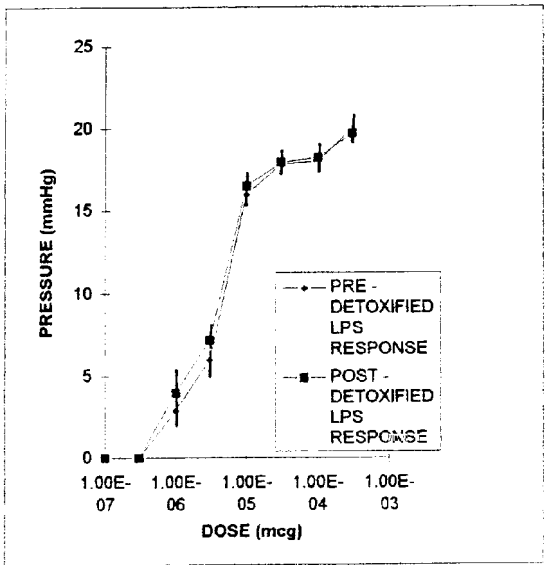

Figure 2. Comparison of reactivity of perfused half lung to methacholine pre and one hour post- detoxified LPS exposure. (*indicates $p<0.05$). Values are means +/- S.E.M. n=6. Paired students t-test.

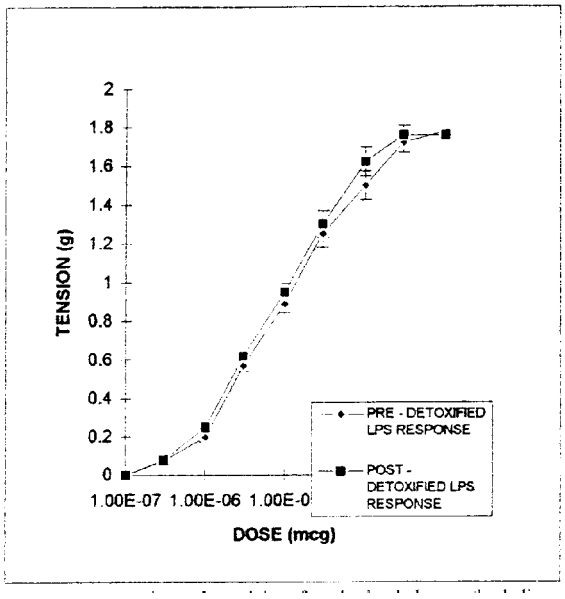

Figure 4. Comparison of reactivity of tracheal spiral to methacholine one hour post detoxified LPS exposure. (*indicates $p<0.05$). Valuess are means +/- S.E.M. n=6. Paired students t-test.

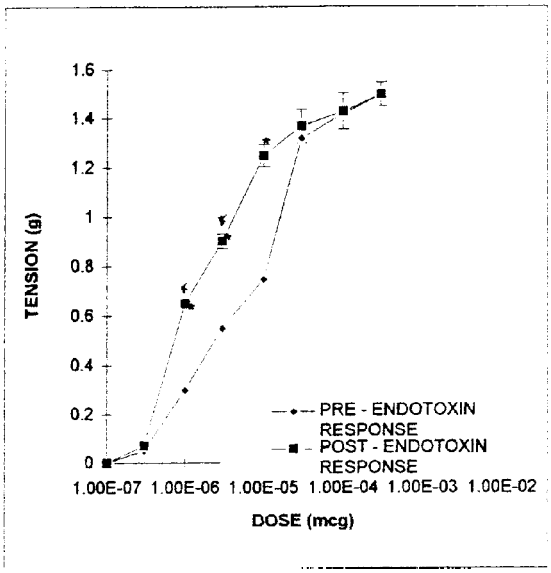

Figure 3. Change in reactivity of tracheal spirals to methacholine one hour post - LPS exposure. (*indicates $p<0.05$). Values are means +/- S.E.M. n=6. Paired students t-test.

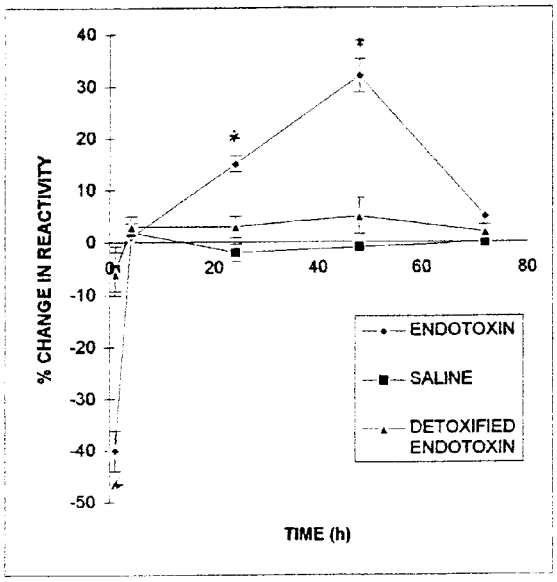

Figure 5. The effect of a single exposure of LPS, d - LPS or saline on the reactivity of guinea pig airways to methacholine at various time points post-exposure. ($p<0.05$, n=7).