

# A Subpopulation of Mac-1 (CD11b/CD18) Molecules Mediates Neutrophil Adhesion to ICAM-1 and Fibrinogen

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**Abstract.** We report that a subpopulation (10%) of the Mac-1 (CD11b/CD18) molecules on activated neutrophils mediates adhesion to ICAM-1 and fibrinogen. We describe a novel mAb (CBRM1/5) that binds to an activation-specific neopeptide on a subset of Mac-1 molecules on neutrophils and monocytes after stimulation with chemoattractants or phorbol esters but does not recognize Mac-1 on resting myeloid cells. CBRM1/5 immunoprecipitates a subpopulation of

Mac-1 molecules from detergent lysates of neutrophils, binds to immunoaffinity-purified Mac-1, and localizes to the I domain on the  $\alpha$  chain of Mac-1. Because CBRM1/5 recognizes a fraction of Mac-1 on activated neutrophils, but still blocks Mac-1-dependent adhesion to fibrinogen and ICAM-1, we suggest that only a small subset of Mac-1 molecules is competent to mediate adhesion.

**U**NDER normal conditions, circulating neutrophils flow through the vascular system making few attachments to the endothelium. Within minutes of an exposure to an inflammatory stimulus, neutrophils roll along the adjacent vessel wall, adhere firmly to the endothelium, and then traffic into the parenchyma (8). Recent experiments have identified the molecules on neutrophil and endothelial cell surfaces that facilitate emigration. Members of the selectin family mediate the initial rolling (47, 48, 82) and members of the integrin family strengthen neutrophil attachment to the endothelium (47, 82), and then promote transmigration to the underlying tissue (7, 72).

The subfamily of integrins that mediates neutrophil attachment to endothelium includes three members, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18) which share a common  $\beta$  subunit that is noncovalently associated with unique but closely related  $\alpha$  chains (42, 74). These leukocyte integrins mediate several additional adhesive events that are crucial for immune system function. They promote the adhesion that is required for T lymphocyte target cell lysis (15), T lymphocyte proliferation (15), natural killing (43), lymphocyte and monocyte adhesion to endothelial cells (25, 36, 83), neutrophil homotypic aggregation, and neutrophil chemotaxis (6).

Experiments by several groups have demonstrated that the adhesiveness of the leukocyte integrins is not constitutive, but rather is controlled by intracellular signals transduced from other surface receptors. For example, LFA-1 on resting T lymphocytes is unable to bind to its counter-receptor ICAM-1 until a signal has been delivered through the antigen receptor (26, 78). The signal to adhere is transient, in that within 30 min of stimulation, T lymphocytes lose their abil-

ity to bind ICAM-1. Similarly, Mac-1 on neutrophils does not bind its cellular or soluble ligands including ICAM-1 (20), fibrinogen (4, 86), factor X (3), or iC3b (84) until a specific signal (e.g., chemotactic factor, cytokine, or phorbol ester) has been delivered; stimulation increases the avidity of Mac-1 for its ligands within seconds (6, 11, 17, 84).

While it is clear that activation facilitates leukocyte integrin interaction with ligand, the molecular mechanism for the change in avidity remains poorly understood. Because there is no increase in LFA-1 expression after antigen stimulation or phorbol ester treatment in T cells, the avidity change of LFA-1 for ICAM-1 is believed to occur because of a structural change in the molecule (26). Alternatively, the change in avidity could stem from a signal to cluster adhesion receptors at the cell surface (16). The mechanism for avidity regulation of Mac-1 on neutrophils appears analogous but the analysis is complicated by a greater than 10-fold quantitative increase in expression that follows stimulation (10, 17, 49, 55, 76). However, the change in surface expression that occurs after stimulation does not parallel the kinetics or magnitude of adhesion (11, 49), as ion channel antagonists (80) or temperature conditions (66) that inhibit the quantitative increase in Mac-1 expression do not block Mac-1-dependent adhesion to endothelial cells or in vitro substrates. Thus, Mac-1 is hypothesized to undergo additional conformational changes that facilitate adhesion (11, 49, 59, 80). Studies showing that divalent cations (1, 21, 23, 79) or mAbs against Mac-1 or LFA-1 (22, 38, 40, 61) augment adhesion in the absence of activation, support a model in which direct structural changes to the extracellular regions regulate leukocyte integrin avidity.

If avidity regulation translates into a conformational

change in the extracellular region of an integrin molecule, it should be possible to generate probes that sense only the inactive or active states of the molecule. Indeed, mAbs against the platelet integrin gpIIb-IIIa have been described that react with only activated and not resting platelets (57, 68). In this report, we describe the generation of a novel mAb that reacts with Mac-1 molecules on activated neutrophils, purified Mac-1 substrates, or detergent-solubilized cell lysates. Because CBRM1/5 binds to a subset (10–30%) of Mac-1 molecules on stimulated neutrophils, but still blocks binding to fibrinogen and ICAM-1, we suggest that an activated subset of Mac-1 molecules mediates adhesion.

## Materials and Methods

### Monoclonal Antibodies

The following murine mAbs against human antigens were used: TS1/22 (anti-CD11a, IgG1, ascites) (64); LM2/1 (anti-CD11b, IgG1, protein A-purified) (55); CBRM1/23 (anti-CD11b, IgG2a, protein A-purified) (20a); OKM1 (anti-CD11b, IgG2b, protein A-purified) (85); LPM19c (anti-CD11b, IgG2a, a gift of Dr. K. Pulford, Radcliffe Hospital, Oxford, UK) (77); BLY6 (anti-CD11c, IgG1, ascites) (77); R6.5 (anti-CD54, IgG2a, generous gift of Dr. R. Rothlein, Boehringer-Ingelheim, Ridgefield, CT) (72); (CBRIC1/11 (anti-CD54, IgG1, protein A-purified) (M. S. Diamond and T. A. Springer, unpublished data); and My4 (anti-CD14, IgG2b, purified) (34) (Coulter Immunology, Hialeah, FL). X63 (nonbinding antibody, IgG1) and 3G8 (anti-CD16, IgG1) (29) were used as culture supernatants.

### Protein Purification

Mac-1 was purified from leukocyte lysates by immunoaffinity chromatography after detergent solubilization as previously described (20). Soluble ICAM-1 (sICAM-1, generous gift of Dr. S. Marlin, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT) was purified from supernatants of ICAM-1 transfected CHO cells by immunoaffinity chromatography as previously described (51).

### Tissue Culture, Transfection, and Cell Preparation

Neutrophils were isolated from the whole blood of healthy volunteers by dextran sedimentation at room temperature with Ficoll gradient centrifugation and hypotonic lysis at 4°C as previously described (28, 55). Before  $^{125}\text{I}$ -mAb binding experiments, neutrophils ( $2 \times 10^7$  cells/ml) were stored at 4°C in HBSS, 10 mM Hepes, pH 7.3, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$  (HHMC)<sup>1</sup> in polypropylene tubes (Falcon 2097, Becton Dickinson, Lincoln Park, NJ). For time course and temperature studies, the leukocyte-rich supernatant was used after dextran sedimentation. Cell suspensions were placed on ice immediately and washed four times to remove platelets in HBSS, 10 mM Hepes, pH 7.3, and resuspended at  $5 \times 10^6$  cells/ml in HHMC. Neutrophils were identified during flow cytometry by forward and 90° scatter, and confirmed by immunostaining with a mAb to CD16. Mononuclear cells were isolated from the interface of the Ficoll gradient and washed five times in RPMI 1640, 5 mM EDTA, 2.5% FCS (heat-inactivated, low endotoxin, Hyclone Laboratories, Inc., Logan, UT), and resuspended in L15, 2.5% FCS, 1 mg/ml human  $\gamma$ -globulin (ICN ImmunoBiologicals, Costa Mesa, CA) at  $5 \times 10^6$  cells/ml at 4°C. Mononuclear cells were immunostained according to the protocol for neutrophils (see below). The monocyte subpopulation was determined by the flow cytometric forward and 90° scatter pattern and confirmed by immunostaining with the mAb to the monocyte-specific CD14 antigen.

The generation and selection of CHO cells expressing wild-type and chimeric forms of Mac-1 and p150,95 will be described (20a). They were main-

tained in  $\alpha$ -MEM, 10% dialyzed FCS, 16  $\mu\text{M}$  thymidine, 0.05  $\mu\text{M}$  methotrexate, 2 mM glutamine, and 50  $\mu\text{g}/\text{ml}$  gentamicin.

