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5-Lipoxygenase and endotoxin-induced microvascular albumin exchanges and leucocyte recruitment in guinea-pig lungs

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Abstract

The interference of the 5-lipoxygenase inhibitor, BW B70C ((*E*)-*N*-{3-[3-(4-fluorophenoxy)phenyl]-1(*R*,*S*)-methyl prop-2-enyl}-*N*-hydroxyurea), with *Escherichia coli* lipopolysaccharide (endotoxin)-induced lung leucocyte sequestration and microvascular albumin exchanges was evaluated in the anaesthetised guinea-pig using radioactive tracers, in parallel to the effects on cell counts in the broncho-alveolar lavage fluid, blood tumour necrosis factor (TNF-α) content, secretion of phospholipase A_2 and synthesis of leukotriene C_4 by alveolar macrophages. Intravenous injections of 0.1 or 1 mg/kg endotoxin induced lung leucocyte sequestration but only the higher dose induced an increase in albumin microvascular exchanges and the infiltration of leucocytes towards the airway lumen. Leukotriene B_4, a potential mediator of the 5-lipoxygenase-dependent endotoxin effects, induced a rapid and transient lung leucocyte sequestration and leucopenia associated with a more progressive increase in microvascular exchanges. The 5-lipoxygenase inhibitor, BW B70C, injected i.p. (30 mg/kg) prevented leukotriene C_4 synthesis by alveolar macrophages and reduced leucocyte migration to the airways lumen as well as albumin microvascular leakage but did not affect the endotoxin-induced increase in the blood level of TNF-α and of secreted phospholipase A_2. However, BW B70C failed to modify vascular leucocyte margination induced by 1 mg/kg endotoxin, suggesting that, apart from a role of 5-lipoxygenase, alternative pathways operate in response to endotoxin in guinea-pig.

Keywords: Albumin leakage; Lipopolysaccharide; 5-Lipoxygenase; Leukocyte; Lung; Radioactive tracer

1. Introduction

In situ neutrophil activation and secretion of enzymes, toxic substances and inflammatory mediators may damage the lung vascular endothelium, leading to protein leakage and oedema formation (Haslett et al., 1989; Kaslovsky et al., 1990). In order to be available in the lungs, neutrophils must initially marginate in vessels, then infiltrate the tissues. Accordingly, vascular margination and infiltration in the lung tissue are critical steps which determine the number of leucocytes that will ultimately be available for clearing bacteria and/or as a source of secreted toxic substances that participate in lung pathologies (Haslett et al., 1989).

Bacterial endotoxin (lipopolysaccharide) has marked effects on the lung. It induces pulmonary hypertension, leucopenia, leucocyte sequestration and an increased lung microvascular permeability, leading to non-cardiac pulmonary oedema, associated with increased lung lymph protein clearance (Brigham and Meyrick, 1986). Many of these noxious effects result from the induced formation and release of mediators following the direct and/or indirect activation of neutrophils (Wang et al., 1994; Gonçalves de Moraes et al., 1996), macrophages (Christman et al., 1989), or endothelial (Schleimer and Rutledge, 1986) and other cells. Among these potential mediators, lipoxygenase-dependent metabolites of arachidonate, particularly leukotriene B_4, seem to be very important (Brigham and Meyrick, 1986; Takahashi et al., 1993). Leukotriene B_4 is a chemo-attractant and activating agent for neutrophils (Ford-Hutchinson, 1985), which displays potent inflammatory effects (Rosengren et al., 1991).

In the present investigation, we compared the ability of

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leukotriene B_4 and endotoxin to induce lung leucocyte sequestration, blood volume variations and microvascular albumin exchanges in vivo, using a method in which radiolabelled leucocytes, erythrocytes and serum albumin were injected into anaesthetised guinea-pigs (Bureau et al., 1989, 1994a). In addition, cell counts in broncho-alveolar lavage fluid allowed the evaluation of leucocyte migration to the lungs at different time points. Finally, because of the suggestive evidence for a role of 5-lipoxygenase metabolites as mediators of some effects of the endotoxin, we investigated whether BW B70C, an inhibitor of the 5-lipoxygenase pathway (Yeadon et al., 1993), interferes with vasopermeation, vascular leucocyte margination and infiltration in the guinea-pig lung. It is widely accepted that apart from the lipid mediators, tumour necrosis factor (TNF-α) is important for endotoxin-induced inflammation (Beutler et al., 1985). Secreted type II phospholipase A_2 is an alternative and novel potential mediator of inflammation (Vadas et al., 1993) whose interactions with TNF-α are poorly described. For this reason, and since the sequential release of TNF-α and phospholipase A_2 has been shown during endotoxin-induced inflammation in the guinea pig (De Castro et al., 1995) we investigated whether BW B70C affects the endotoxin-induced increase in blood titres of TNF-α and phospholipase A_2.

2. Material and methods

2.1. Chemicals

Leukotriene B_4 (Cascade Biochem, University of Reading, Reading, UK) was stored at -20°C as a 100 μg/ml stock solution in ethanol. Endotoxin (from *Escherichia coli* 055:B5) was from Difco, Detroit, USA. The 5-lipoxygenase inhibitor, BW B70C ((*E*)-*N*-{3-[3-(4-fluorophenoxy)phenyl]-1(*R,S*)-methyl prop-2-enyl}-*N*-hydroxyurea), a gift from Dr L. Garland (Wellcome Research Laboratories, Beckenham, UK) was ball milled at 4°C overnight to form a suspension in carboxymethylcellulose (0.5 g per 100 ml saline). Heparin was from Choay (Paris, France), pancuronium bromide (Pavulon) from Organon Tecknika (Fresnes, France) and Plasmagel was from Roger Bellon (Neuilly sur Seine, France). Culture medium (RPMI 1640), penicillin, streptomycin, amphotericin and Dulbecco's medium were from Gibco (Paisley, UK), heat-inactivated fetal calf serum (3%) was from Boehringer-Mannheim (Mannheim, Germany). The BCA kit for measuring protein concentration was from Pierce (Rockford, IL, USA) and the Diff Quik stain was from Baxter Dade (Düdingen, Switzerland). Dextran (molecular mass 295 kDa), dextran-coated charcoal, ethyl carbamate, prostaglandin E_2, prostacyclin (prostaglandin I_2), fMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenyl-alanine) and fatty acid-free serum albumin were from Sigma (St. Louis, MO, USA).

