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The C–C Chemokine MCP-1 Differentially Modulates the Avidity of β1 and β2 Integrins on T Lymphocytes

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Summary

The ability of chemokines, particularly MCP-1, to induce integrin-dependent binding of T lymphocytes to endothelial adhesion molecules or extracellular matrix (ECM) components was examined. MCP-1 induced significant adhesion to fibronectin (FN) and to endothelial-secreted ECM but not to purified ICAM-1 or VCAM-1, or to activated endothelium. The MCP-1-induced binding of T lymphocytes to FN was rapid, dose dependent, and resulted from activation of both VLA-4 and VLA-5. Like MCP-1, the chemokines RANTES and MIP-1β induced T lymphocyte binding to FN, but not to ICAM-1. We suggest, therefore, that these T lymphocyte chemokines may be most important, not in initiating integrin-dependent firm adhesion of T lymphocytes to the vascular wall, but rather, in subsequent adhesive interactions during migration into tissue.

Introduction

Transendothelial migration of leukocytes into sites of inflammation is a multistep process mediated by a series of sequential but overlapping interactions (Springer, 1995). In this process, leukocytes in hemodynamic flow tether to and roll along the endothelium of a vessel wall, potentially encountering activating factors that modulate integrin-dependent firm adhesion and arrest of the rolling leukocyte on the vessel wall. Following directional cues from chemoattractants and using integrin molecules for traction, leukocytes then cross the endothelial lining of the blood vessel, traverse through the underlying extracellular matrix (ECM), and enter inflamed tissue. While this model for leukocyte extravasation is widely accepted for neutrophils, it remains to be determined which of these steps are applicable to T lymphocyte emigration from the bloodstream. Particular ambiguity concerns the identity of the signal(s) needed to activate the integrins on T lymphocytes. Strong evidence exists that chemoattractants can provide the signals that modulate the avidity of integrins on neutrophils (Springer, 1995); however, much less is known about the role of these attractant molecules in triggering integrin activation on lymphocytes.

In the past several years, a number of T lymphocyte chemoattractants have been identified (Schall et al., 1990, 1993; Taub et al., 1993a, 1993b, 1995; Tanaka et al., 1993a, 1993b; Carr et al., 1994; Loetscher et al., 1994; Adams et al., 1994). Many of these attractants belong to a family of 8–10 kDa, heparin-binding proteins known as chemokines. The chemokines include the C–C chemokines such as MCP-1, RANTES, MIP-1α, and MIP-1β, which primarily attract monocytes and T lymphocytes, and the C–X–C chemokines such as interleukin-8 (IL-8) and the gro proteins that primarily attract neutrophils (Baggiolini et al., 1994). There is some evidence that chemoattractants may play a role in activating integrins expressed on lymphocytes, as well as neutrophils. First, it has been shown that pertussis toxin inhibits the firm arrest and transmigration of T lymphocytes into inflammatory sites in vivo (Bargatze and Butcher, 1993; Spangrude et al., 1985). This implicates a G protein–coupled signaling event in lymphocyte adhesion and extravasation and suggests a role for chemoattractants, since all chemoattractant receptors thus far identified couple to G proteins (Gerard and Gerard, 1994; Murphy, 1994). Moreover, several chemokines have been shown to up-regulate lymphocyte adhesion to endothelial cells or isolated endothelial ligands (Taub et al., 1993a, 1993b; Tanaka et al., 1993b), although these proadhesive effects were relatively small and required relatively long incubation times.

Interestingly, while both neutrophils and T lymphocytes express β2 integrins that bind to ICAM-1 and ICAM-2 expressed on vascular endothelium, T lymphocytes also express the β1 integrin, VLA-4, which binds to VCAM-1, expressed on stimulated endothelium, as well as fibronectin (FN) expressed in the subendothelial ECM (Hemler, 1990). Thus, unlike most neutrophils, which lack VLA-4 and bind endothelium solely through β2 integrin–mediated interactions with ICAM-1, T lymphocytes can bind endothelium using either β1 integrin–mediated (VLA-4 binding to VCAM-1) or β2 integrin–mediated (LFA-1 binding to ICAM-1 and ICAM-2) interactions. VLA-4 expressed on T lymphocytes has also been found to mediate tethering, rolling, and arrest of T lymphocytes on VCAM-1 (Jones et al., 1994; Alon et al., 1995; Luscinskas et al., 1995; Berlin et al., 1995). While VLA-4 likely works in concert with selectins and β2 integrins to mediate lymphocyte rolling and arrest in vivo (Bargatze et al., 1995), the ability of VLA-4 to mediate spontaneous arrest allows T lymphocytes potentially to bypass the need for chemoattractant-induced firm adhesion, although a chemoattractant would likely still be needed to provide directional cues for transendothelial migration. Thus, the presence of VLA-4 on lymphocytes, but not neutrophils, opens the possibility that the signals for integrin activation on these two cell types might differ significantly.

We have previously demonstrated that MCP-1 acts as a major chemoattractant of T lymphocytes in vitro (Carr et al., 1994). In the experiments presented here, we investigate the role of MCP-1, and other C–C chemokines, in activating T lymphocyte integrins, using a novel and highly sensitive adhesion assay employing a parallel plate flow chamber and controlled detachment conditions. Results demonstrate that MCP-1 induces significant integrin-mediated T lymphocyte adhesion to the ECM protein FN and to endothelial-secreted ECM. In contrast, under the same conditions, MCP-1 fails to induce statistically significant binding to purified ICAM-1

or cytokine-stimulated endothelium. MCP-1 also fails to augment binding to VCAM-1, perhaps owing to the high constitutive binding of T lymphocytes to purified VCAM-1. Therefore, we propose that MCP-1 and other C–C chemokines may be less important for integrin-dependent firm adhesion of T lymphocytes to the vascular wall than for adhesion during or following transendothelial migration into tissue.