### Generation and Labeling of CBRM1/5 IgG and Fab Fragments

The generation of mAbs against Mac-1 will be described in detail elsewhere (20a). In brief, hybridomas were prepared from BALB/c mice that were immunized with immunoaffinity-purified Mac-1. Hybridomas (500) were screened as follows. Purified neutrophils were isolated, separated into two groups, and preincubated at 37°C for 5 min in HHMC. One population was treated with phorbol esters (PMA, 100 ng/ml) for 10 min whereas the other remained untreated. Cell suspensions were placed on ice for 5 min, incubated with hybridoma supernatants, and processed by flow cytometry. In some cases, neutrophils were fixed with 2% paraformaldehyde before incubation with mAbs. CBRM1/5 was identified for its ability to bind PMA-stimulated but not resting neutrophils. It was cloned twice by limiting dilution, and purified from culture supernatant by protein A-Sepharose affinity chromatography after  $\text{NH}_4\text{SO}_4$  precipitation (35). CBRM1/5 was determined to be of the IgG1 subclass (ImmunoPure Monoclonal Antibody Isotyping Kit, Pierce Chemical Co., Rockford, IL).

Fab fragmentation of CBRM1/5 was performed by pepsin digestion followed by cysteine reduction (58). Fab' were separated by  $1.5 \times 75\text{-cm}$  S-200 Sephacryl (Pharmacia Molecular Biology Division, Piscataway, NJ) size-exclusion chromatography. Fab' eluted from the column as a single homogeneous peak with an apparent  $M_r$  of 40,000. Purity of Fab' and Fab' fragments was confirmed by reducing (5%  $\beta$ -mercaptoethanol) and nonreducing (50 mM iodoacetamide) 8% SDS-PAGE. Proteins were visualized by silver staining (56). Unlabeled (Bio-Rad Laboratories, Richmond, CA) or  $^{14}\text{C}$ -labeled (Gibco BRL, Gaithersburg, MD) protein standards included myosin (200,000  $M_r$ ),  $\beta$ -galactosidase (116,000  $M_r$ ), phosphorylase B (97,000  $M_r$ ), serum albumin (66,000  $M_r$ ), ovalbumin (43,000  $M_r$ ), carbonic anhydrase (31,000  $M_r$ ), trypsin inhibitor (21,500  $M_r$ ), and lysozyme (14,000  $M_r$ ).

mAbs (100  $\mu\text{g}$ ) were labeled with  $\text{Na}^{125}\text{I}$  (1 mCi) using 1,3,4,6-tetrachloro-3 $\alpha$ , 6 $\alpha$ -diphenylglycouril (Pierce Chemical Co.) (30). The specific activity of the iodinated mAbs was determined (protein recovery averaged 83  $\mu\text{g}$ ; sp act  $6\text{--}7 \times 10^6$  cpm/ $\mu\text{g}$ ).

### $^{125}\text{I}$ -mAb Binding Assays

Purified neutrophils ( $2 \times 10^7$  cells/ml in HHMC) were either kept on ice or warmed to 37°C for 5 min and stimulated (PMA, 100 ng/ml; fMLP,  $10^{-7}\text{M}$ ) for 10 min. Activated neutrophils were immediately placed on ice and 500,000 cells were aliquoted into u-bottom plates (96-well nontissue culture-treated microtiter plates, Linbro-Titertek, Flow Laboratories, McLean, VA) containing increasing concentrations of iodinated mAb (0.109–112 nM) in the absence or presence of 20–100-fold excess cold mAb. The binding media contained L15, 2.5% FCS, 1 mg/ml human  $\gamma$ -globulin. Neutrophils were incubated with iodinated mAbs for 4 h on ice, washed five times with L15, 2.5% FCS by 21-gauge needle aspiration, lysed with L15, 2.5% FCS, 0.2 N NaOH, and counted for  $\gamma$ -emission. Specific binding was determined by subtracting the cpm in the presence of excess cold mAb. Scatchard plots were generated (65) and site densities were determined using the data point at which there was no additional increase in specific binding of labeled mAb.

### Surface Labeling, Immunoprecipitation, and Gel Electrophoresis

Purified neutrophils ( $2 \times 10^7$  cells) were washed twice in PBS, 1 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{CaCl}_2$ , and resuspended in 2 ml; fMLP ( $10^{-7}\text{M}$ ) was added to some aliquots of neutrophils. The cells were iodinated, lysed, and precleared as previously described (20).

Immunoprecipitations were performed one of two ways: (a) indirectly by incubating 250  $\mu\text{l}$  of mAb (neat supernatant or 20  $\mu\text{g}/\text{ml}$  purified IgG) with 1  $\mu\text{l}$  of 1 mg/ml-purified rabbit anti-mouse IgG (Zymed Laboratories, Inc., South San Francisco, CA) for 4 h at 4°C. Protein A-Sepharose (20  $\mu\text{l}$  of a 1:1 slurry) was added for overnight incubation. Eppendorf tubes were centrifuged, supernatants were aspirated, 50  $\mu\text{l}$  of iodinated neutrophil lysate was added, and the mixture was incubated for 2 h at 4°C while shaking vigorously; or (b) directly by adding 60  $\mu\text{l}$  of a 1:4 diluted lysate to 60  $\mu\text{l}$  of directly coupled mAb-Sepharose (2–3 mg purified mAb per milliliter CNBr-activated-Sepharose CL-4B) and incubated for 2 h at 4°C while

1. Abbreviations used in this paper: fMLP, f-met-leu-phe; HHMC, HBSS, 10 mM Hepes, pH 7.3, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ .

shaking vigorously. The washing and elution of the immunoprecipitates has previously been described (20). Samples were loaded and subjected to 7% SDS-PAGE (44) in the presence of  $\beta$ -mercaptoethanol, and autoradiographed with intensifying screens (46).

Preclearing experiments were performed with directly coupled-Sepharose as follows. After the initial immunoprecipitation with control (mouse IgG) or specific Sepharose (LM2/1, CBRM1/5), the beads were pelleted, the supernatant (60  $\mu$ l) transferred to a second aliquot of the same mAb-Sepharose, and incubated as described above. Subsequently, these beads were pelleted, the supernatants (60  $\mu$ l) transferred to an aliquot of either LM2/1 or CBRM1/5-Sepharose, and incubated. After each round of immunoprecipitation, the beads were washed and the bound protein eluted as previously described (20). Samples were loaded and subjected to 5% SDS-PAGE (44) in the presence of  $\beta$ -mercaptoethanol and autoradiography (46).

### ELISA with Purified Mac-1

Immunoaffinity-purified human Mac-1 was diluted 1:20 in 25 mM Tris-HCl, 150 mM NaCl, 2 mM  $MgCl_2$ , and adsorbed to individual wells of a nontissue culture-treated microtiter plate (Linbro-Titertek, Flow Laboratories) for 1 h at 37°C. Plates were washed five times and blocked for 1 h at 37°C in HHMC supplemented with 1% human serum albumin. Plates were then washed twice with HBSS, 10 mM Hepes, pH 7.3, 10 mM EDTA, 0.05% Tween 20 and twice again with HBSS, 10 mM Hepes, pH 7.3, 0.05% Tween 20. mAb solution (100  $\mu$ l of 20  $\mu$ g/ml of purified mAbs) in HBSS, 10 mM Hepes, pH 7.3, 0.05% Tween 20 supplemented with either 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 1 mM  $MnCl_2$  or 5 mM EDTA (HHTcation) was added to the wells and allowed to incubate for 1 h at 37°C. The plates subsequently were iced for 5 min and washed four times with HHTcation at 4°C. Enzyme-linked second antibody (50  $\mu$ l of 1:400 dilution of HRP goat anti-mouse IgG (Zymed Laboratories Inc.) was added in HHTcation, incubated for 1 h at 4°C, and the plates were washed five times with HHTcation at 4°C. Substrate (2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid) (Zymed Laboratories, Inc.) was added in 0.1 M citrate with 0.05%  $H_2O_2$ , and the results quantitated at 414 nm on an ELISA plate reader (Titertek Multiscan MCC340, Flow Laboratories).

### Flow Cytometry Studies

The time course and temperature studies were performed as follows. After dextran sedimentation, purified neutrophils or leukocyte-rich supernatants ( $5 \times 10^6$  cells/ml in HHMC) were preincubated in 1.5-ml Eppendorf tubes at 0, 25, or 37°C for 5 min before stimulus was added (IL-8, 25 ng/ml [72-amino acid form], PeproTech, Rocky Hill, NJ; fMLP,  $10^{-7}$  M; PMA, 100 ng/ml). After a time point was reached, samples were placed on ice immediately and aliquots were added to individual wells of a u-bottom microtiter plate on ice containing mAb. In some cases, after a time point was reached, cells were fixed immediately with paraformaldehyde (2%) and incubated for 30 min on ice. Immunofluorescence and flow cytometry were performed as described (19). The protocol for conversion of logarithmic fluorescence into fluorescence has been described (67).

