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ISCHEMIA INDUCED NEUTROPHIL ACTIVATION AND DIAPEDESIS IS LIPOXYGENASE DEPENDENT

BY

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Ischemia and reperfusion lead to eicosanoid and neutrophil (PMN) dependent injury. This study tests the role of ischemia induced lipoxygenase activity in mediating PMN activation and diapedesis. Anesthetized rabbits (n = 8) underwent 3 h of bilateral hindlimb ischemia. At 10 min of reperfusion, leukotriene (LT) B4 levels in femoral venous effluent were 0.49 ± 0.05 ng/ml compared to 0.04 ± 0.07 ng/ml in sham animals (n = 10) (p 0.05). Intracellular H2O2 production of circulating PMN assayed flow cytometrically by

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by dichlorofluorescein (DCF) oxidation, increased from a preischemic value of 74 ± 14 fentamoles (fm) DCF/cell to 135 ± 8 fm DCF/cell ($p < 0.05$). PMN were treated with phorbol myristate acetate (PMA) 10^{-7} M. In contrast to a 162% increase in H_2O_2 production prior to ischemia, PMN at 10 min of reperfusion had an enhanced response to PMA of 336% ($p < 0.05$). Addition of authentic LTB4 (0.5 ng/ml) to PMN from sham animals led to their activation, manifested by an oxidative burst, 127 ± 12 fm DCF/cell and an enhanced response of 337% to PMA stimulation. To study diapedesis, plasma collected at 10 min of reperfusion was introduced into plastic chambers taped atop skin abrasions in rabbits (n = 8). After 3 h, 1610 ± 246 PMN/mm^3 accumulated and LTB4 levels in blister fluid were 0.83 ± 0.03 ng/ml, higher than values of 44 ± 23 PMN/mm3 ($p < 0.05$) and 0.04 ± 0.03 ng LTB4/ml ($p < 0.05$) with saline, and 68 ± 16 PMN/mm3 ($p < 0.05$) and 0.19 ± 0.02 ng/ml ($p < 0.05$) with non-ischemic plasma. The introduction of LTB4, 3.3 ng/ml into the chambers resulted in an accumulation of 536 ± 352 PMN/mm3 ($p < 0.05$). Pre-treatment of animals prior to hindlimb ischemia (n = 5) with the lipoxygenase inhibitor diethylcarbamazine abolished PMN activation (51 ± 12 fm DCF/cell) and ischemic plasma induced diapedesis into the plastic chamber (38 ± 18 PMN/mm3). Pretreatment of non-ischemic animals (n = 13) used for the dermabrasion bioassay with diethylcarbamazine abolished diapedesis into the plastic chambers induced by ischemic plasma (n = 5) (32 ± 24 PMN/mm3) or LTB4 (n = 3) (36 ± 28 PMN/mm3). These data indicate that PMN activation after reperfusion of ischemic tissue is mediated by a lipoxygenase product, perhaps LTB4, and that both reperfusion plasma and authentic LTB4 induce diapedesis by stimulating de novo lipoxygenase activity.

