

Modification of the Inflammatory Response to Allergen Challenge after Exposure to Bacterial Lipopolysaccharide

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The potential role of respiratory infections in altering the development of atopy and asthma is complex. Infections have been suggested to be effective in preventing the induction of T-helper 2-polarized allergen-specific immunity in early life, but also to exacerbate asthma in older, sensitized individuals. The mechanism(s) underlying these effects are poorly defined. The aim of this work was to determine the influence of lipopolysaccharide (LPS) exposure on the development of sensitization to allergen and the response to allergen challenge *in vivo*. Piebald-Virol-Glaxo rats were exposed to a single aerosol of LPS 1 d before or 1, 2, 4, 6, 8, or 10 d after sensitization with ovalbumin (OVA). On Day 11 animals were exposed to 1% OVA and responses to allergen were measured 24 h later, monitoring inflammatory cell influx and microvascular leakage into bronchoalveolar lavage (BAL) fluid as well as pulmonary responses to methacholine using the forced oscillation technique. Histologic analysis was included to complement the BAL results. Single aerosol exposure to LPS 1 d before and up to 4 d after intraperitoneal injection of OVA protected against the development of OVA-specific immunoglobulin (Ig) E. LPS exposure 6, 8, or 10 d after sensitization further exacerbated the OVA-induced cellular influx, resulting in neutrophilia and increased Evans Blue dye leakage with no effect on serum IgE levels. In addition, LPS abolished the OVA-induced hyperresponsiveness in sensitized animals when given 18 h after OVA challenge. This study demonstrates that exposure to LPS can modify the development of allergic inflammation *in vivo* by two independent mechanisms. Exposure early in the sensitization process, up to Day 6 after exposure to allergen, prevented allergen sensitization. Exposure to LPS after allergen challenge in sensitized animals abolished the hyperresponsiveness and modified the inflammatory cell influx characteristic of late-phase response to allergen.

Asthma is recognized as a chronic inflammatory disorder of the lungs that results in bronchial hyperresponsiveness, deterioration of lung function, and impaired quality of life if left untreated (1, 2). Allergic sensitization and respiratory symptoms on exposure to allergen form an integral part of the clinical spectrum of asthma in many older children and adults. The histologic appearances of the airways are similar in both atopic and intrinsic asthmatics (3) and serum immunoglobulin (Ig) E levels are raised (4). Al-

though atopy is not always linked with asthma, its importance increases with age (5). Microbial infections early in infancy may protect from the later development of atopy and asthma such that the stimulus for normal postnatal maturation of the immunoinflammatory response may be provided by microbial stimulation (6, 7). At the same time, clinical data indicate that respiratory viral infections are major trigger factors for acute exacerbations of asthma (8–10). There is circumstantial evidence that some bacterial infections, for example *mycoplasma* and *chlamydia* may also play a role in precipitating asthma (11, 12); however, the clinical impression is that bacterial pneumonia in asthmatic patients does not trigger acute exacerbations.

The inflammation characteristics of asthma and respiratory infections are different. Two subsets of T-helper (Th) lymphocytes have been defined and are distinguishable by a differing pattern of cytokine release. Th1-type cells stimulate the recruitment and activity of macrophages and/or mononuclear phagocytes and are involved in the cellular immunity activated by microbial antigens, typically resulting in IgG antibody production without an increase in IgE (13). They produce interleukin (IL)-2, interferon (IFN)- γ , and tumor necrosis factors. Th2 responses are important in the allergic response and asthma, producing the cytokines IL-4, -5, -6, -10, and -13 that are involved in the humoral immune responses of elevated IgE levels and eosinophilia (13). Complex, antagonistic interrelationships exist between the two subsets and the cytokine milieu (13–15).

On the basis of epidemiologic data suggesting that non-wheezing infections may protect against allergen sensitization and also of clinical impressions that bacterial infections do not trigger asthma, we hypothesized that exposure to the bacterial product lipopolysaccharide (LPS) during the usual time course of late-phase allergic reactions would alter the late-phase events. To address this question we used two *in vivo* models of inflammatory lung disease measuring inflammatory cell influx into bronchoalveolar lavage (BAL) fluid (BALF) and leakage of Evans Blue dye from the microvasculature into the BALF, and monitoring the responsiveness to methacholine in Piebald-Virol-Glaxo (PVG) rats sensitized to ovalbumin (OVA). PVG rats also demonstrate neutrophilia and increased vascular permeability after a single aerosol exposure to LPS.

Materials and Methods

Animals

Male PVG rats weighing 200 to 250 grams (Research Centre, Institute for Child Health Research, Perth, Australia) were used and were 10 wk old at the time of data collection. 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*. An-

(Received in original form February 24, 1999 and in revised form December 1, 1999)

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Abbreviations: bronchoalveolar lavage, BAL; BAL fluid, BALF; interferon, IFN; immunoglobulin, Ig; interleukin, IL; lipopolysaccharide, LPS; methacholine, MCh; ovalbumin, OVA; phosphate-buffered saline, PBS; provocative concentration of MCh producing a 150% increase in frequency-dependent tissue resistance above baseline, PC₁₅₀; Piebald-Virol-Glaxo rats, PVG rats; standard error of the mean, SEM; T-helper, Th; input impedance, Z_L.

Am. J. Respir. Cell Mol. Biol. Vol. 22, pp. 604–612, 2000
Internet address: www.atsjournals.org

nual monitoring indicated that the colony was free of known pathogens. The study protocol was approved by the Institutional Animal Ethics Committee.

