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Systemic Administration of Endotoxin Induces Bronchopulmonary Hyperreactivity Dissociated from TNF-α Formation and Neutrophil Sequestration into the Murine Lungs

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Bronchopulmonary hyperreactivity (BHR), an increased responsiveness to nonspecific bronchoconstrictor agents, is a well-known characteristic of bronchial asthma. It has been recently suggested that the severity of this disease is related to the endotoxin content of house dust. In the present report, it is shown that the i.p. administration of bacterial LPS to mice is followed by a marked early dose-dependent BHR in response to methacholine. The microscopic examination showed no ultrastructural lesions of the lungs or of the airways, but a marked neutrophil accumulation in the capillaries, as confirmed by an increase of the lung content in the neutrophil enzyme marker myeloperoxidase. In parallel, high levels of TNF-α were found in plasma as well as its transcripts in the lung tissues. Using immunologic (anti-TNF-α and anti-granulocyte Abs), and pharmacologic (dexamethasone and vinblastine) tools, it is demonstrated that BHR is apparently neither related to the presence of neutrophils in the pulmonary microvasculature nor to the synthesis of TNF-α. *The Journal of Immunology, 1998, 161: 474–480.

Aerosol or intranasal (i.n.) 3 administration of endotoxin (LPS) induces intense lung inflammation, with macrophage activation and recruitment of neutrophils to the interstitium, to alveoli, and to the airways of guinea-pigs (1), rats (2, 3), and mice (4, 5). Neutrophil recruitment is accompanied by an augmented lung vascular permeability (6). Since these are the characteristic features of acute respiratory distress syndrome (ARDS), LPS-induced lung inflammation has been used as a model for this syndrome (6). In the course of studies concerning the role of TNF-α as a potential mediator of acute LPS-induced lung inflammation, we became interested in the functional consequences of lung inflammation and observed that i.n. administration of LPS to mice induces a strain-dependent early (1.5–6 h) increase of airway resistance and a late (>24 h) increase in airway responsiveness to aerosolized methacholine (Lefort et al., manuscript in preparation). Because the direct administration of LPS into the airways induces both the early and late effects, as well as an intense activation of alveolar macrophages and recruitment of neutrophils, we developed an alternative protocol to minimize the early effect of LPS and to properly study the enhanced responsiveness to inhaled methacholine, i.e., to bronchopulmonary hyperreactivity (BHR).

In the present study, we show that i.p. administration of LPS to mice is followed by a minor, if any, immediate effect on the airways, thus allowing the uncovering of a marked, early, dose-dependent BHR in response to methacholine. Using immunologic and pharmacologic tools, we have demonstrated that BHR is apparently not due to TNF-α production nor to neutrophil recruitment to the lung.

Materials and Methods

Materials

The anti-TNF-α Ab, chimeric TN3 19-12γl (CNT3), as well as the control isotype mAb, L2, were kind gifts of Dr. G. Higgs, Celltech Therapeutics, Slough, U.K. LPS (Escherichia coli 055:B5) was from Difco Laboratories, Detroit, MI. Dexamethasone phosphate, hexadecyltrimethyl ammonium bromide (HTAB), EDTA, O-dianisidine dihydrochloride, hydrogen peroxide (H₂O₂), and N-formyl-Nle-Leu-Phe (FNLP) were from Sigma Chemical, St. Louis, MO. HBSS was from Life Technologies, Paisley, U.K. Methacholine (acetyl-β-methacholine chloride) was from Aldrich-Chemie, Steinheim, Germany.

Mice

Six-week-old male C57BL/6 mice were provided by the Centre d’Elevage R. Janvier (Le Genest Saint-Ise, France). Mice were treated i.p. with either LPS (at different concentrations) or an equivalent volume of saline, the solvent for LPS. Later, at specific time intervals, animals were challenged with methacholine for the study of BHR, as described below.

