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COLCHICINE AND METHOTREXATE REDUCE LEUKOCYTE ADHERENCE AND EMIGRATION IN RAT MESENTERIC VENULES[1]

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Abstract--Colchicine and methotrexate are commonly used in the treatment of gout and rheumatoid arthritis, respectively; however the mechanism(s) of action of these drugs remain(s) unknown. The objective of this study was to determine whether colchicine and methotrexate can modify the adhesion and emigration of leukocytes in postcapillary venules that are exposed to inflammatory mediators such as platelet-activating factor (PAF) and leukotriene B_4 (LTB_4). The rat mesentery was prepared for in vivo microscopic observation. Venules with internal diameters ranging between 25 and 35 μm were selected for study. Erythrocyte velocity, vessel diameter, leukocyte rolling velocity, and the number of adherent (stationary for ≥ 30 sec) and emigrated leukocytes were measured during superfusion of the mesentery with bicarbonate-buffered saline (BBS). Repeat measurements of adhesive and hemodynamic parameters were obtained between 50 and 60 min of superfusion with either 100 nM PAF or 20 nM LTB_4 added to the superfusate. In some experiments, 1 μM of either colchicine or methotrexate was added to the superfusate containing either PAF or LTB_4. Both PAF and LTB_4 caused increases in leukocyte adherence and emigration and reductions in leukocyte rolling velocity and venular shear rate. Colchicine effectively prevented all of the adhesive and hemodynamic alterations induced by both inflammatory mediators, while methotrexate was largely effective in preventing the responses elicited by PAF, but not LTB_4. These results indicate that the therapeutic actions of colchicine and methotrexate may result from the ability of these agents to interfere with the adhesion and emigration of leukocytes from postcapillary venules.

INTRODUCTION

Inflammation is a common feature of numerous rheumatic disorders, including gout and rheumatoid arthritis. These disorders are characterized by an accu-

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mulation and subsequent activation of polymorphonuclear leukocytes in synovial fluid. Neutrophils are drawn from the circulation into the joint cavity by directed migration (chemotaxis) of the phagocytic cells to chemoattractants (e.g., leukotriene B_4, platelet-activating factor, and C5a) that are produced and released into synovial fluid (1). These chemoattractants also elicit the expression of adhesion glycoproteins on the surface of neutrophils, thereby allowing the cells to adhere and emigrate from postcapillary venules (2, 3). Once activated, the neutrophils release proteinases and oxygen-derived free radicals, which contribute to joint destruction (1).

The recognition that neutrophils play a central role in the pathogenesis of inflammatory articular syndromes has led to an interest in identifying therapeutic interventions that interfere with the cascade of events that ultimately result in the recruitment and activation of neutrophils in the joint. A number of drugs currently used for the treatment of articular syndromes appear to act by interfering with the ability of the neutrophil to either migrate toward chemoattractants, phagocytose, degranulate, and/or produce superoxide (4). Colchicine has been used in the treatment of gouty arthritis for centuries, while methotrexate has only recently been used to treat patients with rheumatoid arthritis. Although colchicine and methotrexate have antiinflammatory activity, the specific mechanisms underlying the beneficial actions of these drugs remain undefined. A common action shared by colchicine and methotrexate is inhibition of leukocyte chemotaxis in vitro (5–11). The ability of these agents to suppress leukocyte chemotaxis may result from an effect on microtubular function, membrane receptor function, and/or the expression of surface adhesive glycoproteins. The results of a recent study indicate that methotrexate significantly attenuates neutrophil adhesion to monolayers of cultured endothelial cells (12). The possibility that colchicine and methotrexate limit the inflammatory response by interfering with leukocyte adherence in vivo has not been addressed experimentally, despite the fact that the adhesive interactions between leukocytes and endothelial cells in postcapillary venules is a rate-limiting step in the accumulation of neutrophils in synovial fluid. In this study, we examine the influence of both drugs on the ability of leukocytes to adhere and emigrate from postcapillary venules exposed to either leukotriene B_4 (LTB_4) or platelet-activating factor (PAF), chemoattractants that have been implicated in the neutrophil accumulation associated with rheumatoid arthritis (1).

