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Intercellular Adhesion Molecule-1 Is Required for Chemoattractant-Induced Leukocyte Adhesion in Resting, but Not Inflamed, Venules *in Vivo*

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The leukocyte integrins LFA-1 and Mac-1 bind to endothelial intercellular adhesion molecule-1 (ICAM-1). Leukocyte adhesion induced by micropipette injection of formylmethionylleucylphenylalanine (fMLP) or macrophage inflammatory protein 2 (MIP-2) next to a venule in the exteriorized mouse cremaster muscle was almost completely blocked after intravenous injection of the ICAM-1 mAb YN-1. In contrast, after 2-h pretreatment with TNF-α, leukocyte adhesion induced in postcapillary venules by fMLP or MIP-2 was not blocked by the ICAM-1 mAb. Leukocyte adhesion was significantly reduced by mAb GAME-46 to CD18 even after TNF-α treatment. We conclude that ICAM-1 is necessary for neutrophil adhesion to unstimulated endothelium, but not for adhesion to cytokine-stimulated endothelium. Although ICAM-1 is expressed at high levels after TNF-α, ICAM-1 either is not functional or is redundant with other endothelial ligands for β_2 integrins.

Key Words: leukocyte; ICAM-1; Mac-1; LFA-1; mouse; venule; fMLP; MIP-2; chemokine; adhesion molecule.

INTRODUCTION

Integrins are a family of heterodimeric type I transmembrane adhesion molecules which serve as the primary receptors on cells for binding many extracellular matrix proteins and ligands on other cells (Hynes, 1992). Families of integrins are distinguished based on their β subunit, and it is the β_2 (CD18) integrins which play a key role in firm adhesion in the leukocyte cascade (Springer, 1995). β_2 integrins are constitutively expressed on leukocytes and are conformationally activated in order to play an adhesive role (Hughes and Pfaff, 1998). The β_2 integrins interact with members of the immunoglobulin superfamily, such as intercellular adhesion molecule-1 (ICAM-1), and many other ligands (Hynes, 1992). The members of the β_2 integrin family are lymphocyte function-associated antigen-1 (LFA-1, $\alpha_L\beta_2$, CD11a/CD18), Mac-1 ($\alpha_M\beta_2$, CD11b/CD18), p150,95 ($\alpha_X\beta_2$, CD11c/CD18), and $\alpha_D\beta_2$. Humans lacking β_2 integrins (Anderson and Springer, 1987) and gene-targeted mice with a null mutation in the gene encoding the β_2 chain (Scharffetter-Kochanek *et al.*, 1998) have severe inflammatory defects, suggesting that β_2 integrins play a key role in leukocyte recruitment.

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily and a ligand for the β_2 integrins LFA-1 and Mac-1. ICAM-1 is constitutively expressed on the resting endothelium and is upregulated by inflammatory cytokines (Dustin *et al.*, 1986; Springer, 1995; Iigo *et al.*, 1997). ICAM-1 contains five extracellular immunoglobulin-like domains, two of which are binding sites for LFA-1 and

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Mac-1, respectively. Human LFA-1 binds to the first immunoglobulin domain of human ICAM-1, while human Mac-1 binds to the third immunoglobulin domain (Staunton *et al.*, 1990; Diamond *et al.*, 1991). ICAM-1 mediates adhesion of activated neutrophils in flow chamber *in vitro* assays (Lawrence and Springer, 1991). It has been shown that for optimal function, ICAM-1 exists as a homodimeric molecule (Miller *et al.*, 1995). However, gene-targeted mice lacking normal ICAM-1 (Sligh, Jr. *et al.*, 1993) show very subtle inflammatory defects, which are not comparable to the phenotype of β_2 integrin deficient mice (Scharffetter-Kochanek *et al.*, 1998). This suggests that other ligands for CD18 integrins exist.

Interleukin-8 (IL-8) is an α-chemokine that can be produced by neutrophils, fibroblasts, endothelial cells, and other cells. IL-8 causes shape change, chemotaxis, release of granule contents, and upregulation of adhesion molecules in neutrophils (Baggiolini and Clark-Lewis, 1992). Murine macrophage inflammatory protein-2 (MIP-2) is related to human IL-8, binds to the KC receptor CXCR-2 with high affinity, and produces a similar response to IL-8 (Morgan *et al.*, 1997; Bozic *et al.*, 1994; Lee *et al.*, 1995). IL-8 and, by inference, MIP-2 are expected to be presented by proteoglycans on the surface of endothelial cells (Middleton *et al.*, 1997). fMLP is a small, freely diffusible peptide (Schiffmann *et al.*, 1975) and therefore could enter the vascular space by passing through the blood vessel wall.

Leukocytes appear to require slow rolling for efficient transition from rolling to firm adherence (Kunkel *et al.*, 2000; Jung *et al.*, 1998). Previous studies have shown that leukotriene C4 can lead to reduced rolling velocities and increased leukocyte rolling flux, partially through a P-selectin-dependent mechanism (Kanwar *et al.*, 1995). In addition, blocking CD18 integrins has been shown to increase rolling velocity at low shear rates (Perry and Granger, 1991), suggesting that CD18 integrins are involved in reducing rolling velocity. The period of slow rolling appears to allow the leukocyte to interact with the endothelium and bind molecules that may be presented on the endothelial cells. In this model, the leukocyte would sense chemokines bound to the endothelium or chemoattractants present in solution. The sum of these signals received over time may cause activation of the leukocyte and integrin-mediated arrest of the rolling cell. It is not known whether ICAM-1 is involved in the transition from rolling to firm adhesion *in vivo*. Gene-targeted ICAM-1-deficient mice show only a minor increase in leukocyte rolling velocity (Ley *et al.*, 1998) and limited inflammatory defects (Sligh *et al.*, 1993).