Sensitization Procedure

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

Exposure to LPS

Where appropriate, animals were exposed once to nebulized LPS *Salmonella typhimurium* (50 $\mu\text{g} \cdot \text{ml}^{-1}$) for 30 min by allowing them to run freely in a Plexiglas chamber into which the LPS was aerosolized. The aerosol was generated from an ultrasonic nebulizer (De Vilbiss Ultra-Neb 2000; Sunrise Medical, Somerset, PA), the outlet of which was connected to the chamber. The output of the nebulizer was 0.5 $\text{ml} \cdot \text{min}^{-1}$ and the mean particle size was 3.5 μm (manufacturer's specifications). Naive animals were exposed to LPS either 6 or 24 h before measurements were taken, whereas sensitized animals underwent exposure 1 d before or 1, 2, 4, 6, 8, 10, or 12 d after sensitization with allergen. A second group of sensitized animals were exposed to LPS 18 h after OVA challenge.

Allergen Challenge

At 11 d after sensitization, at the peak of their IgE response, the animals were placed in a Plexiglas chamber and challenged with aerosolized OVA (1%) for 30 min using the ultrasonic nebulizer (De Vilbiss).

Animal Preparation

At 24 h after allergen challenge, the animals were anesthetized by intramuscular injection of xylazine (12 $\text{mg} \cdot \text{kg}^{-1}$) and ketamine (40 $\text{mg} \cdot \text{kg}^{-1}$) and a tracheostomy was performed. The femoral vein was cannulated with polyethylene tubing for the intravenous injection of drugs. Evans Blue dye (50 $\text{mg} \cdot \text{kg}^{-1}$) in a volume of 2 ml was administered by intravenous injection over a 2-min period.

Measurement of Respiratory Mechanics

Pulmonary function was measured using an adaptation of the low-frequency forced oscillation technique, in which input impedance (Z_L) was measured using a wave-tube (16, 17). Briefly, measurements were made by applying loudspeaker-generated small-amplitude oscillatory signals from 0.5 to 20 Hz through a 114-cm-long, 1.45-mm inner diameter polyethylene wave tube during a 6-s 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 H_2O to keep the transpulmonary pressure constant during measurements. Z_L was calculated as described by Peták and colleagues (16). To separate airway and tissue mechanics, a model containing a frequency-independent airway resistance and inductance and a constant-phase frequency-dependent tissue resistance (G) and elastance was fitted to the Z_L spectra (17). Impedance points coinciding with the heart rate and its harmonics were omitted from the model fitting because cardiac activity caused low signal-to-noise ratio at these frequency components.

Methacholine Challenge

After an equilibration period of 15 min, baseline was established with four to six Z_L recordings. Cumulative doses of methacholine (MCh) (2 to 16 $\text{mg} \cdot \text{ml}^{-1}$) were administered by inhalation for 90 s using a jet nebulizer (LC PLUS; Pari-Werk GmbH, Starnberg, Germany) driven by compressed air (5 $\text{liters} \cdot \text{min}^{-1}$) and connected to the input port of the ventilator. Z_L data were ensemble-

averaged in baseline condition, whereas individual Z_L curves were fitted at 1-min intervals after MCh administration. Peak responses in G at each dose were used for further analysis. Responses were measured as percentage increase above baseline.

Evans Blue dye (50 $\text{mg} \cdot \text{kg}^{-1}$) was administered by intravenous injection over a 2-min period immediately after the construction of MCh dose-response curves. At the end of the experiment, 1.0 ml of blood was collected via cardiac puncture for the estimation of OVA-specific serum antibody titers.

BAL

At 30 min after administration of Evans Blue, animals were killed with pentobarbitone and the chest was opened. BAL was performed via the tracheal cannula using 3×8 ml of PBS containing lignocaine hydrochloride (0.35%) and bovine serum albumin (BSA) (0.2%). Routinely, greater than 90% of the lavaging fluid was recovered from the lungs. The BALF was centrifuged at $250 \times g$ 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 hemocytometer. The differential cell count was carried out from cytospin preparations using Leishman's BDH stain and counting 200 cells at random under $\times 100$ magnification. The cells were identified by standard morphology.

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, Inc., Winooski, VT). The concentration of dye was extrapolated from a standard curve of Evans Blue dye concentrations (1 to 10 $\mu\text{g} \cdot \text{ml}^{-1}$).

Serum Antibody Measurements

OVA-specific IgE titers were estimated by enzyme-linked immunosorbent assay (ELISA) according to the method of Van Halteren and associates (18). Briefly, microtiter plates (Falcon flexible assay plate 3912; Becton Dickinson, Winooski, CA) were coated overnight at 4°C with mouse anti-rat IgE in PBS (1 $\mu\text{g} \cdot \text{ml}^{-1}$). Doubling dilutions of standards and samples were added and incubated for 3 h. Digoxygenin conjugate OVA was added (1:1,000) and incubated for 1 h, followed by sheep antidigoxygenin horseradish peroxidase conjugate (1:1,000) for 1 h. The peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) was used for color development and the plates were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate AutoReader EL311; Bio-Tek Instruments). Titers are expressed as reciprocal $\log_2$ titers. OVA-specific IgG was measured by a hemagglutination assay using sheep red blood cells conjugated to OVA and titers expressed as reciprocal $\log_2$ titers as previously described (19).

Histologic Analysis

In a separate group of animals histologic examination of the lungs was undertaken to determine whether the inflammatory cell profile obtained using the BALF was representative of tissue inflammation. Sensitization and OVA challenge as well as LPS exposure were performed as described earlier. At 6 h after LPS exposure in naive animals or on Day 12, 24 h after OVA challenge in sensitized animals, the rats were killed and the lungs and the trachea removed immediately and inflation-fixed with 4% paraformaldehyde at 20 cm H_2O via tracheal installation. The right lung and the trachea were embedded in paraffin wax and one 3- μm section per animal was stained with hematoxylin and eosin. Inflammatory cells were identified by standard morphometry. Numbers of cells in tissue and air spaces were counted in 10 random, nonoverlapping parenchymal fields at $\times 100$ magnification under brightfield

TABLE 1
Effects of OVA on inflammatory cell count, microvascular leakage, and serum antibody levels*

