

# A Peptide Derived from the Intercellular Adhesion Molecule-2 Regulates the Avidity of the Leukocyte Integrins CD11b/CD18 and CD11c/CD18

Rui Li,\* Jinglin Xie,\* Carmela Kantor,\* Vesa Koistinen,‡ Dario C. Altieri,§ Pekka Nortamo,\* and Carl G. Gahmberg\*

\*Department of Biochemistry, 00014 University of Helsinki, Helsinki, Finland; ‡Finnish Red Cross Blood Transfusion Service, Helsinki, Finland; §Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

**Abstract.**  $\beta_2$  integrin (CD11a,b,c/CD18)-mediated cell adhesion is required for many leukocyte functions. Under normal circumstances, the integrins are nonadhesive, and become adhesive for their cell surface ligands, the intercellular adhesion molecules (ICAMs), or soluble ligands such as fibrinogen and iC3b, when leukocytes are activated. Recently, we defined a peptide derived from ICAM-2, which specifically binds to purified CD11a/CD18. Furthermore, this peptide strongly induces T cell aggregation mainly mediated by CD11a/CD18-ICAM-1 interaction, and natural killer cell cytotoxicity. In the present study, we show that the same ICAM-2 peptide also

avidly binds to purified CD11b/CD18, but not to CD11c/CD18. This binding can be blocked by the CD11b antibody OKM10. The peptide strongly stimulates CD11b/CD18-ICAM-1-mediated cell aggregations of the monocytic cell lines THP-1 and U937. The aggregations are energy and divalent cation-dependent. The ICAM-2 peptide also induces CD11b/CD18 and CD11c/CD18-mediated binding of THP-1 cells to fibrinogen and iC3b coated on plastic. These findings indicate that in addition to induction of CD11a/CD18-mediated cell adhesion, the ICAM-2 peptide may also serve as a "trigger" for high avidity ligand binding of other  $\beta_2$  integrins.

**T**HE leukocyte-specific  $\beta_2$  integrins (CD11/CD18),<sup>1</sup> consist of three high mol wt heterodimers with specific  $\alpha$  chains (CD11a,b,c) and a common  $\beta$  chain (CD18), and play a prominent role in mediating diverse cell adhesions required for many leukocyte functions (Springer, 1990; Arnaout, 1990; Patarroyo et al., 1990).

CD11a/CD18 lymphocyte function-associated antigen-1 (LFA-1), which is mainly found on mononuclear leukocytes, probably binds to the NH<sub>2</sub>-terminal immunoglobulin domains of the intercellular adhesion molecules (ICAM)-1 (CD54) (Rothlein et al., 1986; Patarroyo et al., 1987; Staunton et al., 1990; Berendt et al., 1992), ICAM-2 (CD102) (Staunton et al., 1989; Nortamo et al., 1991b; de Fougerolles et al., 1991), and ICAM-3 (CD50) (de Fougerolles et al., 1992; Vazeux et al., 1992; Fawcett et al., 1992; Juan et al., 1993), members of the immunoglobulin superfamily. CD11b/

CD18 (Mac-1), which is expressed primarily on cells of the myelo-monocytic lineage, binds to ICAM-1 through the third immunoglobulin domain (Diamond et al., 1991). In addition, CD11b/CD18 also binds to several soluble ligands including the complement fragment iC3b (Beller et al., 1982; Wright et al., 1983), fibrinogen (Wright et al., 1988; Altieri et al., 1988), and factor X (Altieri and Edgington, 1988a), which become insolubilized during activation of the complement and clotting cascades. CD11c/CD18 (p150,95) is enriched on macrophages. The ligands for CD11c/CD18 are poorly characterized. It has been shown that CD11c/CD18 binds to iC3b (Micklem and Sim, 1985; Myones et al., 1988), and recent reports suggest that it also binds to fibrinogen (Loike et al., 1991; Postigo et al., 1991), and at least to one counter-receptor on the surface of endothelial cells (Stacker and Springer, 1991).

The leukocyte integrins need to be activated in order to become adhesive. The molecular mechanisms involved in the activation of CD11/CD18 are incompletely understood. Quantitative as well as qualitative changes occur in these receptors after cell activation. Phorbol ester treatment (Patarroyo et al., 1985; Rothlein and Springer, 1986), or cross-linking the T cell receptor on lymphocytes (Dustin and Springer, 1989) induces a high avidity state of CD11a/CD18

Please address all correspondence to Dr. Carl G. Gahmberg, Department of Biochemistry, P.O. Box 5 (Unioninkatu 35), 00014 University of Helsinki, Helsinki, Finland. Tel: 358 0 1917773. Fax: 358 0 1917769.

