Pulmonary Leukosequestration Induced by Hind Limb Ischemia

HAIM ANNER, M.D., RONALD P. KAUFMAN, JR., M.D., LES KOBZIK, M.D., C. ROBERT VALERI, M.D.,*
DAVID SHEPRO, PH.D.,† and HERBERT B. HECHTMAN, M.D.

Lower torso ischemia leads to acute respiratory failure, an event associated with the accumulation of inflammatory cells in the lungs. This study tests whether ischemia-induced eicosanoid synthesis leads to polymorphonuclear leukocyte (PMN) accumulation in the lungs. Anesthetized rats (N = 51) were randomized into five groups: nonischemic sham rats (N = 10); the remaining four groups were rats made ischemic for 4 hours with bilateral thigh tourniquets treated just before tourniquet release with saline vehicle (N = 17): the thromboxane (Tx) synthase inhibitor OKY-046 (Ono Pharmaceutica, Osaka, Japan) 2 mg/kg intravenously every 2 hours (N = 8); the lipoxygenase inhibitor diethylcarbamazine (DEC) (Sigma, St. Louis, MO) 0.2 mg/kg/min intravenously (N = 8); the platelet-activating factor receptor antagonist SRI (Sandoz Inc., East Hanover, NJ) 63-072 3 mg/kg intravenously every 30 minutes (N = 8). Four hours after ischemia, plasma TxB_2 levels in the ischemic placebo-treated group was 3570 ± 695 pg/mL, compared with 495 ± 73 pg/mL in sham rats ($p < 0.001$). Lung microscopy showed foci of proteinaceous exudate in alveoli and 121 ± 10 PMN/20 high power fields (HPF) compared with 59 ± 9 PMN/20 HPF in the sham group ($p < 0.001$). One day after ischemia PMN accumulations remained elevated at 119 PMN/20 HPF. Pretreatment with OKY-046 led to reduced TxB_2 levels of 149 ± 17 pg/mL, normal lung histology, and 83 ± 13 PMN/20 HPF, a value similar to that of the sham group and lower than that of the placebo-treated group ($p < 0.05$). Treatment with DEC yielded TxB_2 levels of 1419 ± 492 pg/mL, which was lower than that of the placebo group ($p < 0.05$) but higher than that of the sham group ($p < 0.05$). Microscopy showed normal lungs with 79 ± 7 PMN/20 HPF lower than the placebo group ($p < 0.05$). SRI 63-072 did not inhibit Tx synthesis or leukosequestration in the lungs. Platelet counts decreased in all groups relative to sham animals ($p < 0.05$). The results indicate that Tx synthesis induced by ischemia moderates PMN accumulations in the lungs. Inhibition of lipoxygenase is believed to prevent PMN accumulations both by limiting leukotriene-induced Tx synthesis as well as by limiting production of chemoattractants.

From the Department of Surgery, Brigham and Women's Hospital, and Harvard Medical School, The Naval Blood Research Laboratory, Boston University School of Medicine, and The Biological Science Center, Boston University,† Boston, Massachusetts*

LOWER TORSO ISCHEMIA stimulates the release of a circulating agent(s), which leads to the accumulation of blood cells including polymorphonuclear leukocytes (PMN) in the lungs.[1,2] Neutrophil entrapment is considered to be the final event resulting in the local release of toxic agents and acute respiratory failure.[3]

Previous studies of ischemia have concluded that pulmonary injury occurs when blood cell microemboli are mechanically sieved by the lungs.[4] Since synthesis of the proaggregator and chemoattractant TxA_2 is stimulated by ischemia,[5] the current study was designed to test its role in blood cells, particularly neutrophil accumulations in the lungs. Further, the role of two other chemoattractants and activators of neutrophils, the leukotrienes (LT) and platelet-activating factor (PAF),[6-8] were included in the study to test their roles in pulmonary leukosequestration.

Methods

Fifty-seven male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), weighing an average of 500 g, were anesthetized with 60 mg/kg of pentobarbital sodium administered by intraperitoneal injection. A jugular venous catheter was inserted for supplementary doses of anesthesia as well as fluid and drug administration. The volume of fluid given in all experiments was 2 mL/kg/h.

