

Modification of Acute and Late-Phase Allergic Responses to Ovalbumin with Lipopolysaccharide

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Key Words

Lipopolysaccharide · Allergic sensitization · Lung inflammation · Rats

Abstract

Background: We have previously shown that lipopolysaccharide (LPS) exposure in sensitised animals 18 h after ovalbumin (OVA) challenge inhibits OVA-induced airway hyper-responsiveness (AHR). In the present study, we investigated the effect of LPS on OVA-induced acute and late-phase allergic responses in sensitised rats when challenged with OVA. **Methods:** Rats were sensitised with OVA and 11 days later challenged with 1% OVA in the presence or absence of LPS (0.5–50 µg/ml) given in the same nebulizer. Acute responses to OVA were measured each minute for 30 min after challenge. In a separate group of animals, late-phase responses to OVA were determined at 24 h. At the end of each study, Evans blue dye was injected and animals sacrificed 30 min later. Bronchoalveolar lavage was obtained to monitor inflammatory cell migration and microvascular leakage. **Results:** OVA challenge in sensitised animals produced an acute response with changes in lung me-

chanics peaking 10.0 ± 0.9 min after OVA and returning to baseline within 30 min. This was followed 24 h later by increased responses to methacholine chloride (MCh), inflammatory cell influx and increased Evans blue leakage into the lungs. Presence of 5 or 50 µg/ml LPS in the nebulizer during OVA challenge altered the kinetics of the acute-phase response, with an immediate decrease in lung function (time to peak decreased from 10.3 ± 1.2 to 1.8 ± 0.2 and 2.2 ± 0.3 min, respectively: $p < 0.001$, $n = 6$) and a dose-dependent attenuation of late-phase AHR, cellular influx ($n = 5$, $p < 0.001$) and Evans blue leakage ($n = 5$, $p < 0.001$) at 24 h. **Conclusions:** In summary, co-administration of OVA with LPS modifies both the acute and late-phase responses to the allergen, inducing an earlier acute change in lung function and a dose-dependent inhibition of late-phase responses to the allergen.

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Introduction

Current theories on how asthma is initiated focus on an imbalance in T helper (Th) cells between type 1 (Th1) and type 2 (Th2) responses. The development of Th2

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1018–2438/02/1292–0119\$18.50/0

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memory by T lymphocytes consolidates the atopic phenotype. Clinical asthma, however, is thought to result from repeated environmental allergen challenge leading to persistent airway inflammation and tissue remodeling. The so-called 'hygiene hypothesis' suggests that decreasing levels of exposure to infections [1, 2] and/or commensal microbial stimuli [3] in developed countries, particularly during the induction of primary Th1/Th2 responses to aeroallergens in early life [3, 4], may contribute to an increased asthma prevalence. Bacterial lipopolysaccharide (LPS), a constituent of the outer membrane of Gram-negative bacteria, which is present in domestic and occupational environments [5, 6] has been suggested as one potential mediator of these effects.

The potential importance of LPS as a trigger of asthma has been suggested by numerous studies showing reduced lung function and bronchial obstruction associated with increased acetyl- β -methacholine chloride (MCh) responsiveness after LPS inhalation [5, 7, 8]. Co-exposure of allergen with bacterial product might be expected to occur with natural exposures, as common antigens are known to contain LPS. A study by Michel et al. [9] has reported that in common house dust, house dust mite antigen makes up ~ 0.13% of dust weight while LPS makes up ~ 0.03% and they have shown a significant correlation between domestic LPS exposure and clinical severity of asthma [9]. In another study, contamination of allergen extracts with endotoxin (LPS) caused neutrophilia in broncho-alveolar lavage (BAL) fluid after segmental broncho-provocation with allergen in asthmatic patients and abolition of their late-phase response to allergen [10].

In our laboratory, we have shown that whilst early exposure of rats to aerosolized LPS protects against the development of ovalbumin (OVA)-specific IgE, exposure to LPS 18 h after OVA challenge completely abolished late-phase airway and parenchymal hyper-responsiveness to MCh and significantly reduced the influx of inflammatory cells into the BAL fluid of sensitised rats [11]. Those studies have demonstrated that LPS exposure can have complex effects on the consequence of allergen challenge depending on the timing of bacterial exposure.

In the present study, we investigated the effects of LPS exposure on acute and late-phase responses to allergen when given together with allergen as might be expected to occur with natural exposures or when allergens are contaminated with bacterial products. We used an in vivo rat model of allergic inflammation to measure changes in respiratory mechanics, cellular infiltration and microvascular leakage using the BAL fluid recovered from the animals immediately following or 24 h after OVA exposure.

Methods

Animals

Male Piebold-Virol-Glaxo (PVG) rats weighing 200–250 g (Research Centre, Institute for Child Health Research, Perth, Australia) were studied. They were barrier housed in a clean animal house environment, kept on an OVA-free diet and had access to water and food ad libitum. Regular monitoring ensures that the colony is kept free of known pathogens. The study protocol was approved by the Institutional Animal Ethics Committee.

Sensitisation Procedure

Eight-week-old rats were actively sensitised on day 0 by a single intraperitoneal injection with 100 μ g OVA in phosphate-buffered saline (PBS), along with 50 ng of the IgE-selective adjuvant ricin.

Measurement of Lung Function

Animal Preparation. For measurement of lung function, animals were anaesthetised by intramuscular injection of xylazine (12 mg/kg) and ketamine (40 mg/kg) immediately after or 24 h after OVA challenge in sensitised animals to measure acute and late-phase responses to OVA, respectively. Tracheotomy was performed and the femoral vein cannulated with polyethylene tubing for the intravenous injection of drugs. The other femoral vein was also cannulated in the rats receiving MCh infusion.