The substrate for fluorometric determination of secreted phospholipase A_2 activity was 1-hexadecanoyl-2(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol (Molecular Probes, Eugene, OR, USA). It contains a fluorescent group linked to the fatty acid in the *sn*-2-acyl position (Radvanyi et al., 1989).

The reagents for determination of TNF-α were the recombinant human TNF-α as a standard (Boehringer-Mannheim) and fibrosarcoma cells (WEHI 164 clone 13 line, kindly provided by Dr F.J. Zijlstra, Erasmus University, Rotterdam, Netherlands).

The reagents for radioimmunoassays were the antibody against leukotriene C_4 (kindly provided by Drs. F. Kohen and U. Zor, Weizmann Institute, Rehovot, Israel), 14,15(n)-[^{3}H]leukotriene C_4 and scintillation mixture ACS from Amersham (Amersham, UK).

The radiotracers and the reagents for radiolabelling the cells were tropolone (2-hydroxy-2,4,6-cyclo-heptatrienone) from Sigma (stored at -20°C as a 4.4×10^{-3} M stock solution in saline); iodine-131 albumin, indium-111 chloride, sodium pyrophosphate decahydrate and stannous chloride provided by CIS Biointernational (Gif sur Yvette, France) and technetium-99m pertechnate (^{99m}Tc) a gift from the Service Central des Radio-Isotopes, Hôpital Necker (Paris, France).

2.2. Equipment

The animals were ventilated with a Harvard small animal respirator (UK). A gamma detector (Na-I crystal, $2'' \times 2''$, Aptec, USA) was used to measure simultaneously the radioactivities of technetium-99m (^{99m}Tc), indium-111 (^{111}In) and iodine-131 (^{131}I) in the lung region. The probe was collimated with lead to permit the observation of a limited field (diameter of the aperture = 1 cm). Three energy windows were set from 113 to 157 keV, 160 to 276 keV and 330 to 411 keV for counting the gamma emission of ^{99m}Tc, ^{111}In and ^{131}I, respectively. Counts for each isotope were corrected for the overlap between the radioactivities of the different isotopes. A card (Intertechnique, Soft Interwin, Les Ulis, France) installed in a computer was connected to the gamma detector to store and operate calculations on radioactivity measurements. A gamma well-type counter (1282 Compugamma, LKB Wallac, Turku, Finland) was used to measure blood sample radioactivities, to correct them for overlap and to store results for further calculations. Details of the procedure were described earlier (Bureau et al., 1989).

A scintillation counter (1212 Rackbeta, LKB Wallac) was used to measure the ^{3}H radioactivity of labelled leukotriene C_4.

Fluorescence was monitored with a Jobin and Yvon JY3D spectrofluorometer.

2.3. Isolation and radiolabelling of the cells

Erythrocytes and leucocytes were radiolabelled with ^{99m}Tc and ^{111}In, respectively. Erythrocytes were labelled according to the procedure described in the kit from CIS

Bio International (Gif sur Yvette, France) and by Bureau et al. (1994a). Autologous leucocytes were recovered from blood according to Sweatman et al. (1987). Two ml of arterial blood were collected in a tube containing 0.5 ml of acid citrate dextrose (ACD) as an anticoagulant (2.5 g citrate trisodium + 1.4 g citric acid + 2 g glucose per 100 ml) and an equivalent volume of Plasmagel was reinjected into the animal. Blood was mixed with 1 ml of dextran (molecular mass 295 kDa) 6% (w/v in saline 0.85% NaCl), then allowed to sediment for 40 min. The plasma fraction was centrifuged ($800 \times g$) and the remaining erythrocytes were eliminated from the cell pellet by hypotonic lysis. The cell suspension was washed with Dulbecco's buffer free of Mg^{2+} and Ca^{2+} ($1400 \times g$ for 10 min). The percentage of neutrophils among blood leucocytes was $42.9 \pm 3.5\%$ before and $61.4 \pm 3.5\%$ (mean $\pm$ S.E.M., $n = 13$) after separation. Lastly, leucocytes were radiolabelled with ^{111}In-tropolonate, according to Dampure et al. (1982), as described previously (Bureau et al., 1994a). At each step prostaglandin E_2 ($1–5 \times 10^{-5}$ M) was added to ensure cytoprotection. At the end of the procedure the leucocytes were resuspended in autologous plasma prepared from the citrated blood with 10^{-6} M prostacyclin. Prostaglandins injected with the bolus of radiolabelled leucocytes were probably largely metabolised when the i.v. injection of leukotriene B_4, endotoxin or saline was performed 2–3 h later (see Section 2.4.1). Thus the endotoxin-induced leucopenia was statistically the same in animals which received prostaglandins with the bolus of radiolabelled leucocytes and in those which were not injected with radiolabelled cells (results not shown). This also indicates that radiolabelled leucocytes responded like non-radiolabelled cells to an inflammatory stimulation.

2.4. In vivo experiments

Guinea-pigs were anaesthetised (ethyl-carbamate, 1.5 g/kg i.p), paralysed with pancuronium (2 mg i.p.) and ventilated (0.1 ml room air/100 g; 70 cycles/min). Catheters were inserted into the carotid artery and into the jugular vein. Leukotriene B_4 was administered at 3.3 μg/kg and endotoxin at 0.1 and 1 mg/kg i.v. The 5-lipoxygenase inhibitor, BW B70C (medium: 0.5% carboxymethylcellulose in saline), was injected i.p. at 30 mg/kg 1 h before endotoxin (Yeadon et al., 1993).