MATERIALS AND METHODS

Surgical Procedure. Forty male Sprague-Dawley rats (180–220 g) were maintained on a purified laboratory diet and fasted for 24 h prior to the experiment. The animals were initially anesthetized with thiobarbital (12 mg/100 g body weight); then a tracheotomy was performed to

facilitate breathing during the experiment. The right carotid artery was cannulated and systemic arterial pressure was measured with a Statham (Oxnard, California) P23A pressure transducer connected to the carotid artery cannula. Systemic blood pressure and heart rate were continuously recorded with a Grass physiologic recorder (Grass Instruments, Massachusetts). A midline abdominal incision was made and a segment of the midjejunum was exteriorized through the abdominal incision. All exposed tissue was moistened with saline-soaked gauze to minimize evaporation and tissue dehydration.

Intravital Microscopy. Rats were placed in a supine position on an adjustable Plexiglas microscope stage and the mesentery was prepared for microscopic observation as described previously (13). The mesentery was draped over an optically clear viewing pedestal that allowed for transillumination of a 2-cm^2 segment of tissue. The temperature of the pedestal was maintained at 37°C with a constant temperature circulator (Fisher Scientific, model 80). Rectal and mesenteric temperatures were continuously monitored using an electrothermometer. The exposed bowel wall and mesentery were covered with Saran Wrap (Dow Chemical Co., Indiana); then the mesentery was suffused with warmed bicarbonate-buffered saline (pH 7.4) that was bubbled with a mixture of 5% O_2–5% CO_2–90% N_2. An intravital microscope (Nikon Optiphoto, Japan) with a ×40 objective lens (Leitz Wetzlar L20/0.32, Germany) and a ×15 eyepiece was used to observe the mesenteric microcirculation. The mesentery was transilluminated with a 12-V, 50-W direct current-stabilized light source. A video camera (Hitachi, VK-C150, Japan) mounted on the microscope projected the image onto a color monitor (Sony, PVM-2030, Japan), and the images were recorded using a video cassette recorder (Panasonic, NV8950, Japan). A video time–date generator (Panasonic, WJ810) projected the time, date, and stopwatch function onto the monitor.

Single unbranched venules with diameters ranging between 25 and 35 μm and a length > 150 μm were selected for study. Venular diameter (D_v) was measured either on- or off-line using a video image-shearing monitor (IPM, LaMesa, California). The number of adherent leukocytes was determined off-line during playback of videotaped images. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period equal to or greater than 30 sec (13). Adherent cells were expressed as the number per 100 μm length of venule. The number of emigrated leukocytes was also determined off-line during playback of videotaped images. Any interstitial leukocytes present in the mesentery at the onset of the experiment were subtracted from the total number of leukocytes that accumulated during the course of the experiment. Leukocyte emigration was expressed as the number per microscopic field (1.7×10^{-2} mm^2 or 150 μm length of venule). Rolling leukocytes were defined as those white blood cells that moved at a velocity less than that of erythrocytes in the same stream. Leukocyte rolling velocity was determined from the time required for a leukocyte to traverse a given distance along the length of the venule.

Centerline red blood cell velocity (V_{RBC}) was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, Texas) that was calibrated against a rotating glass disk coated with red blood cells. Venular blood flow was calculated from the product of mean red blood cell velocity (V_{mean} = centerline velocity ÷ 1.6) (14) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (τ) was calculated based on Newtonian definition: $\tau = 8(V_{mean}/D_v)$ (15).