The animals were divided into five groups. In all animals, except the sham controls (N = 10), arterial tourniquets were applied to both thighs after a 2-minute elevation of the hind limbs to minimize the amount of retained blood. The animals were then placed prone, and after 4 hours the tourniquets were removed. Thirty minutes before tourniquet release, animals were randomized to receive: saline vehicle (N = 23); the thromboxane (Tx) synthase inhibitor OKY-046 (Ono Pharmaceutica, Osaka, Japan), 2 mg/kg by intravenous bolus, repeated every 2 hours (N = 8); the lypoxygenase

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Reprint requests and correspondence: Herbert B. Hechtman, M.D., Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

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inhibitor diethylcarbamazine (DEC)[9] (Sigma, St. Louis, MO), 0.2 mg/kg/min in a continuous intravenous infusion (N = 8); the platelet-activating factor (PAF) receptor antagonist SRI 63-072[10] (Sandoz Inc., East Hanover, NJ) in repeated intravenous boluses of 3 mg/kg every 30 minutes (N = 8). Treatment in all animals was continued for 4.5 hours when the experiment was ended.

After 4 hours of reperfusion, a midline laparotomy was performed and 5 mL of blood withdrawn from the inferior vena cava in syringes containing ethylene diamine tetracetic acid and aspirin. Platelets were counted by means of phase microscopy. The blood was then centrifuged at 1500 × g at 4 C for 20 minutes, the plasma separated and stored at −20 C until assayed. Plasma TxB_2 and 6-keto-$PGF_{1\alpha}$ were measured by radioimmunoassay.[11-13]

The rats were killed with intravenous potassium chloride, and the lungs harvested for light microscopy. This was done *via* a midsternotomy. Through a tracheostomy, gluteraldehyde was introduced into the lungs and kept under a constant hydrostatic pressure equivalent to a 20-cm column of water for 20 minutes. The trachea was then ligated, and the lungs removed and bathed in gluteraldehyde for 24 hours. Sections were taken from identical lung regions and were stained with hematoxylin and eosin. Leukocytes were counted in 20 high power fields (HPF). Sham control animals were studied (N = 10) where all procedures were done except the hind limbs were not made ischemic. Four animals of the sham group and three of the placebo-treated group were used to determine wet-to-dry lung weight ratios of the bloodless lung at the conclusion of the experiment, that is, after 4 hours of hind limb ischemia and 4 hours of reperfusion. Lastly, plasma TxB_2 levels, lung weights, and PMN entrapment were assayed in placebo-treated rats 24 hours (N = 3) and 48 hours (N = 3) after ischemia.

Results are presented as the mean ± SE. Significance between means was tested by an analysis of variance and nonpaired t-test. Significance was accepted if $p < 0.05$.

Animals used in this study were maintained in accordance with the guidelines of the Committee of 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 (DHEW publication no. [NIH] 78-23, revised 1978).

Results

Four hours of reperfusion after a 4-hour period of hind limb ischemia resulted in a rise of plasma TxB_2 in the placebo-treated animals to 3579 ± 695 pg/mL, compared with 495 ± 73 pg/mL in the nonischemic sham group ($p < 0.001$) (Fig. 1). There was also a rise in 6-keto-$PGF_{1\alpha}$ to 285 ± 50 pg/mL compared with sham treatment values of 156 ± 50 pg/mL ($p < 0.05$) (Fig. 2).