Treatment	n	OVA-Specific IgE (1/log ₂ titer)	OVA-Specific IgG (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	11	3.50 ± 0.24	3.59 ± 0.43	0.87 ± 0.08	0	0.72 ± 0.07	0.12 ± 0.02	0.03 ± 0.01	1.98 ± 0.10
Sens/sal	8	7.32 ± 0.29 [§]	4.32 ± 0.43	0.64 ± 0.14	0	0.44 ± 0.02	0.18 ± 0.04	0.02 ± 0.01	1.95 ± 0.02
Sens/OVA	11	7.03 ± 0.35	4.15 ± 0.40	4.16 ± 0.19 [†]	0.16 ± 0.03 [†]	1.59 ± 0.10 [†]	2.15 ± 0.15 [†]	0.26 ± 0.03 [†]	4.36 ± 0.21 [†]

*Inflammatory response to OVA in sensitized animals 24 h after allergen exposure (sens/OVA group). Serum OVA-specific IgE and IgG were measured. The total and the differential inflammatory cell count as well as microvascular leakage (assessed by Evans Blue dye) was studied using the BALF. Results are expressed as means ± SEM.

[§]P < 0.01 versus naive.

[†]P < 0.01 versus sensitized and saline-challenged animals (sens/sal group).

illumination. All histologic analyses was performed on the same region of each lung, the right upper lobe, and measurements were performed blind by the same operator (M.K.T.).

Study Groups

Influence of LPS exposure on late-phase events after OVA challenge. Six sensitized animals were exposed to a single aerosol challenge of saline or LPS 18 h after OVA challenge on Day 12 and inflammatory parameters as well as responses to inhaled MCh were determined 6 h later; that is, 24 h after initial allergen exposure.

Influence of LPS exposure on primary allergic sensitization. Groups of animals were exposed to saline or LPS 1 d before or 1, 2, 4, 6, 8, or 10 d after sensitization with intraperitoneal injection of OVA. They were then challenged with 1% OVA on Day 11 and their serum and BALF collected 24 h later.

Histologic analysis. Six key groups of rats (4 in each group) were exposed to saline or LPS in naive or sensitized animals to complement the BAL data with parenchymal sections. These animals were not lavaged.

Drugs and Materials

OVA (Grade V), ricin, LPS *S. typhimurium*, acetyl-β-MCh chloride (MCh), lignocaine hydrochloride, Evans Blue dye, Trypan Blue, and Leishman's BDH stain were obtained from Sigma Chemical Company (St. Louis, MO). Mouse (monoclonal) antirat IgE was supplied by Biosource (Camarillo, CA); digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester, sheep antidigoxigenin-POD Fab fragments by Roche Diagnostics (Basel, Switzerland); and TMB peroxidase substrate and Solution B by Kirkegaard & Perry Laboratories (Gaithersburg, MD). 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), and pentobarbitone sodium (Lethabarb) from Virbac (Peakhurst, Australia). Paraformaldehyde and Depex mounting medium were purchased from BDH Laboratory Supplies (Poole, UK).

Statistical Analysis

Measurement of the difference in the total and the differential cell counts, as well as Evans Blue leakage and serum antibody measurements between different treatment groups were made by the simple unpaired two-tailed Student's *t* test. The effects of LPS treatment in naive and sensitized animals on MCh responses were assessed by calculating the provocative concentration of MCh producing a 150% increase in frequency-dependent tissue resistance above baseline (PC₁₅₀) by linear interpolation on a semilogarithmic MCh dose-response curve. In this particular animal model aerosolized MCh produces predominantly a tissue response (16), thus only tissue mechanics were used for statistical

analysis. Comparisons between groups were made on log transformed data using one-way analysis of variance with Student-Newman-Keuls correction for multiple comparisons. All results are expressed as means ± standard error of the mean (SEM). *P* < 0.05 was regarded as statistically significant.

Results

Inflammatory Models

Response to allergen in sensitized animals 24 h after challenge. Intraperitoneal sensitization of naive animals to OVA resulted in increased OVA-specific serum IgE on Day 12 (*P* < 0.01, *n* = 8; Table 1). OVA-specific serum IgG antibody was unchanged. Exposure of these animals to 1% OVA on Day 11 resulted in a greater than 4-fold increase in inflammatory cell influx into the BALF 24 h later (*P* < 0.01, *n* = 11) as a result of increased numbers of eosinophils (*P* < 0.01), macrophages (*P* < 0.01), lymphocytes (*P* < 0.01), and neutrophils (*P* < 0.01). This cellular influx was associated with a significant increase in the concentration of Evans Blue in BALF (*P* < 0.01; Table 1).

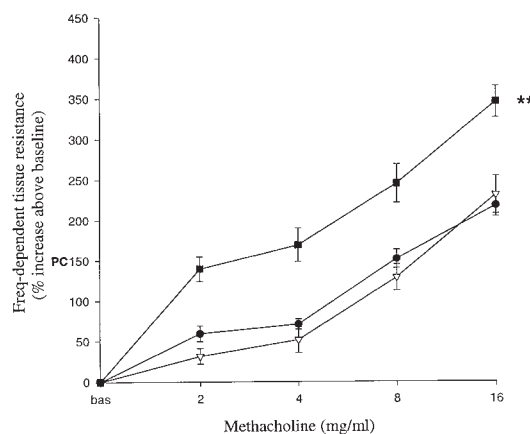


Figure 1. Effects of OVA in sensitized animals. Cumulative dose-response curves to inhaled MCh (2 to 16 mg · ml⁻¹) in naive (filled circles) (*n* = 9) or in sensitized and OVA-challenged animals (sens/OVA) (filled squares) (*n* = 11). Results are measured as a percentage increase in frequency-dependent tissue resistance and are expressed as means ± SEM. ***P* < 0.01 versus sensitized and saline-challenged animals (sens/sal) (open inverted triangles) (*n* = 10).