2.4.1. Radioactivity measurements

The method used to evaluate simultaneously leucocyte sequestration, blood volume and albumin leakage in the lung has been described in detail (Bureau et al., 1989, 1994a). Briefly, autologous leucocytes obtained from 2 ml of blood were radiolabelled with ^{111}In and reinjected into the animal via the jugular catheter. 2–3 h later autologous erythrocytes radiolabelled with ^{99m}Tc and ^{131}I human serum albumin were also injected i.v. The radioactivity of the three tracers was measured externally with a gamma detector placed on a lung region at the contact of the thorax and on blood samples in a gamma-type well counter. Blood samples were collected starting 30 min after the injection of radiolabelled erythrocytes and albumin, before and after i.v. bolus injection of the agonists (endotoxin at 0.1 or 1 mg/kg or leukotriene B_4 at 3.3 μg/kg). Parameters evaluated from the blood and lung were the radioactivities of erythrocytes, as an index of lung blood volume, of extravascular albumin, as an index of albumin exchanges through the endothelium and of non-circulating leucocytes, as an index of their lung recruitment.

In the same experiments, variations in albumin and leucocyte radioactivity in blood, resulting from their exchanges in the different organs, were also evaluated. Values are given as percent of the corresponding basal blood radioactivity.

2.4.2. Determination of TNF-α

TNF-α concentration was measured in blood plasma by cytotoxicity on fibrosarcoma cells, according to a procedure previously described (De Castro et al., 1995). Inter- and intra-assay variability was always below 30%. The blood sample for this assay was collected 1 h after i.v. injections of endotoxin (1 mg/kg) or saline to animals treated or not with BW B70C.

2.4.3. Determination of secreted phospholipase A_2

Secreted phospholipase A_2 was determined fluorometrically as described by Radvanyi et al. (1989). Briefly, the substrate (1-hexadecanoyl-2(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol) forms vesicles in the aqueous medium and the fluorescence of the monomer is negligible. After hydrolysis by phospholipase A_2, the fluorescent fatty acid liberated is not soluble in the medium but binds tightly to serum albumin, producing fluorescent emission. The fluorescence was monitored in a spectrofluorometer equipped with a xenon lamp, using excitation and emission wavelengths of 345 and 398 nm, respectively. The concentration of hydrolysed substrate was proportional to the slope of the fluorescence emission. The sensitivity of the assay was 50 pmol substrate hydrolysed/ml enzyme sample. Inter- and intra-assay variability was always below 25%. According to the time course of phospholipase A_2 secretion (De Castro et al., 1995), the blood sample for this assay was collected 6 h after the i.v. injection of endotoxin (1 mg/kg) or saline into animals treated or not with BW B70C.

2.4.4. Collection of broncho-alveolar lavage fluid

In these experiments i.v. injections were performed through the saphenous veins. At the time of the experiment animals were anaesthetised and their airways were washed with 50 ml of NaCl 0.9% using 5 ml fractions. These fractions were injected slowly with a syringe through a

tracheal cannula. After 5 s, the lavage fluid was recovered gently. The fractions were mixed and the cell content was evaluated microscopically using a Mallassez haemacytometer. Differential leucocyte counts were performed in cytospin preparations of the broncho-alveolar lavage fluid after staining with the Diff Quik set. At least 300 cells were counted under an oil immersion objective by two investigators unaware of the design.

2.5. Ex vivo activation of alveolar macrophages

The cell pellets obtained from centrifuged broncho-alveolar lavage fluid ($470 \times g$, 10 min, room temperature) were resuspended in a culture medium (RPMI 1640) supplemented with heat-inactivated foetal calf serum (3%), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) at a concentration of 10^6 cells/ml and were poured into 35-mm diameter tissue culture wells (1 ml per well). Then cells were allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were discarded and the remaining monolayers (> 95% alveolar macrophages) were incubated for an additional hour in serum-free RPMI medium. The cells were then stimulated with fMLP (10^{-6} and 10^{-7} M) for 10 min at 37°C. Supernatants were kept at −20°C for leukotriene C_4 radioimmunoassay.

2.6. Determination of leukotriene C_4

The radioimmunoassay for measurement of the leukotriene C_4 concentration in macrophage supernatants was performed according to Aehringhaus et al. (1982). The monoclonal anti-leukotriene C_4 antibody employed was 10% cross-reactive with leukotriene D_4 and less than 0.1% with leukotriene A_4 and leukotriene B_4. Inter- and intra-assay variabilities were always below 10%. Samples (100 μl) were incubated with the antibody and 14,15(n)-[^{3}H]leukotriene C_4 for 24 h at 4°C. Dextran-coated charcoal was used to separate unbound ligand by centrifugation ($3000 \times g$, 10 min, 4°C) and the antibody-leukotriene C_4 complex remaining in the supernatants. Radioactivity was measured, using a scintillation counter, in aliquots of the supernatant (0.5 ml) mixed with 4.5 ml of an aqueous scintillation mixture (ACS).

Leukotriene C_4 concentration was expressed as pg/million cells and the number of adherent cells was determined after protein assay using the BCA kit. The conversion factor routinely used was 200 μg protein corresponding to 10^6 cells.

2.7. Statistics

The various groups of experiments were compared using the non-parametric test of Fisher.