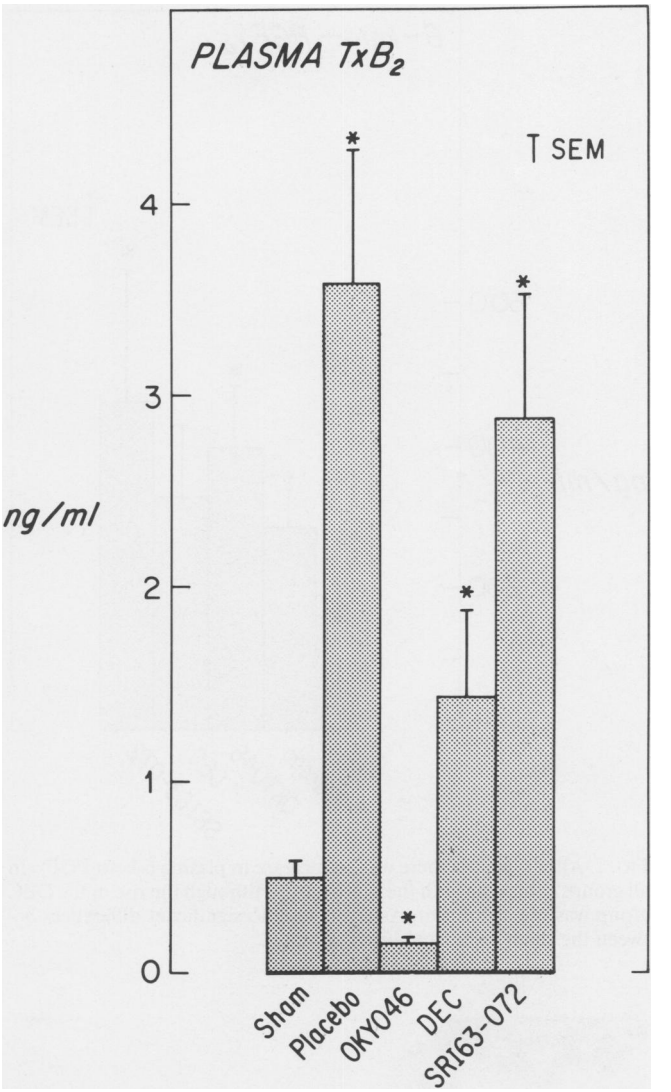

FIG. 1. Four hours of reperfusion after bilateral hind limb ischemia led to a rise in plasma TxB_2 levels compared with those of sham animals. OKY-046 prevented this phenomenon, whereas DEC modified it. The PAF antagonist, SRI 63-072, was without effect. Asterisks refer to significant differences between the sham group and other groups.

Microscopic examination of the lungs of sham animals showed normal histologic features (Fig. 3), whereas placebo-treated ischemic animals demonstrated accumulations of PMNs in the capillary beds throughout the lung parenchyma (Fig. 4). Most neutrophils were contained within vessels, but foci of inflammatory cells and proteinaceous exudate within alveolar air spaces were also present. The accumulation of PMNs in the lungs was in excess of what could be attributed to local differ-

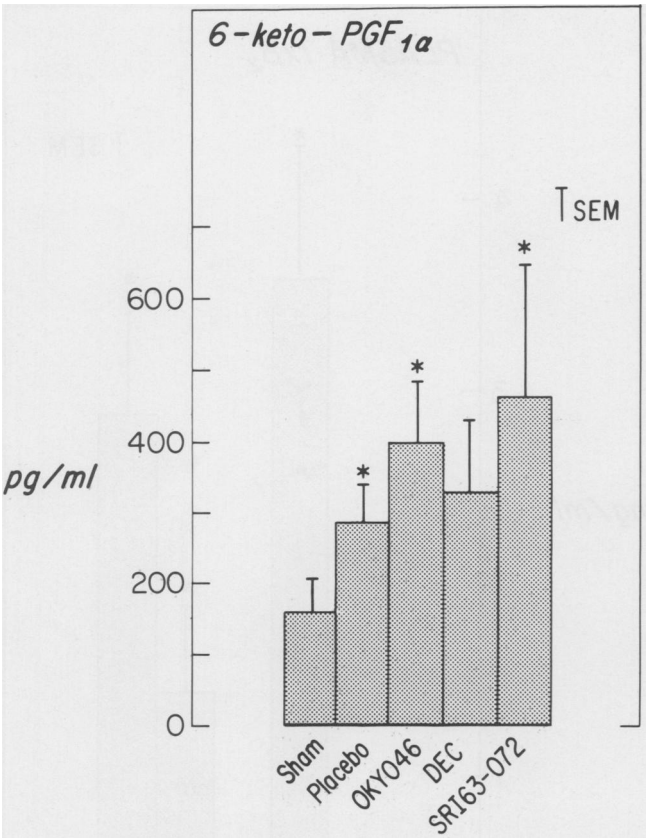

FIG. 2. After ischemia there was an increase in plasma 6-keto-$PGF_{1\alpha}$ in all groups compared with the sham group, although the rise in the DEC group was not significant. Asterisks refer to significant differences between the sham group and other groups.