### Adhesion Assays

Purified fibrinogen (2 mg/ml in PBS [Sigma Chemical Co., St. Louis, MO]) or purified sICAM-1 (200  $\mu$ g/ml in PBS) were spotted (25  $\mu$ l) onto 6-cm bacterial Petri dishes (Fisher 1007) for 90 min at room temperature. Plates were blocked with the detergent Tween 20 as previously described (20). Neutrophils ( $4 \times 10^6$  cells in 1 ml) were resuspended in HBSS, 10 mM Hepes, pH 7.3, 1 mM  $MgCl_2$ , and preincubated with mAbs for 10 min at room temperature. Subsequently, cells were added to the dishes in the presence or absence of fMLP ( $10^{-7}$  M, final vol of 3 ml), and allowed to adhere for 4 min. Nonadherent cells were removed by 12 washes with a Pasteur pipette after gentle swirling with the same buffer supplemented with 0.5% BSA. Binding was quantitated by scoring the number of adherent cells of at least four different fields using an ocular grid at 100 $\times$  magnification. The percent inhibition by mAb was determined upon comparison with the media control.

The binding of ICAM-1<sup>+</sup> L cells is a modification of previously described protocols (19). Briefly, purified Mac-1 was diluted and adsorbed (30  $\mu$ l) to 6-cm Petri dishes. After a 90-min incubation at room temperature, nonspecific binding sites were blocked with 0.5% heat-treated BSA. After removal from tissue culture plates with trypsin-EDTA (Gibco Laboratories, Inc., Grand Island, NY), ICAM-1<sup>+</sup> L cells were washed twice and resuspended in PBS, 2 mM  $MgCl_2$ , 0.5% heat-treated BSA ( $0.5$ – $1.0 \times 10^6$

cells/ml). 30 min before the binding assay, the Petri dishes or cells were preincubated at room temperature with mAb (1:200 dilution of ascites or 20  $\mu$ g/ml of purified mAb) in PBS, 2 mM  $MgCl_2$ , 0.5% heat-treated BSA (2 ml). Cells (1 ml) were added to Petri dishes, and incubated for 60 min at 37°C. The Petri dishes were then swirled and nonadherent cells were removed by tipping the plate at an angle, removing the binding buffer with a Pasteur pipette, and adding fresh media (3 ml) to the edge of the dish. The procedure was repeated five times. Subsequently, the number of adherent cells was determined by light microscopy at 100 $\times$  using an ocular grid. The percent binding was normalized to a media control.

## Results

### Monoclonal Antibody to a Neopeptide on Mac-1

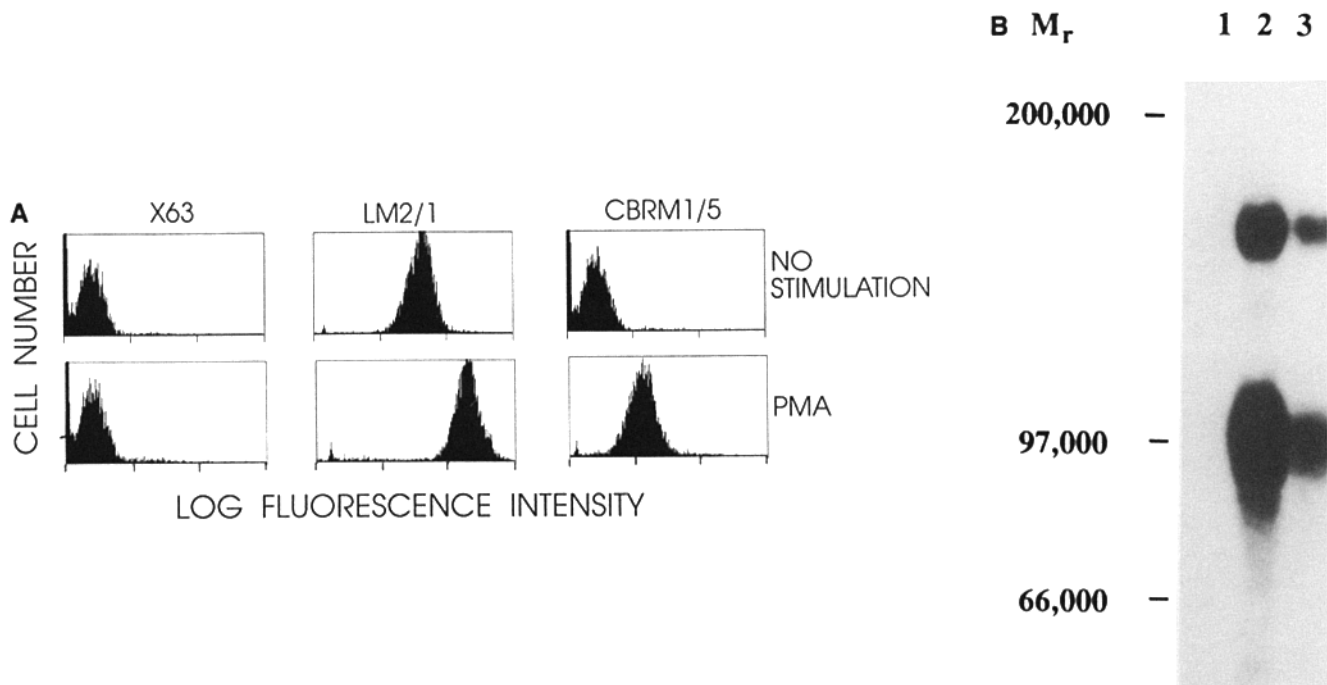
If a conformational change in Mac-1 was involved in the regulation of its adhesiveness, specific epitopes should be expressed on activated but not resting cells. To generate mAbs that recognize these activation-dependent epitopes, immunoaffinity-purified Mac-1 that was judged functional by its capacity to bind several ligands (18, 19) was used to immunize BALB/c mice. Hybridoma supernatants were screened for their differential ability to bind to resting and phorbol ester-activated neutrophils. One mAb, CBRM1/5, showed little binding to resting neutrophils but bound to neutrophils that were stimulated with phorbol esters (Fig. 1 A). CBRM1/5 bound to activated cells at a significantly lower level compared to previously characterized mAbs to Mac-1. Immunoprecipitation from detergent lysates of surface-labeled neutrophils (Fig. 1 B) or transfected COS cells (data not shown) confirmed that CBRM1/5 recognized Mac-1.

The lower level of expression of the CBRM1/5 epitope on PMA-stimulated neutrophils was not due to insufficient amounts of mAb, as studies by flow cytometry suggested that saturation was reached at 20  $\mu$ g/ml (data not shown). This was confirmed by binding experiments with <sup>125</sup>I-CBRM1/5 and <sup>125</sup>I-LM2/1. CBRM1/5 bound specifically to activated (fMLP or PMA) neutrophils but not to resting neutrophils (Fig. 2 A and C, note difference in scale; Table I). Despite the lower absolute numbers of binding sites, the binding of CBRM1/5 was saturable, and of comparable affinity ( $K_d$  = 15 nM) to LM2/1 ( $K_d$  = 2 nM). Neutrophils stimulated with fMLP or PMA had a total Mac-1 site density of 139,000 and 197,000 sites/cell (determined with LM2/1) but a CBRM1/5 neopeptide site density of 13,000–57,000 sites/cell, respectively (Table I). Bivalent binding of CBRM1/5 to Mac-1 was not strictly required because monovalent Fab' fragments of CBRM1/5 bound specifically to activated neutrophils and displaced ( $K_i$  of Fab' = 200 nM) bivalent CBRM1/5 IgG (Fig. 2 E, and data not shown).