BHR determination

Mice were placed in a whole-body plethysmographic chamber (Buxco Electronics, Sharon, CT) to analyze the respiratory waveforms. After a 4-min stabilization, mice received methacholine for 20 s by aerosolization (3 × 10⁻² M in the aerosolator, Aldrich-Chemie). The resulting airways resistance was expressed as Penh = [Te (expiratory time)/40% of Tr (relaxation time) − 1] × Pef (peak expiratory flow)/Pif (peak inspiratory flow) × 0.67, according to the manufacturer’s instructions. Results were expressed as ΔPenh, corresponding to the difference between the basal and...
maximal values. It is notable that upon LPS administration, the basal values increased at most (at 3 h) by 0.5 arbitrary units, i.e., by <10% of the values obtained upon methacholine challenge.

**Analysis of airway inflammation**

To collect bronchoalveolar lavage fluid (BALF), animals were anesthetized with 12 mg/kg of sodium pentobarbital i.p., trachea were cannulated, and lungs were washed eight times with 0.5 ml saline to provide 4 ml of BALF. Aliquots of each BALF were used to evaluate the total and differential cell number. BALF supernatants were collected by centrifugation and stored at −20°C until assayed for TNF-α levels. Blood was also collected over anticoagulant, and plasma was prepared by centrifugation. Samples were then stored and processed as for BALF supernatants. In some experiments, FNLP (2 mg/kg) was applied i.n. 3 h after i.p. administration of LPS or saline.

For histologic analysis, lungs were fixed in paraformaldehyde, and serial 5-μ sections were performed followed by staining with hematoxylin/eosin.

**Measurement of TNF-α production by enzyme immunometric assay**

Levels of TNF-α in the BALF were determined by an enzyme immunoassay. This method relies on the reaction of thiogroups of mAb Fa’s fragments with maleimido groups previously introduced into acetylicholinesterase (AChE) as previously described (7). As for anti-IL-5 Abs (8), anti-TNF-α mAb MP6-XT22, and MP6-XT3 were purified from ascitic fluids (cloned hybridomas kindly provided by Dr. P. Minoprio, Institut Pasteur, Paris, France), using the affinity chromatography method on a protein G column (HiTrap affinity columns, Pharmacia Biotechnology, Uppsala, Sweden) after precipitation by ammonium sulfate. Characteristics of these anti-rat anti-murine TNF-α mAbs were described in details elsewhere (9).

Immunometric assays were performed in 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark), coated with 10 μg/ml of the rat anti-murine TNF-α mAb, MP6-XT3, as described previously (10). The one-step procedure used for immunometric assays involved the simultaneous addition of 100 μl of TNF-α standards (7.8–100 pg/ml) or samples, and 100 μl of the second rat anti-murine TNF-α mAb, MP6-XT22-AChE conjugated, at a concentration of 10 Eillman U/ml. After incubation for 18 h at 4°C, the plates were extensively washed and solid-phase bound AChE activity was determined colorimetrically by adding 200 μl of Ellman’s medium. Absorbance was read at 405 nm with an automatic microplate reader (Dynatech MR 5000; Dynatech Laboratories, Saint Cloud, France). The lower limit of detection of this assay was 15 pg TNF-α/ml sample.

**TNF-α mRNA transcript levels**

Lungs were isolated and thoroughly washed with saline via the pulmonary artery, and poly(A)+ mRNAs were isolated using the Poly(A) tract system (Promega Corp., Madison, WI) and an ultratraxor (T25; Janke and Kunkel, IKA-Laortechnik, Stanfen, Germany) for homogenization. Intradermal RT-PCR was performed using specific primers for TNF-α (sense, CTTGTGGGAGGCGGACCACGCCT; antisense, CTCAAGCG CTGAAGTGGCTCCCTCTCTC) and for β-actin (sense, GGAATCTCTAT GTGGTTGAGACG; antisense, GGGAGGACATAGCCCTCGATA GAT) as control. The cDNAs were synthesized in 25 μl using 5 μg of total RNAs. 0.5 μg oligo(dT(12-14)), or hexamers as primers, 0.5 U RNAse (Promega France, Charbonnières, France) and 200 U M-MTLV reverse transcriptase RNase H- (Promega) in the manufacturer’s buffer, for 1 h at 42°C. Hot-start PCR was performed on a Peltier thermal cycler apparatus type A (Molecular Dynamics, Sunnyvale, CA), comparing TNF-30 min with 0.5 µg of murine rTNF-α (Innugenex, Los Angeles, CA) emulsified in adjuvant (Hunter TiterMax; CytRx, Norcross, GA). The animals were bled 7 days after the second injection, and Igs were prepared from the serum by precipitation with 40% saturated ammonium sulfate (v/v). The presence of specific Abs was checked in vitro by testing the inhibition of the cytotoxic effect of TNF-α on WEHI cells. Following the third injection, animal were bled every 3 wk for several mo, and sera were pooled. Precipitated Igs were diazylated overnight against PBS and stored at 4°C at a final concentration of 9 mg/ml (No. H42031).