ences in blood volume or vascular congestion. Quantitative counts of PMN in the lungs were 121 ± 10 PMN/20 HPF, higher than the sham group value of 59 ± 9 PMN/20 HPF ($p < 0.001$) (Fig. 5). Wet-to-dry weight ratios of the lungs in placebo-treated and sham groups were 4.97 ± 0.24 and 4.89 ± 0.05, respectively.

Pretreatment with OKY-046 blocked ischemia induced TxB_2 synthesis. After 4 hours of reperfusion, TxB_2 levels were 149 ± 17 pg/mL, lower than the sham group ($p < 0.001$). Few abnormalities were noted on microscopy. Accumulation of neutrophils was 83 ± 13 PMN/20 HPF, similar to that of the sham group and reduced compared with that of placebo-treated animals ($p < 0.05$) (Fig. 5). In the DEC-treated group, TxB_2 levels were 1419 ± 492 pg/mL, a value higher than that of sham animals ($p < 0.05$) but lower than that of the placebo-treated group ($p < 0.05$) (Fig. 1). Histologic features of the lungs were virtually normal, and leukocyte count in the lungs was 79 ± 7 PMN/20 HPF (Fig. 5), lower than that of the placebo-treated group ($p < 0.05$). The PAF antagonist, SRI 63-072, had no significant effect on TxB_2 levels, 2912 ± 635 pg/mL, nor on leukosequestration in the lungs, 110 ± 26 PMN/20 HPF, both measurements being higher than levels in sham animals and similar to levels in placebo-treated animals.

Platelet counts decreased significantly in all four ischemic groups compared with that of sham animals ($p < 0.05$) (Fig. 6). Platelet aggregates were not seen in histologic sections.

