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LEUKOTRIENE B4 AND THROMBOXANE A2 ARE ESSENTIAL COFACTORS IN CD 18 DEPEENDENT NEUTROPHIL DIAPEDESIS

BY

G. GOLDMAN, R. WELBOURN, C.R. VALERI, D. SHEPRO,
AND
H.B. HECHTMAN

NAVAL BLOOD RESEARCH LABORATORY
BOSTON UNIVERSITY SCHOOL OF MEDICINE
615 ALBANY STREET
BOSTON, MA 02118

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Previous studies have indicated that thromboxane (Tx) and leukotriene (LT)B4 act synergistically to induce neutrophil (PMN) adhesion in the microvasculature. This study tests the ability of Tx to induce LTB4 synthesis which then leads to activation of PMN and endothelial adhesion receptors. Tx-mimic (U 46619, 1 ug/ml) was administered into abraded skin chambers placed on the back of rabbits (n=6). After 3 h LTB4 was synthesized in the blister fluid, 385 pg/ml, a value higher than

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levels in saline treated blisters 10 pg/ml ($p < 0.05$). The LTB4 generation following Tx-mimic was correlated ($p < 0.05$, $r=0.70$) with neutrophil diapedesis. These averaged 645 PMN/mm^3, higher than saline values of 20 PMN/mm^3 ($p < 0.05$). Intravenous (IV) treatment of other rabbits (n=4) with the lipoxygenase inhibitor diethylcarbamazine 60 mg/kg followed by 40 mg/kg/h prevented Tx-mimic induced LTB4 synthesis (10 pg/ml) and diapedesis (19 PMN/mm^3) (both $p < 0.05$). IV treatment of yet other rabbits (n=4) with the anti-CD 18 monoclonal antibody R 15.7, 1 mg/kg abolished Tx-induced diapedesis (3 PMN/mm^3) ($p < 0.05$). In contrast, local administration of the protein synthesis inhibitor actinomycin D 3 ng, to prevent expression of endothelial adhesion proteins, limited TNF but not Tx-induced diapedesis. The data indicate that Tx-induced diapedesis is mediated by the generation of LTB4 and activation of neutrophil CD 18 but not endothelial adhesion receptors.

ABSTRACT

Previous studies have indicated that thromboxane (Tx) and leukotriene (LT) B_4 act synergistically to induce neutrophil (PMN) adhesion in the microvasculature. This study tests the ability of Tx to induce LTB_4 synthesis which then leads to activation of PMN and endothelial adhesion receptors. Tx-mimic (U 46619, 1μg/ml) was administered into abraded skin chambers placed on the back of rabbits (n=6). After 3 h LTB_4 was synthesized in the blister fluid, 385 pg/ml, a value higher than levels in saline treated blisters 10 pg/ml ($p < 0.05$). The LTB_4 generation following Tx-mimic was correlated ($p < 0.05$, $r=0.70$) with neutrophil diapedesis. These averaged 645 PMN/mm^3, higher than saline values of 20 PMN/mm^3 ($p < 0.05$). Intravenous (IV) treatment of other rabbits (n=4) with the lipoxygenase inhibitor diethylcarbamazine 60 mg/kg followed by 40 mg/kg/h prevented Tx-mimic induced LTB_4 synthesis (10 pg/ml) and diapedesis (19 PMN/mm^3) (both $p < 0.05$). IV treatment of yet other rabbits (n=4) with the anti-CD 18 monoclonal antibody R 15.7, 1mg/kg abolished Tx-induced diapedesis (3 PMN/mm^3) ($p < 0.05$). In contrast, local administration of the protein synthesis inhibitor actinomycin D 3 ng, to prevent expression of endothelial adhesion proteins, limited TNF but not Tx-induced diapedesis. The data indicate that Tx-induced diapedesis is mediated by the generation of LTB_4 and activation of neutrophil CD 18 but not endothelial adhesion receptors.