Results

MCP-1 Differentially Induces T Lymphocyte Adhesion to FN Compared with ICAM-1 or VCAM-1

In preliminary experiments using a traditional static adhesion assay (Diamond et al., 1990), we examined the ability of MCP-1 to induce integrin-dependent binding of T lymphocytes to purified preparations of the vascular ligands ICAM-1 and VCAM-1 or to cytokine-stimulated human umbilical vein endothelial cell (HUVEC). Despite gentle washing conditions, soluble MCP-1 at a wide range of concentrations and incubation times was found to have no effect on T lymphocyte adhesion to either purified ligand or to the endothelium (data not shown). Moreover, coimmobilization of MCP-1 on heparin–bovine serum albumin with ICAM-1 also failed to induce T lymphocyte binding to ICAM-1 (data not shown). In contrast, however, soluble MCP-1 was found to induce a small, but reproducible, increase in binding of T lymphocytes to FN (data not shown).

More sensitive adhesion assays were performed using a parallel-plate flow chamber, which allowed controlled detachment forces to be applied to adherent cells. Cells were introduced into the chamber and allowed to bind in stasis to immobilized adhesion proteins. Cells were then subjected to incremental increases in flow shear, and the cells that remained bound were quantitated at each shear. All cellular interactions with the substrate were recorded on videotape for later analysis. Unlike static assays in which the only readout of adhesion is the final number of cells remaining bound following exposure to relatively high but poorly controlled and undefined detachment forces, the parallel-plate flow assay allows the effects of MCP-1 to be monitored at the single cell level at each shear applied. The incremental application of known shear detachment forces permits subsets of cells with low, medium, and high adhesion strengths to be readily monitored by their resistance to detachment. Maximal MCP-1-induced binding to FN was found to occur between 4–7 min of stimulation; at later times, the ratio of stimulated to resting T lymphocytes bound to FN decreased, while at shorter times relatively fewer cells bound (data not shown). Based on these data, we chose to analyze the effects of MCP-1 on T lymphocyte adhesion following 6.5 min of stimulation.

In experiments examining FN binding, MCP-1 was found to induce binding of greater than 15% of the T lymphocytes, a 4- to 6-fold increase over resting cells (Figure 1A). Some interactions between stimulated or resting cells and FN were found to be relatively weak, as can be seen by the rapid detachment of a population of cells at low shear stress. However, the majority of MCP-1-stimulated T lymphocytes remained firmly bound to FN, even at very high shear stresses, indicating a strong adhesive interaction. A small fraction of unstimulated T lymphocytes also demonstrated shear-resistant binding to FN, which suggests the presence of a subset of cells with constitutive FN-binding activity within this population of resting T lymphocytes. In contrast, using identical flow conditions, in the presence of MCP-1 there was on average only a modest increase in T lymphocyte binding to ICAM-1 over the range of shear forces studied (Figure 1B). The effects on binding to ICAM-1 were not seen in all experiments and were not statistically significant. These results were further verified by performing the controlled detachment assays at 37°C, for varying incubation periods, or using variable ICAM-1 concentrations; again, MCP-1 was found to have no statistically significant effect on T lymphocyte binding to ICAM-1 (data not shown). Phorbol myristate acetate (PMA) was included as a positive control for stimulated binding to the substrate in all experiments. The binding of PMA-stimulated T lymphocytes to ICAM-1 typically ranged from approximately 45%–25% over the range of shears tested. When ICAM-1 binding experiments were performed at 37°C or for longer incubation periods (>10 min), PMA-induced binding of T lymphocytes was increased to >60% at low shear stress (data not shown). Dose-response curves for PMA stimulation of binding to fibronectin and ICAM-1 were similar, with near maximal stimulation of binding at 0.5 ng/ml and 1.5 ng/ml, respectively (data not shown).

Unlike the low background binding of resting T lymphocytes to FN or ICAM-1, the binding of resting T lymphocytes to VCAM-1 was very high and MCP-1 was not observed to augment further this constitutive binding at any of the applied shears (Figure 1C). To test whether this high constitutive T lymphocyte adhesion was related to the density of the VCAM-1 substrate, the concentration of immobilized ligand was decreased. Interestingly, while VCAM-1 titration resulted in decreased binding of resting cells, there was still no detectable enhancing effect of MCP-1 on T lymphocyte binding (data not shown). Since VCAM-1 supports tethering in flow as well as firm adhesion of T lymphocytes, we studied the ability of MCP-1 to modulate T lymphocyte binding to VCAM-1 in shear flow. MCP-1 had no effect on the number of cells that tethered to VCAM-1 at 1.0 dyne/cm^2 (Figure 1D), nor did MCP-1-stimulated cells that tethered exhibit any additional shear resistance (Figure 1d). Thus, while MCP-1 was able to induce significant T lymphocyte binding to FN, it was unable to stimulate significant binding to ICAM-1, nor was it able to augment the high constitutive tethering and firm adhesion of T lymphocytes to VCAM-1, even when these effects were studied in a highly sensitive controlled shear detachment assay.