Experimental Protocols. After all parameters measured on-line (arterial pressure, erythrocyte velocity, venular diameter) were in a steady state, images from the mesenteric preparation were recorded on videotape for 10 min. Immediately thereafter, the mesentery was superfused with either 100 nM PAF (Sigma Chemical, St. Louis, Missouri) or 20 nM LTB_4 (G.D. Searle Research, Skokie, Illinois) dissolved in bicarbonate-buffered saline. The mesentery was superfused with PAF or LTB_4 for 60 min, with video recordings and repeat measurements of all parameters made during the final 10 min. The control responses to PAF ($N = 8$) or LTB_4 ($N = 6$) were determined in 14 rats. In additional experiments, either colchicine or methotrexate (both obtained from Sigma) was added to the PAF- or LTB_4-containing bicarbonate-buffered saline to attain a final concentration of

1 μM, a level normally detected in patients receiving methotrexate (16). The same protocol was used as described above, i.e., baseline measurements followed by superfusion with the experimental solution for 60 min, with repeat measurements taken between 50 and 60 min.

Flow Cytometry Studies. Human neutrophils were separated from whole blood using gradient separation on Histopaque-1077 (Sigma) and dextran sedimentation as previously described (17). This procedure yields a neutrophil population that is 98% viable (propidium iodide) and 95–98% pure. The freshly isolated neutrophils were incubated for 20 min with 10^{-7} M (maximal effective dose) of either PAF or LTB_4 in the presence or absence of 1 μM colchicine or methotrexate. Flow cytometric (FCM) analysis of CD11/CD18 expression by neutrophils was performed on an EPICS 753 flow cytometer/sorter (Coulter Electronics, Hialeah, Florida) for the simultaneous accumulation of immunofluorescence in addition to forward angle and 90° light scatter signals. Dead cells and debris were excluded by forward angle and 90° light scatter gating or, in some experiments, by the exclusion of dead cells that incorporate propidium iodide (18). Cell preparations were stained as described elsewhere (19) using anti-CD18 mAb (IB_4) as a primary reagent (20) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Southern Biothech, Birmingham, Alabama) as the secondary reagent. Controls included cells stained with the secondary reagent alone, or cells stained with an irrelevant-isotype-matched control mAb. Twenty-five thousand cells were analyzed in each experiment and each experiment was repeated three times.

Statistics. The data were analyzed using standard statistical analyses, i.e., analysis of variance with the Scheffe's (posthoc) test. All values are reported as mean $\pm$ standard error. Statistical significance was set at $P < 0.05$.

RESULTS

Figure 1 summarizes the responses of leukocyte adherence in rat mesenteric venules to superfusion with either PAF or LTB_4 in the presence or absence of colchicine or methotrexate. Under baseline conditions, the number of adherent leukocytes (per 100 μm length of venule) was 3.9 $\pm$ 0.4. Superfusion for 60 min with PAF and LTB_4 significantly increased the number of adherent leukocytes to 29.5 $\pm$ 4.7 and 39.3 $\pm$ 7.7 per 100 μm venule length, respectively. The addition of colchicine to the superfusion solution significantly attenuated leukocyte adherence responses to PAF (13.6 $\pm$ 3.7) and LTB_4 (5.5 $\pm$ 1.7). Methotrexate was also effective in attenuating the leukocyte adherence response to PAF (9.3 $\pm$ 2.4), however, it did not blunt the adhesion response to LTB_4 (56 $\pm$ 8.4).

Under baseline conditions (Figure 2), the number of emigrated leukocytes per microscopic field was 3.2 $\pm$ 0.6. Increases in the number of emigrated leukocytes were noted 60 min after superfusion with either PAF (19.3 $\pm$ 2.8) or LTB_4 (11.7 $\pm$ 2.9). Colchicine significantly attenuated the leukocyte emigration elicited by PAF (11.6 $\pm$ 2.8) and LTB_4 (1.7 $\pm$ 0.6). Methotrexate significantly inhibited the emigration response to PAF (5.3 $\pm$ 2.6) but not to LTB_4 (16.3 $\pm$ 7.9).