INTRODUCTION

Thromboxane (Tx) A_2 synthesis by the lungs in response to remote ischemia or HCl-aspiration leads to neutrophil (PMN) accumulations at the site of synthesis. Thus, local pulmonary inhibition of Tx synthesis or Tx receptors, by aspiration of Tx antagonists attenuated neutrophil sequestration (1). Following ischemia or acid aspiration there is also leukotriene (LT)B_4 synthesis. Surprisingly, selective lung inhibition of this eicosanoid by local aspiration of the lipoxygenase antagonist, diethylcarbamazine also led to reduction in neutrophil adhesion (2). The sequential and/or synergistic action of Tx-LTB_4 on PMN adhesion is not clear. Several lines of evidence suggest that LTB_4 may serve as a cofactor of Tx action in the induction of PMN adhesion: 1. Tx-mimic treated PMN led to their increased adhesion in vitro to pulmonary microvascular endothelium, an event that was prevented with an LT receptor antagonist (3); 2. inhibition of LT synthesis or LT receptors limited diapedesis induced by ischemic-plasma which contained high concentrations of TxB_2, a less active prostanoid then TxA_2 but one capable of inducing diapedesis (4); 3. in the setting of acid aspiration, an LT receptor antagonist while not reducing plasma Tx levels limited lung PMN adhesion (5). The reverse may also be true, that is Tx may serve as cofactor for LTB_4 action. Thus, inhibition of Tx synthesis reduced diapedesis induced by authentic LTB_4 in rabbit skin blisters (6). These data indicate that both thromboxane synthesis and lipoxygenase activity are essential for diapedesis in vivo to be completed.

The process of neutrophil adhesion and diapedesis is a key event in neutrophil-dependent microvascular injury. It has been shown that interaction between the CD 18 neutrophil adhesion receptor and its endothelial ligand, the intercellular adhesion molecule-1 (ICAM-1) is a necessary step for PMN to release H_2O_2 and granule contents, thereby altering the vascular barrier (7,8). Two aspects of PMN-endothelial adhesion exist. The first is invoked by PMN stimulation with chemoattractants such as LTB_4 or C5a and leads to activation or upregulation of CD 18 (9).

The second aspect relates to endothelial activation with expression of ICAM-1 or other adhesion receptors such as the endothelial leukocyte adhesion molecule-1 (ELAM-1) (10,11). This process requires cytokines and is dependent upon protein synthesis.

The aim of the current study was to examine our hypothesis that Tx and LTB_4 are essential cofactors in PMN diapedesis and specifically to test one aspect of this hypothesis that Tx may induce LTB_4 synthesis which in turn will mediate diapedesis. The study further tests the role of neutrophil adhesion receptors and newly synthesized endothelial adhesion proteins in regulating this eicosanoid mediated event. The results indicate that TxA_2 induces LTB_4 synthesis that in turn promotes neutrophil diapedesis. Neutrophil migration in this setting is regulated by CD 18 interaction with basally expressed but not de novo synthesized endothelial cell adhesion proteins.

METHODS

Animal Preparation

Eighteen New-Zealand white male rabbits weighing approximately 3 kg were used. Anesthesia was achieved with intramuscular ketamine, 35 mg/kg and intravenous xylazine 5 mg/kg and maintained with xylazine 2 mg/kg every 30 minutes. Saline, 0.3 ml/kg/h was infused via a carotid arterial cannula introduced aseptically via a small neck incision of the day of the experiment. All animals were placed of 37 °C heating pads.