Characterization of the Effect of MCP-1 on T Lymphocyte Adhesion to FN

The controlled shear detachment assay was used to study MCP-1-induced T lymphocyte binding to FN in further detail. T lymphocytes were stimulated with increasing concentrations of MCP-1 and cells remaining bound at low (1.2 dyne/cm^2), medium (6.0 dyne/cm^2),

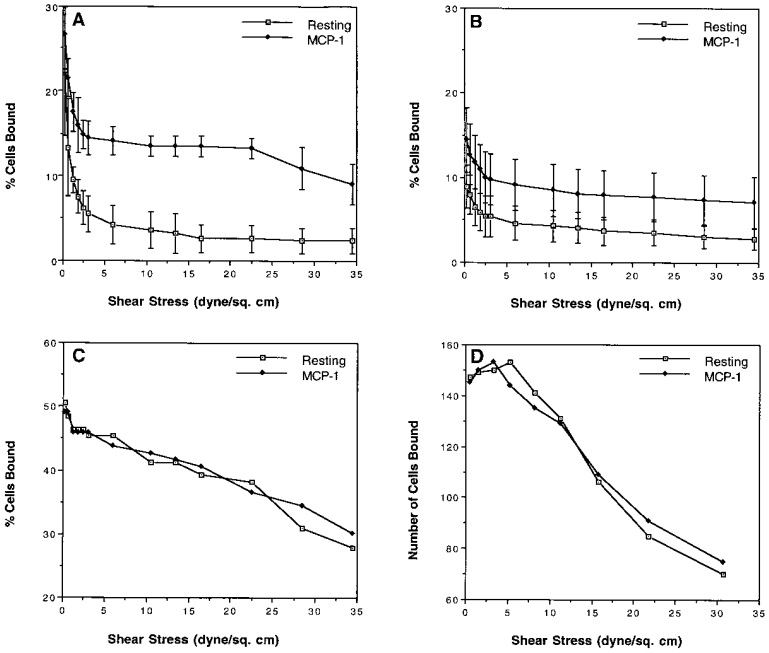

Figure 1. MCP-1 Induces Shear-Resistant Binding of T Lymphocytes to Immobilized FN, but Not ICAM-1 or VCAM-1 in a Controlled Shear Detachment Assay

T lymphocytes with or without MCP-1 (100 ng/ml) or PMA (50 ng/ml) were flowed into a parallel plate flow chamber and allowed to attach to FN (A) (30 μg/ml), ICAM-1 (B) (30 μg/ml), or VCAM-1 (C) (20 μg/ml) under static conditions at room temperature for 6.5 min or (D) were allowed to attach to VCAM-1 under physiologic flow conditions (1.0 dyne/cm^2) for 6.5 min. Following attachment, flow was initiated and increased in 2- to 2.5-fold increments every 10 s. Within an experiment, the same field of view was used and all experiments were recorded on videotape. The number of cells remaining bound at each interval was determined by analysis of the videotape and is expressed as the percentage of input cells remaining bound. The data in (A) and (B) is the average of at least three experiments and error bars represent standard error of the mean. Data in (C) and (D) are representative of one of three experiments. PMA was included as a positive control in each experiment and was found to induce binding, over the range of shears shown, which decreased from an average of 58.0%–52.3% on FN (A), 41.1%–24.3% on ICAM-1 (B), and 73.7%–69.1% on VCAM-1 (C). Statistical analysis using a paired two-tailed student's t-test showed that MCP-1 stimulation induced statistically significant binding ($p < 0.05$) to FN, but not to ICAM-1 or VCAM-1.

and high (22.5 dyne/cm^2) shear stresses, representing cell populations bound with increasing strengths of adhesion, were analyzed. MCP-1 was found to induce dose-dependent T lymphocyte binding to FN (Figure 2). MCP-1-stimulated binding was statistically significant compared with binding of resting cells at 50 and 100 ng/ml at the lower shears and at 100 ng/ml at higher shear. These concentrations are in agreement with concentrations that we have previously shown to induce maximal T lymphocyte transendothelial chemotaxis (Carr et al., 1994). In contrast with the results on FN, MCP-1 failed to induce statistically significant binding to ICAM-1 at any of the concentrations tested (data not shown).

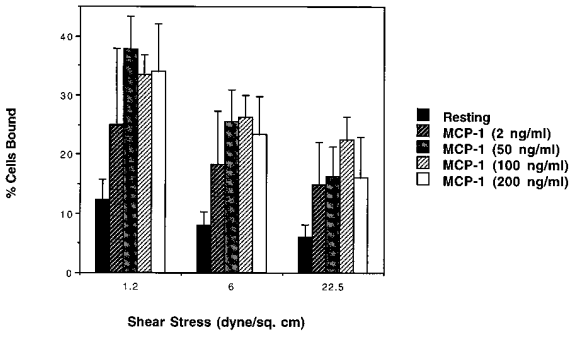

Figure 2. MCP-1 Induces Concentration-Dependent Binding of T Lymphocytes to FN in a Controlled Shear Detachment Assay

Experiments were performed as in Figure 1 with MCP-1 added at the concentrations indicated. Representative low, medium, and high shear stress are shown. PMA was included as a positive control and induced 47.3%, 42.2%, and 36.1% binding at 1.2, 6, and 22.5 dyne/cm^2, respectively. Statistical analysis using a paired two-tailed student's t-test showed that MCP-1 stimulation induced statistically significant ($p < 0.05$) binding to FN at 50 and 100 ng/ml. Data are the average of three independent experiments. Error bars represent SEM.

