Little is known about the possible role of leukocytes in the pathogenesis of vasospasm. We hypothesized that vasoactive products released by leukocytes might produce constriction of atherosclerotic arteries. To test this hypothesis, we infused fmet-leu-phe (fMLP), a peptide that activates leukocytes to release their vasoactive products, into the perfused hind limb of normal and atherosclerotic cynomolgus monkeys. Infusion of fMLP did not change resistance of large arteries in normal monkeys. In contrast, fMLP produced pronounced constriction of large arteries in atherosclerotic monkeys. To determine whether leukotrienes, platelet-activating factor, or prostaglandin E\(_2\) (PGE\(_2\)), which are released by leukocytes, may contribute to leukocyte-induced vasoconstriction in atherosclerotic monkeys, we injected leukotriene D\(_4\), platelet-activating factor, and PGE\(_2\) intra-arterially into the perfused hind limb. Leukotriene D\(_4\) and platelet-activating factor had minimal effects on large arteries in both normal and atherosclerotic monkeys. PGE\(_2\) produced marked constriction of large arteries in atherosclerotic, but not normal, monkeys. Thus, pronounced constriction in atherosclerotic, but not normal, arteries during infusion of fMLP suggests that products released by leukocytes may mediate vasoconstriction in atherosclerotic vessels. Vasoconstrictor responses to PGE\(_2\) are profoundly potentiated by atherosclerosis, which suggests that PGE\(_2\) may contribute to leukocyte-induced vasoconstriction. (Circulation Research 1989;65:1078-1086)

Observations in patients\(^1\) and experimental animals\(^2,3\) suggest that vasospasm is most likely to occur when atherosclerosis is present. Mechanisms by which atherosclerosis predisposes arteries to spasm are not clear. Aggregation of platelets, with release of vasoactive products, has been implicated in the pathogenesis of spasm.\(^3,4\) Little is known, however, about a possible role for leukocytes in the pathogenesis of spasm.

Atherosclerotic lesions contain many leukocytes, predominantly monocyte-macrophages, which are attached to the endothelium and are in the vessel wall.\(^5,6\) Monocyte-macrophages release a variety of vasoactive substances, including thromboxane A\(_2\), prostaglandin E\(_2\) (PGE\(_2\)), peptidoleukotrienes, and platelet-activating factor (PAF).\(^7\) Furthermore, there may be decreased production of prostaglandin I\(_2\) (PGI\(_2\)) and increased synthesis of PGE\(_2\) and thromboxane A\(_2\) in atherosclerotic vessels compared with normal vessels.\(^8,9\) Thus, it is possible that leukocytes, either as blood-borne leukocytes or monocyte-macrophages in the vessel wall, may play a role in spasm of atherosclerotic arteries.

The tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) is a potent activator of polymorphonuclear leukocytes\(^10\) and monocyte-macrophages.\(^11,12\) fMLP induces leukocyte degranulation with resultant stimulation of arachidonic acid metabolism through interaction with a specific receptor.\(^13\) A recent study of the isolated perfused rabbit heart demonstrated that injection of fMLP in noninfarcted hearts produced minimal coronary vasoconstriction, but after myocardial infarction, fMLP stimulated the release of thromboxane A\(_2\), PGE\(_2\), and peptidoleukotrienes with resultant coronary vasoconstriction.\(^14\) The response to fMLP was attributed to activation of inflammatory cells that are present in the infarcted hearts.\(^14\)

In this study, we tested the hypothesis that, when leukocytes are activated by fMLP, vasoactive products released by leukocytes may produce constriction in atherosclerotic vessels. Recent work from our laboratory has shown that vasoconstrictor responses to thromboxane A\(_2\), a major product of...
leukocytes, are augmented by atherosclerosis. We also tested the hypothesis that responses to three major products released by activated leukocytes, PGE₂, leukotriene D₂ (LTD₂), and PAF are altered by atherosclerosis in a direction that would favor vasoconstriction.