Forced Oscillation Technique. Lung mechanics was measured using low-frequency forced oscillation technique as previously described [11] in which input impedance (Z_L) was measured using a wave tube. Briefly, measurements were made by applying loudspeaker-generated small-amplitude oscillatory signals from 0.5 to 20 Hz through a wave tube during a 6-second apnoeic period. A three-way tap was used to switch the animal from the respirator to a loudspeaker-in-box system at end-expiration. The mean pressure in the loud-speaker was adjusted to 2.5 cm H₂O to keep the transpulmonary pressure constant during measurements. To separate airway and tissue mechanics, constant-phase model containing a frequency-independent airway resistance (R_{aw}) and a frequency-dependent tissue resistance (G) and elastance (H) was fitted to Z_L spectra [12].

Acute-Phase Response

Acute responses to inhaled 1% OVA were determined 11 days after sensitisation, when serum anti-OVA IgE antibodies are at peak levels. Following an equilibration period and establishment of baseline, responses to OVA in naive or in sensitised rats were measured in the presence or absence of LPS (*Salmonella typhimurium*; 0.5–50 μ g/ml) in the same jet nebulizer (LC PLUS, Pari-Werk, Starnberg, Germany). The aerosol mixture was inhaled for 90 s and lung function measured each minute for 30 min thereafter. Responses were characterized as the time-to-peak response (in minutes) and area under the curve (AUC) from plots of lung function versus time using ANADAT (RHT Infodat, Montreal, Canada).

Late-Phase Response

In a separate group of animals, also studied on day 11 after sensitization, animals were placed in a Plexiglass chamber and challenged with a single dose of aerosolized 1% OVA for 30 min using the ultrasonic nebulizer (De Vilbiss Ultra-Neb™ 2000, Sunrise Medical, Somerset, Pa., USA). Output of the nebulizer was 0.5 ml/min and the mean particle size was 3.5 μ m (manufacturer's specifica-

tions). Late-phase responses to OVA were determined 24 h after OVA challenge in the presence or absence of LPS.

After an equilibration period, four to six measurements of Z_L were made, ensemble-averaged and the constant-phase model fitted to determine baseline mechanics. MCh was infused at rates of 2, 4, 8, or 16 $\mu\text{g/kg/min}$ using an infusion pump (Stoelting 220VAC, Wood Dale, Ill., USA). At each dose, four successive measurements of Z_L were made, 1 min apart, commencing 90 s after MCh administration. The constant-phase model was fitted to individual Z_L curves. MCh infusion took a total of 25–35 min to complete. Peak responses in R_{aw} or G at each dose were used for analysis. Responses are reported as a percentage increase above baseline (same animal at 0 MCh) for each parameter.

Microvascular Permeability

In both groups of animals, Evans blue dye (50 mg/kg) was administered by intravenous injection, over a 2-min period immediately after measurements of lung function. The amount of Evans blue dye in the lavage supernatant was quantified by measuring the absorbance at 630 nm using a spectrophotometer (Microplate AutoReader EL311, BIO-TEK Instruments, Winooski, Vt., USA). The concentration of dye was extrapolated from a standard curve of Evans blue dye concentrations (1–10 $\mu\text{g/ml}$).

Broncho-Alveolar Lavage

Thirty minutes after Evans blue administration, animals were sacrificed with pentobarbitone and their chest opened. BAL was performed via the tracheal cannula using 3×8 ml of PBS containing lignocaine hydrochloride (0.35%) and bovine serum albumin (0.2%). Routinely, >90% of the lavaging fluid was recovered from the lungs. The lavage fluid was centrifuged at 250 g for 10 min at 4°C , and the cell pellet resuspended in 1.0 ml sterile PBS solution. Total cell count was determined by adding 20 μl of the cell suspension to 20 μl trypan blue stain and counting cells under a light microscope using a Neubauer haemocytometer. The differential cell count was carried out from cytopspin preparations using Leishman's BDH stain and counting 200 cells at random under a magnification of $\times 100$. Cells were identified by standard morphology.

Serum Antibody Measurements

Blood (1 ml) was collected via cardiac puncture for the estimation of OVA-specific serum antibody titres to confirm allergic sensitization. OVA-specific IgE titres were measured by ELISA according to the method of Van Halteren et al. [13]. Briefly, microtitre plates (Falcon flexible assay plate 3912, Becton Dickinson, Mountain View, Calif., USA) were coated overnight at 4°C with mouse anti-rat IgE in PBS (1 $\mu\text{g/ml}$). Doubling dilutions of standards and samples were added and incubated for 3 h. Digoxigenin conjugate OVA was added (1:1,000) and incubated for 1 h, followed by sheep anti-digoxigenin POD conjugate (1:1,000) for 1 h. Peroxidase substrate TMB was used for color development and plates were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate AutoReader EL311, BIO-TEK Instruments). Titres are expressed as reciprocal $\log_2$ titres.