### Kinetics and Temperature Studies

IL-8, fMLP, or PMA prompted a time-dependent increase in expression of the CBRM1/5 epitope that plateaued after 10 min (Fig. 3). In all cases, CBRM1/5 reacted with a subset of the LM2/1-reactive Mac-1 molecules on the cell surface.

mAb binding studies were carried out at different temperatures to determine whether the CBRM1/5 epitope expression correlated with an increase in surface expression or the activation of Mac-1 (Fig. 4). Elevating the temperature from 4 to 37°C increases the surface expression of Mac-1 but does not necessarily promote Mac-1-dependent adhesion (11, 49, 66). This temperature-dependent increase is primed by



**Figure 1.** (A) Immunofluorescence flow cytometry of peripheral blood neutrophils that were unstimulated or treated with PMA (100 ng/ml) for 10 min at 37°C. Neutrophils were stained with either a negative control (X63), a mAb to Mac-1  $\alpha$  subunit (LM2/1), or CBRM1/5. (B) Immunoprecipitation of Mac-1 from Triton-X-100 detergent lysates of  $^{125}$ I-labeled peripheral blood neutrophils. Immunoprecipitates with the following mAbs were subjected to reducing 5% SDS-PAGE and autoradiography: lane 1, CBRIC2/2 (ICAM-2, as control, neutrophils are negative for ICAM-2); lane 2, LM2/1 (Mac-1  $\alpha$ ); lane 3, CBRM1/5 (Mac-1  $\alpha$ ). Molecular weights of protein standards (see Methods) are indicated to the left.

preparative procedures. We found that raising the temperature from 4 to 37°C significantly increased the overall amount of surface Mac-1, whereas expression of the CBRM1/5 epitope remained virtually absent on both monocytes (Fig. 4 A) and neutrophils (Fig. 4 B). In contrast, in both monocytes and neutrophils, fMLP triggered expression of the CBRM1/5 epitope. These experiments suggest that the expression of CBRM1/5 is associated with the activation of Mac-1.

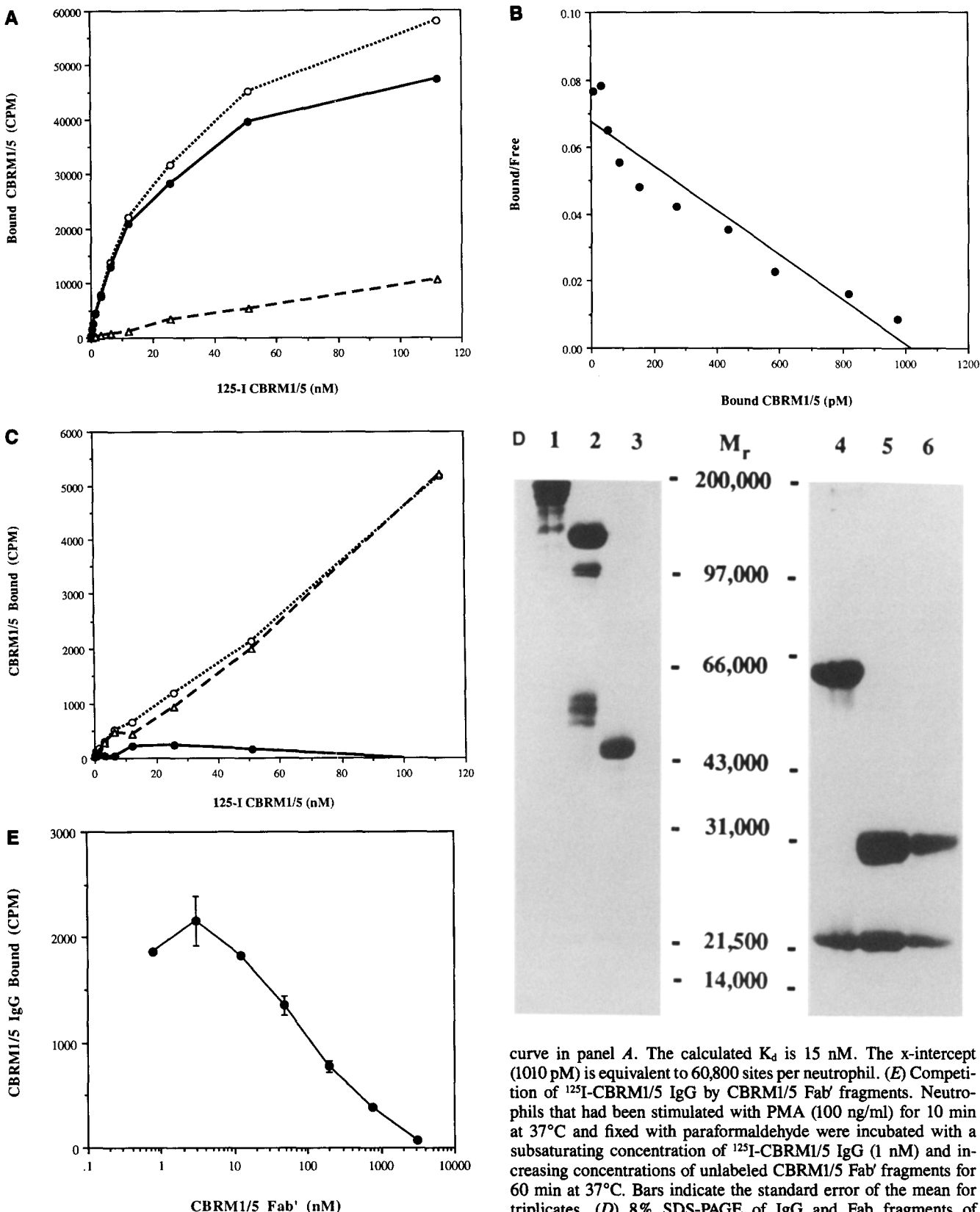
#### Epitope Mapping of CBRM1/5 on Transfected Cells

We examined the expression of CBRM1/5 on CHO cells that were transfected with wild-type and chimeric Mac-1 molecules (Fig. 5). A subset of Mac-1 molecules on transfected CHO cells constitutively expressed the CBRM1/5 epitope. This agrees with our previous observations that the activation mAb NKI-L16 reacted constitutively with a subset of LFA-1 molecules on transfected COS cells (45). CHO cell transfectants that express wild-type Mac-1 are functional in that they rosetted with iC3b-coated erythrocytes, bound *Leishmania* promastigotes, and adhered to purified ICAM-1 (data not shown). CBRM1/5 localized specifically to the I domain of the Mac-1  $\alpha$  chain as it, like LM2/1, bound to CHO cells that expressed the wild-type Mac-1 and chimera X-e-M-b-X which contains the I domain of Mac-1 on a p150,95 frame. In contrast, CBRM1/5 did not bind to wild-

type p150,95 or the reciprocal chimera, M-e-X-b-X, which contains a p150,95 I domain on a Mac-1 backbone (Fig. 5).

#### Effect of Divalent Cations on CBRM1/5 Expression

For integrins to bind to their ligands, divalent cations are required. Adhesion is abolished uniformly by the presence of EDTA and altered quantitatively by the type of divalent cation that is present (1, 18, 23, 33). We tested the CBRM1/5 and LM2/1 epitopes for their sensitivity to different divalent cations. For binding to cell surface (Fig. 6 A) or purified Mac-1 (Fig. 6 B), the presence of divalent cations was required for CBRM1/5 epitope expression but not for LM2/1. 5 mM EDTA abrogated the expression of CBRM1/5 but only slightly reduced expression of LM2/1. Neutrophils stimulated with fMLP expressed the neoepitope better in the presence of  $\text{Ca}^{2+}$  than in  $\text{Mg}^{2+}$  alone, but in other studies, CBRM1/5 was expressed better on neutrophils stimulated with PMA in the presence of  $\text{Mg}^{2+}$  alone (data not shown).  $\text{Mn}^{2+}$ , which is reported to enhance Mac-1 adhesive function in vitro (1), augmented the expression of CBRM1/5 on purified Mac-1 and promoted the expression of the epitope on resting neutrophils. Unlike the Mac-1 on neutrophils, Mac-1 that was purified by immunoaffinity chromatography expressed equivalent levels of the CBRM1/5 and LM2/1 epitopes in the presence of divalent cations. In contrast, Mac-1 that was purified by iC3b-Sepharose chromatography (37)



**Figure 2.** Binding of CBRM1/5 IgG and Fab'. Saturation binding to A, neutrophils that were stimulated with PMA (100 ng/ml) for 10 min at 37°C. and C, untreated neutrophils. Neutrophils were incubated with <sup>125</sup>I-labeled CBRM1/5 either in the absence (○) or presence of excess unlabeled CBRM1/5 (Δ) for 4 h on ice. Specific binding (●) was measured by subtracting the nonspecific from the total binding. (B) A Scatchard plot of the specific binding from the

curve in panel A. The calculated  $K_d$  is 15 nM. The x-intercept (1010 pM) is equivalent to 60,800 sites per neutrophil. (E) Competition of <sup>125</sup>I-CBRM1/5 IgG by CBRM1/5 Fab' fragments. Neutrophils that had been stimulated with PMA (100 ng/ml) for 10 min at 37°C and fixed with paraformaldehyde were incubated with a subsaturating concentration of <sup>125</sup>I-CBRM1/5 IgG (1 nM) and increasing concentrations of unlabeled CBRM1/5 Fab' fragments for 60 min at 37°C. Bars indicate the standard error of the mean for triplicates. (D) 8% SDS-PAGE of IgG and Fab fragments of CBRM1/5. Samples (3–5 μg) of CBRM1/5 IgG (lanes 1 and 4), CBRM1/5 Fab<sub>2</sub> (lanes 2 and 5), or CBRM1/5 Fab' (lanes 3 and 6) were subjected to 8%-SDS-PAGE under nonreducing (50 mM iodoacetamide, lanes 1–3) or reducing (5% β-mercaptoethanol, lanes 4–6) conditions and silver staining. Molecular weights of protein standards (see Methods) under nonreducing and reducing conditions are indicated in the center.