The objective of this study was to examine the role of ICAM-1 in leukocyte adhesion in response to local chemoattractant. Using micropipettes to administer a dose of chemoattractant in close proximity to a venule, we induced the rapid conversion of rolling cells to firmly adherent cells (Morgan *et al.*, 1997; Ley *et al.*, 1993) in two well-characterized models of mild surgical trauma in untreated mice (Ley *et al.*, 1995) and TNF-α induced severe inflammation (Kunkel and Ley, 1996) in the mouse cremaster muscle. Through analysis of the adhesion response (increase in adherent cells) due to microinjection, and with the use of antibodies and gene-targeted mice, we measured the importance of ICAM-1 in firm leukocyte adhesion.

MATERIALS AND METHODS

Monoclonal antibodies, cytokines, and chemoattractants. A hybridoma cell line producing the function-blocking mAb against murine ICAM-1, YN-1 (rat IgG2b), was obtained from American Type Culture Collection (Rockville, MD). YN-1 has previously been shown to bind to ICAM-1 and to block the mixed lymphocyte response (Takei, 1985). An isotype control antibody, IgG2b (clone A95-1), and a CD18 blocking antibody (clone GAME-46) (Driessens *et al.*, 1996) were obtained from PharMingen (San Diego, CA). TNF-α was obtained from R&D Systems (Minneapolis, MN). Two chemoattractants, MIP-2 (200 nM; Austral Biologicals, San Ramon, CA) and fMLP (10 μM; Sigma, St. Louis, MO), were used in these experiments.

Intravital microscopy. Fifty-one male C57BL/6 mice were used in this study and all were at least 8 weeks old and ranged in weight from 21 to 34 g. All mice were anesthetized with an intraperitoneal (ip) injection of ketamine hydrochloride (125 mg/kg, Sanofi Winthrop Pharmaceuticals, New York, NY), xylazine (12.5 mg/kg, TranquiVed, Phoenix Scientific, Inc., St. Joseph, MO), and atropine sulfate 0.025 mg/

kg, Fujisawa USA, Inc., Deerfield, IL). Twenty-five mice were pretreated with an intrascrotal injection of TNF-α. TNF-α (500 ng) was injected in 0.3 ml of saline 2–2.5 h before exteriorizing the cremaster muscle. Once the mouse was anesthetized, cannulation of the trachea, jugular vein, and carotid artery was performed. Blood pressure was monitored throughout the experiment (Model BPMT-2, Stemtech, Inc., Menomonee Falls, WI). In order to maintain body temperature, the mice were laid on a heating pad that was filled with circulating water from a water bath kept at 38°C.

The cremaster muscle was prepared for intravital microscopy as previously described (Ley *et al.*, 1995). In all animals, a major feed–drainage artery–vein pair connected the cremaster to the epididymis. The epididymis and testes were pinned to the side, and the exposed cremaster microcirculation remained well perfused. This surgical procedure took approximately 10 min. After being excised, the cremaster muscle was superfused with thermocontrolled (at 35°C) bicarbonate-buffered saline. Some mice were treated with intravenous injections of 30 or 100 μg of YN-1 or isotype control IgG2b immediately prior to cremaster surgery.

Microscopic observations were made on a Zeiss intravital microscope (Axioskop, Carl Zeiss, Inc., Thornwood, NY) with a saline immersion objective (SW 40/0.75 numerical aperture). A venule was observed for approximately 1 min, then chemokine was microinjected next to the venule for a period of 60 s, after which the venule was observed for another 6 min. Venules ranging from 26 to 63 μm were used for these microinjections and video recordings were made using a CCD camera system (Model VE-1000CD, Dage-MTI, Inc., Michigan City, IN). Data were recorded on a Mitsubishi VHS recorder. The venule centerline blood velocity was measured using a dual photodiode and a digital on-line cross correlation program as previously described (Pries, 1988). The centerline blood velocity was converted to mean blood flow velocity by multiplying by a factor of 0.625 (Lipowsky and Zweifach, 1978). The wall shear rate was estimated as $\gamma_w = 2.12 \cdot 8\nu_b / d_{ven}$. Here, γ_w is the vessel wall shear rate, ν_b is the mean blood flow velocity, d_{ven} is the diameter of the venule, and 2.12 is an empirical factor obtained from velocity profiles observed in the microvasculature *in vivo* (Reneman *et al.*, 1992). Following the observation period, the centerline blood velocity was measured again. If the blood velocity changed significantly during the injection, then observation was halted and any adhesion data were discarded.

Blood samples were taken in 10-μl capillary tubes via the carotid artery throughout the experiment and analyzed for leukocyte concentration using a hemocytometer. Leukostat (Fisher Scientific, Pittsburgh, PA)-stained blood smears were used to obtain differential leukocyte counts.

Microinjections. Injection of MIP-2 or fMLP into the tissue surrounding a venule was used to activate rolling leukocytes and induce firm adhesion. A glass micropipette was pulled from standard borosilicate glass with an outer diameter of 1.0 mm (Stoelting, Wood Dale, IL) on a vertical pipette puller (Stoelting, Wood Dale, IL). The blunt end of the pipette was then fire-polished by briefly passing the end through a flame. The tip of the pipette was then beveled using a micropipette grinder (Model EG-40, Narishige, Sea Cliff, NY) with a 0.3-μm abrasive foil (No. 6775, AH Thomas, Philadelphia, PA). The pipettes were filled with approximately 10 μl of either fMLP (10 μM) or MIP-2 (200 nM). Tubing was threaded through the pipette holder and then secured on the end of the filled pipette. At its opposite end, the tubing was connected to a 10-ml syringe and a three-way stopcock system. The pipette holder was inserted within a piezo-driven micromanipulator (Model DC-3k Märzhäuser-Wetzler, Upper Saddle River, NJ). Using this micromanipulator, the pipette was placed approximately 10–30 μm from a venule wall, so that the beveled tip penetrated the interstitial tissue. The venules that were chosen had a relatively low level of background adherence. After the pipette was in place, the venule was recorded for about 60 s and then air pressure was applied to the pipette by applying pressure to the syringe pump. A successful injection was verified by microscopically observing visible swelling of the interstitial tissue during injection. Pipette contents were injected for a 60-s period. The vessel was recorded during injection and for about 6 min after the injection ended in order to observe an adhesion response over time.