ABSTRACT

Ischemia and reperfusion lead to eicosanoid and neutrophil (PMN) dependent injury. This study tests the role of ischemia induced lipoxygenase activity in mediating PMN activation and diapedesis. Anesthetized rabbits (n=8) underwent 3 h of bilateral hindlimb ischemia. At 10 min of reperfusion, leukotriene (LT)B_4 levels in femoral venous effluent were 0.49 ± 0.05 ηg/ml compared to 0.04 ± 0.07 ηg/ml in sham animals (n=10) ($p < 0.05$). Intracellular H_2O_2 production of circulating PMN assayed flow cytometrically by dichlorofluorescein (DCF) oxidation, increased from a preischemic value of 74 ± 14 fentamoles (fM) DCF/cell to 135 ± 8 fM DCF/cell ($p < 0.05$). PMN were treated with phorbol myristate acetate (PMA) 10^{-7}M. In contrast to a 162% increase in H_2O_2 production prior to ischemia, PMN at 10 min of reperfusion had an enhanced response to PMA of 336% ($p < 0.05$). Addition of authentic LTB_4 (0.5 ηg/ml) to PMN from sham animals led to their activation, manifest by an oxidative burst, 127 ± 12 fm DCF/cell and an enhanced response of 337% to PMA stimulation. To study diapedesis, plasma collected at 10 min of reperfusion was introduced into plastic chambers taped atop skin abrasions in rabbits (n=8). After 3 h, 1610 ± 246 PMN/mm^3 accumulated and LTB_4 levels in blister fluid were 0.83 ± 0.03 ηg/ml, higher than values of 44 ± 23 PMN/mm^3 ($p < 0.05$) and 0.04 ± 0.03 ηg LTB_4/ml ($p < 0.05$) with saline, and 68 ± 16 PMN/mm^3 ($p < 0.05$) and 0.19 ± 0.02 ηg/ml ($p < 0.05$) with non-ischemic plasma. The introduction of LTB_4, 3.3 ηg/ml into the chambers resulted in an accumulation of 536 ± 352 PMN/mm^3 ($p < 0.05$). Pre-treatment of animals prior to hindlimb ischemia (n=5) with the lipoxygenase inhibitor diethylcarbamazine abolished PMN activation (51 ± 12 fM DCF/cell) and ischemic plasma induced diapedesis into the plastic chamber (38 ± 18 PMN/mm^3). Pretreatment of non-ischemic animals (n=13) used for the dermabrasion bioassay with diethylcarbamazine abolished diapedesis into the plastic chambers induced by

ischemic plasma (n=5) (32 ± 24 PMN/mm^3) or LTB_4 (n=3) (36 ± 28 PMN/mm^3). These data indicate that PMN activation after reperfusion of ischemic tissue is mediated by a lipoxygenase product, perhaps LTB_4 and that both reperfusion plasma and authentic LTB_4 induce diapedesis by stimulating de novo lipoxygenase activity.

INTRODUCTION

Impaired perfusion of peripheral tissue is a common clinical event, occurring in settings such as trauma, thrombo-embolism and vascular surgery. Restoration of blood flow is associated with local injury to the previously ischemic tissue as well as remote inflammatory events in the lung (1). The pulmonary microvascular injury is characterized by leukosequestration and increased permeability. This sequence of reperfusion injury is eicosanoid and white blood cell dependent. Thus, high plasma levels of LTB_4 and thromboxane B_2 are found after ischemia (1-3). Further, animals treated with the lipoxygenase inhibitor diethylcarbamazine (DEC) or a thromboxane synthetase inhibitor or who were rendered leukopenic demonstrate attenuation of both local and lung injury (2,3).

This study was designed to test the hypothesis that lipoxygenase products generated during the reperfusion of ischemic tissue, mediate neutrophil activation and enhanced diapedesis. Activation was determined by flow cytometric analysis of the neutrophil oxidative burst while diapedesis was evaluated using an in vivo skin abrasion assay.

METHODS

Animal preparation. Fifty-two New Zealand white male rabbits weighing 3 to 6 kg were used. Initial 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, O.3 ml/kg/h was infused through a carotid arterial cannula placed aseptically via a small neck incision on the day of the experiment. All animals were placed on 37° C heating pads.

Hindlimb ischemia. Anesthetized rabbits (n=8) underwent 3 hours of bilateral hindlimb tourniquet ischemia. Prior to the completion of the ischemic period, the vena cava was ligated

just above the iliac confluence. During the first 10 minutes of reperfusion, the venous return of both hindlimbs was collected from the vena cava distal to its point of ligation (approximately 0.5 ml/min) and discarded. A similar volume of saline was replaced simultaneously via the carotid line. After this 10 minute washout, 4 ml of hindlimb venous return was collected in cooled heparinized syringes containing ethylene diamine tetracetic acid (0.07 mg/ml) and aspirin (0.05 mg/ml) 0.01 ml, and transferred on ice for flow cytometry. Thereafter, another 10 ml sample of blood was collected in a similar manner and centrifuged at 1500 x g for 20 minutes. Aliquots of 0.5 ml of the separated plasma were frozen at -20° C and subsequently used in an in vivo chemotactic assay as well as for the measure of leukotriene (LT)B_4. Prior studies have shown that aspirin in the dose used in plasma did not interfere with the chemotactic assay.