**Table I. Site Density of Mac-1 on Neutrophils**

	<sup>125</sup> I-LM2/1 Sites/Cell	<sup>125</sup> I-CBRM1/5 Sites/Cell
Unstimulated	11,300 ± 1,600	~900 ± 900
fMLP	138,700 ± 21,000	13,200 ± 1,400
PMA	196,900 ± 800	57,200 ± 1,300

The site densities were determined from the amount of mAb bound at saturation. Data (± standard error of the mean) are the average of two independent experiments performed in triplicate on a single donor.

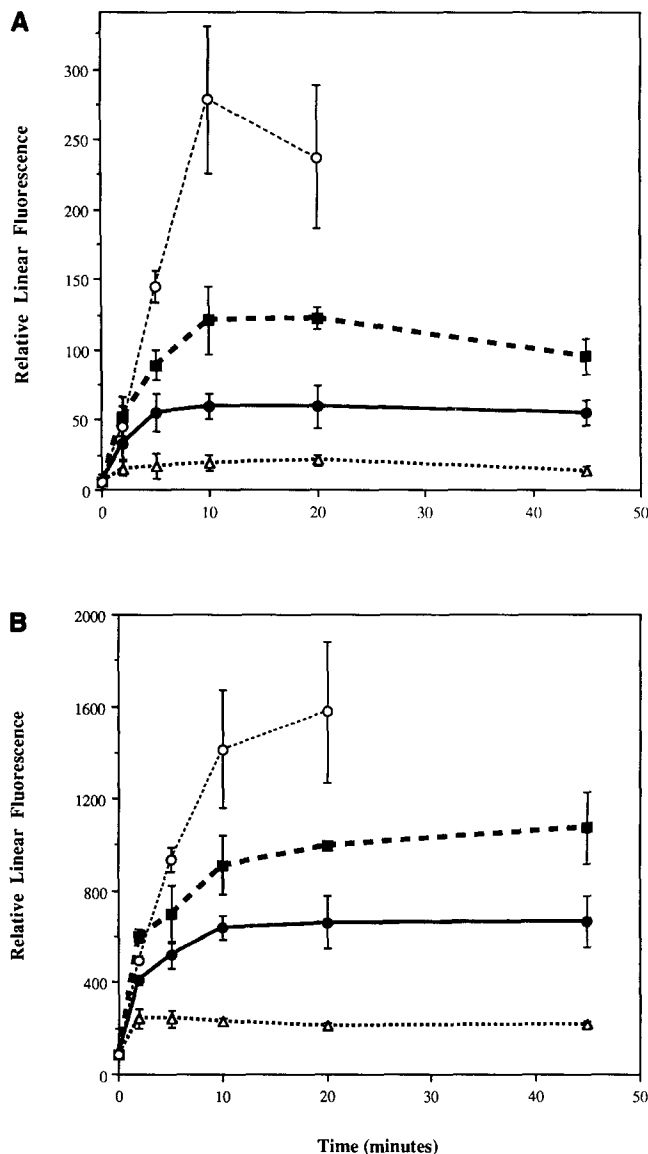
expressed the LM2/1 epitope but lacked the CBRM1/5 epitope, and was unable to sustain ligand binding (Diamond, M. S., K. P. M. van Kessel, S. D. Wright, and T. A. Springer, unpublished observations).

### Structural Subsets of Mac-1

To determine whether CBRM1/5 recognized a subset of Mac-1 molecules, sequential immunoprecipitation experiments were performed (Fig. 7). After preclearing a neutrophil lysate twice with CBRM1/5–Sephacryl so that negligible amounts of CBRM1/5-reactive Mac-1 remained, LM2/1–Sephacryl immunoprecipitated a significant quantity of Mac-1. However, if the lysate was precleared with LM2/1–Sephacryl first, CBRM1/5–Sephacryl was unable to immunoprecipitate any additional Mac-1. These experiments suggest that CBRM1/5 recognizes a subset of the LM2/1-reactive Mac-1 molecules in the cell lysate. Close examination of the immunoprecipitates revealed that the amount of Mac-1 that expressed the CBRM1/5 epitope in solution exceeded that on the surface of activated neutrophils. Detergent solubilization may remove physical or chemical constraints on Mac-1 and prompt some molecules to acquire spontaneous expression of the CBRM1/5 epitope. In support of this, immunoaffinity-purified Mac-1 (Fig. 6 B) and detergent-permeabilized, resting neutrophils both express CBRM1/5 (data not shown).

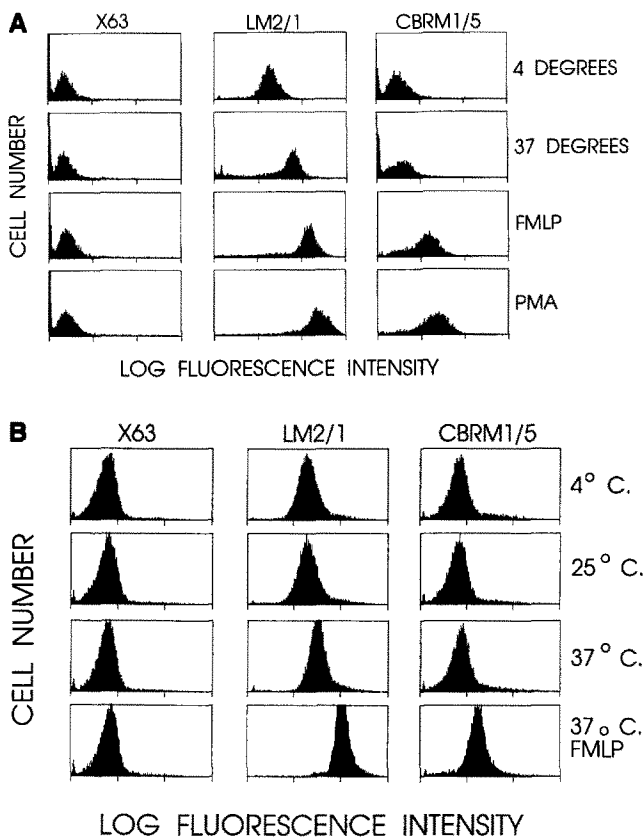
### Functional Effects of CBRM1/5

To determine whether the subset of Mac-1 molecules identified by CBRM1/5 was functionally important, we examined the ability of this mAb to block Mac-1-dependent neutrophil adhesion to ICAM-1. Adhesion of neutrophils to ICAM-1 has been shown previously to depend both on Mac-1 and LFA-1 (20, 71). Bivalent IgG, Fab<sub>2</sub>, or monovalent Fab' fragments of CBRM1/5 inhibited greater than 80% of the Mac-1-dependent binding of fMLP-stimulated neutrophils to ICAM-1 (Fig. 8 A). CBRM1/5 was comparable in effectiveness to LPM19c, a mAb to Mac-1 that binds a similar number of sites per cell as LM2/1 and completely blocks Mac-1–ICAM-1 interaction (Fig. 8 A, [19], and data not shown). As a control, a third mAb to Mac-1, CBRM1/23, had no significant inhibitory effect on neutrophil adhesion to ICAM-1. When CBRM1/5 was added to a blocking mAb to LFA-1 (TS1/22), similar to the case with LPM19c (20), the combination of mAbs virtually abolished neutrophil binding to ICAM-1. The ability of CBRM1/5 to inhibit Mac-1-dependent neutrophil interaction with ICAM-1 was confirmed in binding assays with purified Mac-1 (Fig. 8 B). Preincubation of purified Mac-1 with CBRM1/5 completely abolished adhesion of L cells transfected with ICAM-1.



**Figure 3.** Kinetics of expression of Mac-1 epitopes. Neutrophils that were activated at 37°C for the indicated times with no stimuli (open triangles), fMLP (10<sup>-7</sup>M, solid squares), IL-8 (25 ng/ml, solid circles), or PMA (100 ng/ml, open circles) were immunostained with A, CBRM1/5 or B, LM2/1 and subjected to immunofluorescence flow cytometry. Data are expressed as relative linear fluorescence units and represent the mean of three experiments. The bars indicate the standard error of the mean.