Figure 3 presents the responses of leukocyte rolling velocity to PAF and

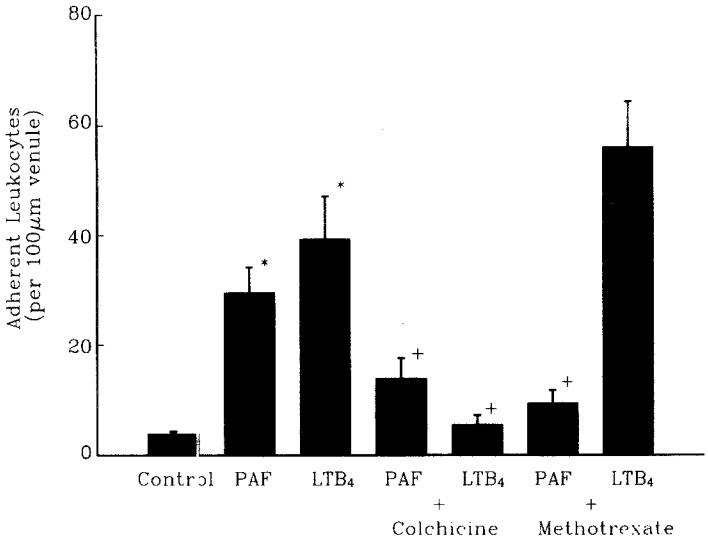

Fig. 1. Effects of colchicine and methotrexate on platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) -induced leukocyte adherence in rat mesenteric venules. Asterisk denotes $P < 0.05$ relative to control value; a plus indicates $P < 0.05$ relative to corresponding inflammatory mediator.

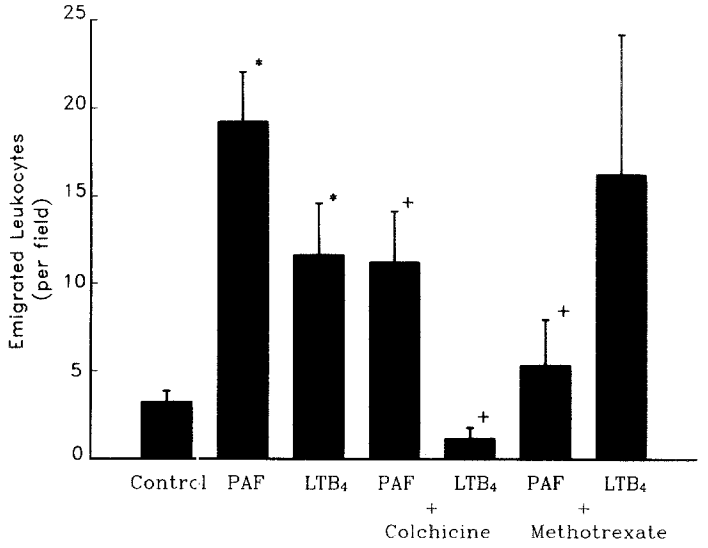

Fig. 2. Effects of colchicine and methotrexate on platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) -induced leukocyte emigration in rat mesenteric venules. Asterisk denotes $P < 0.05$ relative to control value; a plus indicates $P < 0.05$ relative to corresponding inflammatory mediator.

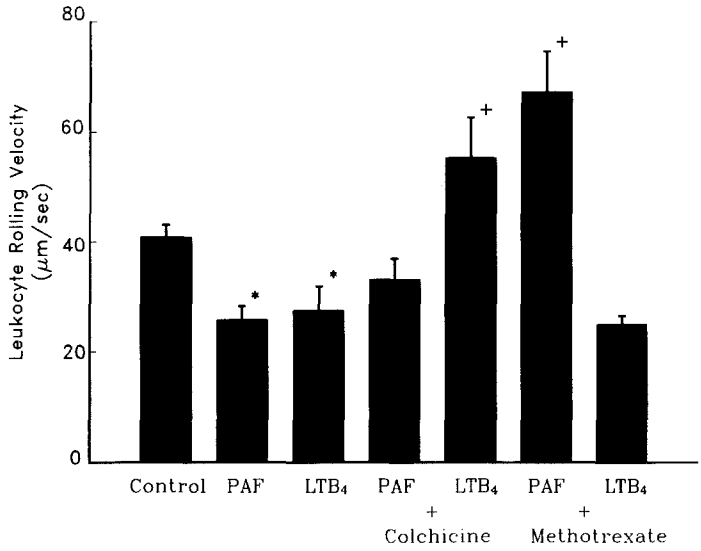

Fig. 3. Effects of colchicine and methotrexate on platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) -induced alterations in leukocyte rolling velocity in rat mesenteric venules. Asterisk denotes $P < 0.05$ relative to control value; a plus indicates $P < 0.05$ relative to corresponding inflammatory mediator.