Sham rabbits. The preparation was the same as above including vena cava ligation but without ischemia (n=10). Plasma from the inferior vena cava of these animals was collected and used in the chemotactic and LTB_4 assay.

Skin abrasion blister chambers. Chemotactic responses were measured by a modification of the technique of Otani (4). After anesthesia a 20 x 25 cm area of the back of rabbits used only to assay chemotaxis (n=34) was clipped with electric shears (Wahl Clipper Company, Sterling IL). The clipped region was coated with sodium thioglycolate (Lemon Scented Nair, Carter Products, New York, NY) for 15 min, washed with tap water, rinsed with 100 ml of 0.25% acetic acid and then re-rinsed with tap water. The animals were permitted to rest for 24 to 36 hours to permit any non-specific inflammatory response to subside before chemotactic studies were performed. Animals exhibiting skin sensitivity were excluded from the study.

On the day of the experiment, the animal was re-anesthetized and a circular area of hairfree skin was outlined with a template having a 9/32 inch diameter (Rapi-Design Template No. 40,

Rapi-Design Products, Burbank CA). This area was gently abraded with an electric ink eraser (Petty Electric Ink Eraser, Pierce Corp., River Falls, WI) until uniform glistening was produced. This normally took 15-20 seconds. The debris from the abraded area were removed by pressing adhesive tape (Blender M, 3M Surgical Products, St. Paul MN) over the site. On rare occasions, when abrasion led to trace bleeding, the site was abandoned.

Plastic, "unit dose" blister chambers (Rexhaus Corporation, Westfield Industrial Park, Westfield MA) with a volume capacity of 0.5 ml were placed over the abraded areas and secured with Steridrape (3M Surgical Products, St. Paul MN). Usually 18 to 24 blister chambers could be affixed to each rabbit. Injections into the chambers were made with a 27-gauge needle. At the conclusion of the experiment, fluid was withdrawn from the chambers and neutrophils counted with a hemocytometer. Animals were allowed to rest for 14 days before they were re-used.

Preparation of Test Solutions. Authentic LTB_4 (Sigma Chemical Co., St. Louis MO) was reconstituted in saline and frozen at -70° C in 0.5 ml aliquots until used at a final concentration of 3.3 ηg/ml. All manipulations involving LTB_4 were performed with apparatus washed with methanol and air-dried. LTB_4 was injected from foil-covered syringes into blister chambers blackened with an indelible marker to prevent light inactivation.

Preparation of Diethylcarbamazine.

DEC-N,N Diethyl-4-methyl•1-piperazinecarboxamide, (Sigma Chemical Co., St. Louis MO) was administered intra arterially 40 mg/kg/h after dissolution in 0.9% sodium chloride.

Leukotriene B_4 assay. Concentrations of LTB_4 in plasma and blister fluid were measured in duplicate by radioimmunoassay using a rabbit antibody and standards supplied by Seragen (Cambridge MA). Cross reactivity of LTB_4 antibody with other leukotrienes, TxB_2, the PG's and their metabolites was less than 1%.