TABLE 2
Effects of LPS on inflammatory cell count, microvascular leakage, and serum antibody levels*

Treatment	n	Total Cells (10 ⁶ /ml)	Neutrophils (10 ⁶ /ml)	Macrophages (10 ⁶ /ml)	Lymphocytes (10 ⁶ /ml)	Eosinophils (10 ⁶ /ml)	OVA-Specific IgE (1/log ₂ titer)	OVA-Specific IgG (1/log ₂ titer)	Evans Blue (mg/ml)
Naive	11	0.87 ± 0.08	0.03 ± 0.01	0.72 ± 0.07	0.12 ± 0.02	0	3.50 ± 0.24	3.59 ± 0.43	1.98 ± 0.10
LPS6	10	4.72 ± 0.34 [†]	4.14 ± 0.30 [†]	0.39 ± 0.05 [†]	0.19 ± 0.05	0	3.42 ± 0.25	3.92 ± 0.53	3.47 ± 0.44 [†]
LPS24	9	2.39 ± 0.31 [†]	2.04 ± 0.26 [†]	0.25 ± 0.04 [†]	0.09 ± 0.01	0	3.10 ± 0.29	9.10 ± 0.43 [†]	2.52 ± 0.36

Definition of abbreviations: LPS6 = LPS exposure on Day 6; LPS24 = LPS exposure on Day 24.

*Inflammatory response to 50 mg/ml LPS 6 or 24 h after challenge in naive animals. Total and differential inflammatory cell counts and microvascular leakage (assessed by Evans Blue dye) were studied using the BALF. IgE and IgG levels were monitored from serum. Results are expressed as means ± SEM.

[†]P < 0.01 versus naive.

OVA exposure induced hyperresponsiveness to inhaled MCh in sensitized animals, significantly shifting the dose-response curve to the left and decreasing the PC₁₅₀ from 10.12 ± 1.16 to 3.39 ± 0.68 mg · ml⁻¹ (P < 0.01; Figure 1).

Response to LPS in naive animals. Six hours after exposure. Exposure of naive animals to LPS (50 µg · ml⁻¹) induced a greater than 5-fold increase in the total number of inflammatory cells present in the BALF 6 h after initial exposure (P < 0.01, n = 10), predominantly as a result of neutrophil influx (P < 0.01; Table 2), making up 88% of the total cell count. The number of macrophages was reduced 2-fold (P < 0.01); lymphocyte number and serum OVA-specific IgE/IgG antibody levels remained unchanged (Table 2). Evans Blue leakage increased significantly from 1.98 ± 0.14 to 3.47 ± 0.44 mg · ml⁻¹ (P < 0.01; Table 2). Responses to inhaled MCh were significantly potentiated 6 h after LPS exposure, decreasing the PC₁₅₀ from 8.75 ± 1.10 to 2.66 ± 0.37 mg · ml⁻¹ (P < 0.01; Figure 2). **Twenty-four hours after exposure.** At 24 h after initial exposure to LPS (50 µg · ml⁻¹), the total number of cells in BALF remained significantly above the pre-exposure num-

ber (P < 0.01, n = 9); however, this was reduced 2-fold when compared with 6-h exposure (Table 2). A similar pattern of cellular response was observed, neutrophils making up 85% of the cellular population. Macrophage numbers were reduced (P < 0.01) and lymphocyte numbers remained unchanged (Table 2). OVA-specific IgG was significantly increased from 3.92 ± 0.53 to 9.10 ± 0.43 1/log₂ titer 24 h after LPS exposure (P < 0.01; Table 2). The increased Evans Blue leakage (Table 2) and the hyperresponsiveness evident at 6 h after LPS exposure was no longer evident at 24 h after exposure with a PC₁₅₀ of 11.34 ± 1.81 mg · ml⁻¹ (Figure 2).

Influence of LPS Exposure on Allergen-Induced Late-Phase Events

Exposure of sensitized animals to LPS on Day 12, 18 h after allergen challenge (denoted as the sens/OVA/LPS group), resulted in modification of the allergen-induced inflammatory cell profile seen in the BALF 24 h after allergen challenge. LPS further potentiated the allergen-induced inflammatory cell influx into BALF (P < 0.05, n = 6; Table 3). However, in contrast to the allergen challenge in sensitized animals in the absence of LPS (the sens/OVA/sal group), this increase in total cell number was predominantly due to a 20-fold increase in neutrophil influx (P < 0.01) making up greater than 80% of the cellular content. The numbers of both macrophages (P < 0.01) and lymphocytes (P < 0.01) were reduced 3-fold whereas eosinophils were no longer detected in the BALF (P < 0.01; Table 3). The OVA-specific serum antibody counts remained unchanged (Table 3). The allergen-induced leakage of Evans Blue into BALF in sensitized animals was further exacerbated with LPS exposure (P < 0.01; Table 3). LPS attenuated the allergen-induced hyperresponsiveness to MCh in sensitized animals when given 18 h after OVA challenge (Figure 3), shifting the dose-response curve to the right and increasing the PC₁₅₀ from 2.62 ± 0.61 mg · ml⁻¹ in the sens/OVA/sal-challenged control group to 10.77 ± 3.58 mg · ml⁻¹ in the sens/OVA/LPS group (P < 0.05; Figure 3). LPS exposure in the sensitized and saline-challenged (as opposed to OVA) group (denoted as sens/sal/LPS in Figure 3) showed hyperresponsiveness to MCh (PC₁₅₀ 2.09 ± 0.20 mg · ml⁻¹), and these responses were similar in magnitude to those in naive animals exposed to LPS 6 h before measurement (Figure 2).