**Materials and Methods**

Two groups of adult male Malaysian cynomolgus monkeys were studied. Sixteen normal monkeys were fed commercial laboratory chow (Purina monkey chow, Ralston Purina, Richmond, Indiana). Twelve monkeys were fed atherogenic diet, which contained cholesterol (1 mg/c Calorie) and fat (43% of total calories), for 19±0.5 months (mean±SEM). The monkeys weighed 5.4±0.2 kg in the normal group and 6.1±0.2 kg in the atherosclerotic group. At intervals of 3–4 months, the monkeys were sedated with ketamine hydrogen chloride (10 mg/kg i.v.), and a venous blood sample was obtained. A polyethylene catheter was inserted retrogradely to measure pressure. A calibrated pulsatile perfusion pump (model 1210, Harvard Apparatus, South Natick, Massachusetts) was used to perfuse the left iliac artery at constant flow with blood from the abdominal aorta, and iliac perfusion pressure was measured continuously. When the pump was stopped, perfusion pressure decreased rapidly to 10–15 mm Hg, which indicates that vascular isolation was adequate. Baseline perfusion pressure of the hind limb was established by adjusting blood flow so that perfusion pressure was similar to the animal’s mean arterial pressure. The difference between iliac pressure and dorsal pedal pressure at constant flow indicates resistance of large arteries of the limb. The method has been described in detail previously.

We studied the effects of PGE₂, PGI₂, the chemo tactic peptide fMLP, PAF (1-arachidonylethanolamine, β-acetylaop-alkyl), all from Sigma Chemical, St. Louis, Missouri, and LTD₄ (Merck-Frosst Laboratories, Pointe Claire-Dorval, Quebec, Canada).

PGE₂ (3×10⁻¹⁰ and 3×10⁻⁹ mol) and PAF (2×10⁻¹⁰ and 7×10⁻¹⁰ mol) were injected as a 0.1-ml bolus into the iliac perfusion tubing. PGI₂ (1×10⁻⁹ and 3×10⁻⁹ mol/min) and fMLP (10⁻⁹, 10⁻⁷, and 10⁻⁵ mol/min) were infused at 0.1 ml/min for 4 minutes into the iliac perfusion tubing. fMLP was administered after a 20-minute interval between doses. Higher doses of fMLP (>10⁻⁵ mol/min), which produce a systemic response with a decrease in arterial pressure, were not used in the protocol. The maximal response to intra-arterial infusions of 10⁻⁹-10⁻¹ mol/min fMLP was determined. Maximal responses occurred at 10⁻⁹ mol/min in four normal and four atherosclerotic monkeys, at 10⁻⁸ mol/min in one normal and one atherosclerotic monkey, and at 10⁻⁷ mol/min in four normal and five atherosclerotic monkeys. When a vascular response to fMLP occurred, it was rarely possible to elicit a subsequent response at the same or higher dose, even after waiting 20–60 minutes. This phenomenon may be related to the prolonged binding of fMLP to the leukocyte receptor.

**Studies In Vitro**

Isolated vascular ring preparation. The iliac artery contralateral to the limb that was studied in vivo was isolated and excised. Vessels were cut into 5-mm ring segments and were suspended in a vertically oriented organ bath in 25 ml Kreb’s buffer containing (mM) NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, EDTA calcium 0.026, and glucose 11.1, pH 7.40, aerated with 95% O₂-5% CO₂ and maintained at 37° C. All studies were performed in the presence of propranolol (10⁻⁷ M). Tension was recorded with a linear force transducer (model FTO3c, Grass Instruments, Quincy, Massachusetts) or by an oscillographic recorder.

Over a period of 1 hour, the resting tension of the vascular ring was gradually increased until the optimal tension for generating force during isometric contraction was reached. At each tension, the...
vessel was exposed to potassium chloride (100 mM), and the tension was recorded. After each dose of potassium chloride, the baths were washed with fresh buffer. The resting tension was increased until additional doses of potassium chloride failed to increase the constrictor response. The vessels were left at this optimal resting tension throughout the remainder of the study.

**Protocols.** We examined cumulative responses to fMLP (10^{-10}–10^{-4} M) in vascular rings. fMLP was diluted with distilled water so that less than 0.1 ml was added to the bath for each dose. Vessels were preconstricted with a concentration of prostaglandin F_{2a} (Sigma Chemical) that produced 30–50% of a predetermined maximal constrictor response. Between each concentration-response curve, vessels were washed three times with fresh buffer and were allowed to reequilibrate for at least 30 minutes. At the completion of each experiment, the vascular segment was immersed in fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for approximately 5 minutes while still mounted on the isolated-ring apparatus. The vessel was then removed from the apparatus and maintained in fixative at 4°C for at least 24 hours. The vessels were then examined by scanning electron microscopy to confirm the presence of endothelium as described previously.17