3. Results

3.1. Leukotriene B_4-induced lung leucocyte sequestration, blood leucopenia and changes in lung microvascular albumin exchanges and blood volume

Leukotriene B_4 (3.3 μg/kg i.v.) induced a rapid, reversible and intense leucopenia accompanied by leucocyte sequestration in the lungs (Fig. 1A). Leucopenia was less

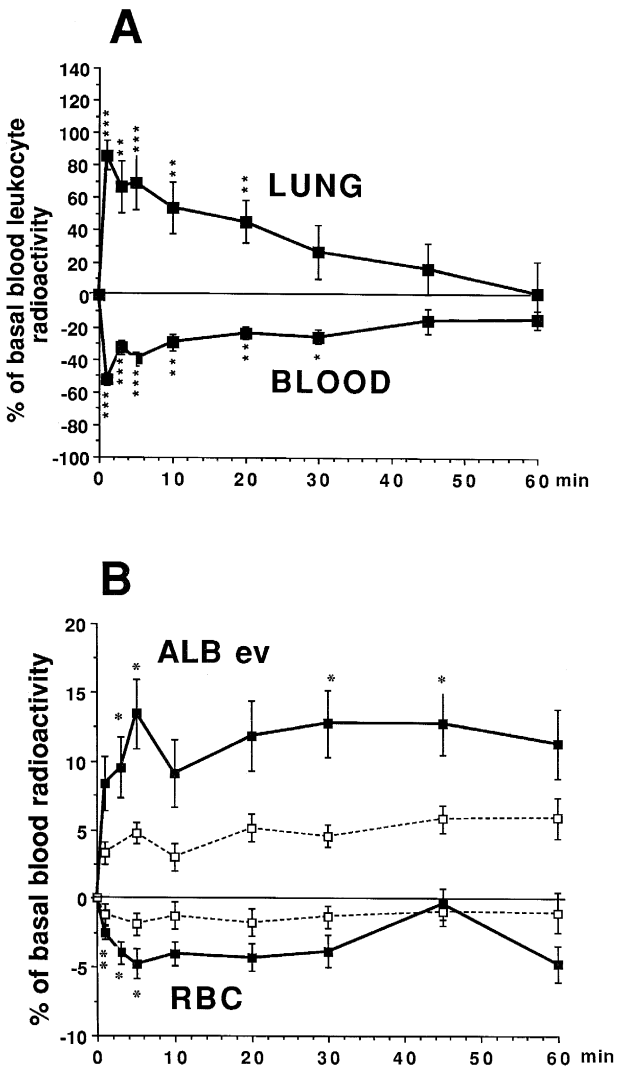

Fig. 1. Leukotriene B_4-induced lung leucocyte sequestration, blood leucopenia and changes in lung microvascular albumin exchanges and blood volume. (A) Values are means ± S.E.M. of the radioactivity bound to leucocytes sequestered in the lungs and circulating in the blood as percent of basal blood leucocyte radioactivity at different times after the i.v. injection of leukotriene B_4 (3.3 μg/kg). Curves for saline-injected animals varied between −30.1 and −2.9% for sequestration and between −10.1 and 10.1% in the blood. (B) Values are means ± S.E.M. of the lung radioactivity bound to extravascular albumin (ALB ev) and to erythrocytes (RBC) as percent of the corresponding basal blood radioactivity. Significance of the difference between animals injected with leukotriene B_4 ($n = 17$) (■), or saline ($n = 10$, not shown in panel A) (□): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

marked than sequestration (maximum value of leucopenia 52.3 ± 3.7 vs. 86.2 ± 8.9% for sequestration), a difference which is probably accounted for by the return of sequestered leucocytes to the circulation.

As seen in Fig. 1B, leukotriene B_4 increased the lung content of radiolabelled extravascular albumin and decreased the radioactivity of erythrocytes, indicating an augmentation of albumin leakage and a reduction of the pulmonary blood volume, respectively. These effects developed more slowly and were less reversible than those on leucocytes.

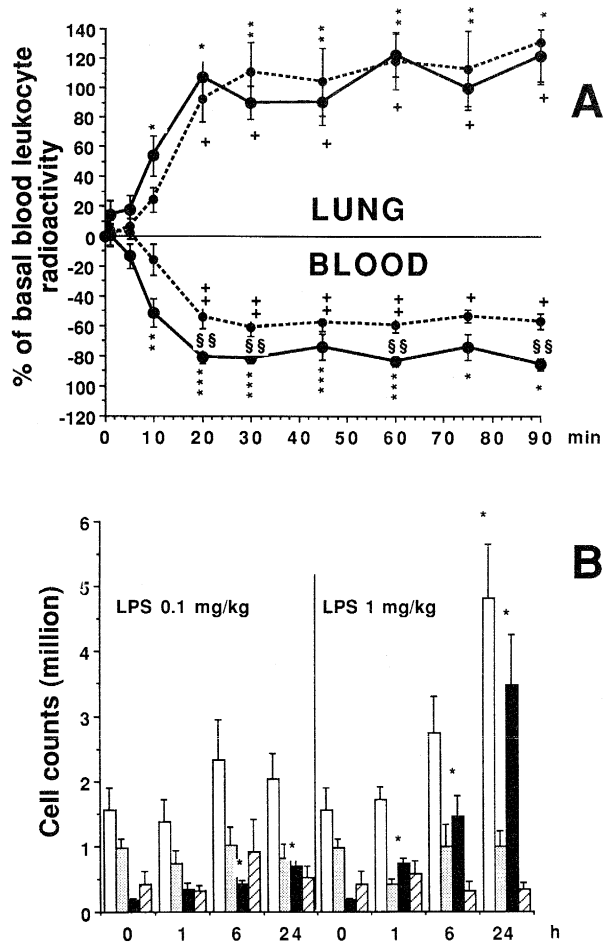

Fig. 2. Endotoxin-induced lung leucocyte sequestration blood leucopenia and changes in leucocyte counts in bronchoalveolar lavage fluid. (A) Values of the radioactivity bound to leucocytes sequestered in the lung and circulating in the blood and given as percent of basal blood leucocyte radioactivity are shown as means ± S.E.M at different times after endotoxin 0.1 and 1 mg/kg i.v. injections. Curves for saline-injected animals are as in Fig. 1. Radioactivity after endotoxin 0.1 mg/kg (---●---), after endotoxin 1 mg/kg (●). Significance of the difference between animals injected with endotoxin 1 mg/kg ($n = 10$) or saline: * $P < 0.05$, ** $P < 0.01$; between animals injected with endotoxin 0.1 mg/kg ($n = 10$) or saline: $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$; between animals injected with endotoxin 1 mg/kg or endotoxin 0.1 mg/kg: $^{\$}$ $P < 0.05$, §§ $P < 0.01$. (B) Counts are given as means ± S.E.M. ($n = 6$) for leucocytes (white columns) including eosinophils (stippled columns), neutrophils (black columns) and lymphocytes (hatched columns) for control saline-injected animals (time 0) and 1, 6 and 24 h after the i.v injection of 0.1 mg/kg endotoxin (left panel) or 1 mg/kg endotoxin (right panel). Significance of the difference between animals injected with endotoxin or saline: * $P < 0.05$.

