Fractalkine and CX₃CR1 Mediate a Novel Mechanism of Leukocyte Capture, Firm Adhesion, and Activation under Physiologic Flow

By Alan M. Fong,* Lisa A. Robinson,‡§ Douglas A. Steeber,‡ Thomas F. Tedder,‡ Osamu Yoshie,‖ Toshio Imai,‖ and Dhavalkumar D. Patel∗‡

From the ∗Department of Medicine, the ‡Department of Immunology, and the §Department of Pediatrics, Duke University Medical Center, Durham, North Carolina 27710; and the ‖Shionogi Institute for Medical Science, Settsu 566, Japan

Summary

Leukocyte migration into sites of inflammation involves multiple molecular interactions between leukocytes and vascular endothelial cells, mediating sequential leukocyte capture, rolling, and firm adhesion. In this study, we tested the role of molecular interactions between fractalkine (FKN), a transmembrane mucin-chemokine hybrid molecule expressed on activated endothelium, and its receptor (CX₃CR1) in leukocyte capture, firm adhesion, and activation under physiologic flow conditions. Immobilized FKN fusion proteins captured resting peripheral blood mononuclear cells at physiologic wall shear stresses and induced firm adhesion of resting monocytes, resting and interleukin (IL)-2–activated CD8⁺ T lymphocytes and IL-2–activated NK cells. FKN also induced cell shape change in firmly adherent monocytes and IL-2–activated lymphocytes. CX₃CR1-transfected K562 cells, but not control K562 cells, firmly adhered to FKN-expressing ECV-304 cells (ECV-FKN) and tumor necrosis factor α–activated human umbilical vein endothelial cells. This firm adhesion was not inhibited by pertussis toxin, EDTA/EGTA, or antiintegrin antibodies, indicating that the firm adhesion was integrin independent. In summary, FKN mediated the rapid capture, integrin-independent firm adhesion, and activation of circulating leukocytes under flow. Thus, FKN and CX₃CR1 mediate a novel pathway for leukocyte trafficking.

Key words: leukocyte migration • chemokines • cell adhesion • fractalkine • chemokine receptors

The recruitment of leukocytes from the circulation into sites of inflammation is a dynamic process involving multiple regulated steps (1, 2). The initial step involves leukocyte contact and rolling on endothelium, and is predominantly mediated by the selectins (3, 4). Subsequently, activation through pertussis toxin (PTX)-sensitive G protein-coupled receptors leads to upregulation of integrin adhesiveness and activation-dependent stable arrest (5–7). Chemokines are soluble, cell-selective molecules that regulate the activation step of leukocyte migration (5–7).

Fractalkine (FKN) is a unique, transmembrane, mucin-chemokine hybrid molecule expressed on the cell surface of IL-1- and TNF-activated endothelium (8). FKN shares high homology with the CC family of chemokines, but has an insert of three amino acids between the two NH₂-terminal cysteine residues, conferring a CX₃C structural motif (8). In vitro, FKN has been shown to have multiple activities including signal transduction through the PTX-sensitive G protein-coupled receptor CX₃CR1 (also called V28) (9) and adhesion of monocytes, NK cells, and T cells in static binding assays (8, 9). In addition, the soluble form of FKN is chemotactic for monocytes, NK cells, and T lymphocytes (8–10). FKN’s unique structure and multiplicity of molecular activities led us to hypothesize that it may regulate several pathways involved in leukocyte migration.

Here, we report that FKN on endothelium interacting with CX₃CR1 on leukocytes can mediate the initial capture, firm adhesion, and activation of circulating leuko-
cytes. Thus, we describe a new pathway for leukocyte migration.

Materials and Methods

Cells. Transfections, and Culture Conditions. Resting PBMCs were isolated from whole blood by centrifugation over lymphocyte separation medium (Organon Teknika, Durham, N C) as described previously (11). IL-2–activated PBLs were generated by culturing PBMCs in RPMI containing 10% FBS and 400 U/ml IL-2 (R & D Systems, Inc., Minneapolis, M N ) for 5 d as described previously (9) and harvesting the nonadherent cells.