Since individual lymphocyte chemokines might be capable of activating specific T lymphocyte subpopulations, the effect of other C–C chemokines on T lymphocyte binding to FN or ICAM-1 was analyzed and compared with binding induced by MCP-1. The chemokines were tested at concentrations previously shown to induce significant levels of T lymphocyte transendothelial chemotaxis (S. J. Roth, M. W. C., and T. A. S., unpublished data). The chemokine RANTES, like MCP-1, was shown to induce a statistically significant increase in binding of T lymphocytes to FN at all shears tested, while MIP-1β induced a significant increase in binding only at the medium and high shears (Figure 3A). In contrast, none of the C–C chemokines, MCP-1, RANTES, MIP-1β, and also MIP-1α, induced a significant increase in binding of T lymphocytes to ICAM-1 compared with that of resting lymphocytes (Figure 3B). Thus, C–C chemokines selectively induce β1 integrin–dependent binding of T lymphocytes to FN but not β2 integrin–dependent binding to ICAM-1. Moreover, the failure of each of the chemokines to stimulate binding to ICAM-1 shows that the lack of β2 integrin activation is not specific to MCP-1.

T lymphocytes use two integrins, VLA-4 and VLA-5, to bind to FN. Monoclonal antibody (MAb) blocking experiments were performed to determine whether MCP-1 was preferentially activating one VLA over the other, or whether both integrins were being activated. MAb to the β1 subunit, shared by both VLA-4 and VLA-5, inhibited MCP-1-induced T lymphocyte binding to FN by greater than 90%, as did MAbs to VLA-4 and VLA-5 when combined (Figure 4). When used separately, MAbs to the α chains of either VLA-4 or VLA-5 partially, but similarly,

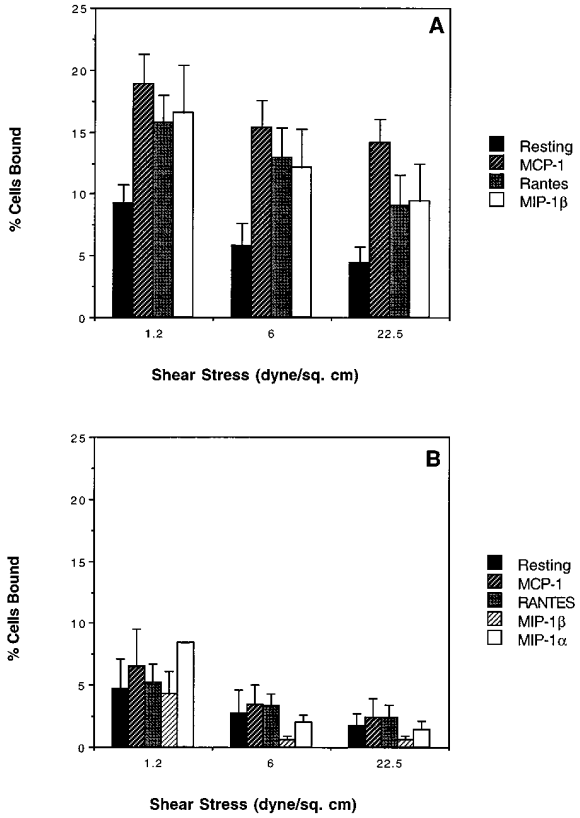

Figure 3. C–C Chemokines Induce Shear Resistant Binding to FN, but Not to ICAM-1, in a Controlled Detachment Binding Assay

Experiments were performed as in Figure 1 with the chemokines at concentrations that had been previously found to induce significant T lymphocyte chemotaxis: MCP-1 and RANTES at 100 ng/ml and MIP-1α and MIP-1β at 10 ng/ml. PMA was included as a positive control and induced 66.6%, 59.5%, and 56.4% binding to FN (A) and 50.9%, 39.9%, and 27.2% binding to ICAM-1 (B) at 1.2, 6, and 22.5 dyne/cm^2, respectively. Representative low, medium, and high shear stresses are shown. Statistical analysis using a paired two-tailed student's t-test showed that MCP-1 and RANTES induced significant adhesion to FN ($p < 0.05$) at each shear tested; MIP-1β induced significant adhesion to FN only at the medium and high shear stresses. None of the C–C chemokines induced significant adhesion to ICAM-1. Data are the average of three independent experiments. Error bars represent SEM.

inhibited MCP-1-induced T lymphocyte binding, suggesting that both VLA-4 and VLA-5 are activated and required for optimal stable binding to FN. These integrin antibodies were also found to reduce the low level binding of resting cells (data not shown), indicating that constitutive T lymphocyte binding to FN was β1 integrin dependent. Immunofluorescent flow cytometry of T lymphocytes stimulated with MCP-1 for 10 min showed that this binding was not due to increased expression of either VLA-4 or VLA-5 (data not shown). These results were further confirmed using peptides which block VLA-5-dependent but not VLA-4-dependent adhesion to FN (Ferguson et al., 1991). RGD peptide was found to block binding to FN at levels similar to those observed in the presence of anti-VLA-5 antibody, while an RGE peptide control had no effect (data not shown).