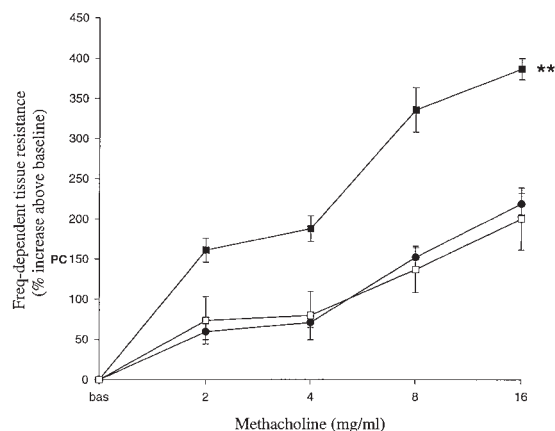


Figure 2. Effects of LPS in naive animals. Cumulative dose-response curves to inhaled MCh (2 to 16 mg · ml⁻¹) in naive animals (filled circles) (n = 9) exposed to 50 µg · kg⁻¹ LPS 6 (filled squares) (n = 10) or 24 (open squares) (n = 9) h before data collection. Results are measured as a percentage increase in frequency-dependent tissue resistance and expressed as means ± SEM. **P < 0.01 versus naive animals.

TABLE 3

*Effects of LPS in naive animals on inflammatory cell count, microvascular leakage, and serum antibody levels**

Treatment	n	Total Cells (10 ⁶ /ml)	Neutrophils (10 ⁶ /ml)	Macrophages (10 ⁶ /ml)	Lymphocytes (10 ⁶ /ml)	Eosinophils (10 ⁶ /ml)	OVA-Specific IgE (1/log ₂ titer)	OVA-Specific IgG (1/log ₂ titer)	Evans Blue (mg/ml)
Sens/sal/LPS	6	4.90 ± 0.39	4.32 ± 0.37	0.34 ± 0.04	0.24 ± 0.03	0	6.99 ± 0.37	4.15 ± 0.34	3.65 ± 0.42
Sens/OVA/sal	6	4.53 ± 0.30	0.31 ± 0.06	1.79 ± 0.09	2.23 ± 0.26	0.20 ± 0.04	6.82 ± 0.47	3.82 ± 0.47	4.05 ± 0.33
Sens/OVA/LPS	6	7.07 ± 0.92 [‡]	5.81 ± 0.96 [§]	0.55 ± 0.04 ^{†§}	0.71 ± 0.11 ^{†§}	0 [§]	5.65 ± 0.37	5.15 ± 0.52	5.79 ± 0.18 ^{†§}

*Inflammatory response to OVA in sensitized animals exposed to LPS 50 mg/ml (or saline) 18 h after allergen challenge. Total and differential inflammatory cell counts and microvascular leakage (assessed by Evans Blue dye) were studied using the BALF. IgE and IgG levels were monitored from serum. Results are expressed as means ± SEM.

[†] $P < 0.05$ and [‡] $P < 0.01$ versus sens/sal/LPS.

[§] $P < 0.05$ and [§] $P < 0.01$ versus sens/OVA/sal.

Influence of LPS Exposure on Primary Allergen Sensitization

Exposure of naive animals to 50 $\mu\text{g} \cdot \text{ml}^{-1}$ LPS 24 h before the sensitization (on Day 1) inhibited their allergen-induced increase in OVA-specific serum IgE levels ($P < 0.01$, $n = 6$; LPS-1 group in Table 4). OVA-specific serum IgG was significantly increased ($P < 0.01$). Challenge of these animals with OVA on Day 11 did not result in the allergen-induced inflammatory cell influx previously reported in sensitized animals 24 h after OVA challenge (Table 1). The total inflammatory cell count and the concentration of Evans Blue in their BALF were similar to those of sensitized and saline-challenged animals (the sens/sal group; Table 4) and were not significantly different from those of naive animals (Table 1). A similar profile of response was obtained in the group of animals that was exposed to LPS 1, 2, or 4 d after intraperitoneal injection with allergen (Table 4). Exposure of LPS 24 h before sensitization (the LPS/sens/OVA group)

resulted in complete inhibition of allergen-induced hyperresponsiveness to MCh (PC_{150} 11.42 ± 3.17 $\text{mg} \cdot \text{ml}^{-1}$), these responses being similar to that of the sensitized and saline-challenged (sens/sal) group (Figure 4).

Exposure of rats to a single aerosol challenge of LPS 6 d after intraperitoneal allergen injection did not alter the serum antibody levels in sensitized animals (Table 4). However, dramatic changes were seen in the inflammatory response measured after OVA challenge. LPS exposure on Day 6 (LPS6) further potentiated the allergen-induced cellular influx into BALF ($P < 0.01$, $n = 5$) in sensitized animals, predominantly as a result of a 25-fold increase in neutrophil influx ($P < 0.01$). Eosinophil numbers were increased 3-fold ($P < 0.01$) whereas both macrophage ($P < 0.01$) and lymphocyte ($P < 0.01$) numbers were reduced (Table 4). Allergen-induced Evans Blue leakage was further potentiated ($P < 0.01$). The pattern of inflammatory response and cellular content of BALF, leakage of Evans Blue dye, and serum OVA-specific IgE and IgG levels obtained from animals exposed to LPS on Day 8 or on Day 10 after sensitization was similar to Day 6 results (Table 4).

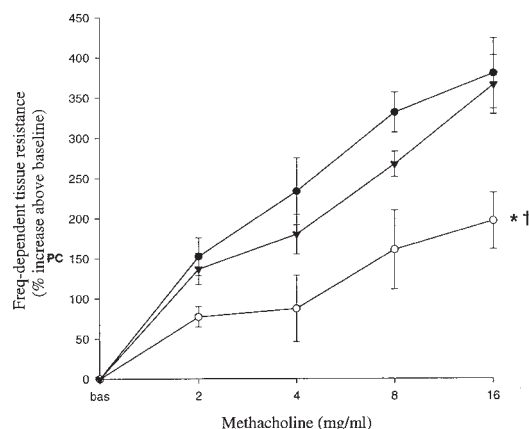


Figure 3. Effects of LPS 18 h after OVA challenge in sensitized animals. Cumulative dose-response curves to inhaled MCh (2 to 16 $\text{mg} \cdot \text{ml}^{-1}$) in sensitized and OVA-challenged animals exposed to 50 $\mu\text{g} \cdot \text{kg}^{-1}$ LPS (sens/OVA/LPS) (open circles) ($n = 5$) or saline (sens/OVA/sal) (inverted triangles) ($n = 6$) 18 h after OVA challenge. Results are measured as a percentage increase in frequency-dependent tissue resistance and are expressed as means ± SEM. * $P < 0.05$ versus sens/sal/LPS (filled circles) ($n = 6$); that is, sensitized animals exposed to LPS 18 h after saline challenge as opposed to OVA and [†] $P < 0.05$ versus sens/OVA/sal.