Flow Cytometry. Intracellular generation of H_2O_2 by blood neutrophils was quantitated using flow cytometry and dichlorofluorescein-diacetate (DCFH)(5). DCFH is a non-fluorescent compound which is oxidized to the highly fluorescent dichlorofluorescein (DCF) within neutrophils undergoing a respiratory burst. Leukocytes were isolated from blood by dextran (6% in 0.9% saline) sedimentation, 0.3 ml per 3 ml blood, for 45 minutes at room temperature (Dextran T 500, Pharmacia. Piscataway NJ). Small aliquots of leukocyte rich sediment (0.01 ml) were added to 1 ml of a balanced salt solution (BSS). This solution contained NaCl (124 μM), KCl (5.8 μM), dextrose (10 mM) and hydroxyethylpiperazine ethanesulphonic acid (20 mM) and was titrated with NaOH to pH 7.4 prior to use. The BSS also contained 100 μM DCFH (Molecular Probes, Eugene OR) and either buffer or phorbol myristate acetate (PMA) 10^{-7}M. The concentration of DCFH was 100μM, an amount which saturated leukocytes in samples from sham or experimental animals. After incubation for 20 minutes at 37°C the samples were placed on ice and analyzed with an Ortho Diagnostics System 2151 Cytofluorograf flow cytometer using the 488 nm excitation line of an argon laser at 125 mw output. In preliminary experiments, it was found that any centrifugation, vortexing or even vigorous pipetting led to increases in baseline oxidation in neutrophils, so these procedures were eliminated in the final protocol. The polymorphonuclear leukocytes within each sample were identified by light scattering. After electronic gating, the green fluorescence of these cells in unstimulated and PMA stimulated samples was recorded (3,000-5,000 neutrophils per sample). In some experiments, the fluorescence values obtained by flow cytometry were calibrated with samples of pure rat neutrophils. These cells were obtained by lavage, 4 hours after intraperitoneal glycogen. The cells were resuspended in BSS, 10^6 cells/ml and labelled with DCFH with or without PMA treatment. Measurements were conducted in a fluorometer (Perkin-Elmer, Norwalk CT). Using a standard curve of reagent

DCF (Sigma Chemical Co., St. Louis MO) the amount of DCFH oxidized to DCF by H_2O_2 in these samples was quantitated, allowing conversion of mean fluorescence channel number to fentamoles of DCF produced per cell. This value was equivalent to fentamoles of H_2O_2 produced (5).

Skin Capillary Blood Flow. Using the laser doppler (Laserflo, Model BPM 403, TSI, Inc., St. Paul, MN) skin blood flow was measured. Flow was recorded in ml/100g/min (6).

Protocol. Thirty minutes prior to ischemia or sham ischemia, rabbits received saline (n=18) or intravenous DEC (n=5). Rabbits used for the chemotaxis assay were also given saline (n=21) or DEC (n=13). Their blisters were treated with 0.5 ml volumes of: saline (n=5), LTB_4 3.3 ηg/ml (n=6) sham plasma (n=7) or ischemic plasma (n=16).

Data are expressed as mean ± SEM in text and figures. An analysis of variance, paired and non-paired Student's t-test were used to determine significance. 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

LTB_4 levels in femoral venous effluent following 3 h of hindlimb ischemia and 10 min of reperfusion were 0.49 ± 0.05 ηg/ml, higher than sham values of 0.04 ± 0.07 ηg/ml ($p < 0.05$). Diethylcarbamazine treatment prior to ischemia prevented the increase in plasma LTB_4 and maintained levels equivalent to the sham group 0.04 ± 0.01 ηg/ml.

Flow Cytometry. Ten minutes following reperfusion the intracellular neutrophil H_2O_2 production increased relative to preischemic values ($p < 0.05$, Fig. 1). When aliquots of sham neutrophils were treated with PMA 10^{-7}M there was increased oxidative activity while PMA treatment of neutrophils 10 minutes after ischemia led to a greatly enhanced response ($p < 0.05$). Treatment with DEC prior to ischemia decreased the oxidative activity of the neutrophil at 10 minutes of reperfusion to levels similar to sham neutrophils with and without PMA (both $p < 0.05$). Neutrophils from sham animals treated with DEC showed oxidative activity similar to saline treated sham animals. Addition of authentic LTB_4 (0.5 ηg/ml) to neutrophils from sham animals led to their activation and to an enhanced response to PMA, similar to values following ischemia (Fig. 1).