1. *Abbreviations used in this paper:* BSA, bovine serum albumin; CD11/CD18, leukocyte-specific  $\beta_2$  integrins; CD11a/CD18 (LFA-1), lymphocyte function-association antigen-1; ICAM, intercellular adhesion molecule.

for ICAM-1, without any substantial increase in lymphocyte surface expression of this integrin. CD11a/CD18 can also be activated by monoclonal antibodies reacting with a variety of leukocyte cell surface glycoproteins (van Kooyk et al., 1989; Koopman et al., 1990; Kansas and Tedder, 1991), evidently through "inside-out" signaling. In addition, certain monoclonal antibodies against the  $\alpha$  chain of CD11a/CD18 has been shown to be able to induce CD11a/CD18-dependent homotypic T cell adhesion (Koopman et al., 1992; Landis et al., 1993), indicating that integrin activation could also be induced by direct ligand binding from the outside of the cells.

Unlike CD11a/CD18, the amount of cell surface CD11b/CD18 and CD11c/CD18 on granulocytes and monocytes can be rapidly upregulated by translocation of these two receptors from an intracellular pool to the cell surface in response to cell activation (Miller et al., 1987; Bainton et al., 1987). However, the change in CD11b/CD18 surface expression that occurs after stimulation does not parallel the kinetics or magnitude of cell adhesion (Buyon et al., 1988; Lo et al., 1989). Thus, CD11b/CD18 is hypothesized to undergo additional qualitative conformational changes that facilitate adhesion (Buyon et al., 1988; Lo et al., 1989; Philips et al., 1988; Vedder and Harlan, 1988). The phorbol ester and adenine nucleotide ADP-induced functional modulation of CD11b/CD18 appears to involve allosteric or qualitative remodeling of the receptor characterized by the formation of activation-dependent neoantigenic epitopes (Diamond and Springer, 1993; Altieri and Edgington, 1988b). Alternatively, the enhanced avidity of CD11b/CD18 could be due to a signal to cluster the receptors in the plane of the membrane (Detmers et al., 1987).

Recent work has demonstrated that ICAM-1, ICAM-2, and ICAM-3 provide important costimulatory signals via their adhesive interactions with the CD11a/CD18 complex during the CD3/TCR-mediated activation of resting T cells (van Seventer et al., 1990; Damle et al., 1992; Hernández-Caselles et al., 1993). Furthermore, ICAM-1 has been shown to be able to induce the high affinity state of CD11a/CD18 characterized by expression of the mAb 24 epitope (Cabanas and Hogg, 1993), which has been thought to be a "reporter" of the activated state of CD11a/CD18 (Dransfield et al., 1990). However, there is no direct evidence for inducing a high avidity state of leukocyte integrins by direct ligand binding.

ICAM-2 is the second ligand found for CD11a/CD18 (Staunton et al., 1989). The external portion of this molecule consists of two immunoglobulin domains, which have 34% identity in amino acid sequences with the two NH<sub>2</sub>-terminal domains of ICAM-1 and ICAM-3. To identify the binding site(s) in ICAM-2 for CD11a/CD18, we recently synthesized several peptides from the first immunoglobulin domain of ICAM-2, and characterized a 22-amino acid long peptide, P1, which specifically binds to CD11a/CD18 (Li et al., 1993b). Furthermore, the peptide strongly stimulates blood T cell aggregation mainly mediated by CD11a/CD18-ICAM-1 interaction, and natural killer cell cytotoxicity (Li et al., 1993a). In the present study, we report that the ICAM-2 peptide also binds to purified CD11b/CD18, but not to CD11c/CD18. The peptide strongly induces CD11b/CD18-ICAM-1-mediated homotypic cell adhesion of monocytic cell lines, and CD11b/CD18 and CD11c/CD18-mediated binding of leukocytes to fibrinogen and iC3b.

## Materials and Methods

### Cell Culture

The endothelial cell line Eahy926 was cultured in DMEM (Sigma Chem. Co., St. Louis, MO) containing hypoxanthine/aminopterin/thymidine, and 10% of FCS (Flow Laboratories, Irvine, Scotland). Eahy926 cells were stimulated by incubating them with 10 ng/ml of TNF- $\alpha$  (Boehringer Mannheim, Mannheim, Germany) overnight (Nortamo et al., 1991a). The monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD) (Tsuchiya et al., 1980) was maintained in continuous culture in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% FCS, 2 mM L-glutamine (Biological Industries, Kibbutz Bet Haemek, Israel), 10 mM Hepes, and 50  $\mu$ M 2-mercaptoethanol (Fluka Chemie AG, Switzerland). The monocytic cell line U937 (Sundström and Nilsson, 1976) was cultured in RPMI 1640 supplemented with 10% FCS, 100 u/ml of penicillin, 100  $\mu$ g/ml of streptomycin and 2 mM of L-glutamine.