LTB_4 in the presence and absence of either colchicine or methotrexate. Leukocyte rolling velocity in rat mesenteric venules under control condition was 40.9 ± 2.2 μm/sec, a value similar to that previously reported for cat mesenteric venules (12). Leukocyte rolling velocity was significantly reduced by superfusion of the mesentery with either PAF (25.9 ± 2.5 μm/sec) or LTB_4 (27.6 ± 4.3 μm/sec). The PAF-induced reduction in leukocyte rolling velocity was blunted by colchicine (33.2 ± 3.8 μm/sec) and prevented entirely by methotrexate (67.5 ± 7.4 μm/sec). The LTB_4-induced reduction in leukocyte rolling velocity was prevented by colchicine (55.4 ± 7.3 μm/sec), yet it was unaffected by methotrexate (25.1 ± 1.6 μm/sec).

Figure 4 summarizes the changes in venular wall shear rate induced by PAF and LTB_4 in the presence or absence of either colchicine or methotrexate. Venular shear rate in the control period was 502 ± 41/sec. Shear rates tended to fall during superfusion with either PAF (301 ± 62/sec) or LTB_4 (336 ± 40/sec); however, the reduction observed with LTB_4 did not reach statistical significance ($P = 0.12$). The shear rates measured during superfusion with colchicine were not significantly different from baseline either in the presence of PAF (448 ± 80/sec) or LTB_4 (502 ± 106/sec). Methotrexate increased wall shear rate above control values in the presence of PAF (665 ± 70/sec), but the change was not statistically significant ($P = 0.13$). Methotrexate did not affect

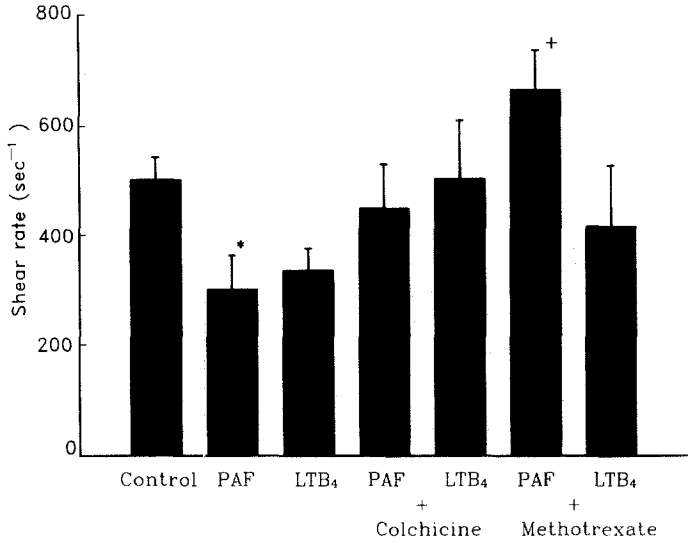

Fig. 4. Effects of colchicine and methotrexate on platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) -induced alterations in venular shear rate. Asterisk denotes $P < 0.05$ relative to control value; a plus indicates $P < 0.05$ relative to corresponding inflammatory mediator.

shear rate in the presence of LTB_4 (415 $\pm$ 112/sec). The changes in shear rate observed in all experiments resulted from corresponding changes in erythrocyte velocity; venular diameter did not differ from baseline (30 $\pm$ 0.5 μm) in mesenteric preparations superfused with either PAF or LTB_4 in the presence of either colchicine or methotrexate.