Skin Abrasion Blister Chambers

Chemotactic responses were measured by a modification of the technique of Otani and Palder (6,12). Briefly, clear plastic chambers (0.25 ml capacity, Rexhaus Corp., Westfield Industrial Park, Westfield, MA) were affixed to dermabraded areas of the back of anesthetized rabbits. Rabbits were treated intravenously with saline (n=6) or diethylcarbamazine (DEC)

(n=4) (Sigma, Chemical Company, St Louis, MO) 60 mg/kg bolus followed by 40 mg/k/h throughout the experiment); or purified anti-CD 18 mAb, R15.7 1mg/kg (n=4), (kindly provided by Dr. R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield CT). Their blisters were filled with ether saline or the Tx-mimic U46619 (Upjohn, Kalamazoo, MI) 1μg/ml. The Tx mimic was the stable endoperoxide analogue, 9,11 dideoxy-11a,9 a-epoxymethano-$PGF_{2\alpha}$. It was provided in ethanol. After dilution the final concentration of ethanol was less than 0.1%. In other rabbits (n=4) treated with IV saline, blisters were filled with agonist-antagonist combinations. The agonists were: Tx-mimic or tumor necrosis factor-α (TNF-α) 10^4 U (Amgen Biologicals, Thousand Oaks, CA) and the antagonists were actinomycin D, 0.12 μg/ml (Aldrich Chem. Co., Milwaukee, WI) or an equal volume of saline. The agents were added as follows: agonist-0.9 ml/antagonist-0.1 ml, divided into 4 aliquots, each for a blister. Injections into the blisters were made with a 27-gauge needle. In each experiment after 3 hours, LTB_4 was assayed in chamber fluid and diapedesis was estimated by counting the number of PMN with a hemocytometer.

LTB_4 Assay

Blister fluid was collected in cooled (4°C) heparinized syringes. This fluid was centrifuged at 1500 x g for 20 minutes (PR-2, International Equipment Co., Needham Heights, MA) and frozen at -20°C for latter LTB_4 assay. This was measured in duplicate by RIA using rabbit antibody and standards obtained from Seragen (Cambridge, MA). Cross reactivity of LTB_4 antibody with other leukotrienes, hydroxyeicosatetraenoic acid (HETE), di-HETE, TxB_2, the prostaglandins and their metabolites was less than 1%.

Data are expressed as mean ± SE in text and figures. A one way analysis of variance was used to compare results and if positive a Student's t-test was performed. Significance was accepted if $p < 0.05$.

Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Welfare, Publication No. 78-23 [National Institute of Health]), revised, 1978.

RESULTS

Three hours after the introduction of Tx-mimic, generation of LTB_4 in the blister fluid was noted, 385 ± 37 pg/ml, higher than values with saline, <10 pg/ml (Fig. 1, $p < 0.05$). Further, with Tx-mimic there were 645 ± 45 PMN/mm^3, higher than saline values of 20 ± 4 PMN/mm^3 (Fig. 1, $p < 0.05$). Leukotriene B_4 synthesis was correlated with PMN accumulations (Fig. 2, $p < 0.05$). Treatment of rabbits with DEC prevented Tx-mimic induced LTB_4 synthesis < 10 pg/ml and diapedesis 19 ± 3 PMN/mm^3 (Fig. 1, both $p < 0.05$). Treatment with the anti CD 18 mAb R15.7 completely inhibited Tx induced diapedesis, 1-3 PMN/mm^3 (Fig. 3, $p < 0.05$). Blisters treated with Tx-mimic 1μg/ml, diluted with saline 9:1 to a final concentration of 0.22μg/ml per blister, showed diapedesis of 450 ± 42 PMN/mm^3. Similar values were noted when the putative antagonist actinomycin D was added to Tx-mimic, 480 ± 70 PMN/mm^3. To demonstrate that actinomycin D was an effective protein synthesis inhibitor it was shown to limit TNF-α induced diapedesis from 230 ± 50 PMN/mm^3 (with saline as control antagonist) to 35 ± 12 PMN/mm^3 (Fig. 3, $p < 0.05$).

DISCUSSION

The results of this study indicate first that Tx induces LTB_4 synthesis that in turn mediates neutrophil diapedesis. Secondly, the process of diapedesis in response to an extraluminal chemoattractant is regulated by activation of neutrophil but not endothelial adhesion receptors. The

first conclusion is based on the fact that Tx-mimic induces LTB_4 synthesis in the blister fluid as measured after 3 hours. Further, inhibition of LTB_4 synthesis with diethylcarbamazine prevents neutrophil migration (Fig. 1). In addition, a significant correlation was found between LTB_4 generation and PMN accumulations in the abraded skin chamber (Fig. 2).