**Preparation of the anti-granulocyte Abs**

RB6-8C5 (anti-Ly-6G) is a rat IgG2b mAb (11) that binds selectively to and depletes mouse neutrophils and eosinophils (but not lymphocytes or macrophages) (12). This anti-granulocyte mAb was purified from ascitic fluids (cloned hybridomas kindly provided by Dr. G. Millon, Institut Pasteur) through precipitation with 45% saturated ammonium sulfate (v/v). Following dialysis at 4°C against PBS, Igs were filtered (0.22 µm) and then stored at 4°C at a final concentration of 5 mg/ml. One i.p. administration of 200 µg of such a preparation to mice led within 24 h to a complete absence of circulating neutrophils, which last for 5 days (data not shown).

**Determination of lung myeloperoxidase (MPO) activity**

Lung tissue MPO activity was determined following a previously described method (13) with minor modifications. After bronchoalveolar lavage, the lungs were removed from the thorax, blotted with gauze to remove blood, and frozen at −20°C until assay. In some experiments, before being removed, lung vessels were flushed to discard circulating blood. The left atrium was thus open, and 5 ml of saline were gently perfused into the right ventricle. Collected lungs were then homogenized for 30 s (Potter-Elvehjem glass homogenizer, Thomas, Philadelphia, PA) at 4°C in 1 ml PBS. The corresponding extracts were centrifuged (10,000 × g, 10 min, 4°C), and supernatants containing hemoglobin were discarded. The pellets were resuspended in 1 ml PBS supplemented with HTAB (0.5%) and EDTA (5 mM) and homogenized again. Following centrifugation, 50 µl of supernatants were placed in a test tube with 200 µl PBS-HTAB-EDTA, 2 ml HBSS, 100 µl O-dianisidine dihydrochloride (1.25 mg/ml), and 100 µl H2O2 (0.05% = 0.4 mM). After 15 min of incubation while shaking at 37°C, the reaction was stopped by the addition of 100 µl NaNO2 (1%). The MPO activity was determined as change in absorbance at 460 nM.

**Statistical analysis**

Each point corresponds to the mean ± standard error of 3 to 6 values obtained from distinct mice. Statistical differences were determined using the one-way analysis of variance (ANOVA), and p < 0.05 was considered significant. Individual groups were compared using the unpaired Student’s t test. Significance is indicated by an asterisk (*) on the figures when p < 0.05.

**Results**

**Dose response and time course of LPS-induced airways hyperresponsiveness**

We studied first the effects of the i.p. administration of LPS on the intensity of the airway responses to aerosolized methacholine. As illustrated in Figure 1A, the direct bronchoconstrictor effects of methacholine alone were minor at the concentration used (first column). In contrast, the injection of LPS induced, after 3 h, a dose-dependent augmentation of the responses to methacholine. ∆Penh was significantly increased at a dose of 0.3 mg/kg, reaching a plateau at 1 mg/kg LPS. The kinetics of LPS-induced BHR were also studied, using 1 mg/kg LPS. Under those conditions, BHR was observed as early as 90 min (Fig. 1B) and reached a plateau at 4.5 h. Normal responsiveness was restored within 24 h.

**Effect of LPS on neutrophil sequestration in the lung**

Microscopic examination showed no ultrastructural lesions of the lungs or the airways nor neutrophil recruitment to the alveoli from
LPS-treated mice, but showed a marked accumulation of neutrophils lining the endothelium of the capillaries (Fig. 2). In agreement, the BALF of mice treated with LPS through the i.p. route were also neutrophil free, while a dramatic increase in the number of neutrophils was observed following i.n. administration of the same dose of LPS, thus validating the efficacy of LPS (data not shown). Since a strong correlation between the number of intra-vascular neutrophils and the lung content in the enzyme marker MPO has been described (14), this parameter was measured to quantify the total neutrophil sequestration to the lungs. Three h after i.p. injection of LPS, the absorbance at 460 nm was of 0.83 ± 0.07 for LPS-treated mice, as compared with 0.04 ± 0.01 for control (p < 0.05; n = 21), a 20-fold increase.