**Morphological studies.** The monkeys were killed with intravenous potassium chloride. The iliac and femoral artery vessels were removed, examined for gross lesions, and fixed by immersion in 10% buffered formalin. Specimens were taken from the proximal iliac artery for histological study of paraffin-embedded transverse sections. Sections were stained with hematoxylin-eosin and Verhoeff-van Gieson's stain. Morphometric determination of the size of the intima and media was performed with an image analyzer, as described previously.16

**Statistical analysis.** Mean values were analyzed with ANOVA general linear models procedure from SAS (SAS Institute, Inc., Cary, North Carolina). Comparisons of morphometric data were determined by the use of nonparametric Wilcoxon rank-sum test. Student's t test for unpaired groups was used for analysis of baseline hemodynamics, leukocyte count, and maximal fMLP responses. In the experiments with isolated vessels, Student's t test for unpaired groups was used to compare concentration-
response curves at each concentration of drug. Statistical significance was considered as \( p < 0.05 \).

**Results**

**Plasma Lipids and Leukocyte Count**

Plasma total cholesterol was 114±8 mg/dl (mean±SEM) in normal monkeys and 567±40 mg/dl in atherosclerotic monkeys. Plasma triglycerides were less than 40 mg/dl in both groups.

Total peripheral leukocyte blood count was 10,800±1,200/mm³ (mean±SEM) in normal monkeys and 11,600±4,700/mm³ in atherosclerotic monkeys. The differential leukocyte count was similar in normal and atherosclerotic monkeys.

**Morphologic Changes**

In atherosclerotic monkeys, morphologic changes were similar to those described previously. There was dense fibrofatty intimal thickening with focal intimal necrosis of the iliac arteries and the proximal part of the femoral arteries. Intimal thickening in the midportion of the femoral arteries consisted largely of fatty streak lesions. Areas of intimal thickening were noted to contain many monocyte-macrophages (Figure 1).

Morphometry demonstrated increases in intimal area in the iliac and femoral arteries in atherosclerotic monkeys. Intimal area of the iliac artery was 0.02±0.01 mm² in normal monkeys and 1.57±0.36 mm² in atherosclerotic monkeys (\( p < 0.05 \) vs. normal monkeys). Intimal area of the femoral artery was 0.01±0.01 mm² in normal monkeys and 1.55±0.31 mm² in atherosclerotic monkeys (\( p < 0.05 \) vs. normal monkeys). Medial area was not significantly different between groups.

**Hemodynamic Studies**

**Baseline values.** Large-artery resistance was significantly greater in atherosclerotic than in normal monkeys (Table 1).

**Responses to fMLP.** The chemotactic peptide fMLP had minimal effects in the limb of normal monkeys (Figure 2, left panel). In atherosclerotic monkeys, fMLP produced vasoconstriction (\( p < 0.05 \) vs. normal monkeys; Figures 2 and 3).

There was minimal constriction of the large-artery segment in response to fMLP in normal monkeys (Figure 2, right panel). In contrast, there was marked constriction in atherosclerotic monkeys (\( p < 0.05 \) vs. normal monkeys). The vasoconstrictor response to fMLP was prolonged (usually 20–30 minutes) in atherosclerotic monkeys.

**Responses to LTD₄.** LTD₄ produced vasodilatation in the limb of normal monkeys (Figure 4). The dilator response to LTD₄ was impaired in atherosclerotic monkeys (\( p < 0.05 \) vs. normal monkeys). LTD₄ did not alter resistance of the large-artery segment in normal or atherosclerotic monkeys (data not included), which indicates that the dilator response to LTD₄ occurs in small vessels.

**Responses to PGE₂.** PGE₂ produced vasodilatation in the limb of normal monkeys (Figure 5 and left panel of Figure 6). The dilator response to PGE₂ was impaired in atherosclerotic monkeys (\( p < 0.05 \) vs. normal monkeys) (Figures 6 and 7). PGE₂ produced minimal constriction of the large-artery segment in the limb of normal monkeys (Figure 6, right panel). There was striking potentiation of constrictor responses of the large artery segment in atherosclerotic monkeys (\( p < 0.05 \) vs. normal monkeys) (Figure 6).

**Responses to PGI₂.** To test the specificity of impaired vasodilator responses to PGE₂ and LTD₄,
effects of PGI₂ were examined. Vasodilator responses to PGI₂ in the limb were not significantly different in normal and atherosclerotic monkeys (Figure 8). PGI₂ had minimal effects on large arteries of the limb in both groups (data not included).