To determine whether MCP-1 stimulation of T lymphocytes required an intact actin cytoskeleton to modulate

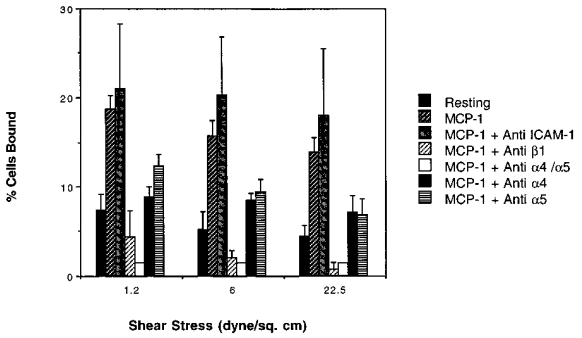

Figure 4. Antibodies to VLA-4 and VLA-5 Inhibit MCP-1-Induced Binding of T Lymphocytes to FN in a Controlled Detachment Binding Assay

Experiments were performed as described in Figure 1. MAbs (1:30 dilution of MAb 13, A4-PUJ1 and A5-PUJ1 ascites or 30 μg/ml of purified R6.5, concentrations shown by FACS to be saturating) were preincubated with cells for 10 min prior to addition of MCP-1 (100 ng/ml); antibody was included throughout the assay. Representative low, medium, and high shear stresses are shown. PMA was included as a positive control and induced 59.1%, 55.7%, and 54.8% binding at 1.2, 6, and 22.5 dyne/cm^2, respectively. Data are the average of three independent experiments. Error bars represent SEM.

integrin affinity for FN, lymphocytes were preincubated with cytochalasin B to disrupt actin microfilaments (Yahara et al., 1982; Cooper, 1987). A high concentration (20 μM) of cytochalasin B was found to abolish shear-resistant MCP-1-induced adhesion of T lymphocytes to FN (Figure 5). This concentration of cytochalasin B also slightly decreased the binding of resting T lymphocytes to FN. However, a 10-fold lower concentration of cytochalasin B caused insignificant inhibition of T lymphocyte adhesion in this assay. Interestingly, when identical concentrations of cytochalasin B were included in chemotaxis assays, migration of T lymphocytes to MCP-1 was significantly inhibited by both concentrations of cytochalasin B (M. W. C., unpublished data), suggesting

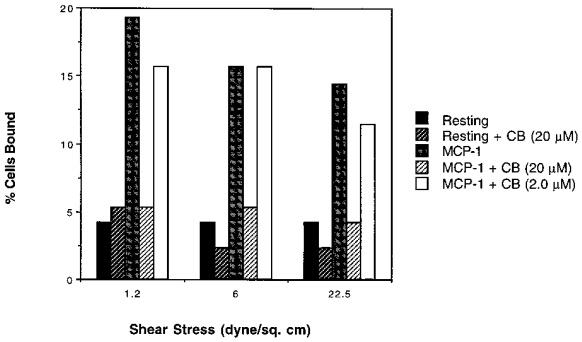

Figure 5. Cytochalasin B Inhibits MCP-1-Induced Binding of T Lymphocytes to FN in a Controlled Detachment Binding Assay

Experiments were performed as described in Figure 1 using FN at 30 μg/ml. Cytochalasin B (20 or 2.0 μM) was preincubated with cells for 30 min prior to addition of MCP-1 (100 ng/ml) and was included throughout the experiment. Cytochalasin B was also included in experiments with resting cells. The data presented here is representative of one of three independent experiments with similar results. Representative low, medium, and high shear stresses are shown. PMA was included as a positive control and induced 43.2%, 41.0%, and 40.3% binding at 1.2, 6, and 22.5 dyne/cm^2, respectively.

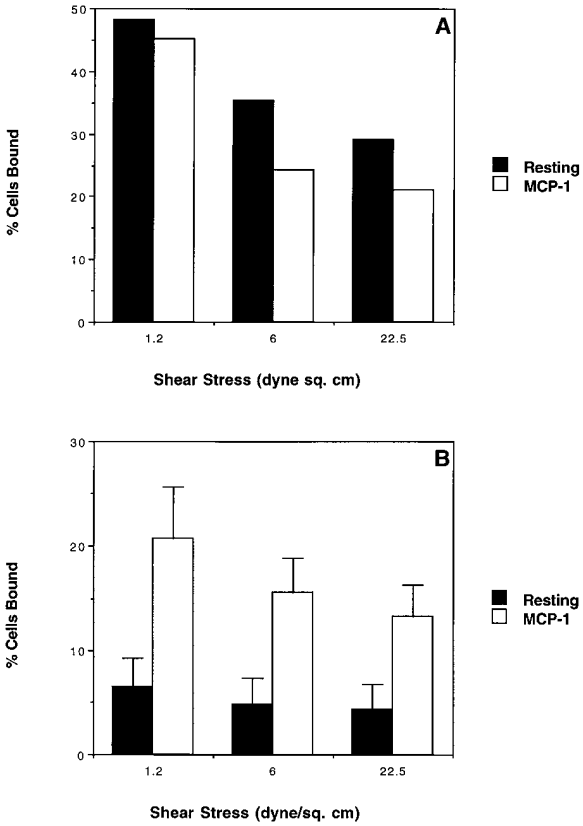

Figure 6. MCP-1 Fails to Augment Binding of T Lymphocytes to TNFα-Stimulated HUVEC but Does Induce Binding to Subendothelial ECM in a Controlled Detachment Binding Assay

Experiments were performed as described in Figure 1 using HUVEC (A) stimulated for 5 hr with 200 U/ml of TNFα or endothelial matrix (B) secreted by HUVEC cultured for 2 weeks, then detergent solubilized. Data in (A) is representative of one of three independent experiments with similar results, while data in (B) is an average of four independent experiments. Representative low, medium, and high shear stresses are shown. PMA was included as a positive control and induced 90.9%, 75%, and 54.5% binding to the HUVEC at 1.2, 6, and 22.5 dyne/cm^2, respectively and 47.6%, 36.2%, and 25.4% binding to the ECM at the same respective shears.