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetech Corp. (San Diego, CA), and grown in EGM™ medium (Clonetics Corp.). ECV-304 cells were obtained from the American Type Culture Collection (Rockville, M D) and the generation of FKN-expressing ECV-304 cells (ECV-FKN) has been described previously (9). ECV-304 and ECV-FKN cells were maintained in M199 medium (GIBCO BRL, Gaithersburg, M D) and the generation of FKN-expressing ECV-304 cells was performed as described previously (9). Expression of FKN on HUVECs was determined by expression of chemokine receptors CCR1, CCR2, CCR3, CCR4, CCR5, CCR7, CCR8, CXCR1, CXCR2, CXCR3, CXCR4, and CXCR1. Cell surface expression of CXCR1 was also tested by indirect immunofluorescence (IF) as described below. To inhibit signal transduction through CXCR1, cells were treated with PTX at 500 ng/ml for 30 min at 37°C. To inhibit integrin function, cells were preincubated with 5 μM each of EDTA and EGTA in flow buffer (see below) for 15 min before use in flow assays, and both EDTA and EGTA were added to the flow buffer. Integrins were also blocked by preincubation of K562-neo or K562-CXCR1 cells for 10 min with the anti-∞ integrin antibody 4B4 (anti-∞CD29, IgG1) (12) or the anti-∞ integrin antibody H52 (anti-∞CD18, IgG1) (13) before use in flow assays. Anti-integrin antibodies were used as ascites fluid diluted 1:100.

Recombinant Proteins. FKN, thymus and activation regulated chemokine (TARC), and EBl1 ligand chemokine (ELC) fused with secreted placental alkaline phosphatase (FKN-SEAP, TARC-SEAP, and ELC-SEAP), and a control SEAP protein were produced as described previously (9, 14, 15). Monocyte chemotactic protein (MCP)-1–SEAP was also produced essentially as described previously (14). In brief, the DNA fragment containing the coding region of MCP-1 was amplified from M CP-1 cDNA by PCR. After digestion with SalI and XbaI, the MCP-1 fragment was subcloned into SalI–XbaI-Bal sites of pDR EF–SEAP(His)6. His y vector in order to express MCP-1 as a soluble fusion protein linked through five amino acid residues (Ser–Arg–Ser–Ser–Gly) with SEAP tagged with six histidine residues (His)6. 293 EBl1-A1 cells (Invitro) were transfected with the expression vector pDR EF–MCP-1–SEAP(His)6 using lipofectamine (GIBCO BRL). The transfected cells were incubated for 3–4 d in DMEM supplemented with 10% FBS. The supernatant containing each SEAP(His)6 chimera was collected by centrifugation, filtered (0.45 μm), and stored at 4°C after adding 20 mM Hepes (pH 7.4) and 0.02% sodium azide. 25-mm round glass coverslips were coated with fusion proteins as described previously (9). In brief, 10 μg/ml anti-SEAP antibody BB6 (Sigma Chemical Co., St. Louis, MO) in buffer (50 mM Tris pH 9.5) was placed on the coverslip and incubated overnight at 4°C. The coverslips were blocked in PBS with 1% BSA at room temperature for 2 h. After washing, 10 nM FKN-SEAP, SEAP, MCP-1–SEAP, TARC-SEAP, or ELC-SEAP was added and incubated at room temperature for 1 h. Coverslips were washed three times before use in the flow chamber.

Hydrodynamic In Vitro Flow Chamber Experiments. Parallel plate flow chamber assays were performed as described previously (16). In brief, protein-coated glass coverslips were assembled in a parallel plate laminar flow chamber and placed on the stage of an inverted phase-contrast microscope. K562-neo or K562-CXCR1 cells were suspended at a density of 106 cells/ml in flow buffer (PBS containing 0.75 mM CaCl2, 0.75 mM MgCl2, and 0.5% bovine serum albumin). Cell suspensions were perfused through the chamber at a 5- or 10-min period at a wall shear stress of 0.25 dynes/cm2 via a syringe pump (Harvard Apparatus, South N atick, MA). At the end of the 5- or 10-min perfusion period, flow buffer was perfused through the chamber at 1.85 dynes/cm2 for 3 min and at 10 dynes/cm2 for 3 min, and multiple ×100 fields were visualized. The entire experiment was recorded using a CCD video camera (Hitachi Denshi, Ltd., Woodbury, N Y ) and a Sony SuperVHS video recorder (Sony Electronics, Inc., San Jose, CA). For some experiments, cell suspensions were initially passed through the chamber at a low shear stress (0.25 or 0.5 dynes/cm2), followed by flow buffer at progressively increasing wall shear stresses of 0.5, 1.0, 1.85, 5.0, and 10 dynes/cm2 at 2-min intervals to determine the stability of adhesive interactions. The number of adherent cells was determined by analyzing the videotapes and counting 16 fields each with a 0.16-mm2 area.