Drugs and Materials

Ovalbumin (grade V), ricin, LPS *S. typhimurium*, MCh, Evans blue dye, trypan blue and Leishman's BDH stain were obtained from Sigma, St. Louis, Mo., USA. Mouse (monoclonal) anti-rat IgE was supplied by Biosource (Camarillo, Calif., USA), digoxigenin-3-O-

methylcarbonyl-(aminocaproic acid-N-hydroxysuccinimide ester, sheep anti-digoxigenin-POD Fab fragments from Boehringer Mannheim (Mannheim, Germany) and TMB peroxidase substrate and Solution B from Kirkegaard & Perry Laboratories (Gaithersburg, Md., USA). BSA was from CSL (Parkville, Australia) and RPMI 1640 from GIBCO BRL (Glen Waverley, Australia). Ketamine (Ketamil®) was purchased from Troy Laboratories (Smithfield, Australia), xylazine (Rompun®) from Bayer (Pymble, Australia), pentobarbitone sodium (Lethobarb®) from Virbac (Peakhurst, Australia) and lignocaine hydrochloride from Delta Synthetic (Glen Waverley, Australia).

Statistical Analysis

Differences in total and differential cell counts, Evans blue leakage and serum antibody measurements between groups were compared by non-parametric analysis of variance (ANOVA) with the Student-Newman-Keuls correction for multiple comparisons. Similar analysis was carried out to determine the effect of LPS on baseline lung function during acute response to OVA (assessing AUC and t-peak measurements). The effect of LPS on OVA-induced late-phase response in sensitised animals was assessed by calculating the provocative concentration (PC) of MCh producing a 300% increase above baseline in airway resistance ($\text{PC}_{300R_{aw}}$) or a 150% increase above baseline in frequency-dependent tissue resistance (PC_{150G}) by linear interpolation on a semilogarithmic MCh dose-response curve. Comparisons between groups were performed on log-transformed data (for PC values) and real values (for responses to the highest dose of MCh) using ANOVA with Student-Newman-Keuls post-hoc analysis. All results are expressed as means $\pm$ SEM. $p < 0.05$ was regarded as statistically significant.

Results

Effect of LPS on Allergen-Induced Acute-Phase Response to OVA

Lung Function. Exposure of sensitised animals to a single aerosol challenge of OVA resulted in an acute response characterised by increased airway (R_{aw} ; $n = 6$, $p < 0.01$) and parenchymal (G or H ; $n = 6$, $p < 0.01$) responses to OVA measured as increased total AUC (table 1). Maximal response in both compartments occurred approximately 10 min after OVA challenge (table 2). Concurrent administration of LPS (5 or 50 $\mu\text{g/ml}$) with OVA reduced the time-to-peak response for R_{aw} ($n = 6$, $p < 0.001$; fig. 1a), G ($n = 6$, $p < 0.001$; fig. 1b) and H ($n = 6$, $p < 0.001$; table 2). LPS had no effect on the magnitude of parenchymal responses to OVA; however, the highest dose of LPS (50 $\mu\text{g/ml}$) reduced R_{aw} : AUC ($n = 6$, $p < 0.05$; table 1). The lowest dose of LPS (0.5 $\mu\text{g/ml}$) had no effect on the magnitude (table 1) or onset (table 2) of OVA-induced responses in sensitised animals. Exposure to LPS (50 $\mu\text{g/ml}$) in naive animals or in sensitised animals without OVA challenge produced no response and no change in lung function (table 2).

Cellular Inflammation. Intraperitoneal sensitisation of naive animals to OVA resulted in increased OVA-specific serum IgE on day 12 from a mean titre of 3.12 ± 0.42 to 8.15 ± 0.44 $\text{l}/\log_2$ ($n = 6$, $p < 0.001$; table 3). Presence of LPS during OVA challenge had no effect on OVA-specific IgE, the total number of inflammatory cells, the number of eosinophils, macrophages or lymphocytes recovered

from the lavage or the concentration of Evans blue in the lavage when measured up to 1 h after OVA challenge (table 3). The presence of LPS 50 $\mu\text{g}/\text{ml}$ with OVA produced a 3-fold increase in the number of neutrophils during this acute phase compared to OVA alone ($n = 5$, $p < 0.05$; table 3).

Table 1. Effect of LPS on AUC in sensitised animals exposed to OVA

Treatment	R_{aw} : AUC	G: AUC	H: AUC
Naive/LPS50	67.3 ± 41.4	74.0 ± 23.5	107.7 ± 18.5
Sens/LPS50	50.2 ± 22.8	78.3 ± 28.9	61.2 ± 24.8
Sens/OVA	$834.9 \pm 215.4^{**}$	$2,011.3 \pm 466.1^{**}$	$754.2 \pm 220.3^{**}$
OVA + LPS0.5	586.6 ± 170.1	$2,020.4 \pm 449.4$	744.1 ± 134.1
OVA + LPS5	894.7 ± 276.8	$1,212.2 \pm 191.8$	233.1 ± 222.7
OVA + LPS50	$353.4 \pm 71.1^*$	$1,918.8 \pm 172.0$	536.4 ± 74.1

Acute effects of LPS on baseline airway (R_{aw}) and parenchymal (G or H) responses in naive ($n = 5$, naive/LPS50) or sensitised animals exposed to LPS 50 $\mu\text{g}/\text{ml}$ ($n = 6$; sens/LPS50) or in sensitised and OVA-challenged animals ($n = 6$; sens/OVA) given LPS 0.5 $\mu\text{g}/\text{ml}$ ($n = 6$; OVA + LPS0.5), LPS 5 $\mu\text{g}/\text{ml}$ ($n = 6$; OVA + LPS5), or LPS 50 $\mu\text{g}/\text{ml}$ ($n = 6$; OVA + LPS50) in the same nebulizer during OVA challenge. Responses were measured immediately after OVA exposure. Differences between groups were examined by calculating the area under the MCh dose-response curve (AUC) and results expressed as means $\pm$ SEM of n number of animals.