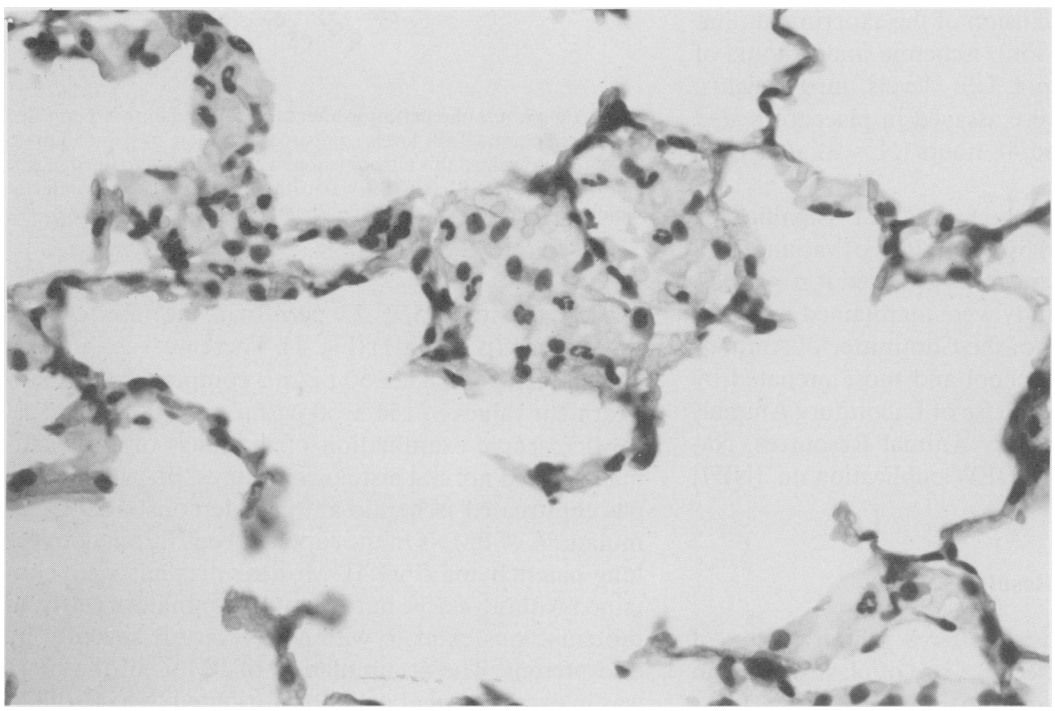

FIG. 3. Histologic specimens of the lungs in placebo-treated animals showed accumulations of PMNs in the capillary bed, mostly within vessels. Occasionally, foci of inflammatory cells and proteinaceous exudate were present within alveolar air spaces as seen in the central portion of the figure.

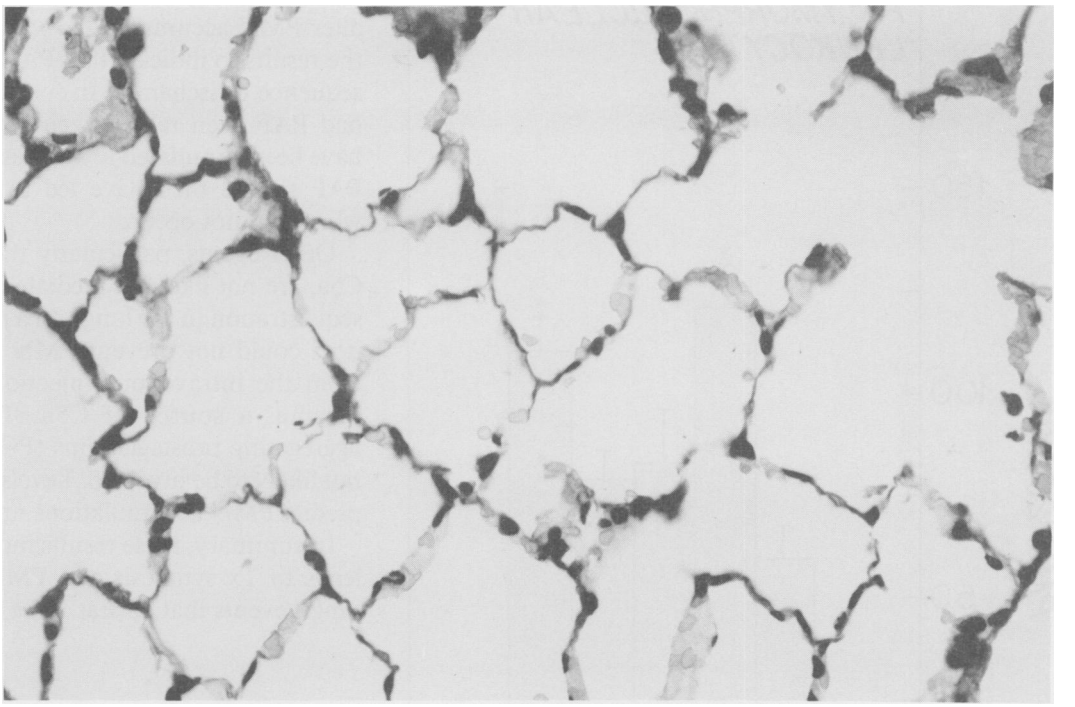

FIG. 4. The histologic features of lungs of sham animals were normal.

Placebo-treated animals that were studied 24 hours after release of the thigh tourniquets had normal TxB_2 levels of 436 ± 105 pg/mL but lung leukocyte counts were still high at 119 ± 1 PMN/20 HPF. After 48 hours, TxB_2 levels were 763 ± 118 pg/mL and lung leukocyte counts were 94 ± 6 PMN/20 HPF.