3.2. Endotoxin-induced lung leucocyte sequestration, blood leucopenia, variation in leucocyte counts in broncho-alveolar lavage fluid and changes in lung microvascular albumin exchanges and blood volume

As shown in Fig. 2A, endotoxin induced a significant lung leucocyte sequestration at 0.1 and 1 mg/kg i.v., the differences between effects of the two doses not being statistically significant. In contrast, leucopenia, which started approximately 10 min after the injection of endotoxin, was less intense with 0.1 than with 1 mg/kg, the differences between the effects of the two doses being significant for most of the time points.

Cell counts in the broncho-alveolar lavage fluid were performed 0, 1, 6 and 24 h after the i.v. injection of 0.1 or 1 mg/kg endotoxin. In Fig. 2B, leucocyte numbers represent the addition of eosinophils, neutrophils and lymphocytes, the macrophages being excluded. At 0.1 mg/kg, endotoxin failed to modify leucocyte counts measured 1 h after stimulation, but increased them slightly after 6 and 24 h. However, at the same time points, the increases in neutrophil counts were statistically significant. At 1 mg/kg, endotoxin induced a significant neutrophil recruitment into the broncho-alveolar lavage fluid after 1 h with a further increase later. At its highest value, the number of leucocytes collected in the broncho-alveolar lavage fluid represented 2.52 ± 0.44% of the total blood leucocyte content. These values were of 0.81 ± 0.17%, 0.71 ± 0.18% and 0.89 ± 0.11% 1 h after the injection of saline, 0.1 mg/kg endotoxin and 1 mg/kg endotoxin respectively (mean ± S.E.M, $n = 6$).

As shown in Fig. 3, a significant reduction of lung erythrocyte radioactivity, indicating a reduction of blood volume, was observed after the i.v. injection of 0.1 or 1 mg/kg of endotoxin. This effect was of lower magnitude with 0.1 than with 1 mg/kg of endotoxin. A significant increase of trans-endothelial albumin exchanges, as indicated by the variation of the lung content of radiolabelled extravascular albumin, was observed only for 1 mg/kg of endotoxin.

3.3. Interference of BW B70C with endotoxin-induced leukotriene C_4 synthesis by alveolar macrophages and not with endotoxin-induced increase in blood levels of TNF-α and phospholipase A_2

To test the ability of BW B70C to prevent lipoxygenase activation in the lungs, macrophages were collected from the broncho-alveolar lavage fluid 6 h after i.p. administration of BW B70C at 30 mg/kg. The synthesis of leukotriene C_4 by these macrophages was evaluated before and after stimulation with fMLP at 10^{-6} M and 10^{-7} M.

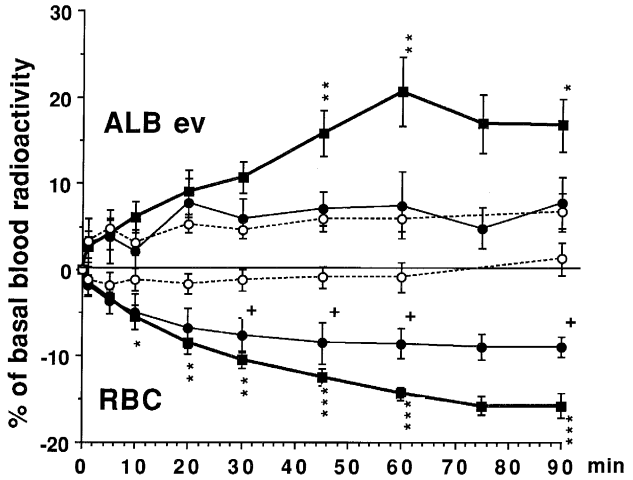

Fig. 3. Endotoxin-induced variations of the lung contents in extravascular albumin (ALB ev) and in erythrocytes (RBC). Values are means ± S.E.M. of the lung radioactivity bound to extravascular albumin and to erythrocytes, as percent of the corresponding basal blood radioactivity, for animals injected with endotoxin 1 mg/kg (■), endotoxin 0.1 mg/kg (●) or saline (---○---). Significance of the difference between animals injected with endotoxin 0.1 mg/kg ($n = 10$) or saline ($n = 10$): $^{+}$ $P < 0.05$; between animals injected with endotoxin 1 mg/kg ($n = 10$) or saline: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

As shown in Fig. 4, the synthesis of leukotriene C_4 by macrophages from control animals stimulated with fMLP was low and close to the detection limit of the method.

