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Immunology

Leukotriene B_4 action on endothelium mediates augmented neutrophil/endothelial adhesion

(inflammation)

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ABSTRACT **The attachment of human polymorphonuclear leukocytes (PMN) to bovine endothelial monolayers is increased by pretreatment of the endothelial cells with leukotriene B_4 (LTB_4). The response is dose dependent with a maximum at 10^{-6} M, requires no more than 1 min of preincubation for the maximal effect, and decays linearly over 30 min once the LTB_4 is removed. Preincubation of PMN with LTB_4 does not augment subsequent adhesion to a plastic substratum or an untreated monolayer of endothelial cells and eliminates the augmented attachment to LTB_4-pretreated endothelial cells. The simultaneous exposure of both cell types during the adhesion interaction gives a biphasic dose-related increment, suggesting that the inhibition of the neutrophil adherence is less marked than with a pretreatment interval. Thus, the LTB_4-dependent adherence of PMN to endothelial cells is selectively mediated by endothelial cells, is dose related, is reversible upon removal of the agonist from the endothelial cells, and is opposed by the direct action of LTB_4 on the PMN.**

Each of the leukotrienes derived by oxidative metabolism from arachidonic acid has been shown to alter the microvascular environment when administered *in vivo* (1). The sulfidopeptide leukotriene 5*S*-hydroxy-6*R*-S-glutathionyl-7,9-*trans*-11,14-*cis*-icosatetraenoic acid (leukotriene C_4, LTC_4) (2) and its peptide cleavage products 5*S*-hydroxy-6*R*-S-cysteinylglycyl-7,9-*trans*-11,14-*cis*-icosatetraenoic acid (leukotriene D_4, LTD_4) (3–5) and 5*S*-hydroxy-6*R*-S-cysteinyl-7,9-*trans*-11,14-*cis*-icosatetraenoic acid (leukotriene E_4, LTE_4) (6) augment vasopermeability after intradermal injection into guinea pig and human skin (6–8) and after topical application to the buccal mucosa of the hamster (9). 5*S*,12*R*-Dihydroxy-6,14-*cis*-8,10-*trans*-icosatetraenoic acid (leukotriene B_4, LTB_4) (10, 11) mediates adherence of neutrophilic polymorphonuclear leukocytes (PMN) when applied to endothelial cell surfaces in the hamster cheek pouch (9) and elicits the migration of PMN from the vascular compartment into dermal tissue of the rhesus monkey and the human (8, 11) when administered by intradermal injection.

It has been assumed that the principal target of LTB_4 in increasing PMN-endothelial cell adherence is the PMN (9), but this cellular mechanism has not been directly demonstrated. In the present study, we evaluated the adherence interaction while the two cell types were treated with LTB_4 simultaneously or after pretreatment of one or both cell types. The agonist action of LTB_4 in mediating dose-dependent increased adherence of PMN to endothelium is endothelial cell selective.

MATERIALS AND METHODS

Cells. Endothelial cells were isolated from calf aortae (12). Fat and connective tissues were mechanically removed from each aorta, and the lumen was washed with Hanks' balanced salt solution (HBSS) buffered with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.4) and containing penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (0.25 μg/ml). The aortae were incubated in buffered HBSS containing 1 mg of collagenase per ml for 20 min at 37°C, and the freed endothelial cells were recovered by perfusion with buffered HBSS. The dispersed endothelial cells were washed twice by sedimentation at 150 × *g* for 5 min at 4°C, were resuspended at a concentration of 10^4 cells per ml in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 5% NuSerum (Collaborative Research, Waltham, MA), containing penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (0.25 μg/ml), and were plated into 24-well cluster dishes. The cells were grown to confluency as primary cultures or as clones of primary cultures and were not used after the fifth passage. That the cells at each passage were endothelial was confirmed morphologically by their cobblestone appearance under light microscopy (12), immunochemically by staining for factor VIII (13), and biochemically by demonstration of angiotensin-converting enzyme activity (14).

PMN were isolated from heparinized venous blood of normal human donors. After the addition of 1 vol of 25% dextran solution to 4 vol of blood, erythrocytes were sedimented at 1 × *g*, and the leukocyte-rich plasma was aspirated. The leukocytes were sedimented at 150 × *g* for 8 min at 4°C, resuspended for 30 sec in a small volume of distilled water to lyse the remaining erythrocytes, and washed twice by sedimentation and resuspension in buffered HBSS. More than 98% of the cells were viable by trypan blue exclusion, and 90–95% were identified as neutrophils after staining with Giemsa. PMN at 10^7 cells per ml of buffered HBSS were radiolabeled by incubation with ^{51}Cr (330 mCi/mg; 1 Ci = 37 GBq; New England Nuclear) at 10 μCi/ml for 1 hr at 4°C and washed by centrifugation at 150 × *g* for 5 min at 4°C.