We also examined the effect of CBRM1/5 on Mac-1 binding to a second ligand, fibrinogen. The binding of neutrophils that are stimulated with fMLP to fibrinogen has been shown to be mediated by Mac-1 (86). CBRM1/5 almost completely abolished fMLP-stimulated neutrophil adhesion to purified fibrinogen (Fig. 8 C). CBRM1/5 IgG and Fab' blocked adhesion to fibrinogen ~90%, and half-maximal blocking was observed at ~0.5 μg/ml and 10 μg/ml of IgG and Fab', respectively (Fig. 8 C). Again, other mAbs to Mac-1 (CBRM1/23) used at similar concentrations showed little inhibitory effect (data not shown). Thus, the 10% subset of Mac-1 molecules on neutrophils after fMLP stimulation that



**Figure 4.** The effect of temperature on Mac-1 expression on **A**, peripheral blood monocytes and **B**, neutrophils. Cells were incubated at 4, 25, and 37°C in the absence or presence of stimuli (fMLP,  $10^{-7}$ M; PMA, 100 ng/ml) for 10 min, and then immunostained with either a negative control (X63), a mAb to Mac-1  $\alpha$  subunit (LM2/1), or CBRM1/5. Representative histograms show the log fluorescence intensity versus cell number.

is identified by CBRM1/5 mediates at least 90% of the adhesive activity of Mac-1.

## Discussion

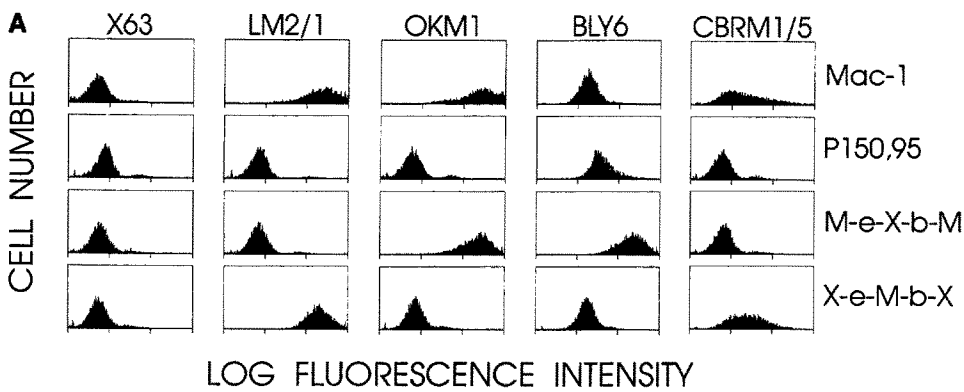
We have characterized a novel mAb, CBRM1/5, that binds to a subpopulation of Mac-1 molecules on activated but not resting peripheral blood neutrophils and monocytes. This mAb recognizes an activation-dependent neopeptide and acts as a reporter for the activation state of Mac-1. Our results demonstrate that the subset of Mac-1 molecules that express the CBRM1/5 neopeptide mediates adhesion to at least two of its characterized ligands, ICAM-1 and fibrinogen. Evidence for this includes the following: (a) CBRM1/5 does not react by immunofluorescence with Mac-1 on the surface of resting neutrophils or monocytes. However, stimulation with the chemoattractant fMLP, the chemoattractive cytokine IL-8, or phorbol esters rapidly promotes expression of the epitope on a subset of Mac-1 molecules. The time course of expression of the neopeptide and adhesive activity of Mac-1 (6, 17) are parallel as both occur within 2 min. (b) The quantitation of site densities by saturation binding experiments with  $^{125}$ I-labeled mAbs to Mac-1 demonstrates that resting neutrophils express  $\sim 12,000$  LM2/1 binding sites per cell but no specific CBRM1/5 sites per cell. Treat-

ment with fMLP and PMA increases the overall site density of Mac-1 (as reported by LM2/1) greater than 10-fold as has been previously described (10, 49, 55, 76), and induces expression of the CBRM1/5 neopeptide on 10–30% of these molecules (13,000–57,000 sites/cell after stimulation) (c) Sequential immunoprecipitation confirms that CBRM1/5 recognizes a structural subset of Mac-1 molecules on neutrophils. Lysates that are precleared of the CBRM1/5 epitope contain residual Mac-1 molecules that are immunoprecipitated by LM2/1 but lysates that are precleared of Mac-1 containing the LM2/1 epitope do not contain residual quantities of CBRM1/5 precipitable material. (d) Functional studies reveal that despite binding to only 10% of the Mac-1 molecules on neutrophils stimulated with fMLP, CBRM1/5 (IgG or Fab fragments) inhibits almost completely (>85%) the Mac-1-dependent adhesion to ICAM-1 and fibrinogen. Additional experiments confirmed the blocking capacity of CBRM1/5, as it abrogates adhesion of ICAM-1<sup>+</sup> L cells to purified Mac-1.

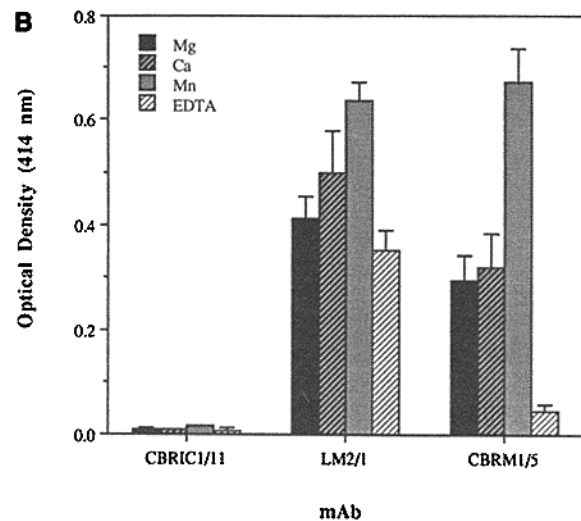
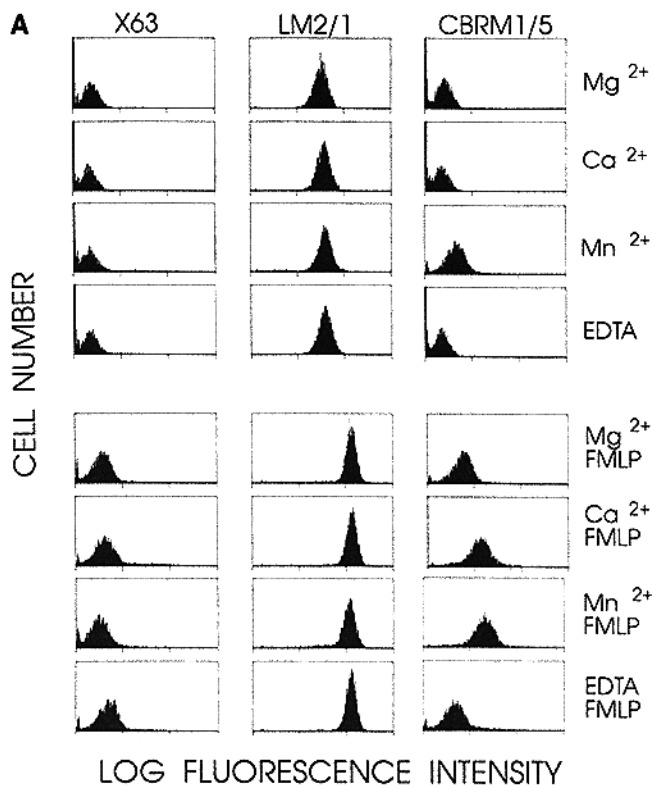
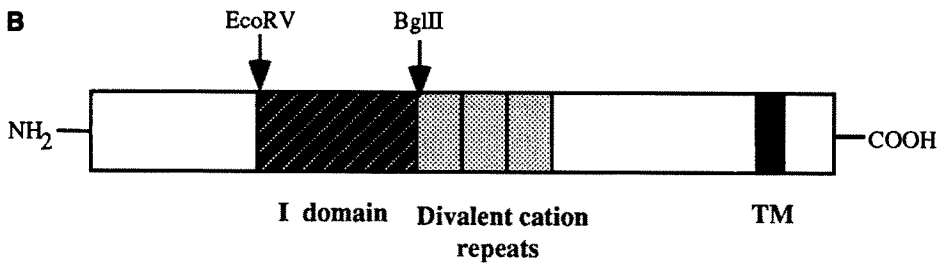
While several mAbs have been characterized that block Mac-1-dependent adhesion to ICAM-1 and fibrinogen (5, 19, 72, 86), CBRM1/5 is unique, as it recognizes the subset of Mac-1 molecules that mediates ligand interaction. CBRM1/5 maps to the I domain on Mac-1 which appears to contain the major recognition site for ICAM-1 and fibrinogen (20a). However, not all mAbs that bind to subsets of Mac-1 molecules recognize functional epitopes. We have generated a second mAb (CBRM1/19, anti-CD18) that binds to activated but not resting neutrophils; however, it does not block function (Diamond, M. S., and T. A. Springer, unpublished results). The finding of structurally distinct subsets of Mac-1 molecules may explain why neutrophils do not show significant changes in the redistribution of surface Mac-1 after activation with fMLP (16, 70).