### Peptide Synthesis

Peptides were synthesized on a model 430A peptide synthesizer, using Fmoc-chemistry (Applied Biosystems, Inc., Foster City, CA). The structures were confirmed by FAB-mass spectrometric analysis (JEOL SX-102). The sequence of the ICAM-2 peptide P1 is GSLEVNCSSTTCNQPEVG-GLETS. The amino acids of the control peptide P8 are the same as those of P1, but in random order, except that the two conserved cysteine residues are in the right positions (Li et al., 1993b). After synthesis, the P1 and P8 peptides were reduced with dithiothreitol (1:1.5 by wt/wt, in 0.1 M NaHCO<sub>3</sub>, pH 8.0) for 1.5 h at room temperature, and purified by high pressure liquid chromatography, using a reverse phase column (Waters C<sub>18</sub>). A COOH-terminal tyrosine was added to the peptides to enable <sup>125</sup>I-labeling (Li et al., 1993b).

### Monoclonal Antibodies

A panel of monoclonal antibodies that bind to immunochemically and functionally distinct epitopes on the leukocyte integrins CD11a,b,c/CD18 was used. The monoclonal antibody 7E4 (Nortamo et al., 1988) reacts with the common  $\beta$  chain of the three leukocyte integrins. Antibody TSI/22 (Sánchez-Madrid et al., 1982) recognizes an epitope on the  $\alpha$  subunit of CD11a/CD18. Monoclonal antibodies OKM1, OKM10 (Wright et al., 1983), LM2/1 (Miller et al., 1986), and 60.1 (Wallis et al., 1986) are specific for spatially separate epitopes localized on the  $\alpha$  chain of CD11b/CD18. Antibodies 3.9 (Myones et al., 1988) and 2E1 (from the 5th International Workshop on Human Leukocyte Differentiation Antigens) react with different epitopes on the  $\alpha$  subunit of CD11c/CD18. Antibody LM609 recognizes an epitope on the  $\alpha$  chain of the vitronectin receptor ( $\alpha_v\beta_3$ ) and the recognition requires association of the  $\alpha$  and  $\beta$  subunits (Cheresh and Spiro, 1987). The ICAM antibodies were LB-2 (ICAM-1) (Clark et al., 1986), B-T1 (ICAM-2) (Diaclone, Besancon Cedex, France) and CG106 (ICAM-3) (Cordell et al., 1994). A mouse IgG1 negative control was from Dakopatts A/S, Denmark.

### Chemicals

ADP, 2-deoxy-D-glucose (DG) and cytochalasin B used were from Sigma Chem. Co., dibutyl cAMP from Boehringer GmbH.

### Purification of the Complement Fragment iC3b

The complement protein C3 was isolated from human plasma as described (Koistinen et al., 1989), dissolved in 0.01 M Na-5,5'-diethylbarbiturate, 0.15 M NaCl, 0.02% NaN<sub>3</sub>, pH 7.3, and converted to C3b by incubating with 0.5% (wt/wt) trypsin (Sigma) for 5 min at room temperature, followed by addition of 1.5% (wt/wt) soybean trypsin inhibitor. C3b was converted to iC3b by incubating with 0.15 mg/ml factor H and 0.012 mg/ml factor I for 1 h at 37°C. iC3b was isolated from the mixture on a Mono Q column by eluting with a 0.05–0.4 M NaCl gradient in 0.01 M phosphate, 5 mM EDTA, pH 7.5.