$^{**} p < 0.01$ vs. sens/LPS50 and $^* p < 0.05$ vs. sens/OVA.

Table 2. Effect of LPS on t-peak in sensitised animals exposed to OVA

Treatment	R_{aw} : t-peak min	G: t-peak min	H: t-peak min
Naive/LPS50	NR	NR	NR
Sens/LPS50	NR	NR	NR
Sens/OVA	10.3 ± 1.2	10.0 ± 0.9	10.5 ± 0.7
OVA + LPS0.5	10.3 ± 0.5	10.7 ± 0.6	10.8 ± 0.5
OVA + LPS5	$1.8 \pm 0.2^*$	$1.8 \pm 0.2^*$	$1.7 \pm 0.2^*$
OVA + LPS50	$2.2 \pm 0.3^*$	$2.5 \pm 0.2^*$	$3.3 \pm 0.2^*$

Acute effects of LPS on baseline airway (R_{aw}) and parenchymal (G or H) responses in naive ($n = 5$, naive/LPS50) or sensitised animals exposed to LPS 50 $\mu\text{g}/\text{ml}$ ($n = 6$; sens/LPS50) or in sensitised and OVA-challenged animals ($n = 6$, sens/OVA) given LPS 0.5 $\mu\text{g}/\text{ml}$ ($n = 6$; OVA + LPS0.5), LPS 5 $\mu\text{g}/\text{ml}$ ($n = 6$; OVA + LPS5) or LPS 50 $\mu\text{g}/\text{ml}$ ($n = 6$; OVA + LPS50) in the same nebulizer during OVA challenge. Responses were measured immediately after OVA exposure. Differences between groups were examined by calculating the time taken to reach maximal response (t-peak) in minutes and results expressed as means $\pm$ SEM of n number of animals. $^* p < 0.001$ vs. sens/OVA. NR = No response.

Fig. 1. Effect of LPS on acute-phase response to OVA challenge. Lung function in naive (●; n = 5) or in sensitised and OVA-challenged animals (○; n = 6) 24 h after OVA exposure in the presence of LPS 5 µg/ml (▼; n = 6) in the same nebulizer during OVA challenge. Results are measured as percentage increase in R_{aw} (a) or frequency-dependent parenchymal resistance (G, b) and are expressed as means $\pm$ SEM of n number of experiments. ^a $p < 0.001$ vs. t-peak in sensitised and OVA-challenged animals.

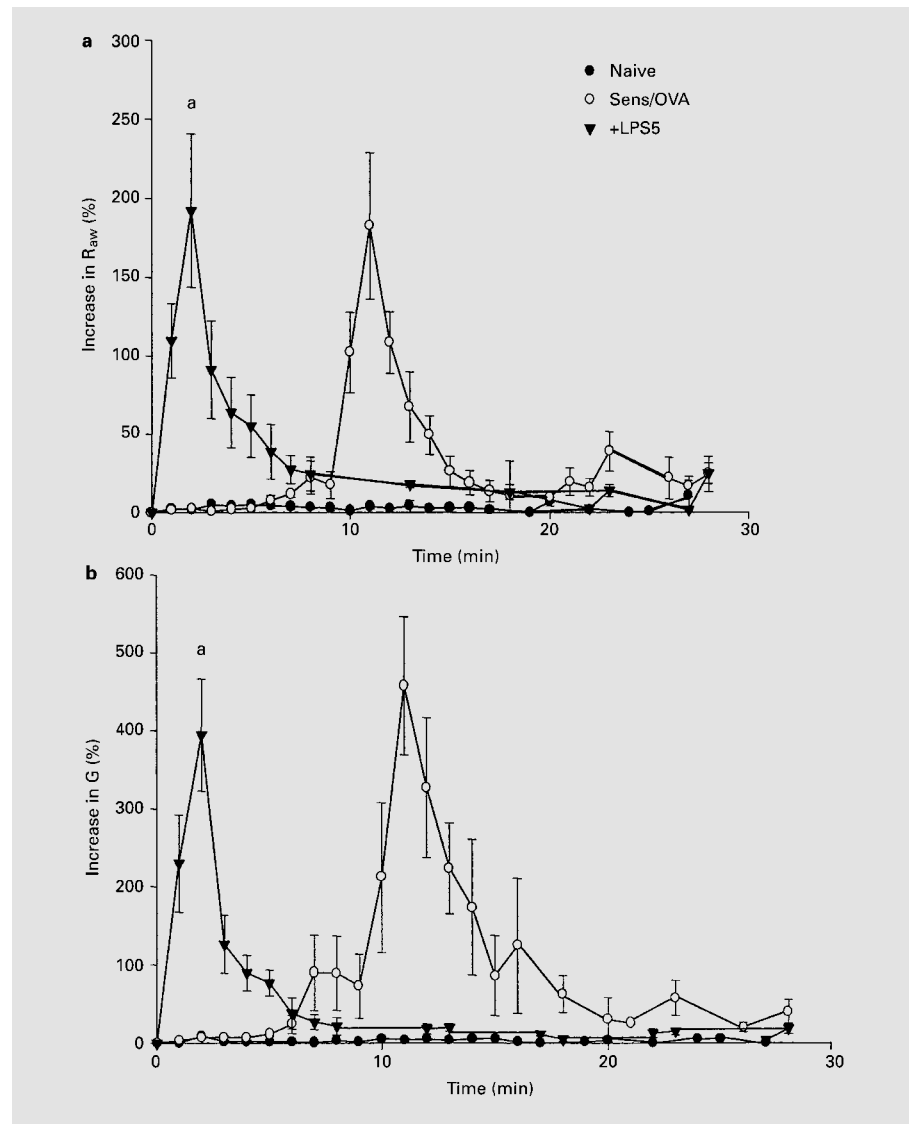


Table 3. Effect of LPS on acute-phase cellular inflammation when given during OVA challenge