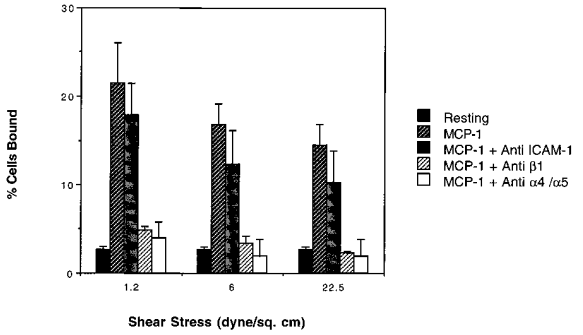

Figure 7. Antibodies to VLA-4 and VLA-5 Inhibit MCP-1-Induced Binding of T Lymphocytes to Subendothelial ECM in a Controlled Detachment Binding Assay

Experiments were performed as described in Figure 1 using endothelial matrix secreted by HUVEC cultured for 2 weeks, then detergent solubilized. MAbs (1:30 dilution of antibody 13, A4-PUJ1 and A5-PUJ1 ascites or 30 μg/ml of purified R6.5, concentrations shown by FACS to be saturating) were preincubated with cells for 10 min prior to addition of MCP-1 (100 ng/ml) and antibody was included throughout the assay. Representative low, medium, and high shear stresses are shown. PMA was included as a positive control and induced 76%, 58%, and 40% binding at 1.2, 6, and 22.5 dyne/cm^2, respectively. Data are the average of three independent experiments. Error bars represent SEM.

that migration is more dependent on the presence of a fully intact actin cytoskeleton than is integrin-dependent adhesion, and that MCP-1-dependent modulation of integrin adhesiveness and MCP-1-dependent chemotaxis may operate through separate pathways.

Effects of MCP-1 on T Lymphocyte Binding to Stimulated HUVEC or HUVEC-Secreted ECM

Using the controlled shear detachment assay, MCP-1-induced interactions between T lymphocytes and cytokine-stimulated HUVEC, shown by immunofluorescent staining to express high levels of ICAM-1, VCAM-1, and E-selectin, were analyzed. Constitutive adhesion of resting T lymphocytes to stimulated HUVEC was high and further stimulation with MCP-1 had no effect on shear-resistant lymphocyte adhesion (Figure 6A). In contrast, neither resting nor MCP-1-stimulated T lymphocytes bound at significant levels to unstimulated HUVEC (data not shown). In other experiments, MCP-1-stimulated or resting cells were allowed to tether to stimulated HUVEC under physiologic flow conditions. MCP-1 was found to have no augmenting effect on the shear resistance of these tethered cells (data not shown). Thus, even when T lymphocytes were allowed first to form rolling adhesions on the HUVEC, stimulation by MCP-1 did not further augment binding to the endothelium.

In parallel experiments, the effect of MCP-1 on T lymphocyte adhesion to HUVEC-secreted ECM was assessed. Immunofluorescent staining of the ECM indicated that the major constituents were FN, laminin, and several types of collagen (Roth et al., 1995). MCP-1 was found to induce significant T lymphocyte binding to ECM (Figure 6B). As was seen in binding experiments to purified FN, β1 integrin–blocking MAbs almost completely inhibited binding to ECM, as did a combination of MAbs to VLA-4 and VLA-5 (Figure 7), suggesting that FN is the major ligand involved in chemokine-induced T lymphocyte interactions with ECM. Moreover, these results on ECM demonstrate MCP-1-induced T lymphocyte binding to a physiologically relevant naturally occurring substrate.

Discussion

The data presented here fail to support the widely accepted hypothesis that chemokines serve to activate T lymphocyte integrins in order to mediate firm binding and arrest of lymphocytes on the vascular adhesion proteins, ICAM-1 and VCAM-1, prior to migration into surrounding tissue. These experiments do, however, show a clear role for chemokines, particularly MCP-1, in modulating lymphocyte adhesion to ECM proteins secreted by endothelium, specifically FN. This MCP-1-induced binding to FN was shown to be dose

dependent and to occur rapidly, within 4–7 min of exposure to the chemokine. Additional experiments demonstrated that MCP-1 activates both the VLA-4 and VLA-5 integrins, which together contribute to the MCP-1-dependent increase in T lymphocyte binding to FN. This binding was dependent on the presence of an intact cytoskeleton, suggesting that MCP-1 stimulation results in postligand stabilization events that are required to mediate firm adhesion.

Our findings on HUVEC-secreted ECM are in agreement with those presented in a recent paper in which RANTES and MIP-1β were found to induce binding of $CD4^+$ T lymphocytes to subendothelial ECM (Gilat et al., 1994). In those experiments, however, a 30 min incubation with chemokine was necessary to detect an adhesive effect; this is unlike our results in which soluble chemokine was found to activate β1 integrin–mediated adhesion in as little as 4 min. This shorter timepoint is presumably more physiologic than the 15–30 min incubations typically used to measure lymphocyte adhesion in static binding assays (Taub et al., 1993a, 1993b; Tanaka et al., 1993b; Gilat et al., 1994), since integrin-dependent adhesion strengthening is believed to occur within minutes following stimulation (Springer, 1995).