After 3 hours, blister chambers filled with plasma from ischemic limbs showed LTB_4 levels of 0.83 ± 0.03 ηg/ml, higher ($p < 0.05$) than: 0.19 ± 0.02 ηg/ml noted with sham plasma; 0.04 ± 0.09 ηg/ml with saline; and 0.04 ± 0.06 ηg/ml with plasma from ischemic animals pretreated with DEC (Fig. 2). In blister chambers filled with authentic LTB_4, the initial concentration of 3.3 ηg/ml decreased to 1.1 ± 0.1 ηg/ml after 3 hours (Fig. 2). Plasma from ischemic animals was chemotactic. Thus, ischemic plasma led to the accumulation of 1610 ± 246 PMN/mm^3 in the blister chamber, higher ($p < 0.05$) than: the sham plasma value of 68 ± 16 PMN/mm^3; saline value of 44 ± 23 PMN/mm^3; and LTB_4 value of 536 ± 352 PMN/mm^3.

Systemic treatment with diethylcarbamazine of animals used for harvesting ischemic plasma or of animals used for the diapedesis assay led to reduced PMN accumulations in response to all chamber additives. Thus, plasma from DEC treated ischemic animals led to the accumulation of only 38 ± 18 PMN/mm^3, lower than plasma from non-DEC treated animals ($p < 0.05$)(Fig. 2). Similarly, DEC treatment of animals used for the diapedesis assay reduced PMN

accumulations: in response to ischemic plasma to 32 ± 24 PMN/mm^3 ($p < 0.05$); in response to addition of LTB_4 to 36 ± 28 PMN/mm^3 ($p < 0.05$); and in response to addition of saline to 28 ± 14 PMN/mm^3 ($p < 0.05$). DEC IV did not affect the blood flow of the abraded skin chambers as measured with the laser doppler. Baseline skin blood flow was defined arbitrarily as $100 \pm 3\%$ and increased to $102 \pm 4\%$ with DEC administration. One hour after DEC administration it was $104 \pm 5\%$. Finally, LTB_4 concentrations in ischemic plasma were closely related to the neutrophil oxidative burst and the numbers of neutrophils counted in blister fluid (Fig. 3).

DISCUSSION

The results of this study indicate that hindlimb ischemia leads to the synthesis of a lipoxygenase product(s), activation of circulating neutrophils and the appearance of an agent in plasma which has chemotactic properties. That LTB_4 is causally related to these events is suggested first by the observation that levels of LTB_4 rise at the same time that the neutrophils undergo an oxidative burst, that is 10 minutes after reperfusion. Secondly, the LTB_4 concentrations found in this study have been shown by others to activate neutrophils (7). Thirdly, addition of authentic LTB_4 to sham PMN led to an oxidative burst to levels similar to those noted after reperfusion. Fourthly, there is a strong correlation between LTB_4 concentrations in ischemic plasma and the oxidative burst of neutrophils and the number of neutrophils that accumulate in the blister pack (Fig. 3). The source of raised plasma LTB_4 observed in this study is not defined although we believe it to be the neutrophil. Activated neutrophils are known to synthesize lipoxygenase products, whereas neutrophil depletion in the setting of ischemia prevents the rise in plasma LTB_4 during reperfusion (8). Finally, lipoxygenase inhibition prior to ischemia prevents the rise in

plasma LTB_4 as well as neutrophil activation. The data, however, are not conclusive in documenting a causal role of LTB_4. Other lipoxygenase products such as hydroxyeicosatetraenoic acid (HETE) could be operative. In addition, LTB_4 could induce thromboxane (Tx) synthesis which might be the direct neutrophil activator. Weight is given to this latter postulate by studies which show that many LTB_4 effects are mediated by TxA_2 (9,10). Further, we have demonstrated that inhibition of Tx synthesis or Tx receptors will prevent ischemia induced neutrophil activation (11).