Treatment	n	OVA-specific IgE 1/log ₂ titer	Total cells 10 ⁶ /ml	Eosinophils 10 ⁶ /ml	Macrophages 10 ⁶ /ml	Lymphocytes 10 ⁶ /ml	Neutrophils 10 ⁶ /ml	Evans blue mg/ml
Naive/LPS50	5	3.12 $\pm$ 0.42	1.24 $\pm$ 0.27	0	1.02 $\pm$ 0.22	0.19 $\pm$ 0.04	0.04 $\pm$ 0.01	2.08 $\pm$ 0.22
Sens/LPS50	6	7.65 $\pm$ 0.37**	1.26 $\pm$ 0.32	0	0.40 $\pm$ 0.10**	0.82 $\pm$ 0.26**	0.05 $\pm$ 0.01	2.22 $\pm$ 0.33
Sens/OVA	6	8.15 $\pm$ 0.44**	1.06 $\pm$ 0.11	0	0.26 $\pm$ 0.03**	0.76 $\pm$ 0.09**	0.04 $\pm$ 0.01	2.29 $\pm$ 0.27
+ LPS0.5	6	7.49 $\pm$ 0.34	1.23 $\pm$ 0.33	0	0.30 $\pm$ 0.08	0.88 $\pm$ 0.23	0.05 $\pm$ 0.01	2.54 $\pm$ 0.41
+ LPS5	6	7.15 $\pm$ 0.34	1.34 $\pm$ 0.30	0	0.33 $\pm$ 0.07	0.97 $\pm$ 0.22	0.05 $\pm$ 0.01	2.12 $\pm$ 0.42
+ LPS50	5	7.72 $\pm$ 0.57	1.36 $\pm$ 0.26	0	0.43 $\pm$ 0.17	0.80 $\pm$ 0.11	0.13 $\pm$ 0.03*	2.48 $\pm$ 0.35

Serum OVA-specific IgE, total and differential cell count and microvascular leakage (assessed by Evans blue) in naive (naive/LPS50) or sensitised (sens/LPS50) animals exposed to LPS 50 µg/ml or in sensitised and OVA-challenged animals (sens/OVA) in the presence of LPS 0.5 µg/ml, LPS 5 µg/ml or LPS 50 µg/ml in the same nebulizer during OVA challenge. Results are expressed as means $\pm$ SEM of n number of animals per treatment group. * $p < 0.05$ vs. sens/OVA and ** $p < 0.001$ vs. naive/LPS50.

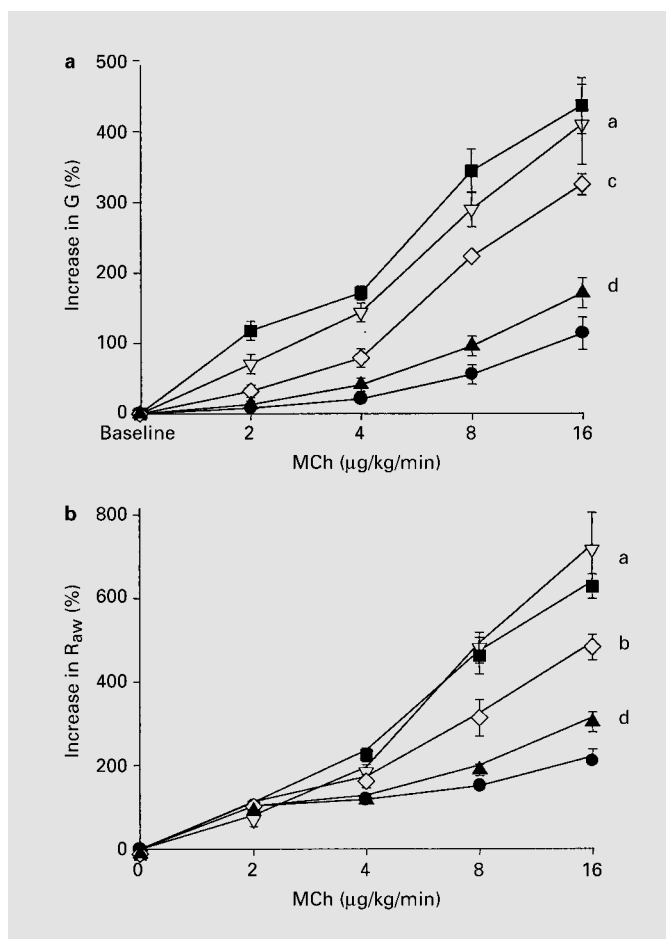


Fig. 2. Effect of LPS on lung function given during OVA challenge. Dose-response curves to intravenous MCh (2–16 μg/kg/min) in naive (●; n = 5) or in sensitised and OVA-challenged animals (▽; n = 5) 24 h after OVA challenge in the presence of LPS 0.5 μg/ml (■; n = 5), LPS 5 μg/ml (◇; n = 6), or LPS 50 μg/ml (▲; n = 5) in the same nebulizer during OVA challenge. Results are measured as a percentage increase in frequency-dependent tissue resistance (G) (a) or R_{aw} (b) and are expressed as means $\pm$ SEM of n number of experiments. ^a p < 0.05 vs. naive and ^b p < 0.05, ^c p < 0.01 and ^d p < 0.001 vs. sensitized and OVA-challenged animals.