The protein kinase C activator PMA induced both β1 and β2 integrin–dependent binding, although at suboptimal doses PMA activated VLA-4 and VLA-5 at slightly lower concentrations than for LFA-1. In contrast, chemokines differentially induced β1 compared with β2 integrin–dependent binding of T lymphocytes at all doses examined. These findings suggest that integrins may require distinct physiologic activation signals to interact with their respective ligands. However, we have not defined whether the underlying mechanism is attributable to differential sensitivity of the integrins to signals in general or selective action of chemokine signaling pathways on β1 as opposed to β2 integrins, both mechanisms may contribute. As mentioned previously, our lab and others have shown that T lymphocytes can interact in shear with VCAM-1 without exogenous stimulation, although stimulation can induce development of firm adhesion so that cells arrest rather than roll on VCAM-1. Thus, VLA-4 expressed on peripheral T lymphocytes appears to exist in a constitutively active form and we found that addition of MCP-1 was seemingly unable to augment further T lymphocyte binding to VCAM-1. Data on the effects of MCP-1 on T lymphocyte binding to FN, however, clearly demonstrate that MCP-1-stimulated binding to FN is at least partially dependent on activation of the integrin VLA-4. Thus, it appears that MCP-1 is only able to regulate some VLA-4 binding activities. This finding is in agreement with work by other groups who find that VLA-4 exists in multiple activation states (Masumoto and Hemler, 1993; Jakubowski et al., 1995). In the model proposed by Hemler and colleagues, VLA-4 is thought to exist in inactive, partially active, or fully active states. In the inactive state, VLA-4 is unable to bind to either of its two ligands, VCAM-1 or FN. In the partially active state, it is only able to bind to VCAM-1, but not FN, and, finally, in the fully activated state, VLA-4 is able to bind both ligands. Thus, binding to VCAM-1 requires a lower threshold of activation than does binding to FN. This model, together with data presented here, supports the idea that VLA-4 expressed on peripheral T lymphocytes exists in a partially active state. Addition of an activator, such as MCP-1 or another chemokine, is required to convert this VLA-4 into the fully active state, allowing T lymphocytes to firmly bind FN.

MCP-1, under these conditions, had only modest or no ability to activate the β2 integrin, LFA-1, as no statistically significant MCP-1-induced T lymphocyte binding to the LFA-1 ligand, ICAM-1, was observed. Although MCP-1 was found to have a modest activating effect on LFA-1 in some experiments, this was not a consistent finding, since in other experiments MCP-1 had no effect compared with control. This lack of effect does not appear to be due to an inability to find optimal T lymphocyte binding conditions, since conditions that supported lymphocyte binding to FN and VCAM-1 failed to support binding to ICAM-1. Experiments varying MCP-1 concentration, ICAM-1 concentration, time of incubation, type of chemokine, and assay temperature, all failed to show chemokine-stimulated T lymphocyte binding to ICAM-1. PMA stimulation did induce significant binding, indicating a suitable ICAM-1 substrate and activatable lymphocytes. These findings suggest that the regulation of β2 integrins differs between neutrophils and T lymphocytes, with β2 integrins on neutrophils, but not T lymphocytes, being regulated by chemokines, at least with the known T cell chemokines examined here. This differential response may be due, in part, to the high expression of chemokine receptors on neutrophils ($2–9 \times 10^4$ receptors/cells) (Baggiolini et al., 1994), while receptors for C–C chemokines are difficult to detect on PBL (Napolitano et al., 1990). Alternatively, the mechanism of chemokine activation for Mac-1, the primary β2 integrin on neutrophils, may be inherently different than the mechanism of chemokine activation for LFA-1, the primary β2 integrin on T lymphocytes.

These data, together with recently published data demonstrating the ability of T lymphocytes to tether, roll, and spontaneously arrest on VCAM-1 (Alon et al., 1995; Berlin et al., 1995; Jones et al., 1994; Luscinskas et al., 1995), suggest that the multistep model of neutrophil extravasation from the bloodstream needs to be refined to apply to the extravasation process of T lymphocytes. T lymphocyte emigration from the bloodstream into chronic inflammatory sites appears initially to involve the cooperative and probably overlapping interactions of both VLA-4 with VCAM-1, and selectins with their respective carbohydrate ligands. The combination of these interactions allows a T lymphocyte free in flow to tether and roll on activated endothelium. We hypothesize that rolling T lymphocytes are able to arrest spontaneously via a semiactivated form of VLA-4, or arrest via β2 integrins in response to factors that remain to be identified. Currently identified chemokines may be most important for directional cues in transendothelial migration, migration through the basement membrane and within tissue, and in regulating adhesion to ECM components. Transendothelial migration of T lymphocytes is inhibited by MAb to β2 integrins or in β2 integrin–deficient patients (Oppenheimer-Marks et al., 1990, 1991; van Kooyk et al., 1993; Kavanaugh et al., 1991; Vennegoor et al., 1992) and it remains unclear what adhesive mechanism regulates the β2 integrin–dependent extension of a pseudopod through the endothelial

layer to initiate the first contact with the subendothelial matrix. However, once this contact is initiated, our data suggest that chemokine-stimulated adhesion to FN is important in completion of transendothelial migration, as well as in migration through ECM in the basement membrane and within tissues.

Experimental Procedures

Reagents

Recombinant human tumor necrosis factor-α (TNFα) was purchased from Genzyme (Cambridge, Massachusetts). Purified recombinant human chemokines were purchased from Peprotech, Incorporated (Rocky Hill, New Jersey) or R and D Systems (Minneapolis, Minnesota).