When isolated neutrophils were exposed to either PAF or LTB_4, agents known to up-regulate the leukocyte adhesion molecule CD11/CD18 (3), an increased fluorescence intensity was detected by flow cytometry. The increase in fluorescence intensity induced by PAF (30%) was not modified by either colchicine (28%) or methotrexate (34%). The responses to LTB_4 (14%) were also unaffected by colchicine (10%) and methotrexate (12%). These results indicate that neither colchicine nor methotrexate alter the up-regulation of CD11/CD18 on neutrophils elicited by PAF and LTB_4.

DISCUSSION

Leukocyte accumulation in synovial fluid is a characteristic response of articular inflammatory disorders such as gout and rheumatoid arthritis (1, 4). The egress of leukocytes from blood into the synovial cavity requires that the leukocyte first make intimate contact with endothelial cells lining postcapillary

venules. The leukocyte-endothelial cell adhesive interactions that occur during inflammation include: leukocyte rolling, adherence, diapedesis, and extravascular migration (2, 21). Leukocyte adhesion begins as a rolling movement along the endothelium in which the leukocyte moves slower than erythrocytes in the same stream of blood. As inflammation progresses, the number of rolling leukocytes increases and leukocyte rolling velocity decreases. As a consequence of these changes, the number of rolling leukocytes that become stationary (adherent) increases and a significant proportion of adherent leukocytes migrate out of the vessel into the adjacent tissue compartment (interstitium or fluid cavity). Although the aforementioned sequence of events was first described over 150 years ago (2), only recently has it been recognized that leukocyte adhesion to microvascular endothelium is: (1) a rate-limiting step in the overall process of inflammation (3, 21), (2) a well-controlled physiological phenomenon that is influenced by both physical and chemical forces acting on leukocyte and endothelial cell surfaces (15, 21), and (3) a realistic target for therapeutic intervention in inflammatory disorders (2).

The results of this study indicate that the beneficial actions of colchicine and methotrexate in articular inflammation may be related, at least in part, to an ability of these agents to interfere with leukocyte adherence and emigration in postcapillary venules. In our studies, two well-characterized lipid mediators (PAF and LTB_4) were used to elicit the leukocyte-endothelial cell adhesive interactions that embody the inflammatory response. Abluminal perfusion of mesenteric venules with either PAF or LTB_4 resulted in a significant reduction in leukocyte rolling velocity and increases in the number of adherent and emigrated leukocytes. Colchicine tended to blunt all of these adhesive interactions; however, the beneficial effects of this agent were most pronounced in the presence of LTB_4. A similar mediator-related effectiveness was noted with methotrexate, which exhibited profound antiadhesive actions in the presence of PAF but not with LTB_4.

The mechanisms by which colchicine and methotrexate attenuate leukocyte–endothelial cell adhesive interactions in vivo remain unclear; however, there are five possible mechanisms that warrant brief consideration: (1) altered membrane receptor function, (2) suppression of mediator-induced up-regulation of leukocyte adhesion glycoproteins, (3) inhibition of superoxide-mediated leukocyte adhesion, (4) production of a metabolite that exhibits antiadhesive properties, and (5) attenuation of shear rate-mediated leukocyte adhesion. Our observation that the effectiveness of colchicine and methotrexate in attenuating leukocyte-endothelial cell adhesive interactions is mediator-related suggests that the drugs may act on specific neutrophil receptors such that the cells are desensitized to either LTB_4 or PAF. Suarez et al. (9) have already proposed that methotrexate may inhibit neutrophil chemotaxis in vivo by reducing the affinity of cell surface receptors for the ligand, LTB_4 or C5a. However, our own obser-

vations with methotrexate would suggest that it does not alter the leukocyte adhesion responses normally elicited by LTB_4 (Figures 1–3).