Data analysis. Vessel diameters, numbers of adherent cells, and rolling velocities were measured using a digital image processing system (Pries, 1988).

Adherent cells were defined as cells which remained stationary for at least 30 s. The basal level of adherent cells for a segment of venule (approximately 200 μm) was determined by recording the venule for a minute before injection. The background level of adherent cells for a venule was subtracted from all adhesion observations for that venule. Therefore, observations were recorded as adhesion above the basal level of adhesion and all data were normalized. Leukocyte rolling velocities were also measured prior to injection and 1, 3, and 5 min from the conclusion of injection by tracking rolling leukocytes for 2 s and recording their positions.

Expression of ICAM-1 on endothelial cells. Whole cremaster muscles were taken from mice that had been injected iv with primary antibody as described (Jung and Ley, 1997). The cremaster muscle was laid flat on a gelatin-coated slide and allowed to air dry for approximately 5–10 min so the edges of the tissue were dry and then fixed in an acetone bath at −18°C for at least 24 h. After fixation, the tissues were permeabilized in 0.05 M Tris buffer with 0.03% saponin. The tissue was then incubated with a biotinylated rabbit-α-rat IgG (Vector Laboratories, Inc., Burlingame, CA) for 90 min and then washed. Next, the tissue was incubated with 0.3% H_2O_2 in 0.05 M Tris buffer for 60 min and then washed. Then the tissue was incubated with ABC reagent (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) before being incubated with peroxidase substrate (DAB SK-4100, Vector Laboratories, Inc.) and counterstained with Giemsa stain (Sigma). Finally, the tissues were washed in xylene for 5 min, mounted using Permount (Fisher Scientific, Pittsburgh, PA), and allowed to dry for 48 h before viewing. Photographs were taken using a 35-mm camera (Olympus) and film for color slides.

Statistical analysis. Adherent cell data and systemic leukocyte counts are presented as means ±SEM. Adhesion data are expressed as "number of adherent leukocytes above baseline ±SEM" in one segment of venule (which is one field of view on the monitor, or approximately 200 μm). Statistical comparison between different adhesion responses (number of adherent cells above baseline at a given time point) was performed using a two-way ANOVA, followed by analysis using the Tukey–Kramer multiple comparison test. The rolling velocity distributions were compared using the Mann–Whitney *U* test. All statistical analysis was performed using NCSS (Kaysville, UT) software package. Statistical significance was set at $P < 0.05$.

TABLE 1
Systemic Leukocyte Counts by Genotype and Treatment

TNF-α	Systemic count	% PMN	% Mononuclear
No	6240 ± 330 (15)	38 ± 2	62 ± 2
Yes	2980 ± 240[a] (29)	36 ± 2	64 ± 2

Note. Data are presented as means ± SEM. Numbers of mice per group are shown in parentheses (*N*).

[a] Significantly different from non-TNF-α-treated wild-type mice ($P < 0.05$).

RESULTS

General observations. While under vivarium conditions, all mice used in this study appeared healthy and did not display any obvious abnormalities. The systemic leukocyte counts in untreated and TNF-α-treated mice (Table 1) were similar to those reported previously (Sligh *et al.*, 1993; Kunkel *et al.*, 1996). Injection of TNF-α caused a reduction of leukocyte counts, most likely due to a systemic increase in adhesion and transmigration of leukocytes, thus reducing the number of leukocytes in the blood (Kunkel and Ley, 1996). However, in both the untreated and the TNF-α models, the systemic leukocyte counts remained constant during intravital microscopy and after mAb treatments. A typical microinjection of fMLP or MIP-2 next to a postcapillary venule elicited a response in which many of the rolling leukocytes were converted to firmly adherent leukocytes by the end of the observation. Figure 1A shows a typical response to MIP-2 in a venule of an untreated WT mouse.

Hemodynamics. The adhesion response and leukocyte rolling were examined in vessels that were hemodynamically similar within a given model. Hemodynamic conditions (centerline blood velocity, wall shear rate, and diameter) in all venules within the untreated model were similar (no significant difference) (Table 2). The same was true in the TNF-α model (Table 3). Within either model, the values measured after a microinjection were no different than values