Phorbol esters which activate protein kinase C will increase neutrophil H_2O_2 production above baseline. Exposure of neutrophils to one chemoactivator has been shown to raise the sensitivity threshold to the influence of another activator (12). The data from this study are confirmatory. Thus, a putative activator such as LTB_4, released in response to ischemia, presumably resets the threshold to PMA stimulation.

The phenomenon of chemoattraction is different from chemoactivation. The latter describes neutrophil stimulation manifest by an oxidative burst with peroxide production. Chemoattraction may involve activation but principally involves directed neutrophil migration. Agents such as LTB_4 may function both as chemoactivators as well as chemoattractants. Thus, application of LTB_4 on the abluminal side of the vasculature such as in the blister pack leads to chemotaxis. In contrast, LTB_4 infused intravenously will function as a chemoactivator, and in so doing may paradoxically inhibit the subsequent ability of LTB_4 to act as a chemoattractant (13). Others have shown that the mixing of chemoattractants with neutrophils will desensitize these cells to subsequent chemoattraction (14).

Plasma collected from ischemic hindlimbs when placed in a blister pack leads to PMN accumulations. However, pretreatment of ischemic animals with a lipoxygenase inhibitor leads

to a reduction of LTB_4 to sham levels and prevents diapedesis induced by ischemic plasma from these DEC treated animals. Even though considerable LTB_4 is present in ischemic plasma, de novo lipoxygenase synthesis appears necessary for chemotaxis. This is shown by the observation that diethylcarbamazine pretreatment of the animal used for the chemotactic assay will prevent neutrophil accumulations. Further, intravenous pretreatment with DEC also prevents LTB_4 induced accumulation of PMN into the blister. These observations suggest that an intact lipoxygenase pathway in circulating neutrophils is essential for their migration. We have not addressed the mechanisms in the current study. However, other studies suggest that either neutrophil adherence receptor (CD 18) modulation or effects on PMN motility could be involved (15,16). It is unlikely that LTB_4 levels represent the sole chemotactic effect of ischemic plasma. This thesis is reinforced by the observation that addition of LTB_4 to the blister in levels higher than those assayed in ischemic plasma led to the accumulation of significantly fewer PMN's (Fig. 2).

These results indicate that ischemia causes the generation of an agent in plasma which leads to diapedesis by stimulating lipoxygenase synthesis. Because of the ability of DEC to prevent synthesis by the ischemic limbs of LTB_4 as well as this lipoxygenase stimulating agent, it is possible that LTB_4 is the lipoxygenase activator and is responsible for a positive feedback loop. Thus, LTB_4 could exert its chemotactic effect by causing the local generation of other lipoxygenase products.

In summary, these data document that ischemia leads to neutrophil activation and the generation of an agent in plasma which by inducing lipoxygenase synthesis can act as a chemoattractant.

Figure 1. Following ischemia, "background" neutrophil oxidative activity assayed by flow cytometry increased relative to sham. Treatment of sham neutrophils in vitro with authentic LTB_4, 0.5 ηg/ml led to a similar oxidative burst. Pretreatment of ischemic animals with diethylcarbamazine modified neutrophil response so that it was similar to the sham group. Neutrophils from sham animals pretreated with DEC (sham+DEC) were not significantly altered. Oxidative activity of neutrophils was enhanced in all groups by treatment with phorbol myristate acetate (PMA). Asterisks indicate $p < 0.05$ when comparing "background" to PMA treatment. Daggers indicate $p < 0.05$ relative to sham or DEC treated animals.