It is now well recognized that adhesive glycoproteins expressed on the surface of both leukocytes and endothelial cells play a critical role in modulating the adhesive interactions between these two cells (2, 3). Although there is low-level constitutive expression of the adhesive glycoprotein complex CD11/CD18 on circulating leukocytes, inflammatory mediators such as PAF and LTB_4 are known to elicit an increased expression of this adhesion molecule in isolated neutrophils (3). Up-regulation of CD11/CD18 is accomplished by receptor-mediated mobilization of secondary (adhesive glycoprotein-containing) granules to the cell surface (21). Since the translocation of CD11/CD18-containing granules to the neutrophil surface may require an intact microtubular system, it is conceivable that microtubule disruptive agents such as colchicine may interfere with the adhesion process by limiting the ability of neutrophils to up-regulate CD11/CD18 in response to PAF and LTB_4. However, our in vitro studies using flow cytometry to quantitate CD11/CD18 up-regulation indicate that neither colchicine nor methotrexate influences the ability of PAF or LTB_4 to mobilize CD11/CD18 to the neutrophil surface.

There is a growing body of evidence that indicates that reactive oxygen metabolites (superoxide and hydrogen peroxide) produced by activated neutrophils promote leukocyte adhesion to cultured endothelial cells (17, 23, 24) and in postcapillary venules (13, 17, 25). Both PAF and LTB_4 are known elicit a respiratory burst from isolated neutrophils, and there is evidence in the literature that indicates that both methotrexate and colchicine inhibit inflammatory mediator-induced superoxide generation by isolated neutrophils (26–28). Thus, it is conceivable that methotrexate and colchicine attenuate leukocyte-endothelial cell adhesive interactions by limiting the production of superoxide and hydrogen peroxide.

In a recent report by Cronstein et al. (12), it was demonstrated that methotrexate attenuates neutrophil adherence to human dermal fibroblasts and umbilical vein endothelial cells. The antiadhesive effects of methotrexate were related to its ability to increase the release of adenosine from fibroblasts and endothelial cells. These investigators have previously demonstrated that adenosine interferes with neutrophil adherence to endothelial cell monolayers (29). Similarly, we have reported that adenosine significantly reduces the increased leukocyte adherence and emigration observed in mesenteric venules exposed to ischemia and reperfusion (30), a process that appears to be mediated by platelet-activating factor (31). It is possible, therefore, that methotrexate administration may provide an effective means for attenuating the acute inflammatory response elicited by ischemia–reperfusion.

The shear forces generated by the movement of a viscous fluid (blood) through microvessels also play an important role in the modulation of leuko-

cyte–endothelial cell adhesive interactions (15). In order for a leukocyte to remain stationary within the vasculature it must bind to endothelium with sufficient strength to withstand the shear rates and associated forces acting to sweep it away. When the shear rate in postcapillary venules is reduced in a graded fashion, the number of adherent leukocytes increases progressively, even in the absence of an inflammatory stimulus (32). In this study, we noted that the increased leukocyte adherence induced by either PAF or LTB_4 was associated with corresponding reductions in venular wall shear rate. Colchicine and methotrexate largely prevented the reductions in shear rate induced by PAF and LTB_4 (Figure 4). While it is tempting to speculate that the antiadhesive effects of colchicine and methotrexate are due to elevated shear rates, the magnitude of the adhesive responses elicited by PAF (29.5 ± 4.7 leukocytes/100 μm vessel length) and LTB_4 (39.3 ± 7.7/100 μm vessel length) in this study are not likely explained by the $<50\%$ reduction in shear rate. Previous studies on mesenteric venules indicate that only 6 ± 1 adherent leukocytes per 100 μm vessel length are observed when shear rate is reduced to <250/sec (32). Thus, any increases in shear rate induced by colchicine or methotrexate would be expected to exert a relatively small effect on PAF- and LTB_4-induced leukocyte adhesion.

In conclusion, the results of this study indicate that both colchicine and methotrexate can significantly attenuate the adhesive interactions between leukocytes and endothelial cells in postcapillary venules during acute inflammation. Our data suggest that modulation of leukocyte adhesion is a novel mechanism of action that should be considered for therapeutic agents used in the treatment of inflammatory joint diseases.

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