Responses to PAF. PAF produced vasodilatation in the limb (reduction in iliac perfusion pressure) in normal and atherosclerotic monkeys (Figure 9). PAF had minimal effects on large arteries of the limb in both groups (data not included).

Responses in Isolated Vessels

Constrictor responses to potassium chloride (100 mM) were greater in normal vessels (3.6±0.5 g) than in atherosclerotic vessels (1.8±0.3 g, p<0.05). Peak responses to prostaglandin F₂α also were greater in normal vessels (4.3±0.9 g) than in atherosclerotic vessels (2.1±0.3 g, p<0.05).

Responses to fMLP. fMLP had minimal effect in iliac arteries from normal and atherosclerotic monkeys. In eight normal monkeys, there was no response to fMLP at 10⁻¹⁰⁻¹⁰⁻⁷ M. Constrictor responses of 9±6% (mean±SEM) were seen at 10⁻⁶ M, 24±14% were seen at 10⁻⁵ M, and 1±1% were seen at 10⁻⁴ M. fMLP 10⁻⁵ M produced profound constrictor responses in two of the eight normal monkeys with no response in the other six monkeys. In 12 atherosclerotic monkeys, there was no response to fMLP at each concentration tested between 10⁻¹⁰⁻¹⁰⁻⁴ M.

Discussion

These data suggest, first, that atherosclerosis affects vascular responses to activation of leukocytes in vivo. fMLP, a peptide which activates leukocytes to release their vasoactive products, produced marked constriction in large arteries in atherosclerotic, but not normal, monkeys. Second, responses to the vasoactive products produced by leukocytes were altered by atherosclerosis: vasoconstrictor responses to PGE₂ are potentiated, and vasodilator responses to LTD₄ are impaired. Third, LTD₄ produces dilatation of small vessels but not large arteries. Impairment of dilator responses to LTD₄ by atherosclerosis provides evidence that atherosclerosis impairs responses of small vessels,
even though small vessels do not develop atherosclerotic lesions.

Importance of Leukocytes

Formation of atherosclerotic lesions is characterized by adherence of blood-borne monocytes to the endothelium. As atherosclerotic lesions develop, cellular components consist primarily of monocyte-macrophages. When activated, these cells can release a variety of vasoactive substances, including thromboxane A$_2$, PGE$_2$, peptidoleukotrienes, and PAF.

To determine whether leukocytes can initiate vascular responses in the limb, we infused fMLP. fMLP is a peptide that activates polymorphonuclear leukocytes and monocyte macrophages by interaction with a specific receptor. Smooth muscle cells, fibroblasts, and epithelial cells do not have receptors for fMLP. Although endothelial cells have receptors for fMLP, receptor binding is involved in membrane transport rather than activation of specific metabolic pathways. Thus, it appears that fMLP may be used to specifically activate leukocytes without direct effects on vascular smooth muscle.

In a previous study, effects of fMLP were examined in rabbits with left ventricular infarction and were followed by ex vivo cardiac perfusion. fMLP released thromboxane, peptidoleukotrienes, and PGE$_2$ and produced coronary vasoconstriction in infarcted, but not normal, hearts. The increased synthesis of prostaglandins in infarcted hearts was temporally associated with the influx of inflammatory cells into the infarcted tissue. The predominant inflammatory cell types seen by histological analysis were polymorphonuclear leukocytes and macrophages. The authors proposed that coronary vasoconstrictor responses to fMLP were mediated at least in part by metabolites of arachidonic acid released from activated inflammatory cells.

Constrictor responses of large arteries during infusion of fMLP in the limb of atherosclerotic monkeys suggest that activation of leukocytes, with release of vasoactive substances, played an important role in the response to fMLP. In this blood-perfused system, fMLP may activate leukocytes that are circulating in the blood, attached to the endothelium, or present in the vessel wall. In a recent study in normal rabbits, fMLP produced coronary vasoconstriction in blood-perfused hearts in vivo and had no effect in the isolated Krebs-Henseleit-perfused heart. This finding suggests that circulating cellular elements mediate vasocon-
Stricter responses to fMLP. Our finding that fMLP has little effect in isolated vessels from atherosclerotic monkeys in vitro also suggests that circulating cellular elements may be of greater importance in the response to fMLP than monocyte-macrophages in the vessel wall.