Effect of LPS on Allergen-Induced Late-Phase Response in Sensitised Animals

Baseline Lung Function. OVA exposure in sensitised animals produced no effect on airway or parenchymal baseline lung function when measured 24 h after OVA. Similarly, presence of LPS with OVA during challenge produced no change in baseline lung function of sensitised animals at 24 h (data not shown).

Responses to MCh. OVA challenge in sensitised animals significantly increased both airway and parenchymal responsiveness to MCh at 24 h (fig. 2). The MCh dose-response curve was shifted to the left when compared to naive controls, producing a greater than 3-fold increase in G (from 114 ± 23 to $410 \pm 56\%$ above baseline; same animal at 0 MCh) at the highest concentration of MCh (16 μg/kg/min; n = 5, p < 0.001; fig. 2a). R_{aw} was also increased 3-fold (from 220 ± 17 to $728 \pm 89\%$ above baseline; n = 5, p < 0.001) after OVA exposure (fig. 2b). $IvPC_{100}G$ was reduced from 13.24 ± 1.89 μg/kg/min in naive animals to 2.69 ± 0.27 μg/kg/min (n = 5, p < 0.001) and $ivPC_{200}R_{aw}$ from 12.93 ± 1.05 to 3.96 ± 0.27 μg/kg/min after OVA challenge (n = 5, p < 0.001; fig. 2b).

Administration of LPS with OVA caused a dose-dependent inhibition of OVA-induced hyper-responsiveness, progressively increasing $ivPC_{150}G$ (fig. 2a) and $ivPC_{300}R_{aw}$ (fig. 2b) with increasing doses of LPS. With LPS 5 μg/ml, $ivPC_{150}G$ was increased from 4.01 ± 0.34 μg/kg/min in sensitised and OVA-challenged animals to 5.54 ± 0.25 μg/kg/min (n = 6, p < 0.01; fig. 2a) while $ivPC_{300}R_{aw}$ was increased from 5.20 ± 0.33 to 7.67 ± 0.83 μg/kg/min (n = 6, p < 0.05; fig. 2b). At this dose of LPS, response to MCh 16 μg/kg/min was reduced to $325 \pm 45\%$ increase above baseline (same animal at 0 MCh) for G and $493 \pm 31\%$ for R_{aw} (n = 6, p < 0.05; fig. 2). LPS 50 μg/ml caused complete inhibition of OVA-induced airway and parenchymal hyper-responsiveness and returned lung function to control with further increase in $ivPC_{150}G$ to 12.98 ± 1.57 μg/kg/min (n = 5, p < 0.001; fig. 2a) and increase in $ivPC_{300}R_{aw}$ to 14.28 ± 1.15 μg/kg/min (n = 5, p < 0.001; fig. 2b). At this dose, LPS further reduced response to MCh 16 μg/kg/min, reducing G to $171 \pm 21\%$ and R_{aw} to $313 \pm 24\%$ increase above baseline (n = 6, p < 0.01). LPS 0.5 μg/ml had no effect on OVA-induced change in lung function (fig. 2).

Cellular Inflammation. Exposure of sensitised animals to OVA resulted in a greater than 4-fold increase in the total number of inflammatory cells in BAL fluid (n = 5, p < 0.001) characterised by increased numbers of eosinophils (n = 5, p < 0.001), lymphocytes (n = 5, p < 0.001), macrophages (n = 5, p < 0.001) and neutrophils (n = 5, p < 0.001; fig. 3). When administered with OVA, LPS caused a dose-dependent inhibition of OVA-induced late-phase cellular influx, reducing the number of eosinophils, lymphocytes, macrophages, as well as neutrophils into the lavage (fig. 3). The highest dose of LPS (50 μg/ml) completely abolished the cellular influx associated with OVA and the cellular profile of BAL in those animals was similar to controls (fig. 3).

Fig. 3. Effect of LPS on OVA-induced cellular inflammation. Total cell count (TCC) and the differential cell count including the number of macrophages (MPH), neutrophils (NPH), lymphocytes (LYMPH) and eosinophils (EOS) recovered from BAL fluid of naive ($\square$; $n = 5$) or of sensitised and OVA-challenged animals ($\blacksquare$; $n = 5$) 24 h after OVA challenge in the presence of LPS 0.5 $\mu\text{g}/\text{ml}$ ($\square$; $n = 5$), LPS 5 $\mu\text{g}/\text{ml}$ ($\square$; $n = 6$), or LPS 50 $\mu\text{g}/\text{ml}$ ($\square$; $n = 5$) in the same nebulizer during OVA challenge. Results are expressed as means $\pm$ SEM of n number of experiments. ^a $p < 0.001$ vs. naive, ^b $p < 0.05$, ^c $p < 0.01$ and ^d $p < 0.001$ vs. sensitised and OVA-challenged animals.