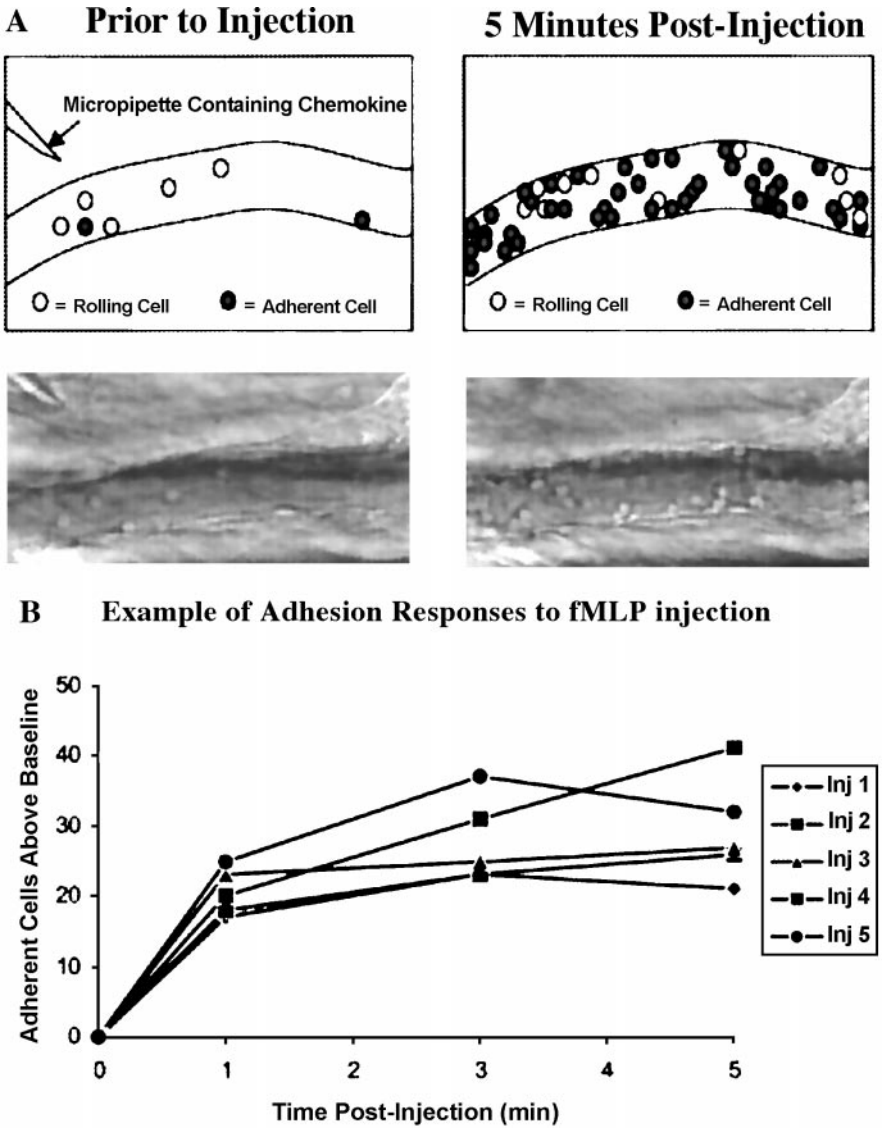

FIG. 1. *Leukocyte adhesion in response to MIP-2 or fMLP microinjection.* Schematic and videomicrograph of a venule prior to microinjection of MIP-2 and 5 min after the conclusion of the microinjection (A). Prior to the injection, the pipette is placed within about 30 μm of the venule and observed for 1 min. At this point, the population of leukocytes primarily consists of rolling cells. At 5 min postinjection, 44 leukocytes have become adherent in this vessel. Example of adhesion responses to fMLP in the untreated model (B). Each line represents the adhesion response to a single microinjection.

measured before an injection (data not shown). Therefore, the microinjections did not alter the hemodynamic parameters associated with a venule. There was no significant correlation between the number of adherent cells 5 min postinjection and the wall shear rate (data not shown). Therefore, all data were pooled and no data were removed due to low or high shear rates. The level of leukocyte adhesion before onset of the microinjection was between 4 and 9 leukocytes per 200 μm of venule in the untreated case (Table 2) and between 16 and 27 leukocytes per 200 μm of TNF-α-treated venule (Table 3). The percent area of endothelial cells covered by adherent leukocytes was below 4% in all groups.

Role of ICAM-1 in leukocyte adhesion in the untreated model. After an injection of chemokine next to a venule of an untreated mouse, the number of adherent leukocytes increased dramatically over a pe-

TABLE 2
Hemodynamic Properties within the Different Groups before and after Injection for Untreated Mice

mAb	Injection	Centerline velocity (mm/s)	Wall shear rate γ (s^{-1})	Baseline adhesion (per 200 μm)	Diameter (μm)	*n*	*N*
None	fMLP	3.1 ± 0.2	922 ± 61	5.1 ± 0.8	35 ± 2	8	5
	MIP-2	2.3 ± 0.4	744 ± 136	6.1 ± 1.2	33 ± 2	7	3
	PBS	2.9 ± 0.6	877 ± 176	5.0 ± 1.9	36 ± 1	9	3
YN1	fMLP	2.6 ± 0.5	830 ± 146	9.2 ± 1.4	34 ± 2	9	4
	MIP-2	2.3 ± 0.7	660 ± 202	5.5 ± 3.1	37 ± 1	4	2
IgG2b	fMLP	2.2 ± 0.5	619 ± 108	3.7 ± 1.4	36 ± 2	8	3
	MIP-2	2.8 ± 0.4	701 ± 105	7.0 ± 3.5	43 ± 2	5	2

Note. There was no statistical difference between the groups of venules (*n*, number of venules in a group; *N*, number of mice in a group; *N*, number of mice in a group).

riod of a few minutes (Fig. 1B). The effect of blocking ICAM-1 was investigated using the function-blocking antibody YN-1. After administration of 30 μg of YN-1, the adhesion response to fMLP was significantly and completely reduced to background levels (7 ± 2 cells, Fig. 2A). Microinjections of PBS vehicle resulted in a small increase of approximately 5 adherent leukocytes. Injections with a chemokine, MIP-2, were conducted to confirm that these results were not restricted to one particular chemoattractant. MIP-2 caused an increase in adherent cells of 30 ± 6 leukocytes. This was reduced to background levels (9 ± 4 cells) after infusing the mouse with 30 μg of YN-1 (Fig. 2B). No decreases in adhesion responses were seen in response to either chemoattractant after administering an isotype control antibody (data not shown).