Histologic Analysis

Histologic assessment of parenchymal sections has shown our BAL results to closely mimic the lung inflammatory response to LPS or allergen exposure. At 24 h after allergen challenge in sensitized animals, parenchymal total inflammatory cell count was significantly increased ($P < 0.01$, $n = 4$; Figure 4, top panel), once again as a result of increased numbers of eosinophils ($P < 0.01$; Figure 4, middle panel), macrophages ($P < 0.01$), lymphocytes ($P < 0.01$), and neutrophils ($P < 0.05$; Figure 4, lower panel) in both tissue and alveolar spaces. LPS exposure in naive animals has been shown to induce a similar increase in cellular influx ($P < 0.01$; Figure 5) and as the lavage results have shown, this is predominantly due to neutrophil influx into the lungs ($P < 0.01$, $n = 4$; Figure 5). Eosinophils were not detected in these animals (Figure 5).

Exposure of sensitized animals to LPS 18 h after allergen challenge resulted in further exacerbation of allergen-induced inflammatory cell influx ($P < 0.05$, $n = 4$; Figure 5) and is in agreement with our BAL data. Similar exacerbated neutrophil influx ($P < 0.01$; Figure 5) and reduced number of macrophages are seen. However, LPS has further potentiated the allergen-induced eosinophil influx

TABLE 4
Effects on inflammatory cell count, microvascular leakage, and serum antibody levels*

Exposure (d)	n	Total Count (10 ⁶ /ml)	Evans Blue (mg/ml)	Neutrophils (10 ⁶ /ml)	Macrophages (10 ⁶ /ml)	Eosinophils (10 ⁶ /ml)	Lymphocytes (10 ⁶ /ml)	OVA-Specific IgE (1/log ₂ titer)	OVA-Specific IgG (1/log ₂ titer)
Sens/sal	8	0.64 ± 0.14	1.95 ± 0.02	0.02 ± 0.01	0.44 ± 0.02	0	0.18 ± 0.04	7.32 ± 0.29	4.32 ± 0.43
Sens/OVA	11	4.16 ± 0.19**	4.36 ± 0.21**	0.26 ± 0.03**	1.59 ± 0.10**	0.16 ± 0.03**	2.15 ± 0.15**	7.03 ± 0.35	4.15 ± 0.40
LPS-1	6	0.75 ± 0.05 [‡]	2.68 ± 0.76 [‡]	0.08 ± 0.02 [‡]	0.40 ± 0.08 [‡]	0 [‡]	0.27 ± 0.03 [‡]	3.49 ± 0.52 [‡]	10.16 ± 0.34 [‡]
LPS1	5	0.90 ± 0.13 [‡]	2.88 ± 0.51 [‡]	0.07 ± 0.02 [‡]	0.49 ± 0.08 [‡]	0 [‡]	0.33 ± 0.07 [‡]	3.92 ± 0.57 [‡]	9.72 ± 0.57 [‡]
LPS2	5	1.04 ± 0.20 [‡]	3.31 ± 0.74 [‡]	0.10 ± 0.03 [‡]	0.49 ± 0.08 [‡]	0 [‡]	0.48 ± 0.15 [‡]	2.92 ± 0.45 [‡]	8.72 ± 0.91 [‡]
LPS4	5	0.88 ± 0.20 [‡]	2.88 ± 0.57 [‡]	0.12 ± 0.02 [‡]	0.50 ± 0.06 [‡]	0 [‡]	0.35 ± 0.17 [‡]	4.52 ± 0.74 [‡]	9.12 ± 1.19 [‡]
LPS6	5	8.36 ± 0.67 [‡]	6.98 ± 0.34 [‡]	6.48 ± 0.8 [‡]	0.62 ± 0.10 [‡]	0.43 ± 0.08 [‡]	0.83 ± 0.23 [‡]	7.32 ± 0.48	3.92 ± 0.55
LPS8	5	8.86 ± 0.52 [‡]	6.23 ± 0.67 [‡]	6.70 ± 0.51 [‡]	0.74 ± 0.21 [‡]	0.54 ± 0.18 [‡]	0.88 ± 0.12 [‡]	7.92 ± 0.67	2.92 ± 0.45
LPS10	5	8.16 ± 0.55 [‡]	5.74 ± 0.38 [‡]	6.23 ± 0.52 [‡]	0.67 ± 0.22 [‡]	0.60 ± 0.16 [‡]	0.66 ± 0.18 [‡]	8.32 ± 0.61	3.32 ± 0.50

*Inflammatory response in sensitized and OVA-challenged animals exposed to LPS 24 h before sensitization (LPS-1 group) or 1, 2, 4, 6, 8 and 10 d after sensitization. Serum OVA-specific IgE and IgG were measured. Total and differential cell counts in the BALF and Evans Blue leakage were studied. Results are expressed as means ± SEM.

***P* < 0.01 versus sens/sal.

[‡]*P* < 0.05 and ^{‡‡}*P* < 0.01 versus sens/OVA.

into the lung parenchyma (*P* < 0.01, *n* = 4; Figure 5), which was not shown by the lavage results (Table 3).