### Binding of <sup>125</sup>I-labeled P1 Peptide to Purified CD11b/CD18 and CD11c/CD18

CD11b/CD18 and CD11c/CD18 were purified from human buffy coat cell

lysates by affinity chromatography on monoclonal antibody LM2/1-Sepharose CL 4B and 3.9-Sepharose CL 4B, respectively, and eluted at pH 11.5 in the presence of 2 mM MgCl<sub>2</sub> and 1% *n*-octyl glucoside (Dustin and Springer, 1989). The purity of the proteins were checked by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (Laemmli, 1970). The heterodimeric forms of the purified integrins were examined by running the preparations once again through the same affinity columns and checking the eluates by SDS-PAGE. The proteins were diluted 1:10 with 25 mM Tris, pH 8.0, 150 mM NaCl and 2 mM MgCl<sub>2</sub>, and attached to flat-bottomed, 96-well microtiter plates (Dynatech Laboratories, VA) by overnight incubation at 4°C. The wells were blocked with 1% BSA for 1 h at room temperature. 12 pmol of <sup>125</sup>I-labeled P1, labeled by using the chloramine-T method (Greenwood et al., 1963), was added to each well in 40 μl of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4 (PBS), containing 2 mM MgCl<sub>2</sub>, 0.5% BSA and 0.02% NaN<sub>3</sub> with or without nonradioactive peptides, monoclonal antibodies, or some soluble ligands for CD11b/CD18, and incubated for 1 h at 37°C. After washing the wells for three times with the binding buffer, the attached <sup>125</sup>I-labeled P1 was solubilized with 1% SDS and counted. Saturation binding of peptide P1 to purified CD11b/CD18 was analyzed in dose response experiments in which increasing amounts of nonradioactive P1 were incubated in CD11b/CD18-coated wells together with 12 pmol of <sup>125</sup>I-labeled P1. Nonspecific binding was defined as the amount of <sup>125</sup>I-P1 bound to CD11b/CD18 in the presence of a 100-fold excess of unlabeled P1, and was subtracted from the total to calculate specific binding.

### Binding of Endothelial Cells to Purified CD11c/CD18

Purified CD11c/CD18 was diluted 1:10 and coated on flat-bottomed, 96-well microtiter plates, and the plates were saturated with 1% BSA as described above. The control plates were treated with BSA only. TNF- $\alpha$ -stimulated Eahy926 cells were removed from the tissue culture flasks with 5 mM EDTA in PBS, washed, and resuspended in DMEM, 40 mM Hepes, pH 7.2, 2 mM MgCl<sub>2</sub> and 5% FCS. Cells ( $5 \times 10^4$ ) in 50 μl were added to each well in the absence or presence of 50 μg/ml of mAb 3.9 or control antibody MIgG, and incubated for 1 h at 37°C. Unbound cells were removed by gentle washing. The binding was quantitated by counting bound cells using 200 $\times$  magnification of four randomly chosen fields from each well.

### Aggregation Assays

Cells were washed with RPMI 1640 medium containing 40 mM Hepes, 2 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub> and resuspended to a concentration of  $10^6$  cells/ml. Aliquots of 100 μl were added to each well of flat-bottomed, 96-well microtiter plates in the absence or presence of peptides, and incubated at 37°C for appropriate time periods. For inhibition of the ICAM-2 peptide P1-induced cell aggregation, cells were preincubated with different monoclonal antibodies or inhibitors for 15 min at room temperature before being treated with the peptide. For quantitative measurement of cell aggregation, the free cells of four randomly chosen areas (2.5 mm<sup>2</sup>) per well were counted. The amount of aggregated cells was expressed as: percent aggregation =  $100 \times [1 - (\text{number of free cells}) / (\text{total number of cells})]$ .

### Flow Cytometry Studies

THP-1 and U937 cells were washed and resuspended in PBS, and treated with preimmune rabbit immunoglobulin to block the Fc receptors on the cells. Aliquots of 100 μl of cell suspensions ( $10^6$  cells) were incubated with 25 μg/ml of different mAbs for 30 min at 0°C. The cells were washed and incubated with FITC-conjugated rabbit-anti-mouse F(ab)<sub>2</sub> (Dako, Copenhagen, Denmark) for 30 min on ice. After washing, the cells were fixed with 1% paraformaldehyde in PBS, and  $5 \times 10^3$  cells were analyzed immediately with a Becton-Dickinson (Immunocytometry System, San Jose, CA) FACScan flow cytometer.