These data are consistent with previous experiments which showed that neutrophil adhesion to an in vitro endothelial monolayer was rapidly increased by treatment of PMN with Tx-mimic. This event was prevented by leukotriene antagonists as well as by an anti CD 18 mAb (3). These previous and present results strongly suggest that Tx induces LTB_4 synthesis that in turn activates neutrophil CD 18 leading to adhesion and diapedesis.

Other experiments have shown that ischemic plasma which contains high concentrations of TxB_2 and LTB_4 led to diapedesis when placed in the skin chamber. Lipoxygenase inhibition with DEC prevented PMN accumulations in the blister (4). Interestingly, in other studies inhibition of Tx synthesis reduced diapedesis in response to authentic LTB_4 placed in the blister (6)(Table 1). The current study is in accord with these observations and shows further the importance of Tx-LTB_4 interaction in determining neutrophil migration. It seems that both TxA_2 and LTB_4 are essential cofactors for diapedesis to be completed. Thus, de novo synthesis of these two eicosanoids has been shown in blister fluid in response to the other agent (Table 1). Further, inhibition of LT synthesis or receptors prevented diapedesis induced by authentic TxB_2 or Tx-mimic. The reverse is also true where inhibition of Tx synthesis or receptors prevented diapedesis induced by authentic LTB_4 (Table 1). Finally, the ability of DEC or the LT receptor antagonist FPL 55712 (13) to prevent PMN accumulations in response to authentic LTB_4 and the similar effectiveness of OKY 046 or the Tx receptor antagonist SQ 29,548 in limiting diapedesis in response to TxB_2 (14) suggests that intact cyclooxygenase and lipoxygenase pathways are required for PMN to diapedese in this setting.

Diapedesis induced by injection of LTB_4 into rabbit skin has been reported to be entirely dependent upon CD 18 receptors (17). The LT antagonists used in this study are not specific for LTB_4 and will inhibit other lipoxygenase products including the peptidoleukotrienes (LTC_4, LTD_4 and LTE_4), agents which promote a non-CD 18 dependent neutrophil-endothelial interaction (15,16). In the current study, the action of these agents is unlikely since PMN adhesion is CD 18 dependent, shown by the ability to prevent PMN accumulations with the anti CD 18 mAb (Fig. 3).

Another pathway of diapedesis could occur via endothelial activation. Tx or LTB_4 may stimulate skin macrophages or mast-cells to synthetize cytokines (18,19). These agents will later activate the microvascular endothelium to increase adhesiveness. It has been shown that LTB_4 is necessary for the generation of tumor necrosis factor (TNF) and interleukin (IL)-1 (20). TNF or IL-1 induce endothelial expression of adhesion molecules such as ICAM-1 or ELAM-1 leading to adhesion and diapedesis. In such situations both DEC (by inhibition of cytokine synthesis) and anti CD 18 mAb (by blocking the ligand of ICAM-1) would prevent cytokine-induced diapedesis. However, cytokine induction of endothelial activation is entirely dependent upon protein synthesis (10,11)). Indeed, actinomycin D significantly reduced TNF-induced diapedesis (Fig. 3). The inability of this protein synthesis inhibitor to affect diapedesis in response to Tx-mimic argues against the action of newly synthesized endothelial adhesion proteins in this setting. It is most likely that LTB_4 in response to Tx-mimic activates CD 18 to interact with basally expressed ICAM-1 (21).

The source of LTB_4 was not defined in this study. Tx-mimic may stimulate lipoxygenase activity in endothelial cells, marginating neutrophils, tissue macrophages or mast-cells (22). These cells because of their unique location adjacent to the vasculature might in the first place activate neutrophil adhesion receptors and secondly create a chemotactic LTB_4-gradient to

induce directed migration of neutrophils.