MAbs

The following previously described murine MAbs against human antigens were used: B1 (anti-CD20, immunoglobulin G2a [IgG2a]) (Stashenko et al., 1980) and MY4 (anti-CD14, IgG2b) (Griffin et al., 1981) from Coulter Immunology (Hialeah, Florida). 3G8 (anti-CD16, IgG1) (Fleit et al., 1989) from AMAC, Incorporated (Westbrook, Maine). OKM1 (anti-CD11β, IgG2b) (Breard et al., 1980) from Ortho Diagnostic Systems (Raritan, New Jersey). R6.5 (anti-ICAM-1, IgG2a) (Smith et al., 1989) produced in our laboratory. A4-PUJ1 (anti-CD49d, IgG1), A5-PUJ1 (anti-CD49e, IgG1), and MAb 13 (anti-CD29, IgG2a) (Akiyama et al., 1989) were gifts from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, Massachusetts).

Preparation of PBL and T Lymphocytes

Human peripheral blood mononuclear cells (PBMCs) were purified from citrate-anticoaggulated whole blood of healthy volunteer donors by dextran sedimentation and centrifugation through Ficoll–Hypaque (1.077) as previously described (English and Anderson, 1974). T lymphocytes were purified from PBMC by negative selection using magnetic cell separation (MACS, Miltenyi Biotec, Sunnyvale, California) (Miltenyi et al., 1990). In brief, PBMCs were incubated for 30 min on ice with 5 μg/ml of MAbs to B cells (B1), natural killer cells (3G8), and monocytes (MY4 and OKM1). The PBMCs were then washed and incubated with MACS rat anti-mouse IgG1 and IgG2a+2b magnetic microbeads (Miltenyi Biotec). Following a 20 min incubation on ice, cells were loaded onto a preëquilibrated magnetic separation column coupled to the MACS magnet. All T lymphocyte purifications were done at 4°C. T lymphocyte preparations routinely had >98% cell viability as determined by trypan blue exclusion and were used in assays on the day they were prepared. Preparations routinely contained greater than 95% pure $CD3^+$ T lymphocytes with less than 1% each of contaminating monocytes, B lymphocytes, or natural killer cells.

Adhesion Assays

Adhesion Substrates

Affinity-purified recombinant 7 domain VCAM-1 (Pepinsky et al., 1992) was a gift from Dr. R. Lobb (Biogen, Cambridge, Massachusetts). Full-length FN was purchased from GIBCO BRL. ICAM-1 was purified by immunoaffinity chromatography as described (Marlin and Springer, 1987). HUVECs were cultured for 1–2 days on tissue culture–treated petri dishes. Confluent HUVEC monolayers were stimulated with TNFα (200 U/ml) for 4–6 hr. ECM substrates were prepared from HUVEC monolayers that were in culture for at least 2 weeks by detergent solubilization with 0.5% Triton X-100 (Pharmacia) in 20 mM NH_4OH. No residual endothelium was detected.

Controlled Detachment Adhesion Assay

Laminar flow adhesion assays were performed as previously described (Lawrence and Springer, 1991; Alon et al., 1995). In brief, adhesion proteins were diluted in 50 mM Tris (pH 9.0) at the indicated concentrations and adsorbed as spots on polystyrene plates for 2 hr at room temperature, washed three times with phosphate-buffered saline, and blocked with 2% HSA in phosphate-buffered saline overnight at 4°C. These plastic plates were assembled as the lower wall in a parallel wall flow chamber and mounted on the stage of an inverted phase–contrast microscope (Diaphot-TMD). Bound cells were quantitated by analysis of images videotaped with a TEC-470 CCD video camera (Optronics, Goleta, California) and a Mitsubishi HS-U67 VHS recorder. T lymphocytes (1×10^6 cells/ml) were resuspended in binding buffer (HBSS, containing 10 mM HEPES [pH 7.4], 1 mM Mg^{2+}, 2 mM Ca^{2+}, and 2 mg/ml HSA) and perfused into the flow chamber. When assessing the effects of soluble activators on T lymphocyte adhesion, the activator was added directly to the cell suspension seconds before being perfused. Perfused cells were incubated under static conditions for 6.5 min at room temperature, then flow was initiated and increased in 2- to 2.5-fold increments every 10 s. The number of cells remaining bound at each interval was determined by careful analysis of the video tape. The same field of view was used for each substrate within each experiment to ensure that the results reflected uniform site density and distribution of the immobilized protein. All adhesion experiments were performed a minimum of three times. PMA control is included in all experiments and is considered to be indicative of the maximal number of cells capable of binding to a given substrate in a given experiment. The data is expressed as the percent of statically interacting cells that remained bound at each shear stress.

For inhibition studies, T lymphocytes were preincubated for 10 min in binding buffer with 30 μg/ml of purified MAb or a 1:30 dilution of ascites. The cell suspension was diluted in a 10-fold volume of binding buffer containing antibody (with or without activator) and immediately perfused into the flow chamber. GRGDSP and GRDESP peptides were preincubated with cells for 20 min at 300 μM prior to infusion and were included in the buffer (with or without activator) at the initiation of the assay. To test the effect of cytochalasin B (CB), cells were incubated with cytochalasin B at a concentration of 2 or 20 μM for 30 s at room temperature and perfused without dilution (with or without activator) into the flow chamber. CB was present in the binding buffer throughout the assay.

Statistical Analysis

Data was analyzed where indicated using a paired two-tailed student's t test.

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