Role of ICAM-1 in TNF-α-induced inflammation. Following stimulation with TNF-α, many adhesion molecules are upregulated. This increase includes molecules that were absent before TNF-α stimulation, such as E-selectin, as well as an upregulation in molecules that were already present, such as ICAM-1 (Jung and Ley, 1997; Dustin *et al.*, 1986). As in the previous model, the role of ICAM-1 was investigated using the mAb against ICAM-1. After TNF-α treatment, the adhesion response to fMLP was 18 ± 4 leukocytes (Fig. 2C) and to MIP-2, 22 ± 4 leukocytes (Fig. 2D) at 5 min postinjection. However, after cytokine stimulation, the adhesion curve did not diminish in animals treated with YN-1. After TNF-α, microinjections of PBS yielded a small, but statistically significant, adhesion response of 9 ± 4 leukocytes (Fig. 2C). To confirm that CD18 integrins were required for leukocyte adhesion in TNF-α-treated venules, we administered the CD18 mAb GAME-46 (30 μg) and performed microinjections with both fMLP and MIP-2. The adhesion response to both chemoattractants was reduced to baseline (Figs. 2C and 2D). These findings

TABLE 3
Hemodynamic Parameters within the Different Groups before and after Injection for the TNF-α Model

mAb	Injection	Centerline velocity (mm/s)	Wall shear rate γ (s^{-1})	Baseline adhesion (per 200 μm)	Diameter (μm)	*n*	*N*
None	fMLP	1.7 ± 0.2	482 ± 51	19.7 ± 2.3	37 ± 2	17	10
	MIP-2	1.8 ± 0.2	491 ± 42	18.8 ± 2.7	39 ± 1	8	5
	PBS	2.9 ± 0.3	694 ± 61	26.8 ± 5.9	44 ± 3	8	3
YN1	fMLP	2.2 ± 0.3	652 ± 85	16.0 ± 1.5	36 ± 2	11	5
	MIP-2	1.9 ± 0.3	523 ± 91	26.1 ± 4.4	40 ± 2	9	3
GAME-46	fMLP	2.0 ± 0.2	591 ± 75	15.1 ± 5.6	37 ± 3	7	2
	MIP-2	2.1 ± 0.2	557 ± 88	12.6 ± 2.4	41 ± 3	7	3
IgG2b	fMLP	2.0 ± 0.4	516 ± 66	20.7 ± 5.8	40 ± 4	6	2
	MIP-2	2.8 ± 0.6	674 ± 129	25.2 ± 3.0	44 ± 3	5	2

Note. There was no statistical difference between the groups of venules (*n*, number of venules in a group; *N*, number of mice in a group).

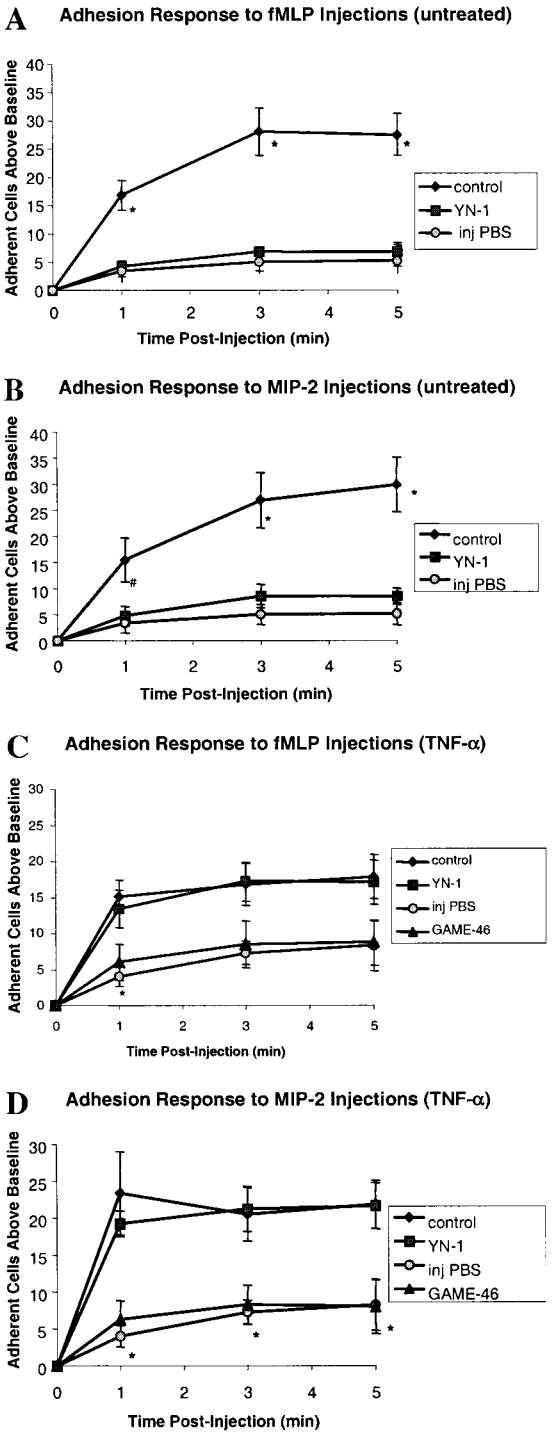

FIG. 2. *Chemoattractant-induced leukocyte adhesion in untreated mice.* Adhesion response to microinjections of fMLP (A,C) and MIP-2 (B,D) in untreated mice (A,B) and in TNF-α-treated mice (C,D). YN-1, infused with mAb YN-1; GAME-46, infused with mAb GAME-46; WT inj PBS, microinjection of PBS. The numbers of injections performed for untreated/TNF-α were (fMLP, 8; MIP-2, 7/fMLP, 17; MIP-2, 8), YN-1 (fMLP, 9; MIP-2, 4/fMLP, 11; MIP-2, 9), GAME-46 (fMLP, 7; MIP-2, 7), and WT inj PBS (9/8). An asterisk signifies a statistically significant difference from all other injections, $P < 0.05$.

show that after TNF-α, chemoattractant-induced leukocyte adhesion is β_2 integrin-dependent, but ICAM-1-independent.