Immunophenotypic Analysis. PBMCs and IL-2–activated PBLs were characterized by three-color flow cytometry as described previously (17) on a FACStarPlus® (Becton Dickinson, San Jose, CA) using CD3–FITC, CD4–PE, CD8–Cy5, CD14–PE, CD16–PE, and CD19–FITC directly conjugated antibodies generously provided by Coulter Corp. (Hialeah, FL). Resting PBMCs and IL-2–activated PBLs that bound to FKN–SEAP–coated coverslips were characterized by two-color IF using CD3–FITC, CD4–PE, CD8–FITC, CD14–PE, CD16–SEAP, and CD19–FITC antibodies. In brief, coverslips were incubated with 50 μl of mAbs diluted in PBS with 1% BSA for 15 min at room temperature and washed. Coverslips were mounted on glass slides and analyzed by IF on an epifluorescent microscope.

Cell surface expression of FKN was assessed by indirect IF using mAb 1D6 to the mucin domain of FKN as described previously (9). Expression of FKN on HUVECs was determined by expression of two-color flow cytometry using mAb 1D6 and directly conjugated CD31–PE mAb kindly provided by Coulter Corp. (Hialeah, FL) as described previously (17). Cell surface expression of CXCR1 was also determined by indirect IF. In brief, 106 cells were incubated with 5 nM chemokine–SEAP fusion proteins in PBS containing 0.05% NaN3 for 30 min at room temperature and washed. After incubation with anti–alkaline phosphatase mAb BB6 and washing, cells were incubated with fluorescein-conjugated goat anti–mouse Ig (Cappell, Gaithersburg, M D), washed, and analyzed by flow cytometry.
Results

FKN Mediates Leukocyte Capture. To determine whether FKN can mediate capture of free flowing leukocytes under conditions of shear stress encountered in vivo, freshly isolated PBMCs were perfused over immobilized FKN-SEAP fusion proteins at physiologic shear stresses. FKN-SEAP (but not SEAP alone) captured leukocytes at wall shear stresses up to 1.85 dynes/cm² (Fig. 1). The capture of leukocytes by FKN was rapid (<33 ms), and no rolling was observed. Despite diminished efficiency of capture with increasing wall shear stress, there was no significant difference in the rate of capture of monocytes compared with lymphocytes. Thus, FKN-mediated leukocyte capture at physiologic wall shear stresses.