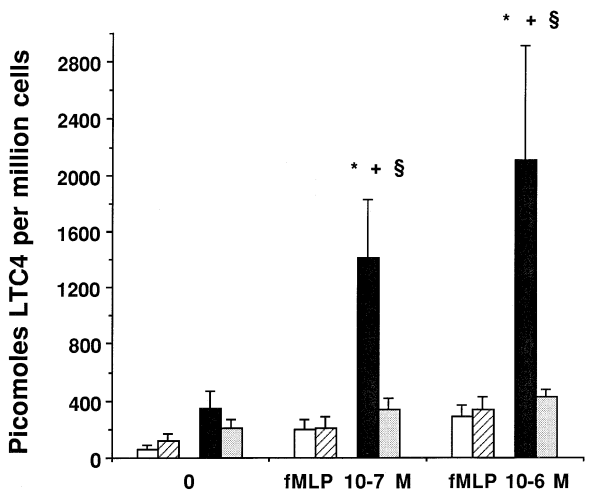

Fig. 4. Interference of BW B70C with leukotriene C_4 release by alveolar macrophages stimulated with fMLP 6 h after i.v. saline or endotoxin injection to guinea pigs. Values are means ± S.E.M. in pmol of leukotriene C_4 released by control or by fMLP-stimulated alveolar macrophages (10^{-7}, 10^{-6} M), recovered from animals 6 h after saline (white columns) ($n = 6$), after BW B70C (30 mg/kg i.p) (hatched columns) ($n = 3$), endotoxin alone at 1 mg/kg (black columns) ($n = 4$), endotoxin at 1 mg/kg associated to BW B70C (stippled columns) ($n = 4$). Significance of the difference between endotoxin- and saline-injected animals: * $P < 0.05$; between endotoxin- and saline-injected animals treated with BW B70C: $^{+}$ $P < 0.05$; between endotoxin-injected animals treated or not with BW B70C: § $P < 0.05$.

Table 1
Effect of BW B70C on endotoxin (1 mg/kg)-induced increase in blood TNF-α and phospholipase A_2 (PLA_2) levels

	Endotoxin	BW B70C + endotoxin	Saline
PLA_2 (pg/ml)	4785 ± 1277 [a]	8028 ± 2115 [a]	705 ± 208
	$n = 6$	$n = 9$	$n = 6$
TNF-α (pmol/ml)	14808 ± 3107 [a]	26858 ± 4526 [a]	10 ± 1
	$n = 8$	$n = 3$	$n = 6$

Values are means ± S.E.M. of the plasma concentration in TNF-α (pg/ml) and in secreted PLA_2 (pmol/ml of hydrolised substrate) for endotoxin (1 mg/kg)-injected animals, for endotoxin-injected animals treated with BW B70C (30 mg/kg i.p.) and for saline-injected animals. Values for endotoxin- and saline-injected animals were significantly different ([a] $P < 0.05$). Treatment of animals with BW B70C did not modify significantly the endotoxin-induced increase in blood levels of TNF-α and PLA_2.

However, the synthesis of leukotriene C_4 by macrophages collected from guinea-pigs pretreated with 1 mg/kg of endotoxin was enhanced at rest and very markedly increased after stimulation with fMLP. Under these conditions, BW B70C reduced significantly the leukotriene C_4 synthesis by macrophages.

Table 1 shows that in addition, inhibition of 5-lipoxygenase by BW B70C augmented non-significantly the blood concentration of TNF-α and of phospholipase A_2 induced by 1 mg/kg endotoxin i.v.

3.4. Interference of BW B70C with the lung leucocyte recruitment and microvascular albumin exchanges induced by endotoxin 1 mg/kg i.v.

By itself, BW B70C failed to modify the slight effects of saline on lung leucocyte sequestration, leucopenia and lung content of extravascular albumin. BW B70C reduced the lung erythrocyte content of saline-injected animals, preventing the study of the effects of lipoxygenase inhibition on the endotoxin-induced reduction of lung blood volume. However, following endotoxin injection, the reduction of the lung content of radiolabelled erythrocytes was similar in animals treated or non-treated with BW B70C (−13.05 ± 1.08% vs. −14.43 ± 0.77% respectively, at 60 min after endotoxin challenge).

The increases in microvascular albumin exchanges caused by 1 mg/kg endotoxin were prevented by BW B70C (Fig. 5A), but the accompanying leucopenia was unaffected (−82.13 ± 1.70 vs. −83.89 ± 3.02% for non-treated animals 60 min after endotoxin challenge). It is noteworthy that lung leucocyte sequestration caused by 1 mg/kg of endotoxin was refractory to inhibition by BW B70C (100.12 ± 17.71 vs. 122.77 ± 14.52% for untreated animals 60 min after endotoxin challenge).

Finally, Fig. 5B shows that BW B70C significantly reduced leucocyte recruitment into the broncho-alveolar lavage fluid 6 h after the i.v. injection of 1 mg/kg