Discussion

Reperfusion after a 4-hour ischemic stimulus led to a prominent rise in plasma TxB_2, which returned to baseline after 24 hours. Similar findings have been noted in dogs after application of a thigh tourniquet and in humans after abdominal aortic cross-clamping during aneurysm surgery.[5,14] Associated with this rise in TxB_2 was an accumulation of leukocytes in the lungs (Fig. 5) that cleared slowly, over the next 2 days. Although there was no increase in the wet-to-dry weight ratio 4 hours after ischemia when PMN accumulations were prominent, there was histologic evidence of microvascular injury manifest by focal proteinaceous exudate into alveolar air spaces (Fig. 3). Previous observations of the pulmonary consequences of lower torso ischemia have documented blood cell accumulations in the lungs, believed to be due to embolization of microaggregates from the ischemic legs.[1,2,4] That platelet counts were noted to fall during neutrophil sequestration in the lungs (Fig. 6) is consistent with the formation and embolization of platelet microemboli. However, histologic observations make this unlikely. Platelet aggregates were not noted by lung microscopy in animals who had PMN accumulations (Fig. 3). Secondly, pharmacologic inhibition of Tx and LT was successful, at least in part, in preventing the PMN accumulations in the lungs but not the decline in circulating platelet numbers (Figs. 5 and 6). It is unlikely that the proaggregators being inhibited, that is TxA_2, would selectively aggregate PMNs and not platelets.

That mechanical filtration of PMN aggregates by the lungs is unlikely makes plausible the thesis that agents such as TxA_2 and LTB_4 lead to pulmonary endothelial–neutrophil interactions.[15] Adherence of PMN to pulmonary endothelium is a necessary initial step. It is possible that LTB_4 acts principally on endothelium.[16] LTB_4-stimulated Tx synthesis by endothelium, an event recently described,[17] may be a critical intermediary step. Thus, LTB_4 when applied to abraided skin or when injected subcutaneously will lead to the local generation of Tx and PMN accumulations, events that can be prevented with Tx synthase antagonists.[18] In other settings where Tx synthesis is stimulated, such as in experimental acid aspiration, PMN accumulations in the lungs are noted.[19] This can be limited by inhibition of Tx synthesis. The precise mechanism of TxA_2 action in these settings is unknown but is believed to involve endothelial–PMN adherence.

It is also theorized that thromboxane may act directly on actin filaments in endothelium in a manner similar

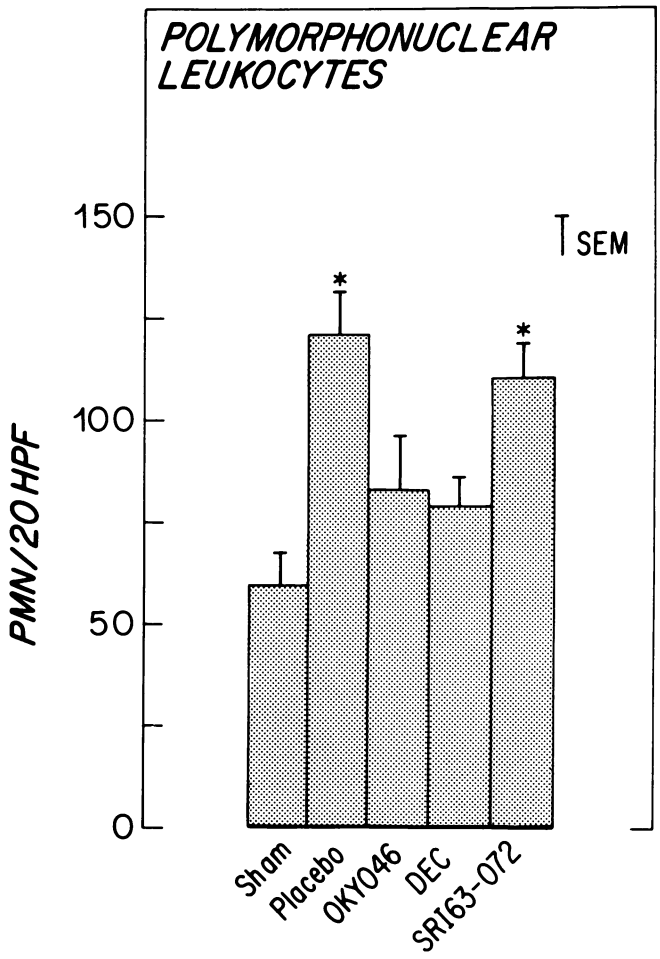

FIG. 5. Leukocyte entrapment was greater in placebo-treated animals than in the sham group. Pretreatment with OKY-046 and DEC significantly reduced pulmonary leukosequestration. Asterisks refer to significant differences between sham-treated animals and other groups.