PBMCs and IL-2-Activated PBLs Firmly Adhere to and Are Adhered by FKN. To test if FKN could support the firm adhesion of normal human leukocytes, both resting PBMCs and IL-2-activated PBLs were perfused over immobilized SEAP fusion proteins at 0.25 dynes/cm² and allowed to capture for 5 min. Firm adhesion was defined as the ability of cells to resist detachment at a wall shear stress of 10 dynes/cm². TARC-SEAP and ELC-SEAP were used as controls. CCR4, the receptor for TARC, is selectively expressed on CD4^+ T cells (14) and CCR7, the receptor for ELC/MIP-3β, is selectively expressed on activated T and B cells (15, 18, 19). Although resting PBMCs and IL-2-activated PBLs adhered firmly to purified FKN-SEAP (Fig. 2 B), they were not captured by (data not shown) nor adherent to (Fig. 2 B) control proteins. Virtually all captured cells remained firmly adherent throughout the experiments (>10 min in duration). As determined by IF, the majority of firmly bound PBMCs were either CD14^+ monocytes (62.5%) or CD8^+ T cells (30%; Fig. 2 C). Resting NK cells bound to immobilized FKN, but the percentage of bound NK cells varied widely between experiments (2.5–15%). Of the IL-2-activated PBLs bound to FKN, 37.5% were NK cells (CD16^+ and/or CD56^+), 56.3% were CD8^+ T cells, and only 6.3% were CD4^+ T cells. Thus, FKN mediated the firm adhesion of resting monocytes, resting and IL-2-activated CD8^+ T cells, and IL-2-activated NK cells strongly adhere to FKN under flow. Immobilized FKN was tested for its ability to capture and induce the firm adhesion of freshly isolated PBMCs in the parallel plate flow chamber. Cells were perfused over immobilized proteins at 0.25 dynes/cm² for 5 min and washed at 10 dynes/cm². Resting PBMCs and IL-2-activated PBLs were characterized by three-color flow cytometry and firmly adherent cells were characterized by two-color IF microscopy. (A) Photomicrographs of PBMCs and IL-2-activated PBLs bound to immobilized SEAP and FKN-SEAP. (B) Numbers of PBMCs and IL-2-activated PBLs remaining bound to immobilized SEAP, FKN-SEAP, TARC-SEAP, and ELC-SEAP at 10 dynes/cm². (C) Percentages of leukocyte cell types binding to FKN-SEAP under flow. The cell types measured and quantified were: CD14^+ monocytes, CD3^+CD16^+56^+ T cells, CD3^+CD8^+ T cells, CD3^-CD8^- T cells, and CD16^-56^- NK cells. The percentage of leukocyte subsets in the starting material as measured by multicolor flow cytometry is depicted by white bars, and the percentage of cell subsets in the FKN-bound fraction as measured by two-color IF microscopy is depicted by black bars. Leukocyte subsets from both resting PBMCs and IL-2-activated PBLs bound firmly and specifically to FKN-SEAP under flow. FKN preferentially bound resting monocytes, resting and IL-2-activated CD8^+ T cells, and IL-2-activated NK cells. The majority of FKN-bound, IL-2-activated PBLs formed pseudopods. Data are representative of three experiments performed. The error bars represent the mean ± SD of the number of cells bound.
ing and activated CD8^+ T cells, and resting and activated CD16/56^+ NK cells under physiologic flow conditions.

Soluble FKN-SEAP fusion proteins bind to and transduce signals through CX3CR1 to mediate a Ca^{2+} flux and chemotaxis (9). Immobilized FKN induced the spreading of bound monocytes (Fig. 2 A), but had no effect on the shape of resting PBLs. Furthermore, FKN induced pseudopod formation in the majority of IL-2-activated PBLs. Thus, FKN can activate resting monocytes and IL-2-activated PBLs.

CX3CR1 is the Leukocyte Receptor for FKN-mediated Capture and Firm Adhesion. Normally, CX3CR1 mRNA is expressed primarily by CD16^+ NK cells and at low levels by CD8^+ T cells and CD14^+ monocytes (9). For other chemokine receptors, IL-2 enhances CX3CR1 expression on both CD4^+ and CD8^+ T cells (9). Since these were the very cell types that were captured by and firmly adherent to purified FKN before becoming activated, FKN-CX3CR1 interactions probably mediated these events. Thus, the ability of FKN-transfected ECV-304 transformed endothelial cells (ECV-FKN; Fig. 3) to capture and induce stable arrest of K562-CX3CR1 (Fig. 3) was examined. CX3CR1-transfected and control cells were allowed to bind to ECV-FKN cells at 0.25 dynes/cm^2 for 10 min and exposed to increasing shear stresses up to 20 dynes/cm^2 (Fig. 4 A). K562-CX3CR1 cells remained bound to ECV-FKN monolayers at wall shear stresses up to 10 dynes/cm^2, and began to release at 20 dynes/cm^2. Thus, FKN on endothelium interacts with CX3CR1 on leukocytes to mediate their capture and firm adhesion.

Figure 3. Expression of FKN in ECV-304 cells and CX3CR1 in K562 cells. The top shows histograms depicting the binding of anti-FKN mAb (solid lines) and control mAb (dashed lines) to untransfected ECV-304 cells and to transfected ECV-FKN cells. The bottom shows histograms depicting the binding of FKN-SEAP (solid lines) and MCP-1-SEAP (dashed lines) to control K562-neo cells and transfected K562-CX3CR1 cells.

Discussion

In the current models of leukocyte migration, chemokines and their receptors transduce signals to the rolling leukocyte to induce cell arrest and firm adhesion by activating...
the adhesive capacity of integrins (1, 2, 5–7). This study demonstrates new roles for chemokines and their receptors in leukocyte migration, and describes a novel mechanism of leukocyte capture, firm adhesion, and activation mediated by the interactions of FKN with CX3CR1.