Experiments performed in previous studies (Kunkel *et al.*, 1996) and the present data in untreated mice show that 30 μg of YN-1 is sufficient to block the function of ICAM-1. However, as ICAM-1 is upregulated after TNF-α treatment (Jung and Ley, 1997), we considered the possibility that not all ICAM-1 was blocked by the mAb. Therefore, we tested an increased dose of YN-1 (100 μg). This higher dose also failed to reduce leukocyte adhesion (Fig. 3). A trend toward reduced leukocyte adhesion was seen at 1 min in venules treated with MIP-2, but the difference did not reach statistical significance.

Immunostaining for ICAM-1. Cremaster muscles were fixed at the conclusion of the experiment and were later immunostained. Successful peroxidase staining for ICAM-1 confirmed that YN-1 had bound ICAM-1 during the experiment. Three different samples of cremaster muscle were stained for primary antibody binding: isotype control antibody (IgG2b), YN-1, and YN-1 after TNF-α stimulation (Fig. 4). As expected, no staining was observed after infusion of the isotype control (Fig. 4A). However, in untreated mice (representing the basal level of ICAM-1 presence), staining was observed with YN-1 (Fig. 4B). After TNF-α treatment, a greater level of staining was observed (Fig. 4C). The darker degree of peroxidase staining suggests that more primary antibody bound to the endothelium during the experiment, thus confirming that ICAM-1 is upregulated after cytokine stimulation (Jung and Ley, 1997).

Rolling velocities. In order to evaluate any difference in the rolling behavior of leukocytes that made the transition from rolling to firm adherence, rolling velocities were measured prior to microinjection (baseline) and 1, 3, and 5 min from the conclusion of the injection. Populations of cells representing each situation were analyzed and cumulative frequency

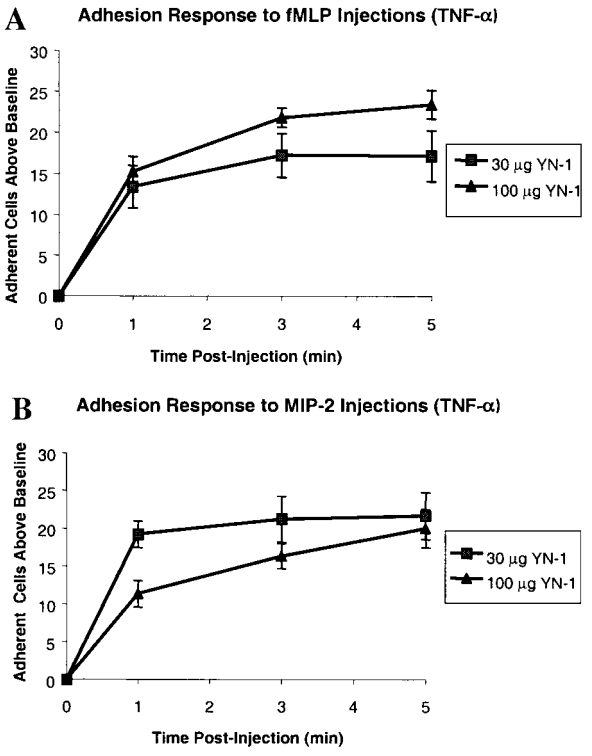

FIG. 3. *Limited effect of blocking ICAM-1.* YN-1 (100 μg) (fMLP, five venules; MIP-2, three venules) was injected iv instead of the usual dosage of 30 μg in TNF-α-treated mice (fMLP, 11 venules; MIP-2, 9 venules) to account for increased ICAM-1 expression. Although some effect of 100 μg of mAb YN-1 was seen at 1 min after MIP-2 microinjection (B), this difference was not seen at other times or at any time after fMLP (A).

histograms were created (Figs. 5A and 5B). Since adherent cells are no longer in the rolling pool, the finding that the slow rolling population of cells (< 10 μm/s) disappeared after application of chemoattractant suggested that slow rolling cells are more prone to becoming adherent than fast rolling cells. As a consequence of this removal of slow-rolling cells, the mean rolling velocity at any time after injection was significantly different from the baseline rolling velocity (Table 4).

To directly test the hypothesis that slowly rolling leukocytes were preferentially recruited in response to chemoattractant, cells were tracked from the moment they began rolling until they adhered to the endothelium, rolled through the field of view, or detached from the endothelium. Examples of cells with high and low initial rolling velocities are shown in Fig. 5. In both the unstimulated (Fig. 5C) and TNF-α treated (Fig. 5D) mice, the cells that entered the venule rolling at a slower velocity became adherent in response to chemoattractant (fMLP) application, whereas fast-rolling cells detached or rolled out of the field of view.

DISCUSSION

This study shows that ICAM-1 has different roles in leukocyte adhesion to unstimulated endothelium and