LTB_4. Synthetic LTB_4 was prepared as described (11, 15) and stored in 10 mM phosphate buffer, pH 6.8/ethanol, 4:1 (vol/vol), under argon. The concentration of LTB_4 for each experiment was determined by scanning ultraviolet absorbance at 269 nm assuming a maximal extinction coefficient of 51,000 $cm^{-1} \cdot M^{-1}$ (Varian spectrophotometer, model 210), and the purity was confirmed by reverse-phase high-performance liquid chromatography (10-μm C_{18} Ultrasil column, 4.6 × 250 mm, Altex-Beckman Instruments) in 65% methanol/34.9% water/0.1% acetic acid, pH 5.6, at a flow rate of 1 ml/min (16).

Adhesion Assay. Adhesion was measured with a monolayer collection assay (17). Endothelial cells at confluency (2×10^5 cells per well) were rinsed twice with buffered HBSS and 1–2 × 10^5 labeled PMN were pipetted onto each endothelial cell culture well. After incubation for 30 min at 37°C, unat-

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Abbreviations: LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; PMN, polymorphonuclear leukocyte(s).

tached PMN were removed by rinsing the monolayers once with 1 ml of buffered HBSS per well. Adhered PMN and the endothelial monolayers in each well were solubilized by the addition of 0.5 ml of 1 M NH_4OH for 1 hr at 25°C, were added to scintillation fluid, and were assayed for radioactivity on a β-scintillation counter with 95% efficiency for ^{51}Cr. The percentage of adherent PMN was calculated as the ratio of cpm remaining adherent to cpm added. Preincubation of PMN, endothelial cells, or both with specific concentrations of LTB_4 was carried out for various time periods with or without washing each cell population before their admixture. The effect of LTB_4 on adherence was expressed as the ratio of adherence of labeled PMN after exposure of either or both cell types to LTB_4 to adherence in the absence of exposure of either cell type to the agonist.

RESULTS

LTB_4 was added to the endothelial cells at concentrations from 10^{-11} to 10^{-6} M, followed immediately by the addition of the PMN. The introduction of LTB_4 at the time of the adherence interaction resulted in a biphasic dose-dependent increase in adherence, with a maximal effect of 2.0 ± 0.09 (mean ± SEM, $n = 5$) times control at LTB_4 concentrations of 10^{-7} to 10^{-8} M and a significant effect at all tested concentrations (Fig. 1). The suggestion of a biphasic dose–response prompted a separate analysis of the dose-related effects of LTB_4 on each of the cell types.

PMN and endothelial cells were preincubated separately or together with incremental concentrations of LTB_4 for 1, 5, or 15 min, washed, admixed, and assessed for adherence after an additional 30-min incubation. When the PMN were pretreated with incremental concentrations of LTB_4 and then washed, there was no increase at any concentration in attachment to endothelial cells or to the plastic substratum of exposed wells after preincubation times of 1 min (Fig. 2) or longer (Fig. 3). In contrast, pretreatment of endothelial cells for 1 min with LTB_4, followed by a wash, elicited a dose-dependent increment in adherence to endothelial cells that was significant at LTB_4 concentrations from 10^{-10} to 10^{-6} M and was maximal at 1.63 ± 0.08 times control (mean ± SEM, $n = 5$) at 10^{-6} M (Fig. 2). Pretreatment of the endothelial cell monolayer with incremental concentrations of LTB_4, followed by a wash, induced identical dose-dependent increments in PMN attachment with a preincubation time ranging from 1 to 15 min (Fig. 3). Pretreatment of each cell population separately with LTB_4 for 1 min, followed by a single wash of each, which took 2 min before admixture, yielded no net adherence relative to control (Fig. 2).