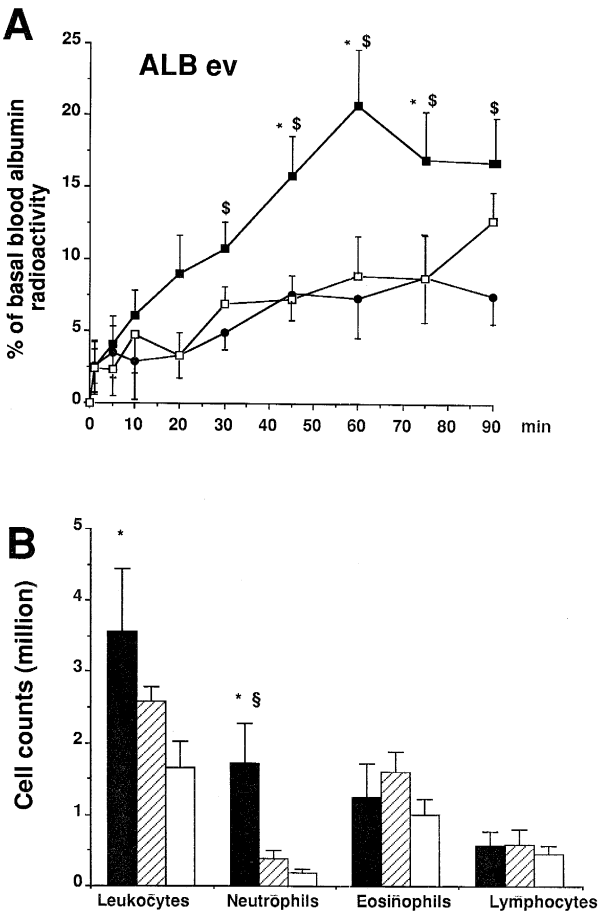

Fig. 5. Modulation by BW B70C of the endotoxin (1 mg/kg)-induced alteration of the lung content in extravascular albumin (ALB ev) and of the cell composition of the broncho-alveolar lavage fluid. (A) Values are means ± S.E.M. of the lung radioactivity bound to extravascular albumin as percent of the basal blood albumin radioactivity for animals injected with endotoxin (1 mg/kg) (■), treated with BW B70C and injected with endotoxin (1 mg/kg) (●) or saline (□). Significance of the difference between animals injected with endotoxin 1 mg/kg, treated with BW B70C ($n = 6$) or non-treated ($n = 10$): * $P < 0.05$; between endotoxin- and saline-injected animals treated with BW B700C: $^{\$}$ $P < 0.05$. (B) Values are means ± S.E.M. ($n = 6$) of leucocyte counts including neutrophil, eosinophil and lymphocyte counts for endotoxin (1 mg/kg)-injected animals (black columns), endotoxin-injected animals treated with BW B70C (hatched columns) or saline-injected animals (white columns), at 6 h post-challenge. Significance of the difference between animals injected with endotoxin, treated with BW B70C ($n = 6$) or non-treated ($n = 6$): $^{\$}$ $P < 0.05$; between animals injected with endotoxin or saline ($n = 6$): * $P < 0.05$.

endotoxin. This reduction was clearly caused by inhibition of neutrophil recruitment.

4. Discussion

In this study the i.v. injection of endotoxin (0.1 and 1 mg/kg) into guinea-pigs induced a marked blood leucopenia and lung leucocyte sequestration, accompanied by a reduced lung blood volume, probably following vasoconstriction. However, during the 90 min of the experiment, only 1 mg/kg endotoxin induced a detectable increase of the extravascular albumin content of lungs, i.e., stimulated the trans-endothelial exchanges in lung vessels. Furthermore, the increases in leucocyte counts in the broncho-alveolar lavage fluid were slight and non-significant with 0.1 mg/kg endotoxin, but were greater 24 h after 1 mg/kg endotoxin. Among leucocytes, the neutrophil count was increased as early as 1 h after endotoxin administration. A first conclusion is that an intense lung leucocyte sequestration, as induced by 0.1 mg/kg endotoxin, is not necessarily associated with their infiltration in the tissues and with an increase of trans-endothelial albumin exchanges.

In the systemic circulation, vascular leucocyte sequestration results mostly from their margination along the endothelium of postcapillary venules. In contrast, vascular lung leucocyte entrapment occurs predominantly in capillaries (Lien et al., 1991). Further, the entire lung leucocyte sequestration includes leucocytes retained in capillaries (whether sticking or not), those infiltrating the tissues and those that have reached the alveolar lumen and can be collected from the broncho-alveolar lavage fluid. In our experiments, 1 h after the injection of 0.1 mg/kg of endotoxin, the lung sequestration of radiolabelled leucocytes was intense, whereas the percentage of leucocytes in the broncho-alveolar lavage fluid represented only 0.7% of the total blood leucocyte content and did not differ from that seen in control experiments. At 1 mg/kg endotoxin, this percentage increased only to 0.9%. This value is probably an underestimate, since lavage recovers only a fraction of the leucocytes present in the air space (Downey et al., 1993). However, it remains possible that a large proportion of lung leucocytes (> 60% being neutrophils) stays in the vascular compartment even during inflammation. Indeed, from the morphometric study of Wang et al. (1994) interstitial neutrophils in the area of the microcirculation represent about 20% of the vascular neutrophils in sheep lungs. During endotoxin infusion both populations increased and the percentage of interstitial neutrophils was still about 20% of the vascular neutrophils, a value which would be still lower if all lung vessels are taken into account. Thus, even under inflammatory conditions, the sequestered pool of leucocytes, as evaluated with our method, includes essentially the leucocytes marginated along the vascular endothelium.

Comparison of the circulating and sequestered pools of radiolabelled leucocytes, evaluated simultaneously, reveals other differences between the two doses of endotoxin. Firstly, the number of leucocytes leaving the blood was lower than the number sequestered by the lungs after 0.1 mg/kg endotoxin. Such a difference may result from the return of leucocytes marginated in other organs to the circulating pool. Secondly, in response to 1 mg/kg of endotoxin, the number of radiolabelled leucocytes leaving the blood was closer to that recruited by the lungs, which means that the return of radiolabelled leucocytes to the

circulation was lower with 1 mg/kg than with 0.1 mg/kg endotoxin, probably because of a stronger interaction between leucocytes and the vascular endothelium. At the higher dose, such an interaction may result from the expression of integrins at the surface of leucocytes and/or of their endothelial counter receptors. The higher dose of endotoxin also induced neutrophil recruitment to the broncho-alveolar lavage fluid, which may result from integrin expression, particularly since Issekutz and Lopez (1993) have shown that the transendothelial migration of neutrophils is CD18-dependent.

Leukotriene B_4, one of the potential mediators of the effects of endotoxin (Brigham and Meyrick, 1986; Ishizaka et al., 1994) is formed from the arachidonate processing by 5-lipoxygenase (Ford-Hutchinson, 1985). Leukotriene B_4 induces the adhesion of leucocytes to the endothelium (Zimmerman et al., 1994; Hoover et al., 1984; Dahlen et al., 1981) and neutrophil migration (Ford-Hutchinson, 1985). In our experiments, contrary to the progressive effects of endotoxin, leukotriene B_4 induced a marked rapid and short-lasting leucopenia and lung sequestration of leucocytes. This suggests that leukotriene B_4 was rapidly metabolized, that its effects on leucocytes were not mediated by the generation of secondary mediators but direct and that leucocytes did not adhere firmly to vascular endothelium.