**Mechanisms of Altered Vascular Responses**

Substances that are released by leukocytes and thus are possible mediators of leukocyte-induced vasoconstrictor responses to fMLP in atherosclerotic monkeys include thromboxane A₂, PGE₂, peptide leukotrienes, platelet-derived growth factor, and hydroxylated eicosatetraenoic acids.

Vasoconstrictor responses to the thromboxane A₂ analogue U46619 are augmented by atherosclerosis. Thus, release of thromboxane A₂ may contribute to leukocyte-induced vasoconstriction.

PGE₂ is a potent vasodilator in the hind limb of rabbits and dogs, but it produces vasoconstriction in isolated bovine, canine, and human coronary arteries. Vascular responses to PGE₂ may be modulated by endothelium since removal of endothelium augments the vasoconstrictor response to PGE₂ in canine basilar arteries. In the present study, vasoconstrictor responses to PG₁₉, which are not endothelium dependent, were preserved in atherosclerotic monkeys. Atherosclerosis produces a functional defect in endothelium-dependent relaxation. It is possible that impairment of endothelium-dependent relaxation by atherosclerosis may contribute to augmentation of vasoconstrictor responses to PGE₂. Thus, PGE₂ may contribute to leukocyte-induced vasoconstriction in atherosclerotic vessels.

Platelets have been implicated in the pathophysiology of vasoospasm. It is unlikely that vascular responses to PGE₂ and fMLP were due to activation of platelets since PGE₂ and fMLP do not appear to produce platelet aggregation. This is possible, however, that activation of leukocytes by fMLP may elicit aggregation of platelets, possibly through release of PAF or thromboxane A₂. This may result in the release of the platelet products thromboxane A₂, serotonin, and adenosine diphosphate. Vascular responses to these agonists are altered by atherosclerosis in a direction that would favor vasoconstriction when platelets aggregate. PAF, which is released by leukocytes, produced vasodilatation in the limb. Thus, PAF does not contribute to fMLP-induced vasoconstriction. The vasoconstrictor response to PAF was similar in normal and atherosclerotic monkeys. This finding is not unexpected, despite impairment of endothelium-dependent relaxation by atherosclerosis because relaxation in response to PAF is not endothelium dependent, except at very high doses.

Vascular responses to LTD₄ in the hind limb have not been evaluated previously in vivo. Studies in vitro indicate that LTD₄ produces coronary vasoconstriction in several species. Recent evidence suggests that vascular responses to leukotrienes are modulated by the endothelium. It is likely that endothelial dysfunction may play an important role in impaired vasodilator responses to LTD₄ in atherosclerotic monkeys.

We have examined separately the vascular responses to several mediators that are released when leukocytes are activated. It is possible that simultaneous release of these vasoactive agents could produce a synergistic response. Thus, sub-threshold doses of individual mediators may have minimal effect, but the simultaneous release of small concentrations of multiple mediators may produce profound vasoconstriction.

Recent evidence suggests that hypercholesterolemia augments macrophage function with enhanced release of products. It is possible that responses to fMLP in atherosclerotic monkeys may be related to enhanced release of vascular products as well as altered responses to these products.

**Implications**

Arterial spasm or enhanced vasoconstriction has a predilection for sites at which atherosclerosis is present. Atherosclerotic lesions contain many leukocytes, which are predominantly monocyte-macrophages attached to the endothelium and within the vessel wall. Our findings indicate that activation of leukocytes, either as blood-borne leukocytes or monocyte-macrophages in the vessel wall, produces pronounced vasoconstriction in atherosclerotic vessels. Leukocyte-induced vasoconstriction may be mediated by thromboxane A₂ and PGE₂, or could be the result of a synergistic response to several vasoactive products. We propose that altered responses to activation of leukocytes may contribute to the susceptibility to vasoospasm that is characteristic of atherosclerosis.

**Acknowledgments**

We thank Dr. Robert Schelper for preparation of the photomicrograph; Marjorie Megan, Pamela Tomkins, Beth Patel, Barry Waack, and Kristin Orgren for expert technical assistance; Dr. Bridget Zimmerman for assistance with statistical analyses;
References


**KEY WORDS** • leukocytes • prostaglandin E₂ • leukotriene D₄ • peripheral circulation • cynomolgus monkeys
Vascular responses to leukocyte products in atherosclerotic primates.
J A Lopez, M L Armstrong, D G Harrison, D J Piegors and D D Heistad

Circ Res. 1989;65:1078-1086
doi: 10.1161/01.RES.65.4.1078

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/65/4/1078