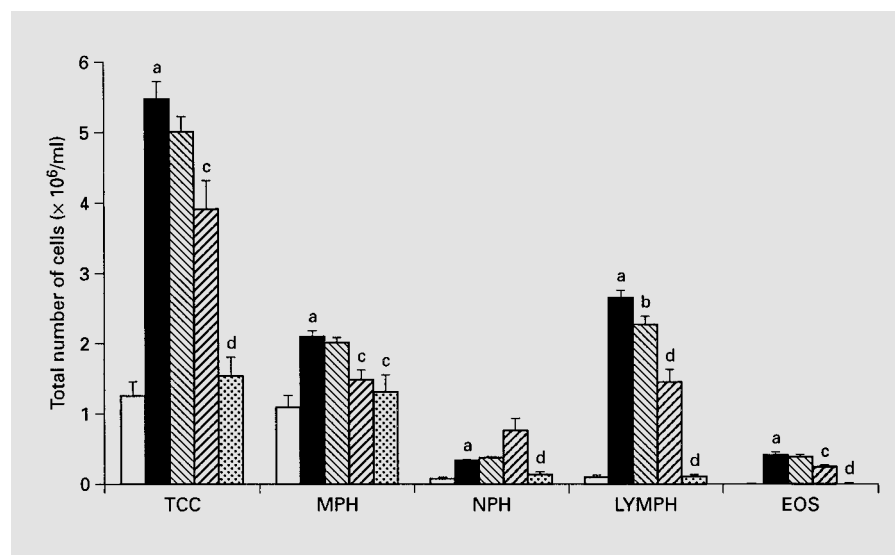
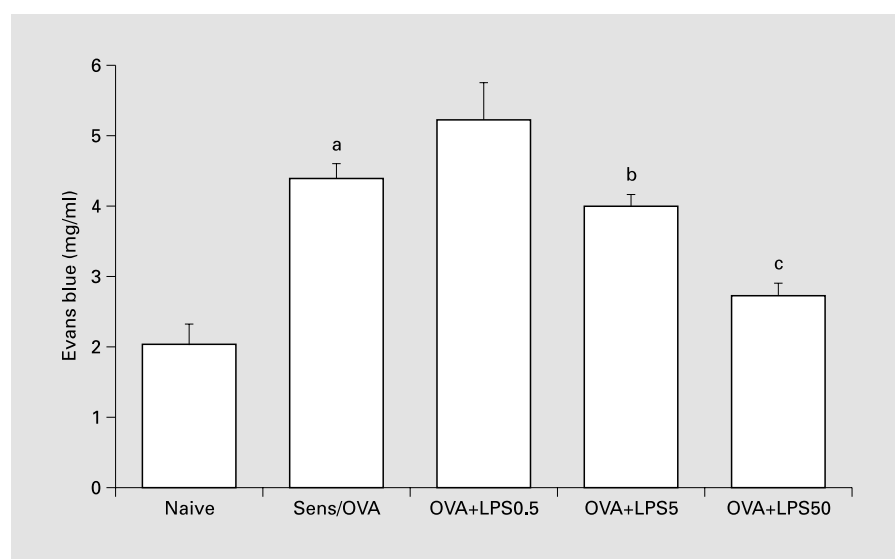


Fig. 4. Effect of LPS on OVA-induced microvascular leakage. Concentration of Evans blue dye in BAL fluid of naive ($n = 5$) or sensitised and OVA-challenged animals ($n = 5$), 24 h after OVA challenge in the presence of LPS 0.5 $\mu\text{g}/\text{ml}$ ($n = 5$), LPS 5 $\mu\text{g}/\text{ml}$ ($n = 6$), or LPS 50 $\mu\text{g}/\text{ml}$ ($n = 5$) in the same nebulizer during OVA challenge. Results are expressed as means $\pm$ SEM of n number of experiments. ^a $p < 0.001$ vs. naive, ^b $p < 0.05$ and ^c $p < 0.001$ vs. sensitised and OVA-challenged animals.



Microvascular Permeability. OVA exposure in sensitised animals induced a greater than 2-fold increase in the concentration of Evans blue in the lavage at 24 h ($n = 5$, $p < 0.001$; fig. 4). LPS administration with OVA caused a dose-dependent inhibition of OVA-induced Evans blue leakage (fig. 4). The highest dose of LPS (50 $\mu\text{g}/\text{ml}$) completely abolished OVA-induced microvascular leakage of Evans blue dye into the BAL ($n = 5$, $p < 0.001$), and those animals were similar to controls (fig. 4).

Discussion

The results of the present study have shown that the contamination of OVA with bacterial LPS during an aerosol challenge in vivo is capable of modifying both the acute and the late-phase responses to OVA in sensitised PVG rats. In these animals, exposure to LPS occurred after the development of OVA-specific IgE. We have shown that with exposure to LPS, the acute change in lung function occurs earlier but the magnitude of this response was essentially unchanged. In contrast, LPS contamination resulted in an inhibition of the late-phase OVA-induced parenchymal and airway hyper-responsiveness

(AHR) to MCh, OVA-induced inflammatory cell influx and microvascular leakage into the BAL fluid at 24 h. This inhibition occurred in a dose-dependent fashion.

In our model, an earlier peak of acute changes in lung function without a change in magnitude of response after exposure to LPS is consistent with rapid mast cell degranulation and release of set quanta of pre-formed pro-inflammatory mediators. Such mediator release may be induced by activation of the CD14 receptor (the high-affinity receptor for LPS) on mast cells [14, 15]. Previous studies have shown that upon activation with LPS plus IFN γ in vitro, mast cell granules downregulate their production of superoxide and nitric oxide (NO) from rodent macrophages by inhibiting TNF α and iNOS mRNA expression in these animals [16]. This effect was evident even at an estimated mast cell to macrophage ratio of 1:40, a relationship likely to occur in vivo.