Discussion

The aim of the present study was to determine the influence of bacterial products on allergen-mediated late-phase events in sensitized animals and on primary allergen sensitization *in vivo*. We characterized allergic inflammation per se in sensitized PVG rats as being associated with elevated serum OVA-specific IgE antibody, with infiltration of inflammatory cells including eosinophils into the parenchyma and BALF, as well as increased microvascular permeability and hyperresponsiveness to inhaled MCh 24 h

after allergen challenge. These findings are in agreement with previous reports (20, 21). In a separate group of animals we also showed that bacterial inflammation, mimicked by exposure to aerosolized LPS, results in increased total cell count in the lavage, predominantly as a result of neutrophil influx which peaked at 6 h after exposure and remained elevated above controls for up to 24 h. Similar inflammatory response is evident in the lung parenchyma after exposure to LPS. LPS-induced Evans Blue leakage and hyperresponsiveness were also seen 6 h after exposure but were no longer apparent after 24 h. These findings are in agreement with those published by Pauwels and co-workers (22).

The effects of exposure to bacterial LPS on the sensitization per se can be seen in Table 4. These results clearly illustrate that if animals are exposed to LPS 1 d before and up to 4 d after sensitization with OVA, LPS inhibits the increase in OVA-specific IgE and upregulates serum IgG levels. As a consequence, no cellular influx or increased Evans Blue leakage into BALF after allergen challenge was evident, and in these animals no hyperresponsiveness to inhaled MCh was evident. When animals were exposed to LPS from Day 6 to Day 10 after sensitization a decreased OVA-specific IgG antibody level and increased serum OVA-specific IgE was seen at Day 12 compared with sensitized animals not exposed to LPS. In these sensitized animals LPS also exacerbated the allergic response to OVA, further exaggerating the Evans Blue leakage and cellular influx, in particular neutrophils and eosinophils. Studies by Michel and coworkers (23) similarly showed that LPS in house dust exacerbates symptoms in atopic asthmatics.

LPS exposure upregulates the production of Th1 cytokines, especially IFN- γ in human T cells (24), and inhibits the expression of Th2 cytokines *in vivo* (25). IFN- γ inhibits the clonal expansion of Th2 cells and suppresses IgE production by human lymphocytes during primary sensitization (26). In our animals exposed to LPS from 1 d before and up to and including 4 d after sensitization, IFN- γ would be expected to inhibit expansion of Th2 cells and

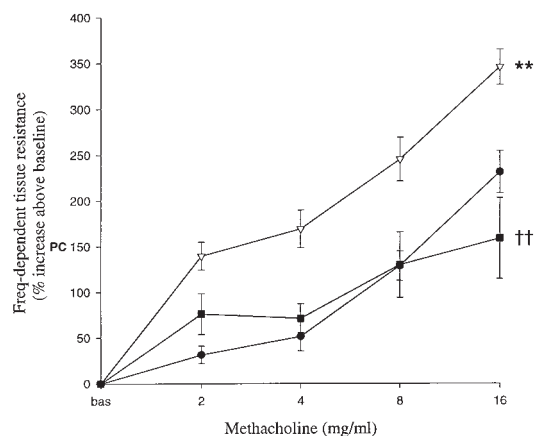


Figure 4. Effects of LPS given before sensitization. Cumulative dose-response curves to inhaled MCh (2 to 16 mg · ml⁻¹) in sensitized and OVA-challenged animals exposed to 50 μ g · kg⁻¹ LPS 24 h before sensitization (LPS/sens/OVA) (filled squares) (*n* = 4). Results are measured as a percentage increase in frequency-dependent tissue resistance and are expressed as means ± SEM. ^{††}*P* < 0.01 versus sens/OVA (inverted triangles) (*n* = 11) and ^{**}*P* < 0.01 versus sens/sal (filled circles) (*n* = 10).

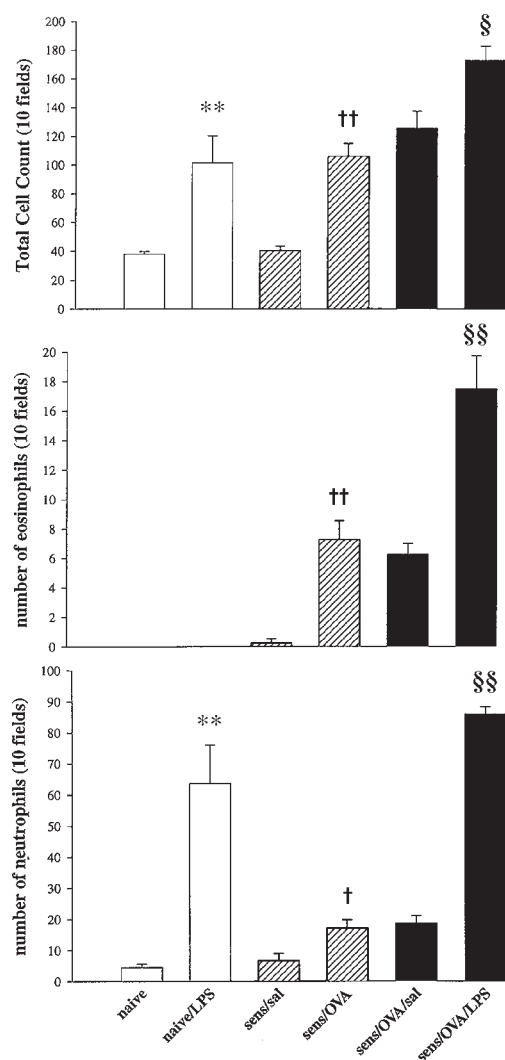


Figure 5. Histologic assessment of lung parenchyma. Effects of LPS 6 h after exposure in naive animals (open bars) ($n = 4$), of OVA in sensitized animals (striped bars) ($n = 4$), and of LPS in sensitized and OVA-challenged animals (filled bars) ($n = 4$) on total inflammatory cell count (top panel) and eosinophil (middle panel) and neutrophil (lower panel) numbers. Results are expressed as means $\pm$ SEM of 10 random, nonoverlapping lung parenchymal fields. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ versus sens/sal; $^{**}P < 0.01$ versus naive; $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ versus sens/OVA/sal.