The results are also consistent with previous observations of Tx and LT activity in experimental models of the adult respiratory distress syndrome (Table 2). For instance, in the setting of hindlimb ischemia and reperfusion there is an extraordinary synergism between cyclooxygenase and lipoxygenase pathways. Inhibition of either Tx or LTB_4 synthesis prevents the later lung leukosequestration and pulmonary edema (23,24). Similarly, following acid aspiration there is generation of Tx and LTB_4. Inhibition of either eicosanoid reduces PMN adhesion in the lungs and limits the protein leak (5,25).

In summary, the data indicate that Tx induces LTB_4 synthesis that in turn mediates neutrophil diapedesis. Activated neutrophil adhesion receptors, the CD 18 complex but not newly synthetized endothelial adhesion proteins regulate this event.

Figure 1. Tx-mimic 1μg/ml placed in blisters induced LTB_4 synthesis and diapedesis. Intravenous treatment with the lipoxygenase inhibitor diethylcarbamazine (DEC), prevented the LTB_4 synthesis and the subsequent neutrophil accumulations. The symbol * indicates $p < 0.05$ relative to Tx-mimic/saline.

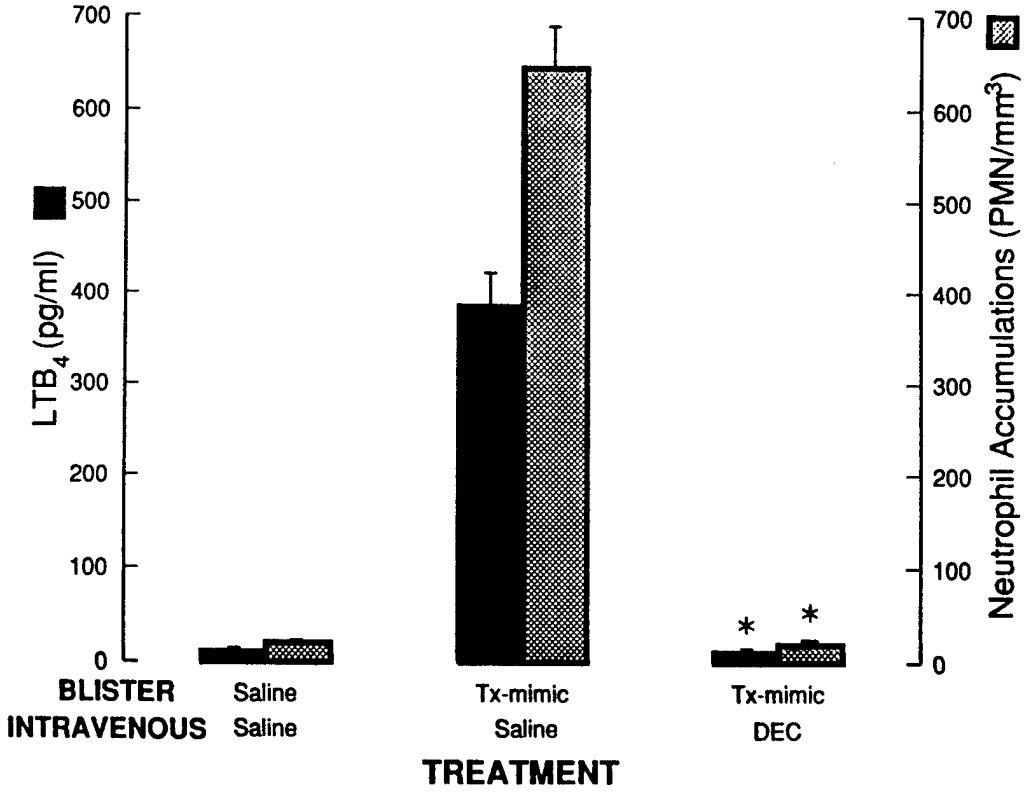

Figure 2. A correlation was found ($r=70$, $p < 0.05$) between the newly formed LTB_4 (in response to Tx-mimic) and neutrophil accumulations.