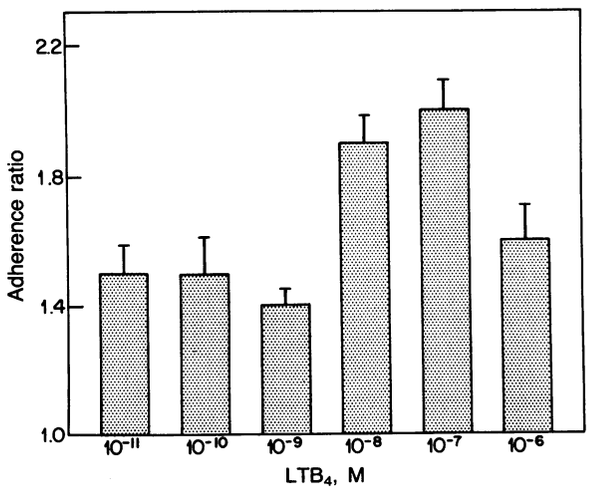

FIG. 1. Dose-related effects of LTB_4 on the attachment of PMN to endothelial monolayers in the continued presence of LTB_4. Data points are the averages of five experiments (mean ± SEM), each conducted in triplicate. Control adhesion was 10.0% ± 1.1% of the cpm added with labeled PMN.

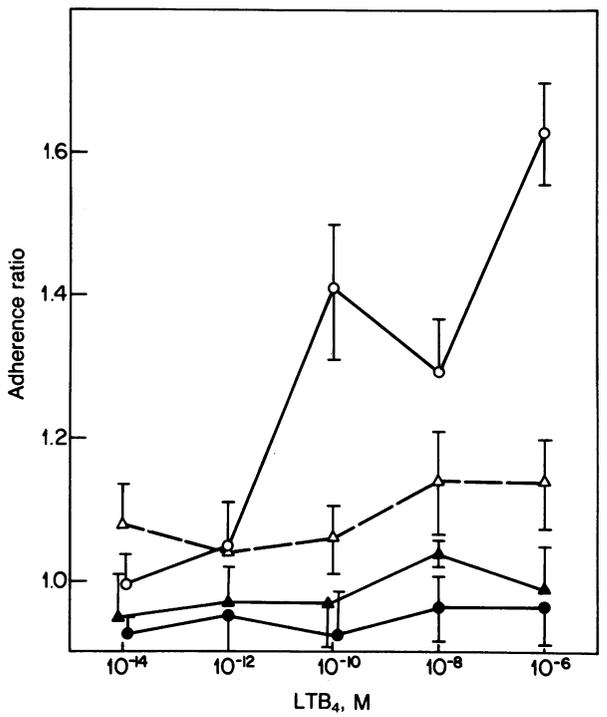

FIG. 2. Dose-related effects of LTB_4 on the attachment of PMN to endothelial monolayers when the endothelial cells (○) or the PMN (△) were pretreated for 1 min. The attachment of PMN to a plastic substratum after LTB_4 pretreatment (●) is also depicted. Data points are the averages of five experiments (mean ± SEM), each conducted in triplicate. Control adhesion was 18.8% ± 2.0% of the cpm added to endothelial monolayers and 17.8% ± 3.6% of the cpm added to the plastic substratum. Also depicted is the attachment of PMN to endothelial monolayers when each was separately pretreated for 1 min, followed by a single wash lasting 2 min (▲).

The duration of the effect of pretreatment of the endothelium for 5 min with 10^{-6} M LTB_4 in enhancing PMN adherence was assessed by washing the endothelial cells once with buffered HBSS and maintaining the culture under routine conditions (buffered HBSS at room temperature) for 1–120 min before adding the PMN. The capacity of endothelial cells to manifest augmented adherence decayed linearly to base line over the initial 30 min after washing to remove LTB_4 (Fig. 4).

DISCUSSION

The capacity of LTB_4 to augment the adherence of endothelial cells and PMN to each other has now been focused to a selective action on the endothelial cells by pretreatment of each cell type separately with LTB_4 before their interaction. The effect of LTB_4 in preparing the endothelial cells for increased PMN adherence is dose dependent and requires no more than 1 min of preincubation to achieve a maximal dose effect (Figs. 2 and 3). The LTB_4-mediated effect persists during ongoing incubation of the agonist with the endothelial cells (Fig. 3) but decays linearly over 30 min once the LTB_4 has been removed by washing (Fig. 4). This may be due to occupied LTB_4 receptors on the endothelial surface becoming internalized and processed over the 30 min or to a low-affinity interaction that is highly reversible. The LTB_4-mediated PMN adherence to endothelial cells is endothelial cell specific, dose related, immediate, and reversible upon removal of the agonist.