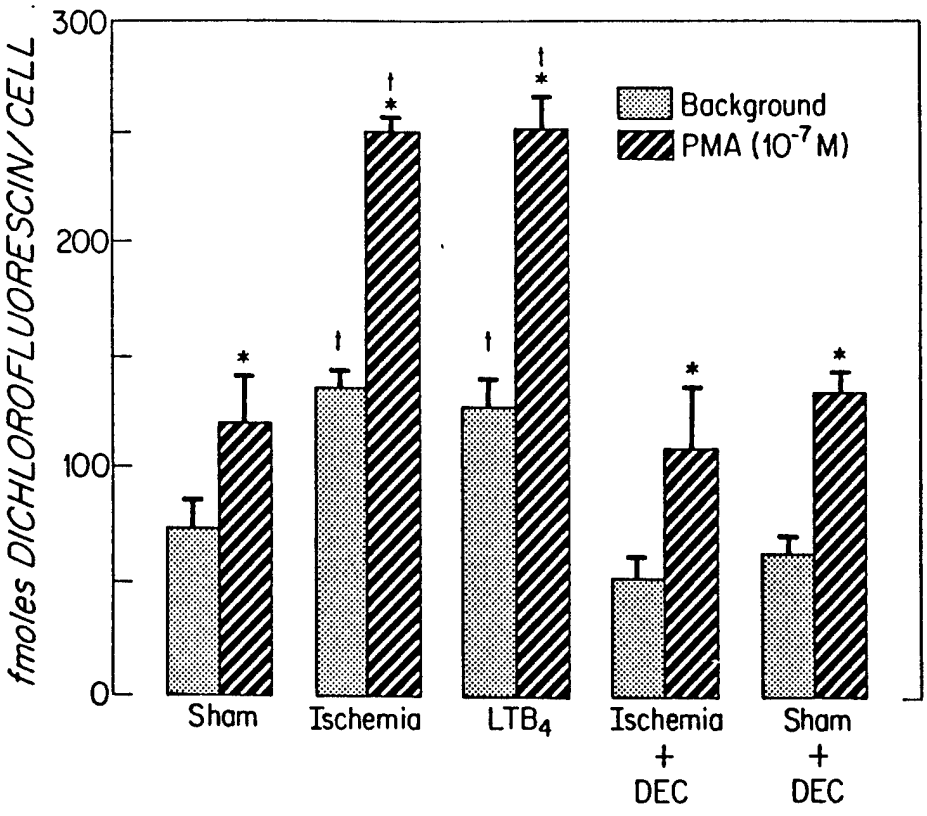


Figure 2. In blister chambers, neutrophil accumulations and LTB_4 levels, 3 hours following introduction of ischemic plasma or LTB_4, were higher than values following introduction of sham plasma from non-ischemic rabbits, or saline. Plasma from rabbits subjected to ischemia but pretreated with IV diethylcarbamazine (DEC) showed minimal diapedesis and LTB_4 production. In data not shown, intravenous DEC pretreatment of rabbits with skin blisters prevented PMN accumulations and LTB_4 synthesis in response to ischemic plasma and to LTB_4. Daggers indicate $p < 0.05$ compared to sham plasma.