Although activation of neutrophils and monocytes with chemotactic factors, cytokines, nucleotides, or phorbol esters increases the avidity of Mac-1 for several of its ligands (3, 4, 19, 59, 72, 84, 86), the molecular basis for this change has remained unclear. The quantitative increase in Mac-1 expression on neutrophils does not correlate directly with adhesive functions (11, 49, 80), although, until now, no direct evidence has been obtained for a structural change. Studies at 4, 25, and 37°C show that an increase in overall Mac-1 expression does not induce the CBRM1/5 epitope on neutrophils or monocytes. Similarly, an increase in Mac-1 surface expression is not sufficient to promote Mac-1-dependent adhesion to ICAM-1, fibrinogen, or other ligands ([11, 80] and Diamond, M. S., unpublished observations). Adhesion to fibrinogen and ICAM-1, and expression of the CBRM1/5 epitope both require cell stimulation. We suggest that activation prompts a subset of Mac-1 molecules to acquire the functionally relevant CBRM1/5 epitope, and to become structurally competent to bind to ICAM-1 and fibrinogen.

Several mAbs against activation-dependent neopeptides on integrin superfamily members have been recently described. mAbs against the platelet integrin gpIIb-IIIa have been reported to monitor structural changes that are induced either by cellular activation or ligand binding (24). Indeed, the initial evidence for a structural change in integrins came from the development of the PAC-1 mAb which recognizes gpIIb-IIIa on ADP, epinephrine, or thrombin-treated but not resting platelets (57, 68). PAC-1 binds to a subset (20–50%)

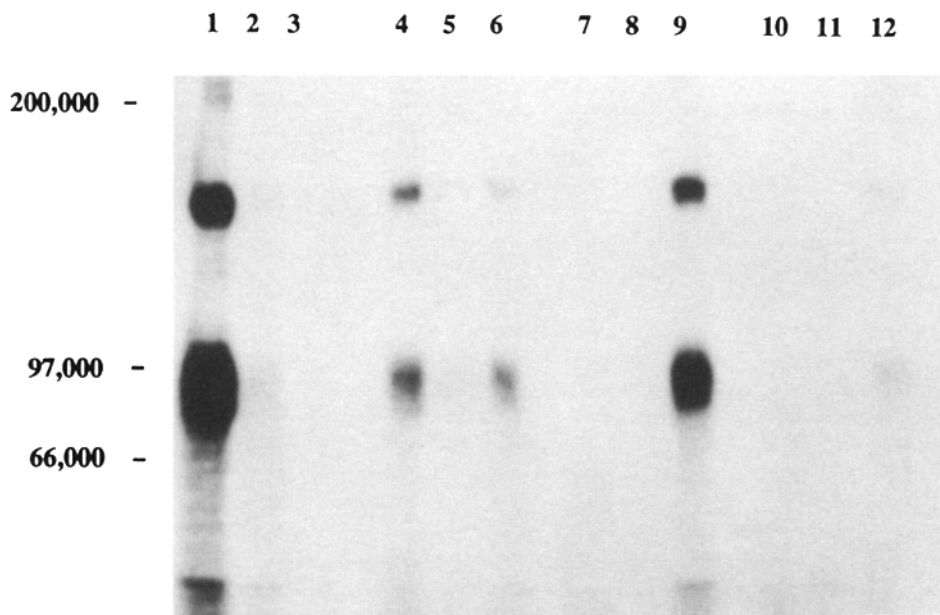


**Figure 5.** Flow cytometric analyses of Mac-1/p150,95 chimeric molecules. (A) CHO cells co-transfected with the  $\beta$  subunit and the indicated Mac-1, p150,95, or chimeric  $\alpha$  subunit were stained with X63 (nonbinding control), LM2/1 (anti-Mac-1  $\alpha$ ), OKM1 (anti-Mac-1  $\alpha$ ), BLY6 (anti-p150,95  $\alpha$ ), and CBRM1/5 (anti-Mac-1  $\alpha$ ). The BLY6 mAb (ascites) gave a higher background staining than other mAbs. It stained Mac-1 transfected and untransfected CHO cells equivalently (data not shown). (B) Schematic of the  $\alpha$  subunit, showing the position of restriction sites used for exchange of I domains between Mac-1 and p150,95.

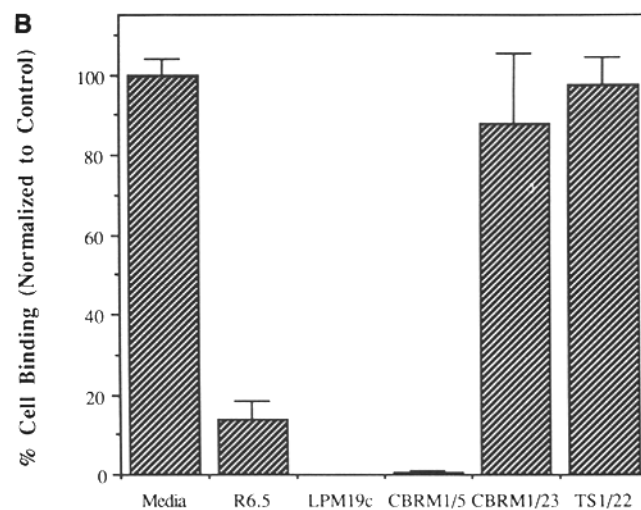
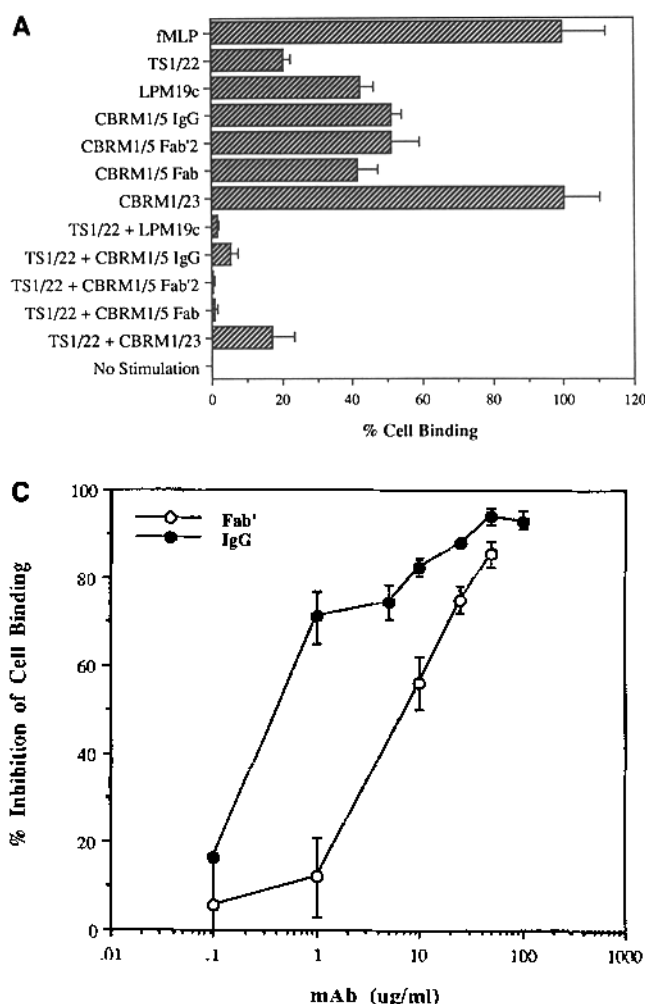


**Figure 6.** Divalent cations affect expression of Mac-1 mAb epitopes. (A) Flow cytometry of neutrophils. Neutrophils were incubated at 4°C (resting) and 37°C (FMLP,  $10^{-7}$ M) for 10 min in HBSS that was supplemented by the indicated divalent cation or chelating agent. Cells were immunostained with either a negative control (X63), a mAb to Mac-1  $\alpha$  subunit (LM2/1), or CBRM1/5. All subsequent washes were performed in the presence of the indicated divalent cation or EDTA. Cells were subjected to immunofluorescent flow cytometry. (B) ELISA of purified Mac-1. Purified Mac-1 was adsorbed to microtiter wells and nonspecific sites were blocked after sequential washes with human serum albumin and Tween 20 in the presence of the indicated divalent cation or EDTA (see Methods). Solid-phase Mac-1 was incubated with mAbs (CBRIC1/11, anti-ICAM-1 as control; LM2/1, anti-Mac-1  $\alpha$ ; CBRM1/5, anti-Mac-1  $\alpha$ ) and developed by an ELISA with HRP coupled goat anti-mouse IgG. This representative experiment was performed in triplicate and the bars indicate the standard error of the mean.