We have shown that FKN alone on the endothelium can mediate leukocyte capture and firm adhesion of monocytes, CD8+ T cells, and CD16/56+ NK cells. Although CX3CR1 mRNA is also expressed by IL-2 activated CD4+ T cells (9), they did not firmly adhere to immobilized FKN in these studies. The reason for this is not clear. Although CX3CR1 on the leukocyte can serve as the receptor for FKN to mediate all of these functions, FKN may also interact with other receptors. In the current models of leukocyte recruitment, selectins capture circulating leukocytes in the primary adhesion step (1–4). The selectins recognize distinct, but closely related, sialylated carbohydrates on their receptors (22–25). For example, L-selectin recognizes mucin-like molecules with extensive O-glycosylation (22–26). Since FKN has a mucin-like domain with many potential O-glycosylation sites, it may interact with selectins. However, in this study, selectins were not necessary for FKN-CX3CR1 adhesion since K562 cells do not express detectable levels of L-, E-, or P-selectin (27 and data not shown). Thus, in this system, leukocyte capture by FKN was selectin independent.

Leukocyte firm adhesion, in the multistep model of leukocyte migration, requires activation by chemokines and is mediated primarily by the integrin family of adhesion molecules and their receptors (1, 2). In this study, FKN interactions with CX3CR1 were sufficiently strong to mediate firm adhesion under conditions of high wall shear stresses (up to 20 dynes/cm²), without the involvement of integrins or other adhesion molecules. Combined with the ability of FKN to capture free-flowing leukocytes at physiologic wall shear stresses (1.85 dynes/cm²), this indicates that FKN and CX3CR1 mediate leukocyte capture and firm adhesion under flow. However, FKN and CX3CR1 may also act in concert with other effectors of the classical multistep pathways of leukocyte migration.

FKN appears to be unique amongst the chemokines in its ability to induce leukocyte firm adhesion. Neither TARC nor ELC fusion proteins were able to induce the firm adhesion of flowing PBMC. FKN naturally has a mucin stalk, whereas the other chemokines do not. The negative results with TARC and ELC may have been due to the fact that they lack mucin stalks. The extension provided by the mucin stalk may be a key factor in allowing FKN to mediate firm adhesion.

FKN expression is induced on primary cultures of human umbilical vein and pulmonary artery endothelium by the proinflammatory cytokines IL-1 and TNF-α (8, 10).

Figure 4. Integrin-independent firm adhesion of K562-CX3CR1 cells to ECV-FKN cells and immobilized FKN-SEAP under physiologic flow conditions. (A) K562-CX3CR1 cells remain firmly adherent to ECV-FKN cells under physiologic wall shear stresses, and this FKN-mediated firm adhesion is PTX insensitive and integrin independent. K562-neo cells and K562-CX3CR1 cells (+ EDTA/EGTA and ± PTX treatment) were perfused over ECV-FKN monolayers for 10 min at 0.25 dyne/cm² and subjected to increasing wall shear stresses up to 20 dyne/cm². Shown are the numbers of firmly adherent cells at various shear stresses. (B) Anti-β1 and β2 integrin mAbs have no effect on the firm adhesion of K562-CX3CR1 cells to ECV-FKN cells. The numbers of adherent K562-neo and K562-CX3CR1 cells at 1.85 dynes/cm² and 10 dynes/cm² in the absence and presence of anti-β1 and β2 integrin mAbs are shown. (C) K562-CX3CR1 cells bind to immobilized FKN under flow. FKN-SEAP, MCP-1-SEAP, and SEAP fusion proteins were immobilized by binding to glass coverslips coated with anti–alkaline phosphatase mAbs and were tested for their ability to support firm adhesion of K562-CX3CR1 cells under flow. Cells were perfused over immobilized SEAP fusion proteins for 10 min at 0.25 dyne/cm², and exposed to a wall shear stress of 10 dynes/cm². Shown are the numbers of K562 and K562-CX3CR1 cells remaining bound to immobilized chemokine-SEAP fusion proteins at 10 dynes/cm². Error bars represent the mean ± SD. Data are representative of three experiments performed.
Further, the mouse homologue of FKN, neurotactin, is expressed at low levels on brain endothelium in normal mice and upregulated on endothelium in inflamed brain in allergic encephalomyelitis (10), suggesting that the FKN pathway may be functional in leukocyte trafficking to inflamed brain. Although trafficking of lymphocytes to brain during peak inflammation in Sindbis virus–infected mice was blocked by antibodies to the β2 integrin LFA-1, lymphocyte entry into the brain during the early inflammatory response was not affected by antibodies to β1 integrins, VLA-4, or CD44 (28), indicating that the lymphocytes migrating to brain during early Sindbis virus infection used a different pathway of leukocyte migration. It is intriguing to hypothesize that the FKN pathway of circulating leukocyte adhesion to endothelium may play an important role in the accumulation of leukocytes on the endothelium of inflamed brain, facilitating subsequent diapedesis and migration.