Conversely, PMN are not conditioned by LTB_4 for in-

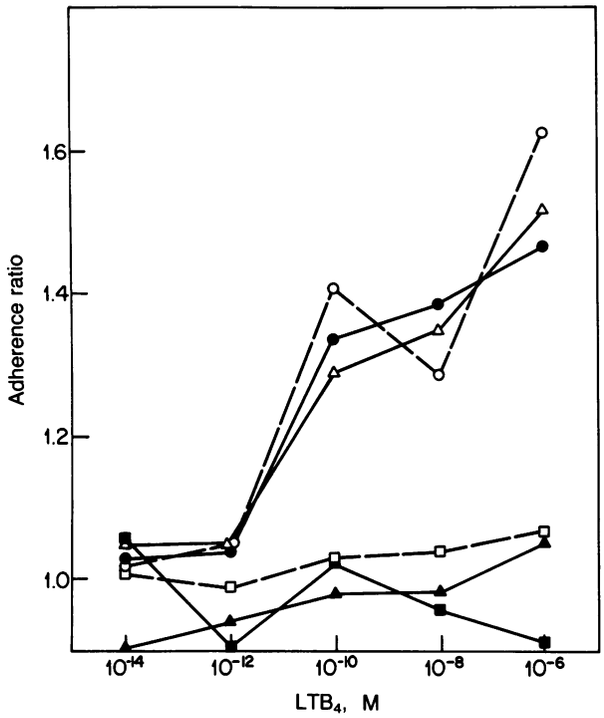

FIG. 3. Effects of incremental concentrations of LTB_4 preincubated with PMN for 1 min (■), 5 min (□), or 15 min (▲) or with endothelial cells for 1 min (○), 5 min (●), or 15 min (△) on subsequent adhesion. Control adhesion in five experiments was 13.1% ± 1.6% (mean ± SEM) with average SEM for experimental points 0.07.

creased adherence to endothelium, but rather are impaired for adherence, as compared to untreated PMN (Figs. 2 and 3). The capacity of human PMN to aggregate in the presence of LTB_4 has been shown by aggregometry (18) and has been inferred from the impeded elution of LTB_4-treated PMN from nylon wool columns (19). The aggregation sites on PMN responding to LTB_4 may be distinct from those involved in endothelial cell adherence. Alternatively, PMN sites for endothelial adherence may have a brief half-life or require a high PMN concentration for expression. It is also possible that LTB_4 induces expression on PMN of an inhibitory locus with respect to endothelial cell binding and that this effect appears less rapidly than the pro-adherence effects on the endothelial cells.

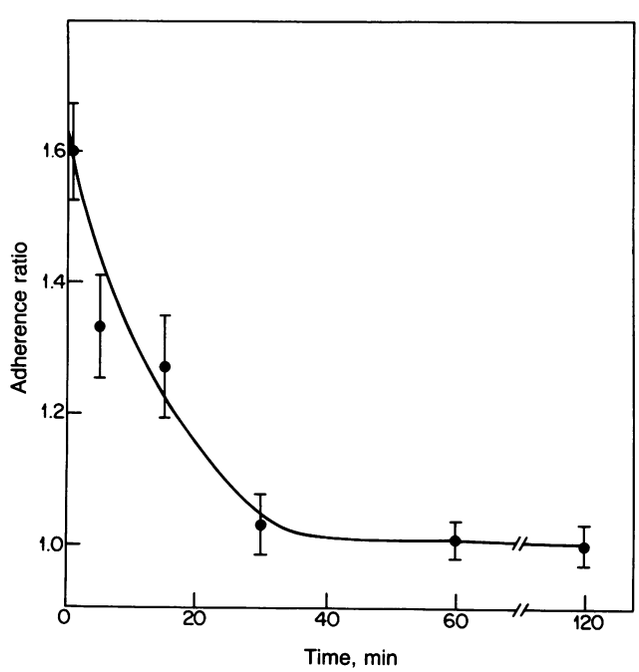

FIG. 4. Decay of the effect of 10^{-6} M LTB_4 on the endothelial cell-mediated augmented adherence. Data points are the averages of three experiments (mean ± SEM), each conducted in triplicate. Control adhesion was 14.6% ± 3.0% of cpm added.

In vivo studies of leukocyte adherence to ear vessel walls in rabbits with prior local injury reveal PMN attachment only on the side of the vessel nearest the injury (20). This is consistent with the action on a vascular element rather than the leukocyte being a primary factor, as demonstrated by the current *in vitro* analysis of the adherence response to LTB_4. That other mediators of host inflammatory responses may also augment the vascular endothelial capacity to bind PMN has been demonstrated for the anaphylatoxic complement fragment C5a and for the synthetic chemotactic tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (21). A possible implication of the present study is that the PMN could contribute to normal physiologic margination by providing LTB_4 and to pathobiologic responses by supplying increased LTB_4 after activation. Depending on its local concentration, LTB_4 either could recruit PMN or could inhibit their accumulation at particular sites on the vessel wall.

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