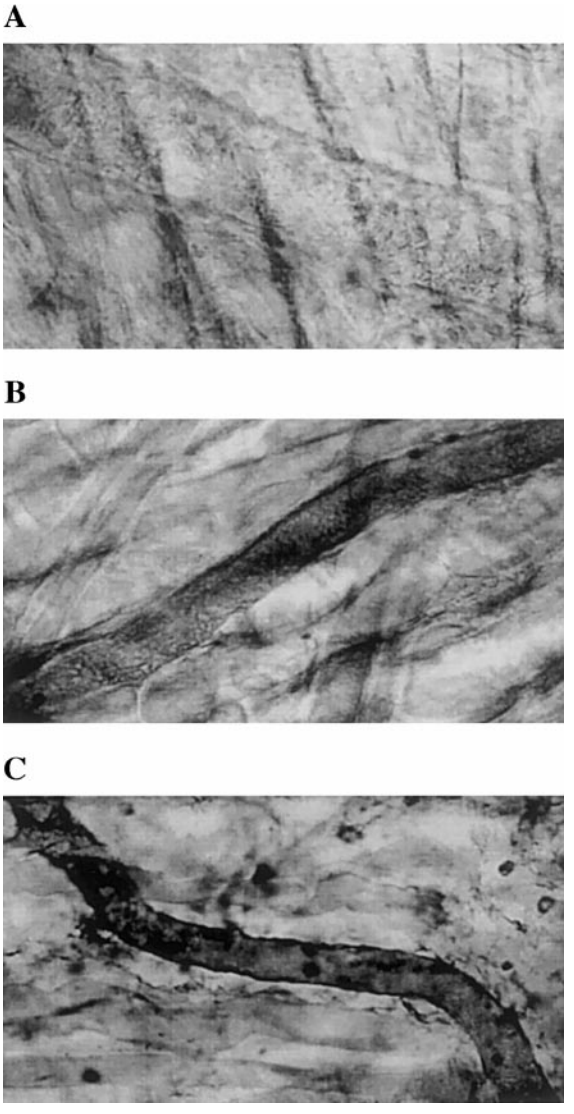

FIG. 4. *Expression of ICAM-1 in mouse cremaster muscle venules.* Peroxidase-based immunostaining of cremaster muscle whole mounts. (A) Isotype control antibody (IgG2b), (B) YN-1 in untreated mice. (C) YN-1 after TNF-α stimulation.

TABLE 4.

Mean Rolling Velocities (40 Cells in Each Group) before Injection and at 1, 3, and 5 min from the Conclusion of Injection of fMLP

Model	Baseline velocity (μm/s)	Minutes after fMLP (μm/s)		
		1	3	5
Untreated	24 ± 2	30 ± 3*	35 ± 4*	40 ± 4*
TNF-α	7 ± 1	42 ± 4**	42 ± 5**	39 ± 5**

Note. Data are given for both the untreated and TNF-α models.

* Statistically significant from baseline rolling velocity in the untreated model.

** Statistically significant from baseline rolling velocity in the TNF-α model.

after cytokine stimulation. Without stimulation, ICAM-1 appears to be essential for mediating firm adhesion of leukocytes in response to chemoattractant application. The adhesion response to microinjection of either fMLP or MIP-2 was reduced to baseline after administration of a function-blocking monoclonal antibody. In contrast, after treatment with TNF-α, ICAM-1 was highly expressed, but no longer necessary to mediate firm adhesion.

Virtually all previous work points to the importance of β_2 integrins on the leukocyte in mediating adhesion and transmigration (Shang and Issekutz, 1998; Issekutz *et al.*, 1999; Scharffetter-Kochanek *et al.*, 1998). Alternate β_2 integrin ligands, such as ICAM-2 (Mizgerd *et al.*, 1998; Shang and Issekutz, 1998) and undefined endothelial Mac-1 ligands (Lo *et al.*, 1989), have been suggested to be responsible for ICAM-1-independent leukocyte adhesion in other experimental systems. Our data suggest that such an ICAM-1-independent but β_2-integrin-dependent pathway of adhesion exists in inflamed microvascular endothelial cells. Based on our data, it is proposed that endothelial ligands for β_2 integrins different from ICAM-1 are probably not constitutively expressed, or they are expressed at low enough levels to have little impact on the adhesion response without cytokine stimulation. After treatment with TNF-α, ICAM-1 is no longer essential for firm adhesion, suggesting that other ligands are induced. ICAM-2 is unlikely to be involved in this binding because its expression is not induced by treatment with inflammatory cytokines (Xu *et al.*, 1996) and may actually be downregulated (McLaughlin *et al.*, 1998). ICAM-2-deficient mice generated by gene targeting and homologous recombination have no inflammatory defect (Gerwin *et al.*, 1999), and blocking ICAM-2 does not reduce neutrophil recruitment in mouse peritonitis (Mizgerd *et al.*, 1998).

Here, we show that blocking ICAM-1 does not change the adhesion response after TNF-α. Therefore, ICAM-1 also does not play a significant role in mediating adhesion or is redundant with other adhesion molecules. There are three plausible explanations for ICAM-1-independent adhesion after stimulation: (1) ICAM-1 is no longer functional, (2) other molecules dominate as CD18 ligands, or (3) other molecules block ICAM-1 function. After TNF-α treatment, various other endothelial surface molecules are upregulated, including P-selectin, E-selectin, and VCAM-1 (Jung and Ley, 1997; Sanders *et al.*, 1992; Hahne *et al.*, 1993). Although the identity of the endothelial adhesion molecules responsible for leukocyte adhesion in cytokine-induced inflammation are unknown, our data show that two fundamentally different pathways of adhesion appear to exist *in vivo*, dependent on the inflammatory status of the tissue.

Potential limitations of the present study include our inability to positively identify the nature of each adherent leukocyte by intravital microscopy. However, in this model, at least 92% of adhering leukocytes have been identified as neutrophils by studying Giemsa-stained whole mounts of cremaster muscles (Kunkel and Ley, 1996). Therefore, a small number of mononuclear cells may inadvertently be included in our analysis, but this number accounts for not more than 8% of adherent leukocytes and cannot explain the different role of ICAM-1 with and without TNF-α. Interestingly, about equal numbers of mononuclear cells are removed from the circulation by treatment with TNF-α, but only neutrophils appear to accumulate in the cremaster muscle. Another limitation could be indirect attachment of leukocytes through secondary capture, i.e., interaction of newly recruited leukocytes with already adherent leukocytes (Bargatze *et al.*, 1994), which is largely independent of ICAM-1. Although secondary capture is a prominent phenomenon in flow chamber systems perfused with red cell-free leukocyte suspensions (Alon *et al.*, 1996), we have previously shown that secondary capture accounts for a very small percentage ($< 1\%$) of leukocyte interactions in inflamed cremaster venules (Kunkel *et al.*, 1998). In the present study, adherent leukocytes cov-