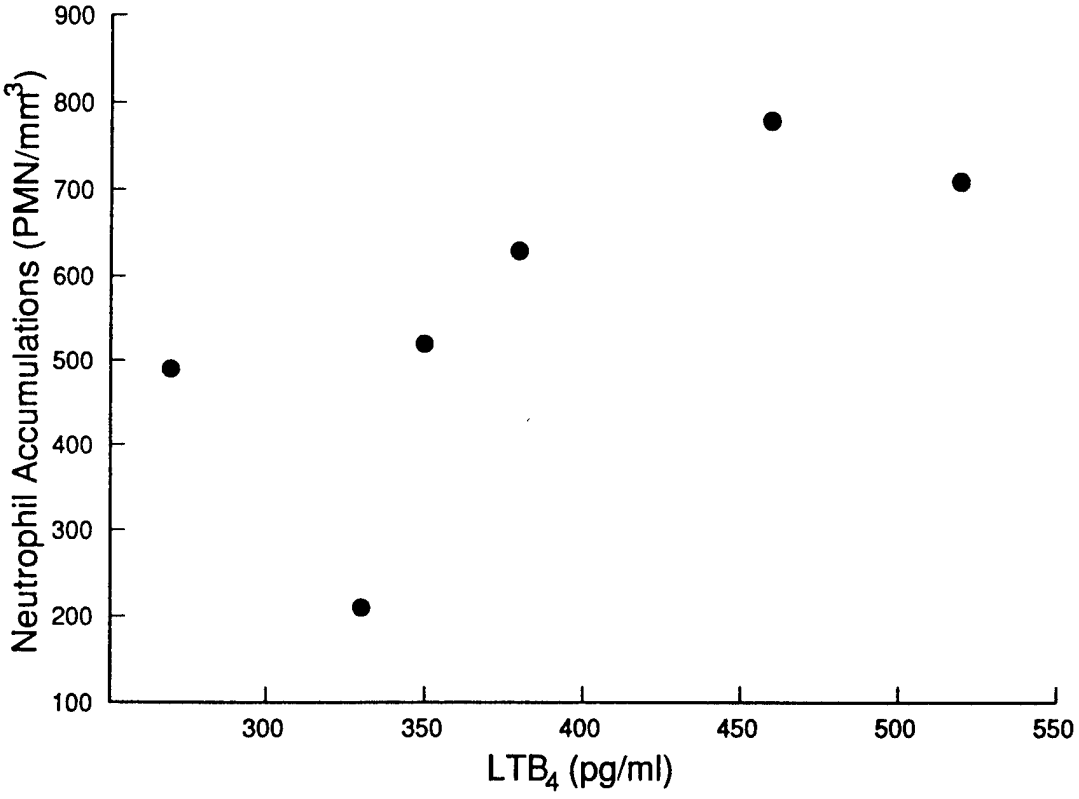

Figure 3. Anti CD 18 mAb (R 15.7) but not the protein synthesis inhibitor actinomycin D, prevented diapedesis in response to Tx-mimic. The symbol * indicates $p < 0.0$ relative to saline treatment.