to vasoactive amines to encourage PMN movement out of the microvasculature by diapedesis.[20] Actin filaments are believed to be important determinants of the size of intercellular clefts between endothelial cells. The opening of these tight junctions occurs when the actin cables are disassembled by agents such as histamine, LTB_4, and TxA_2.[21] This leads to increased permeability to macromolecules as well as to increased PMN diapedesis.[20,22]

The observation that treatment with DEC leads to a lowering of TxB_2 levels may be due to inhibition of LT and LT-induced generation of Tx.[17] The salutory effect of DEC on PMN accumulations may be due both to this reduction in TxA_2 as well as to a decrease in synthesis of the chemoattractant LTB_4. Both eicosanoids may contribute to leukosequestration by moderating the size of gap junctions as well as PMN–endothelial interactions.

The inability of the PAF antagonist SRI 63-072 to alter PMN accumulations was a surprise. We interpret the results to indicate that PAF is not released as a consequence of ischemia. In further support of this thesis, had PAF been released, circulating blood cells should have been stimulated to synthesize TxA_2.[23] Inhibition of PAF should then have led to a fall in plasma TxB_2, which did not occur.

Other agents, particularly the complement fragment C5a, are not likely to mediate ischemia-induced leukosequestration in the lungs. In a previous study, Tx inhibitors could not prevent PMN accumulations resulting from the intravenous injection of zymosan-activated plasma, a source of C5a. The vasodilating, antiaggregating prostaglandins (PG), such as PG_2, are also not likely to be involved. Levels of 6-keto-$PGF_{1\alpha}$ did not predict PMN accumulations nor did the PG/Tx ratio.

In summary, these results indicate that limb ischemia leads to Tx synthesis and PMN accumulations in the lungs, events that appear to be causally related.

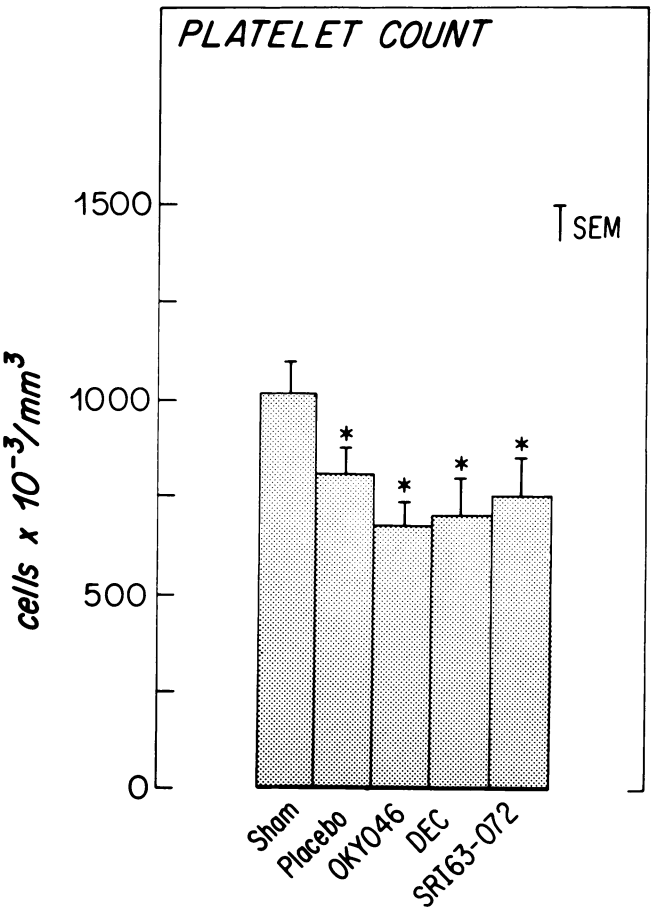

FIG. 6. Platelet counts fell in all ischemic groups. Asterisks refer to significant differences between the sham group and other groups.

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