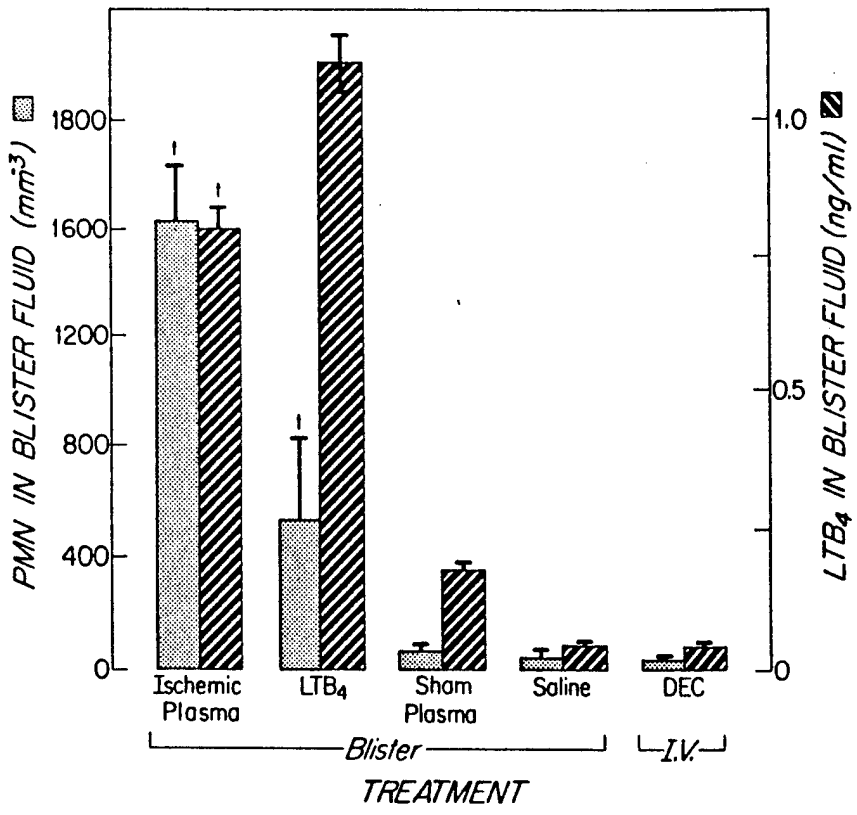
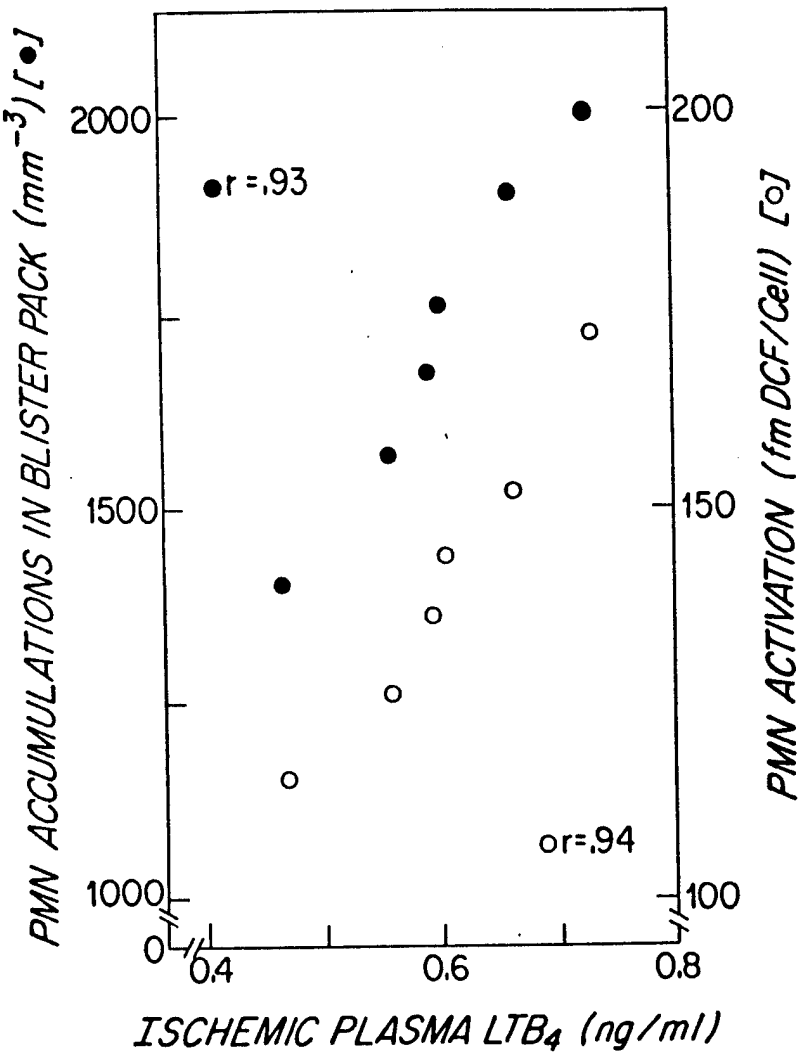


Figure 3. LTB_4 concentrations in ischemic plasma correlated significantly with the neutrophil oxidative burst as measured flow cytometrically and the number of PMN accumulating in the blister chambers. All r values are significant at $p < 0.05$.



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