In this study we report co-exposure of LPS and OVA to cause dose-dependent inhibition of late-phase inflammatory cell influx, microvascular leakage and AHR, 24 h after acute OVA challenge in sensitised animals (fig. 2–4). Similar results have been reported in guinea pigs chronically exposed to an aerosol mixture of OVA and LPS 1 day per week for 3 weeks [17]. Those authors have shown LPS to reduce the absolute number of OVA-induced eosinophils in lung parenchyma, which was further abolished by simultaneous exposure to (1, 3)- β -D-glucan, a component of mould cell wall. Because eosinophil migration into the airways is thought to result from local interleukin (IL)-5 release from OVA-reactive Th2 cells and since alveolar macrophages are known to suppress T cell activation in vitro through their release of NO [18], we postulate that LPS-induced stimulation of NO production by alveolar macrophages may be responsible for Th2 cell inactivation and therefore attenuation of the lack of eosinophil response 24 h after OVA challenge.

It is additionally possible that LPS-induced production of the anti-inflammatory cytokine IL-10 could attribute to this phenomenon. Intratracheal administration of rmIL-10, 10 min before OVA inhalation is effective in reducing eosinophil and lymphocyte influx as well as abolishing late bronchoconstrictor response 24 h after OVA exposure in sensitised Brown-Norway rats [19]. Similar findings have recently been reported in IL-10 knockout mice showing deficiency in IL-10 to play an important role in OVA-induced eosinophilic airway inflammation and AHR [20]. Recently, we have shown that 6 h after LPS exposure in naive animals or 24 h after OVA exposure in sensitised animals, there is an upregulation of iNOS and downregulation of cNOS activity in the lungs of PVG rats

[21]. Both groups of animals displayed AHR to MCh and had cellular infiltration following LPS or OVA challenge in vivo [11]. However, exposure of sensitised and OVA-challenged animals to LPS, 18 h after OVA challenge, resulted in loss of AHR [11] and reversal of OVA-induced changes in NOS isoenzyme activities [21]. Furthermore, we have recently demonstrated that LPS exposure in naive animals induced rapid release of IL-10, evident as early as 1 h after exposure. This release peaked at 3 h with greater than 5-fold increase in IL-10 and the levels remained elevated above baseline at 18 h [21]. These kinetics and rapid time course of IL-10 release are suggestive of pre-formed IL-10 release that may be responsible for inhibition of late-phase responses to OVA at 24 h. Given that IL-10 has been shown to prevent T-cell activation [22] and if responses to OVA in our sensitized animals are T-cell dependent, this may represent a direct mechanism responsible for attenuation of OVA-induced cellular and bronchial responses in these animals.

We have previously established that the inflammatory response to LPS in naive animals peaks 6 h after exposure and is characterised by neutrophil influx, increased airway and parenchymal responses to MCh and increased microvascular permeability [11]. AHR and microvascular leakage are no longer apparent 24 h after LPS exposure [11]. Those results would suggest that the effect of LPS reported in this paper 24 h after OVA challenge is not a result of LPS inflammation per se but rather a result of its complex interaction with the allergen.

Holt et al. [23] recently proposed a two-phase model of asthma, which may be used to illustrate the development of the disease. This model describes the progression from primary allergic sensitisation against environmental allergens during infancy and early childhood (phase 1), to the expression of persistent airway disease as a result of chronic airway inflammation following repeated allergen exposure in later life (phase 2). The potential protective effects of microbial stimuli such as LPS in this two-stage process have been proposed to be mediated principally via enhancement of Th1 function, thus providing feedback inhibition of atopy-associated Th2 immunity [24]. However, the data presented here suggest an additional potential locus of action. In our animal model presented, the effect of LPS on allergic inflammation is shown to be dependent on the dose and timing of bacterial exposure. Our present data suggest that at appropriate dose/time combinations, LPS can abolish the late-phase allergic response to OVA and as a consequence may interrupt the link between allergic sensitisation and the development of chronic inflammation that would otherwise lead to Th2

consolidation and asthma progression with repeated exposure to the allergen.

It is well known that the prevalence of atopy is much higher than the prevalence of allergic respiratory diseases [25] and that being atopic greatly increases one's risk of developing asthma, however we propose that additional environmental co-factors are needed to drive the chronic inflammatory cascade in the atopic individual and hence the expression and persistence of airway disease. Literature in the past has suggested that the environmental exposure to LPS may only exacerbate allergic inflammation in previously sensitised individuals [26, 27]. Longitudinal changes in FEV₁ in swine-confinement operators have been strongly related to long-term and not acute LPS exposure [27]. The doses of LPS we used (0.5–50 µg/ml) correspond to concentrations which can be encountered in occupational and environmental settings [28] and our studies employed single and not chronic aerosol exposures to either allergen or LPS. Our data suggest that an acute exposure to low doses of LPS prevents allergen-induced deterioration in lung function in sensitised individuals and it may be possible that low doses of LPS in sensitised subjects are protective while larger, chronic doses of LPS may prove detrimental.

In summary, these data give valuable insight into the kinetics involved with the protective effects of LPS and suggest that low-dose exposure to bacterial LPS with the allergen, while causing an immediate (albeit transient) bronchoconstrictor effect, may protect sensitised subjects from the late effects of exposure to allergen. How these data may relate to human asthma is currently not known. Epidemiological studies have reported the farming environment to be protective against atopy and asthma [29, 30]. Interestingly, in these studies airborne LPS has been proposed as a causative or protective factor in this environment. Further study is warranted to elucidate the precise mechanism(s) by which the link between the exposure to allergen and late-phase response is broken by LPS exposure. An understanding of this process could lead to the discovery of new therapeutic targets for asthma.

Acknowledgements

This study was supported by a grant from the National Health and Medical Research Council, Australia. M.K.T. holds the Asthma Foundation of WA Inaugural PhD Scholarship. Special thanks goes to the staff at the Research Center, especially Mark Brunini, for their technical expertise.

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