Since these results were obtained without flushing the lung vessels, experiments were repeated with lungs removed after a perfusion of saline (5 ml) through the right ventricle. This procedure reduced by 70% the total hemoglobin content of the tissue. A significant difference was noted between washed or nonwashed lungs from LPS-injected animals in terms of MPO content, i.e., 0.76 ± 0.03 vs 0.70 ± 0.03 (n = 6, p < 0.02). Nonetheless, this difference was only ~10% and still amounted to an increase of 17-fold above the basal levels. Such a result demonstrated that neutrophils sequestered onto the vessels firmly adhered to the endothelial wall.

MPO evaluation confirmed the histologic observations and suggested that LPS triggers a signal at the peritoneal, systemic, or lung level, leading to the accumulation of neutrophils in the pulmonary vasculature, but failing to induce a full signal at the epithelial level to insure BALF invasion. Indeed, under the present experimental protocol, neutrophils, although not invading tissue, were prone to do so. This was demonstrated by instilling i.n. FNLP (a synthetic bacterial peptide that activates macrophages and neutrophils) 3 h after i.p. administration of LPS. As a result, numerous neutrophils invaded the BALF, as shown by increased cell counts in BALF collected 3 h after FNLP challenge, i.e., \(5.03 \pm 1.53 \times 10^5\) neutrophils/lung compared with \(0.05 \pm 0.01 \times 10^5\) neutrophils/lung (n = 3) for animals also receiving FNLP but 3 h after the i.p. administration of saline. The lungs and/or the neutrophils are thus markedly modified by the i.p. administration of LPS in such a way that neutrophils readily invade the tissues.

**Effect of LPS on TNF-α production in blood and gene expression in lungs**

Upon treatment of mice with i.p. LPS, a high level of TNF-α was observed in plasma at 90 min, which was absent at 180 min (Fig. 3, A and B), under conditions in which no TNF-α was found in the
peritoneal lavage or in the BALF (not shown). This enhanced protein production was accompanied by an intense expression of TNF-α transcripts in the lungs (Fig. 4, A and B). It is thus likely that LPS given i.p. is rapidly absorbed into the blood and reaches the lungs.

**Differential modulation of TNF-α production, neutrophil recruitment, and BHR**

At this stage, it seemed possible that LPS-induced BHR, TNF-α gene expression, and protein production were associated. To investigate this hypothesis, three different approaches were used.

First, the recognized anti-inflammatory agent dexamethasone was administered at different doses, and the various parameters used were studied. When dexamethasone was administered s.c. twice at 5 mg/kg, 18 and 1 h before LPS challenge, the generation of TNF-α in plasma and BHR was suppressed (Fig. 3A), as was the transcription of TNF-α (Fig. 4A). By contrast, when dexamethasone was administered twice at 0.625 mg/kg (18 and 1 h before LPS challenge), BHR was not affected, but TNF-α protein production was still suppressed (Fig. 3B) and specific transcript expression practically abrogated (Fig. 4B). Even though both doses of dexamethasone suppressed TNF-α formation, the total MPO activity of the lungs was not reduced. In fact, it was constantly augmented, although not always significantly (Fig. 5). This indicates that TNF-α production can be suppressed without inhibiting BHR or neutrophil accumulation in the lung vasculature.