**Figure 7.** 5% SDS-PAGE analysis of immunoprecipitates from neutrophil lysates. Iodinated neutrophils were lysed in 1% Triton X-100 and 60 $\mu$ l-aliquots were precipitated sequentially with Sepharose coupled to LM2/1 (lane 1), LM2/1 (lane 2), and finally with CBRM1/5 (lane 3) or with CBRM1/5 (lane 4), CBRM1/5 (lane 5), and finally with LM2/1 (lane 6) or with mouse IgG (lanes 7 and 10), mouse IgG (lanes 8 and 11) and finally with LM2/1 (lane 9) or with CBRM1/5 (lane 12). Material eluted from the beads was run under reducing conditions and subjected to autoradiography. Molecular weights of protein standards (see Methods) are indicated to the left.



**Figure 8.** CBRM1/5 mAb blocks binding of Mac-1 to ligand. (A) Neutrophil binding to ICAM-1. Neutrophils were allowed to bind to purified sICAM-1 in 6-cm Petri dishes for 4 min at room temperature in the presence of fMLP ( $10^{-7}$ M) after a 10-min preincubation with the following mAbs: TS1/22 (anti-LFA-1  $\alpha$ , 1:250 dilution of ascites), LPM19c (anti-Mac-1  $\alpha$ , 1:250 dilution of ascites), CBRM1/5 (anti-Mac-1  $\alpha$ , 25  $\mu$ g/ml IgG or Fab'; 50  $\mu$ g/ml of Fab'), CBRM1/23 (anti-Mac-1  $\alpha$ , 25  $\mu$ g/ml). (B) The effect of CBRM1/5 on ICAM-1<sup>+</sup>L cell adhesion to purified Mac-1. ICAM-1<sup>+</sup>L cells were allowed to bind to purified Mac-1 in 6-cm Petri dishes for 60 min at 37°C after a 25-min preincubation with the following mAbs with the dishes: TS1/22 (anti-LFA-1  $\alpha$ ), LPM19c (anti-Mac-1  $\alpha$ ), CBRM1/5 (anti-Mac-1  $\alpha$ ), and CBRM1/23 (anti-Mac-1  $\alpha$ ). One group of ICAM-1<sup>+</sup>L cells was preincubated with a mAb to ICAM-1 (R6.5, 30  $\mu$ g/ml). Data are expressed as the percent cell binding relative to a media control (130 cells/mm<sup>2</sup>). Datapoints are the average of five microscopic fields and the bars indicate the standard error of the mean. (C) Dose-dependent inhibition of neutrophil binding

to fibrinogen by CBRM1/5. Neutrophils were allowed to bind to purified fibrinogen in 6-cm Petri dishes for 4 min at room temperature in the presence of fMLP ( $10^{-7}$ M) after a 10-min preincubation with increasing concentrations of CBRM1/5 IgG or Fab'. The binding was determined by light microscopy (100 $\times$ ) after the unbound cells were removed by serial washes with a Pasteur pipette (see Methods). Data are expressed as the percent inhibition of cell binding relative to a media control (177 cells/mm<sup>2</sup>). Datapoints are the average of five microscopic fields and the bars indicate the standard error of the mean. A–C show representative experiments that were repeated several times.

of gpIIb-IIIa molecules after stimulation and inhibits fibrinogen-mediated platelet aggregation (68). Furthermore, CHO cells that express gpIIb-IIIa are unable to bind soluble fibrinogen or the PAC-1 mAb until a structural change has occurred (57). Additional evidence for structural changes has come from the characterization of a class of mAbs that recognizes ligand-induced binding sites on gpIIb-IIIa (31). The generation of mAbs to multiple conformations of gpIIb-IIIa suggests that cell activation and ligand binding may effect distinct structural changes that facilitate adhesion and mediate function (32). Because PAC-1 and CBRM1/5 share similar binding properties, we suggest that the leukocyte integrins, like the platelet integrins, require similar structural changes to promote adhesion.

Several activation-dependent epitopes on leukocyte integrins have also been described. The mAb 7E3, which was raised originally against gpIIb-IIIa (13), binds to all Mac-1 molecules on ADP-stimulated but not resting monocytes or monocyte cell lines (2). Unlike PAC-1 or CBRM1/5, it has no reported functional effects. The NKI-L16 mAb recognizes the leukocyte integrin LFA-1 on resting T lymphocytes only weakly (45, 79), but binds to T lymphocytes that have been activated with phorbol esters, IL-2, or mAbs that cross-link CD3. NKI-L16 induces homotypic adhesion of B cell lines and T cell clones, and partially restores binding to purified ICAM-1 of COS cells that have been transfected with a wild-type LFA-1  $\alpha$  chain and a defective CD18  $\beta$  subunit (38). However, the NKI-L16 epitope is not sufficient for cell binding since cloned T cells express high levels constitutively but do not aggregate spontaneously (79). The mAb KIM 127 recognizes the common CD18  $\beta$  subunit of the leukocyte integrins and promotes LFA-1- and Mac-1-dependent adhesive events (61). Binding of this mAb may trigger a conformational change that mimics activation and increases avidity for the ligand. In comparison with these activation-dependent mAbs against leukocyte integrins, CBRM1/5 appears unique in that it binds to a subset of molecules on stimulated cells, blocks rather than promotes adhesion, and has an epitope whose presence correlates with, but does not induce, the highly avid form of Mac-1 on neutrophils and monocytes.

Recent studies have begun to address the structural events that effect the increase in avidity. In one model, stimulation of cells promotes or alters the divalent cation binding by an integrin, and this event triggers the high avidity conformation (1, 21, 23). Integrin  $\alpha$  chains contain a region of extensive homology to the divalent cation binding sites on  $\text{Ca}^{2+}$  regulatory proteins (14, 60, 75), and numerous studies demonstrate a requirement for divalent cations in integrin function (1, 9, 23, 27, 33, 39, 41, 50, 52, 63, 73). Furthermore,  $\text{Mn}^{2+}$  is reported to stimulate integrin adhesion in the absence of other activating signals (1, 23, 27, 33, 41, 73). Several activation-dependent neoepitopes on leukocyte integrins have been shown to require the presence of divalent cations. Binding of the mAb 24 (21, 23), which recognizes a common determinant on the  $\alpha$  chain of the three leukocyte integrins, requires either  $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$  but is abolished in the presence of  $\text{Ca}^{2+}$ . In contrast, expression of the NKI-L16 epitope requires  $\text{Ca}^{2+}$ , as it is abolished in the presence of EGTA or EDTA (79). CBRM1/5 is the first example of an activation-specific mAb against an integrin  $\alpha$  subunit whose expression requires divalent cations but is known to bind to a region outside the divalent cation binding repeats. EDTA

abolishes the expression of the CBRM1/5 epitope on both cellular and purified Mac-1. On neutrophils,  $\text{Mn}^{2+}$  increases the CBRM1/5 epitope expression in the absence of stimulation, and thus, may impose structural alterations that mimic cell activation (1, 23). Although these studies suggest that divalent cations modulate integrin function, it remains unclear if they are permissive for ligand binding or whether divalent cations are involved actively in the regulation of adhesion.

While leukocyte emigration into inflammatory sites is a protective response of the host defense system against foreign pathogens or for tissue repair, under some circumstances leukocytes can mediate significant degrees of vascular and tissue injury. Neutrophils have been implicated in the pathogenesis of a number of clinical disorders including adult respiratory distress syndrome, ischemia-reperfusion injury after myocardial infarction, vasculitis, and shock (for review see [12]). Already, several groups have demonstrated that the inflammatory injury mediated by leukocytes can be attenuated by antibodies against the leukocytes integrins (53, 62, 69, 81). mAbs like CBRM1/5, which recognize activated but not resting leukocytes, could be used clinically to inhibit, monitor, or deplete the highly adhesive cells that effect inflammatory injury.

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