the synthesis of OVA-specific IgE. After the fourth day after sensitization a different process is likely to be occurring. In the primary allergic response the OVA allergen is presented to CD4⁺ T lymphocytes largely by dendritic cells (27), resulting in the expansion of OVA-specific Th2 cell clones. These cells produce Th2 cytokines including IL-4 which, in turn, acts on "virgin" B cells to produce memory B cells and IL-4-independent, IgE-producing plasma cells that are no longer sensitive to the inhibitory effect of IFN- γ (28). Our data would suggest that in the first 4 d after sensitization, IFN- γ and/or IL-12 produced by LPS exposure drives the B cells toward IgG antibody production but inhibits class switching through to IgE. The

different pattern of response to LPS seen when 6 or more d are allowed to lapse between primary sensitization and LPS exposure suggests that plasma cells committed to producing IgE are present by Day 6, and these committed IgE-producing cells are resistant to IL-12 and/or IFN- γ produced in response to LPS exposure. LPS must be present before the isotype switch to IgE has occurred by Day 6 to exert its inhibitory effects.

In this paper we report that LPS induced hyperresponsiveness in naive as well as in sensitized but saline-challenged animals. The mechanisms involved in this increased responsiveness to MCh is currently unknown, however a recent report by Andersson and coworkers at an International Meeting of the American Thoracic Society (29) suggests it may be the downregulation of the constitutive nitric oxide synthase (cNOS) isoenzyme that is responsible for this phenomena. These researchers have shown LPS to halve the cNOS activity in the rat lung and the trachea 6 h after intratracheal instillation with LPS. Similarly, data reported by Schuiling and colleagues further point to an involvement of decreased levels of cNOS-derived nitric oxide in the airway hyperreactivity seen after allergen challenge in sensitized guinea pigs (30).

Exposure to LPS 18 h after OVA challenge in sensitized animals further exacerbated the cellular allergic inflammatory response to the allergen. The profile of the inflammatory response was characterized by exaggerated neutrophil influx and microvascular leakage without eosinophil influx into the BALF. In addition, the usual postallergen challenge hyperresponsiveness to inhaled MCh was abolished. This reported dissociation between neutrophil influx and airway hyperresponsiveness has previously been shown in our laboratory using Brown-Norway rats (unpublished observation) and is in agreement with reports of ozone-induced lung inflammation (31, 32).

The mechanism by which LPS is able to normalize responses to MCh in allergic animals may be explained by its stimulation of inhibitory cytokines IL-12 and/or IFN- γ . IL-12 is produced by monocytes and dendritic cells in response to bacterial products, including LPS. In the presence of IL-4, LPS stimulates the production of high levels of IL-12 by human dendritic cells *in vitro* compared with low levels in the absence of IL-4 (33). Schwarze and coworkers have shown IL-12 to inhibit airway hyperresponsiveness to MCh after OVA exposure in sensitized mice (34). This mechanism may be responsible for the lack of hyperresponsiveness observed in our sensitized and allergen-challenged animals exposed to LPS. Further, Schwarze and coworkers suggested that LPS may serve to prevent the influx of eosinophils into the BALF after allergen challenge. This is supported by our results in sensitized animals exposed to LPS 18 h after OVA challenge, where we failed to detect eosinophils in the BALF even though histology has shown eosinophil accumulation in the lung parenchyma, suggesting a possible LPS effect on regulation of eosinophil chemoattractants and/or eosinophil apoptosis. The presence of LPS in aerosolized OVA (given chronically) has been shown to prevent OVA-induced eosinophilia in guinea pigs (35), and contamination of allergen used for bronchial challenge with LPS resulted in alteration of cellular inflammation (36).

To summarize, in this study we have demonstrated that in the PVG rat, exposure to the bacterial product LPS has the ability to prevent sensitization to allergen *in vivo* only if the exposure occurs early in the sensitization process, which in this model means up to Day 6 after primary sensitization with allergen. Exposure to LPS after Day 6 further aggravated the allergic inflammatory response. In our study LPS can thus be seen as having dual effects. First, exposure to LPS after allergen challenge in sensitized animals inhibited MCh hyperresponsiveness and eosinophil influx into the BALF which we can propose to be associated with its stimulation of high levels of IL-12 resulting in inhibition of Th2-driven allergic response (34). LPS also stimulated B-cell activity. Up to and including 4 d after sensitization, LPS was able to stimulate the B cells to differentiate into IgG-producing plasma cells. However, if the isotype switch had already occurred, LPS then directly stimulated those B cells which have been primed to produce IgE.

It is currently accepted that respiratory viral infections are important "trigger factors" in the development of allergic respiratory diseases, particularly asthma (37, 38). Unlike viral infections, bacterial infections do not generally trigger asthma and in fact, early exposure may protect the individual from development of atopy and asthma later in life (8). In this study we have reported additional evidence suggesting a potentially protective role of bacterial exposure against primary allergen sensitization which could result in disease modification involving alterations in the natural history of asthma. This mechanism may contribute to the variations in the frequency of atopy/asthma that have recently been reported between first- and second-world countries, which point to an inverse relationship between disease prevalence and socioeconomic status (39–41). These findings have led to the development of the "hygiene" hypothesis which suggests that decreasing levels of exposure to infections (42, 43) and/or commensal microbial stimuli (6, 8) in developed countries, particularly during the induction of primary Th1/Th2 responses to aeroallergens during early life (8, 44), may be responsible for increased disease prevalence. Bacterial LPS has been suggested as a potential mediator of these effects (8), and in this context it is interesting to note that a recent study has identified a polymorphism in the gene encoding the high-affinity receptor for LPS (CD14) which is associated with atopy intensity (45).

Acknowledgments: This study was supported by the National Health and Medical Research Council, Australia, and the Asthma Foundation of Western Australia.

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