A second approach consisted of a passive immunization against TNF-α. The Ab CTN3 (9.2 mg/ml) was given i.v. (200 μl) and i.n. (40 μl) 18 h before the i.p. administration of LPS, and animals were studied after 3 h. As seen in Figure 6A, BHR was not significantly reduced upon such a treatment. Similarly, a rabbit

**FIGURE 3.** BHR, formation of TNF-α, and their modulation by dexamethasone following i.p. administration of LPS to mice. Experiments were performed as described in Figure 1 using 1 mg/kg LPS. Three different groups were studied at two different time intervals (90 and 180 min) for two different parameters, i.e., BHR (ΔPenh) and TNF-α concentrations in plasma (ng/ml). A. Animals were pretreated twice with either saline (saline-LPS) or dexamethasone (5 mg/kg), 18 and 1 h before LPS challenge (dexamethasone-LPS). Animals from control groups received saline as described above and saline again in place of LPS (saline-saline). B. Groups of animals were constituted exactly as described above with the exception of the dose of dexamethasone (0.625 mg/kg). Results are expressed as mean ± SEM obtained from three to seven distinct animals. *,” dexamethasone-LPS” groups with values significantly different (p < 0.05) from “saline-LPS” groups for each time point (hatched columns).

**FIGURE 4.** TNF-α gene expression in the lung and its modulation by dexamethasone upon i.p. administration of LPS to mice. TNF-α mRNA transcript levels were evaluated in lungs collected from animals under different experimental conditions. As in Figure 3, the same three different groups were studied at two different time intervals (90 and 180 min). A. Animals were pretreated twice with either saline (LPS; two distinct animals) or dexamethasone (5 mg/kg), 18 and 1 h before LPS challenge (LPS + Dexa. – three distinct animals). Animals from the control groups received saline as described above and saline again in place of LPS (S; one animal). B. Groups of animals were constituted exactly as described above except for the dose of dexamethasone (0.625 mg/kg).
administration of TNF-α of LPS (data not shown). In agreement, neither the i.p. nor the i.n. and the lung enrichment in MPO induced by the i.n. administration although not suppressing, the neutrophil recruitment to the BALF although not shown), confirming adequate neutralization. Surprisingly, under these experimental conditions, enrichments of the lungs in MPO were also not affected (Fig. 6A). As a validation of the efficacy of the protocol, these Abs were effective in significantly reducing, in lungs collected from animals under different experimental conditions. Animals received LPS (1 mg/kg) or not following a pretreatment (or not) with dexamethasone (0.625 or 5 mg/kg, 18 and 1 h before LPS challenge). Results are expressed as mean ± SEM obtained from three to four distinct animals.

polyclonal Ab raised against murine TNF-α was inactive against BHR (Fig. 6A), although increased TNF-α contents in the blood of LPS-treated mice were reduced to the background level (not shown), confirming adequate neutralization. Surprisingly, under these experimental conditions, enrichments of the lungs in MPO were also not affected (Fig. 6B). As a validation of the efficacy of the protocol, these Abs were effective in significantly reducing, although not suppressing, the neutrophil recruitment to the BALF and the lung enrichment in MPO induced by the i.n. administration of LPS (data not shown). In agreement, neither the i.p. nor the i.n. administration of TNF-α up to 50 μg/kg induced BHR (not shown).

To verify whether neutrophils are important for LPS-induced BHR, animals were treated with 2 mg/kg vinblastine 96 h before LPS administration. At this time point, neutrophils were depleted from circulating blood, and no MPO activity was found in the lungs after i.p. LPS, which seemed logical in view of their disappearance. Under those conditions, BHR was not modified (Fig. 7). This result strongly suggests that neutrophil accumulation in the vasculature of the lungs and BHR induced by i.p. LPS are not associated. To confirm this concept, we also tested the effects of the anti-granulocyte mAb RB6-8C5. As illustrated in Figure 7, under conditions in which there is a total absence of circulating neutrophils, BHR was again not affected, while MPO activity was fully abrogated.