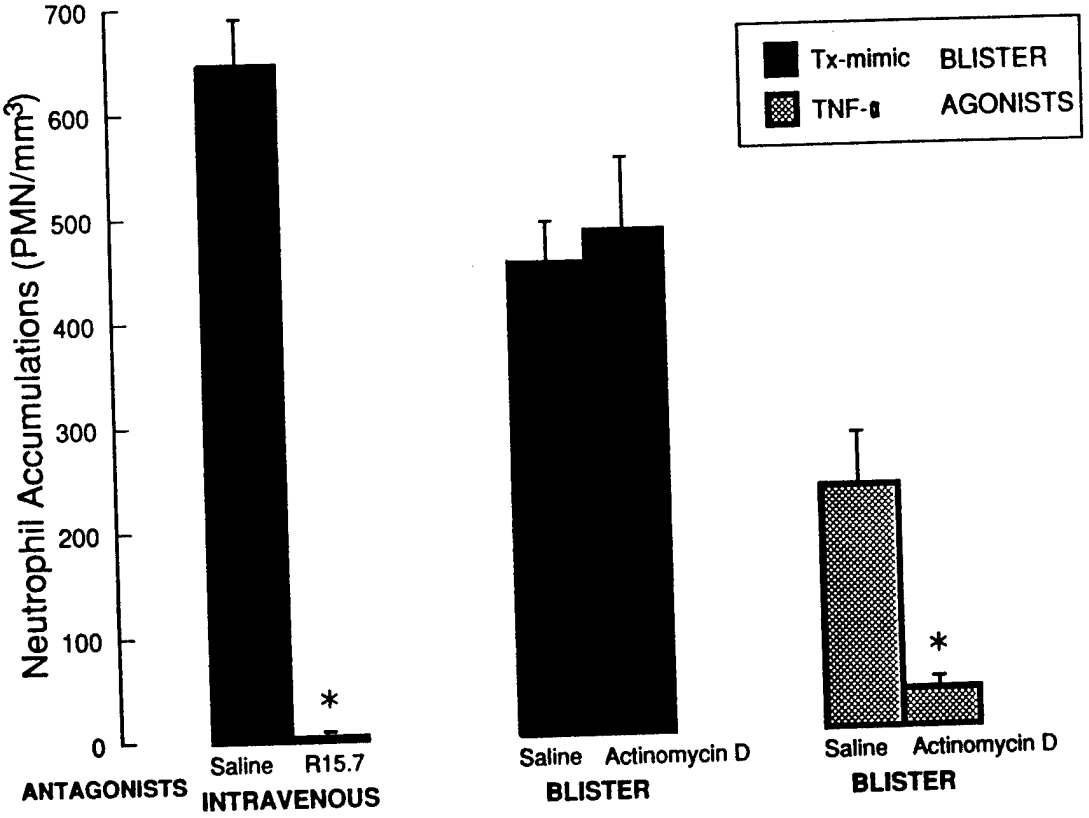

TABLE 1

INTERACTION BETWEEN Tx AND LTB_4: EFFECTS ON EICOSANOID LEVELS AND NEUTROPHIL DIAPEDESIS

	BLISTER TREATMENT			
	LTB_4 $10^{-8*}/10^{-9\dagger}$M	Tx Mimic U46619 $10^{-6\dagger}$M	TxB_2 $10^{-7*}/10^{-8\dagger}$M	Reference
De Novo Synthesis TxB_2	↑↑	---	↑	(4,6,13,14)
De Novo Synthesis LTB_4	↑↑	↑	---	
IV Treatment	***Neutrophil Accumulations***			
Saline	+++	+++	+++	
OKY 046	↓↓	---	↓↓	(6,14)
SQ 29548	↓↓	↓↓	↓↓↓	(14)
DEC	↓↓↓	↓↓↓	↓↓↓	4
FPL 55712	↓↓↓	---	↓↓↓	13
LTB_4 Receptor Antagonist U 75302	↓↓	---	↓↓	26

* Authentic agonist
† Ischemic plasma
--- No data

TABLE 2

MODIFICATION OF PMN LUNG ADHESION WITH Tx AND LT ANTAGONISTS

SETTING	IV TREATMENT	PLASMA LEVEL TX	PLASMA LEVEL LTB_4	EFFECT ON PMN LUNG ADHESION	REFERENCE
HCl-Aspiration	**Saline**	++	++	+++	(5,25)
	OKY 046	0	+	↓↓	(25)
	SQ 29,548	++	+	↓↓	(25)
	DEC	0	0	↓↓	(25)
	FPL 55712	++	++	↓↓	(25)
Hindlimb Ischemia	**Saline**	++	++	+++	(23,24)
	OKY 046	0	---	↓↓↓	(23)
	SQ 29,548	++	---	↓↓↓	(23)
	DEC	0	0	↓↓↓	(24)

BS Baseline
--- No data

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