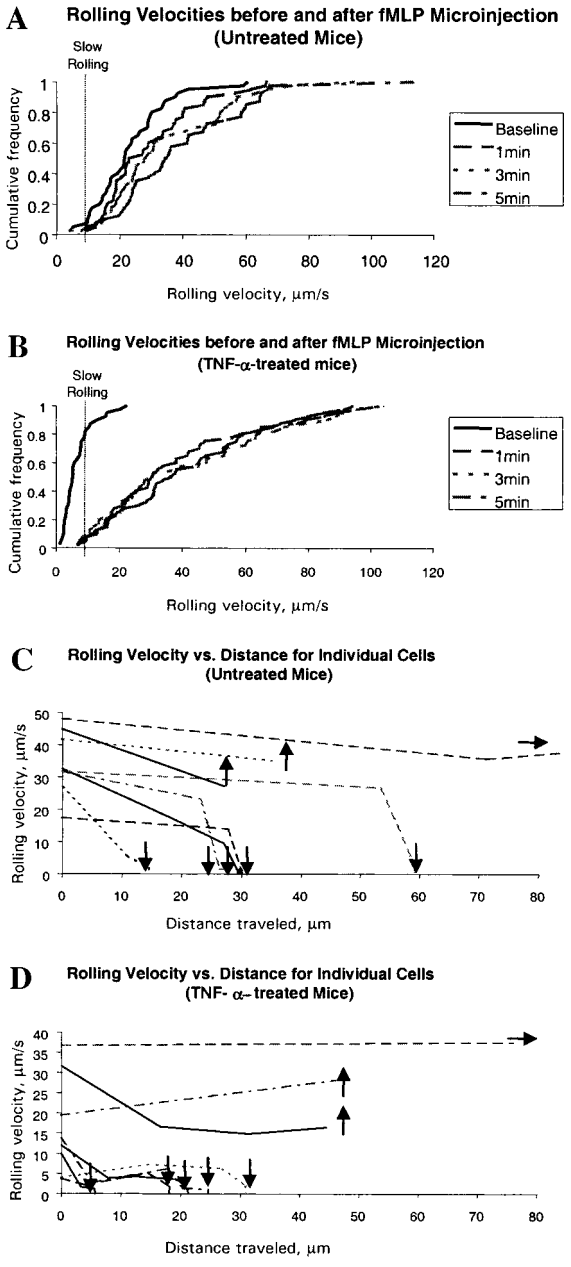

FIG. 5. *Slow rolling leukocytes preferentially adhere in response to chemoattractant.* (A and B) Cumulative frequency histograms of rolling velocity data recorded prior to microinjection (solid line) and 1, 3, and 5 min after fMLP (dashed lines) (n = 40 cells in each curve). The slow rolling population of cells was eliminated after microinjection of fMLP in both untreated (A) and TNF-α-treated mice (B). Rolling velocity as a function of distance traveled (rolled) for individual leukocytes (different dashed and solid lines) in venules of untreated (C) and TNF-α-treated (D) mice in response to microinjection of fMLP. The slower rolling leukocytes show an increased propensity to become adherent (↑ rolling cell detached, ↓ rolling cell became adherent, → rolling cell rolled out of field of view).

ered less than 4% of the endothelial surface area of the inflamed venules. Finally, the cremaster vasculature may not be representative of events in other parts of the body. Indeed, the endothelial cells of skeletal muscle beds may have little in common with endothelial cells of the lung, liver, or kidney, organs relevant in multiorgan failure. Therefore, the present results cannot be interpreted to mean that ICAM-1 is not important in multiorgan failure. Indeed the leukocytes accumulating in and around the cremaster venules after TNF-α do not reflect the changes in systemic counts, suggesting that mononuclear cells must be trapped in other organs under these conditions.

The kinetics of induction of firm adhesion are clearly different between mild inflammation in untreated mice and severe TNF-α-induced inflammation. In the untreated mice receiving surgery only, maximum adhesion in response to chemoattractant takes about 3 min (Figs. 2A and 2B), after which very little additional adhesion occurs. However, in the TNF-α model, maximum adhesion occurs by 1 min (Figs. 2C and 2D). Plausible explanations for this phenomenon include (1) different surface densities of relevant adhesion molecules present on the endothelium, (2) different association and dissociation rates of adhesion molecules, (3) increased permeability of the inflamed endothelium, allowing faster access of the chemoattractant to the intraluminal compartment, or (4) a different state of activation of rolling leukocytes. The β_2 integrin ligands presented after TNF-α treatment may have a much greater surface density, thus increasing the chance that receptor/ligand pairs will come into contact. Beyond β_2 integrin ligands, adhesion may also be supported by molecules, such as E-selectin, that are involved in the slow rolling step of the adhesion cascade (Ley *et al.*, 1998; Kunkel and Ley, 1996). Alternatively, or in addition, CD18 ligands presented after TNF-α treatment could have much higher association rates. This would increase the probability of binding occurring if a receptor/ligand pair came in contact with each other. In the third case, the chemoattractant may reach the rolling leukocyte more efficiently. This process is likely to contribute to the accelerated response, because inflammatory mediators like TNF-α are known to increase microvascular permeability (Yi and Ulich, 1992). In addition, rolling leukocytes entering the field of view may already be partially acti-

vated. Indeed, recent studies have suggested that leukocyte activation occurs during slow rolling (Kunkel *et al.*, 2000). This last explanation is strongly favored by our data showing that slow rolling cells are preferentially recruited to become firmly adherent in response to exogenous chemoattractants (Figs. 5C and 5D), and consequently, injection of a chemoattractant next to a venule eliminated the slow rolling population of cells (Figs. 5A and 5B).

In conclusion, this study shows that slow rolling leukocytes in a model of TNF-α-induced inflammation become preferentially adherent in response to microinjections of chemoattractant through a CD18-dependent, ICAM-1-independent pathway. This pathway is physiologically significant, as suggested by the very different phenotypes of ICAM-1 deficient versus CD18 deficient mice. To better understand this important pathway of leukocyte adhesion, it will be necessary to identify alternative endothelial ligands for CD18 integrins at the molecular level.

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