Discussion

BHR, an increased responsiveness to nonspecific bronchoconstrictor agents, is a well-known characteristic of bronchial asthma (15). It can also be observed following airway infections (16) or inhalation of airborne pollutants (17), two conditions in which bacterial LPS may be involved. Indeed, LPS is a constituent of Gram-negative bacteria and is found in the air environment including in house dust (18). This is particularly relevant because LPS increases bronchial reactivity in asthmatic subjects (19). Further, it has been recently established that the severity of asthma is related to endotoxin in house dust (20). In an experimental guinea pig model, i.n. administration of LPS is followed by intense BHR in response to i.v. serotonin (21). Inhalation of LPS by rats also leads to a dose-dependent increase in airway responsiveness to i.v. serotonin (3), which has been attributed to TNF-α (22). The main consequence of LPS inhalation is the induction of a huge neutrophil recruitment to the air spaces (5). A relationship between airway inflammation and nonallergic BHR has been suggested. Thus, in an experimental dog model, airway responsiveness closely correlated with the number of neutrophils in the airway epithelium (23). Similarly, inhalation of C5a caused lung inflammation and an increase of responsiveness to histamine in rabbits (24). Conversely, other studies suggested that the influx of neutrophils may not be involved. This is particularly the case in a study performed by Pauwels et al. (3), in which no correlations were found between inflammation and BHR in a rat model of inhalation of LPS.

It is of note that i.p. injections of LPS cause neutrophil infiltration in the alveolar spaces in rats (13), but induce neutrophil sequestration in the lung vasculature without any transpulmonary recruitment in mice (13, 25), BALF being thus neutrophil free. This insight led us to administer LPS i.p. to mice, to study the resulting BHR and the role of neutrophils and TNF-α. A further advantage of the model we used is that, in our hands, i.n. administration of LPS to mice induces a direct, long-lasting increase in airway resistance, preventing subsequent evaluation of BHR (Le-fort et al., manuscript in preparation).

The present data clearly show that i.p. LPS augments the reactivity of the lungs to methacholine, a prototype bronchoconstrictor
was not at all mitigated. This dissociation between TNF-α in rats (22). Under our experimental conditions, LPS triggered others (26, 27), pretreatment of mice with dexamethasone, twice at neal fluid decreased exponentially following i.p. treatment, while Hirano (13) showing that the concentration of LPS in the perito-
the lung tissue; it is in agreement with the recent data of as specific transcripts in the lungs. This strongly suggests that LPS synthesis and by an accumulation of neutrophils in the capillaries as observed by light microscopy and confirmed by the increased MPO found in the lung tissues in the absence of recruitment into the BALF.

TNF-α has been implicated as a mediator of LPS-induced BHR in rats (22). Under our experimental conditions, LPS triggered within 90 min a transient appearance of TNF-α in the blood as well as specific transcripts in the lungs. This strongly suggests that LPS rapidly entered the blood stream and reached the competent cells within the lung tissue; it is in agreement with the recent data of Hirano (13) showing that the concentration of LPS in the perito-
neal fluid decreased exponentially following i.p. treatment, while the concentration in the blood increased up to 1 h and returned to a basal level within 4 h. As expected from the data reported by others (26, 27), pretreatment of mice with dexamethasone, twice at 5 mg/kg, totally abrogated the synthesis of TNF-α. Concomitantly, BHR was also suppressed. Nonetheless, experimental conditions were found, using a small dose of dexamethasone (0.625 mg/kg) for which, although TNF-α formation was still abrogated, BHR was not at all mitigated. This dissociation between TNF-α and BHR was substantiated by another approach using two different specific TNF-α Abs, which, at effective doses (TNF-α was no more detectable in blood), failed to modify BHR. These results contrast with the observations of Kips et al. (22). An obvious expla-

agents. This BHR was accompanied by the induction of TNF-α synthesis and by an accumulation of neutrophils in the capillaries as observed by light microscopy and confirmed by the increased MPO found in the lung tissues in the absence of recruitment into the BALF.

FIGURE 7. Effects of neutrophil depletion on BHR and MPO activity in the lung upon i.p. administration of LPS to mice. BHR (ΔPenh) and MPO activity were evaluated in animals under different experimental conditions. Animals were pretreated with either saline (Saline-LPS), vinblastine (Vin-
tblastine-LPS), or an anti-granulocyte mAb (Anti-granulocyte-LPS) before LPS challenge. Animals from control groups received saline twice instead the pretreatment and LPS (Saline-Saline). Results are expressed as mean ± SEM obtained from four to seven distinct animals. *, Treated groups with values significantly different (p < 0.05) from the saline-LPS-treated groups.

BHR is thus apparently neither related to the presence of neu-

thetic factor is most probably essential in triggering BHR when LPS is given i.p. and has yet to be defined.

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