The time course of these effects was similar to that of neutrophil adhesion to venous endothelium following the topical application of leukotriene B_4 to the hamster cheek pouch preparation (Oda and Katori, 1992). In contrast, the vascular effects (microvascular albumin exchanges and reduction of pulmonary blood volume) of leukotriene B_4 were less reversible. An increase in endothelial 'permeability' by leukotriene B_4 has been described (Björk et al., 1982; Yoshimura et al., 1994) or denied (Noonan et al., 1988; Harris et al., 1993). The increase in lung capillary pressure can itself enhance transendothelial albumin exchanges. It is noteworthy that the pattern of leukotriene B_4-induced increase of capillary pressure in an isolated guinea-pig lung preparation (Noonan et al., 1988) is similar to that of the increase in the lung content of extravascular albumin in our experiments.

In order to study the contribution of 5-lipoxygenase to the effects of endotoxin, the selective inhibitor, BW B70C (Yeadon et al., 1993), was injected i.p. at 30 mg/kg. The dose and route were validated, since BW B70C inhibited significantly the production of leukotriene C_4 by ex vivo alveolar macrophages 6 h after endotoxin injection.

Control experiments showed that BW B70C itself induced slight, non-significant changes in the lung contents of sequestered leucocytes and of extravascular albumin. It also decreased the lung red blood cell content, suggesting a reduction of the pulmonary blood flow. Nevertheless, the inhibition by BW B70C of the endotoxin-induced increase in extravascular albumin content was not due to an increased reduction of the blood volume, which was the same in endotoxin-challenged animals irrespective of the treatment with BW B70C. Likewise, the prevention by BW B70C of leucocyte recruitment to the broncho-alveolar lavage fluid cannot be accounted for by a non-specific effect. It has been shown that 5-lipoxygenase inhibition prevents the neutrophil enrichment of the broncho-alveolar lavage fluid induced by the intra-tracheal injection of 1 mg endotoxin to guinea-pigs (Vincent et al., 1993). Consistent with these results, the peptidyl leukotriene receptor antagonist, ONO-1078, reduces neutrophil migration toward the alveolar lumen as well as the exchanges of albumin through the alveolo-capillary barrier in the guinea-pig lung following endotoxin administration (Ishizaka et al., 1994). This antagonist of peptidyl leukotriene formation also inhibits directly neutrophil migration, which means that other powerful chemoattractants like leukotriene B_4 could be involved.

Connections have been described between the 5-lipoxygenase pathway and TNF-α, an essential mediator of endotoxin toxicity. Indeed, products of leukotriene B_4 and leukotriene C_4 enhance TNF-α production by monocytes (Gagnon et al., 1989) and the i.v. injection of endotoxin induces the appearance of TNF-α and of leukotrienes in the rat heart and lung (Tanaka et al., 1994). However, in our experimental model, 5-lipoxygenase failed to interfere with TNF-α synthesis, in agreement with results obtained in rats (Ferrandiz and Foster, 1991) and in mice (Schade et al., 1991). The secreted phopholipase A_2, which may also mediate some effects of endotoxin (Vadas et al., 1993) was similarly unaffected by 5-lipoxygenase inhibition. Thus, one or several metabolites of the 5-lipoxygenase pathway, among which probably leukotriene B_4, remain potential mediators for the increase in trans-endothelial albumin exchanges and neutrophil migration towards the airways induced by 1 mg/kg endotoxin i.v. Vascular leucocyte margination induced by the same dose of endotoxin was nevertheless not prevented by BW B70C. This is probably not accountable for by an insufficient dose of inhibitor, since neutrophil migration was inhibited. More likely, mediators other than 5-lipoxygenase derivatives are involved in leucocyte margination to vessels during the first hour after 1 mg/kg endotoxin. Secreted phospholipase A_2 is probably not involved since the peak of its production occurs 6 h after i.v endotoxin injection (De Castro et al., 1995).

Other mediators involved may be platelet activating factor, interleukin-1, TNF-α, and interleukin-8. Platelet activating factor (PAF) has been shown to mediate the initial endotoxin-induced neutropenia in Brown Norway rats (Couglan et al., 1994) but, in our model, the selective PAF receptor antagonist, SR 27417 (1 mg/kg i.v.), which prevents endotoxin-induced hypotension (observable for doses higher than 1 mg/kg; Bernat et al., 1992), failed to prevent the leucopenia and lung leucocyte sequestration induced by 1 mg/kg endotoxin (Bureau et al., 1994b). TNF-α may be one of the first mediators implicated in

septic shock, since its blood concentrations reach maximum values 1 h after endotoxin administration (De Castro et al., 1995). Production of interleukin-1 is dependent on TNF-α (Brennan et al., 1989). Finally, chemokines such as interleukin-8 are other potential candidates, since in vitro studies showed that interleukin-8 may mediate the TNF-α-induced neutrophil migration through the endothelium (Smart and Casale, 1994). However, during the first hour after endotoxin injection, the production of interleukin-8 is probably too low to contribute to the lung leucocyte recruitment (Martich et al., 1991). Finally, it is important to note that essential differences can occur between species. Indeed, while our results and those of Vincent et al. (1993) indicate that endotoxin-induced neutrophil migration is 5-lipoxygenase dependent in the guinea pig model, it is TNF-α dependent in mice (Gonçalves de Moraes et al., 1996).

In conclusion, we demonstrated that, at 0.1 mg/kg, *E. coli* endotoxin induces leucocyte margination on the lung vessels, whereas 1 mg/kg induces as well leucocyte infiltration into the airway lumen and trans-endothelial albumin leakage. 5-Lipoxygenase derivatives probably mediate leucocyte infiltration in the lung tissue as well as albumin leakage, whereas vascular leucocyte margination, as induced by 1 mg/kg endotoxin in guinea-pig lung, is lipoxygenase-independent.

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