In summary, we have shown that FKN can capture circulating leukocytes and induce their firm adhesion and activation. Furthermore, FKN-mediated firm adhesion is dependent on neither chemokine receptor signaling nor on integrins or other cell adhesion molecules, suggesting an alternate and novel regulatory mechanism for leukocyte trafficking. This novel FKN-mediated pathway may be particularly relevant for the recruitment of monocytes, CD8+ T cells, and NK cells to sites of inflammation.

Figure 5. Expression of FKN by TNF-activated HUVECs and their ability to support arrest of K562-CX3CR1 cells under flow conditions FKN expression on HUVECs, either resting (A) or stimulated with 100 ng/ml TNF-α for 12 h (B), was measured by IF staining with mAb 1D6. Shown are histograms of the reactivity of anti-FKN (1D6) and control (P3) mAbs. Cells were also counterstained with anti-CD31-PE to ensure they were endothelial cells. FKN was expressed on a subset of CD31- TNF-activated HUVECs (C). Comparison of the level of FKN expression by TNF-activated HUVECs and ECV-FKN cells. Shown are histograms of the reactivity of mAb 1D6 with 12-h TNF-activated HUVECs and ECV-FKN cells. (D) K562-CX3CR1 cells bind to TNF-activated HUVECs but not to resting HUVECs K562-neo cells and K562-CX3CR1 cells were perfused over HUVECs and TNF-activated HUVECs monolayers for 5 min at 0.5 dynes/cm² and subjected to a wall shear stress of 1.85 dynes/cm².

Further, the mouse homologue of FKN, neurotactin, is expressed at low levels on brain endothelium in normal mice and upregulated on endothelium in inflamed brain in allergic encephalomyelitis (10), suggesting that the FKN pathway may be functional in leukocyte trafficking to inflamed brain. Although trafficking of lymphocytes to brain during peak inflammation in Sindbis virus–infected mice was blocked by antibodies to the β2 integrin LFA-1, lymphocyte entry into the brain during the early inflammatory response was not affected by antibodies to β1 integrins, VLA-4, or CD44 (28), indicating that the lymphocytes migrating to brain during early Sindbis virus infection used a different pathway of leukocyte migration. It is intriguing to hypothesize that the FKN pathway of circulating leukocyte adhesion to endothelium may play an important role in the accumulation of leukocytes on the endothelium of inflamed brain, facilitating subsequent diapedesis and migration.

In summary, we have shown that FKN can capture circulating leukocytes and induce their firm adhesion and activation. Furthermore, FKN-mediated firm adhesion is dependent on neither chemokine receptor signaling nor on integrins or other cell adhesion molecules, suggesting an alternate and novel regulatory mechanism for leukocyte trafficking. This novel FKN-mediated pathway may be particularly relevant for the recruitment of monocytes, CD8+ T cells, and NK cells to sites of inflammation.

We thank Leona P. Whichard, Jonathan Baron, and Dawn M. Jones for their technical assistance. We also thank Barton F. Haynes and Michael Krangel for helpful discussion and a critical review of this manuscript.

This work was supported by National Institutes of Health grants AR-39162, AI-26872, CA-54464, and HL-50985 and by the Shionogi Institute for Medical Science. L. A. Robinson is supported by the Pediatric Scientist Development Program through a grant from St. Jude Children’s Research Hospital.

Address correspondence to Dhavalkumar D. Patel, Box 3258, 222 CARL Bldg., Duke University Medical Center, Durham, NC 27710. Phone: 919-684-4234; Fax: 919-684-5230; E-mail: patel003@mc.duke.edu

Received for publication 17 March 1998 and in revised form 31 July 1998.
References