### Binding of THP-1 Cells to Fibrinogen or iC3b Coated on Plastic

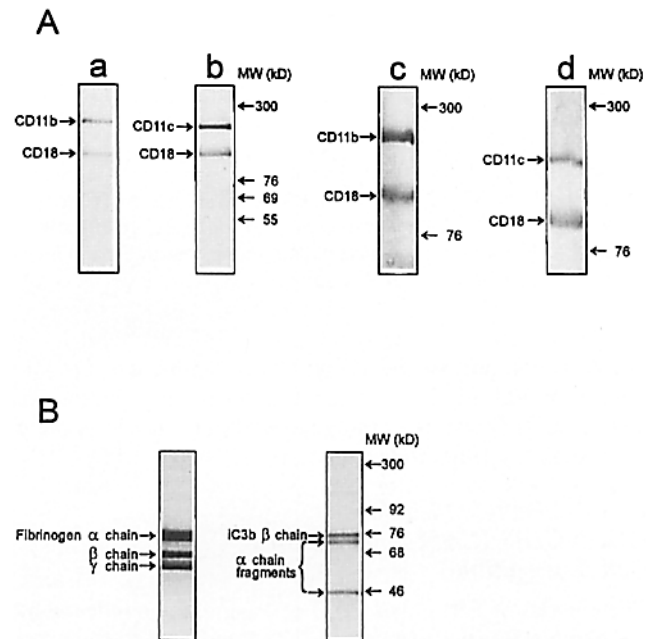
96-well microtiter plates were coated with purified fibrinogen (30 μg/ml in PBS), iC3b (2 μg/ml in PBS), or C3b (2 μg/ml in PBS) for 16 h at 4°C. At the end of incubation, the wells were blocked with 1% BSA in PBS for 2 h at room temperature. 100-μl aliquots of ADP or ICAM-2 peptide-stimulated THP-1 cells at  $2 \times 10^6$ /ml in RPMI 1640 medium supplemented with 40 mM Hepes, 2 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub> were added to each well, and incubated for 30 min at room temperature. For blocking ex-

periments, the peptide-stimulated cells were pretreated with different mAbs for 10 min at 22°C before being added to fibrinogen, iC3b-, or C3b-coated wells. Nonadherent cells were removed by three washes with the binding medium. The binding was quantitated by scoring the number of attached cells with 200 $\times$  magnification of four randomly chosen fields from each well.

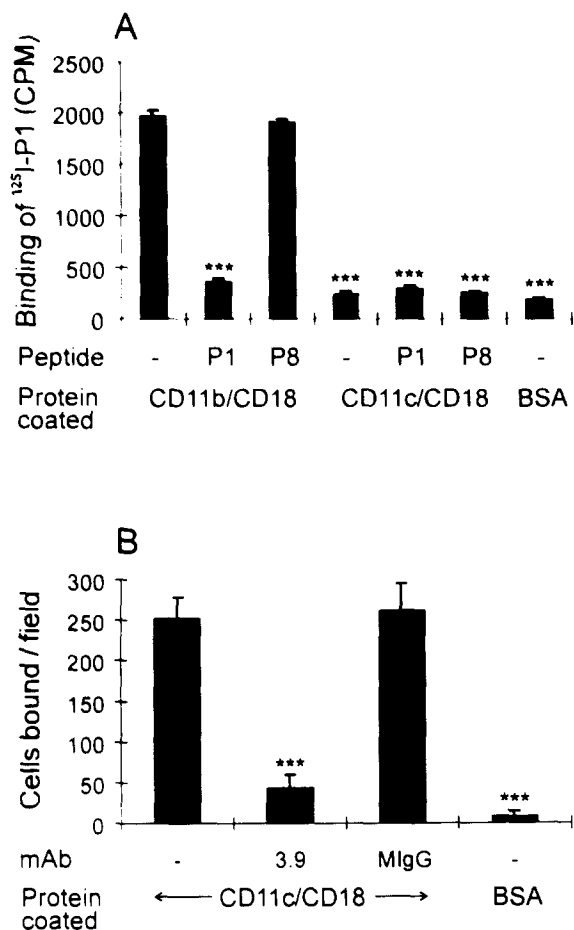
## Results

### Binding of ICAM-2 Peptide to CD11b/CD18

The purified CD11b/CD18 and CD11c/CD18 preparations were checked by polyacrylamide gel electrophoresis in the presence of SDS. The preparations contained the expected CD11b, CD11c, and CD18 polypeptides, and no major impurities were observed (Fig. 1 *A, a* and *b*). Most of the purified integrins was in functional intact heterodimeric forms as examined by running the preparations through the affinity columns once again, and checking the eluates by SDS-PAGE (Fig. 1 *A, c* and *d*). The <sup>125</sup>I-labeled ICAM-2 peptide P1 bound to purified CD11b/CD18 coated on plastic, while little binding was observed to the purified CD11c/CD18 or BSA. The binding of <sup>125</sup>I-labeled P1 to CD11b/CD18 was specifically and almost totally inhibited by unlabeled P1 (Fig. 2 *A*). The control peptide P8 had no effect. The purified CD11c/CD18 was in active form as shown by the fact that Eahy926 cells bound to CD11c/CD18 coated on plastic, and the binding was blocked by the CD11c antibody 3.9 (Fig. 2 *B*). The binding of <sup>125</sup>I-labeled P1 to CD11b/CD18 was saturable (Fig. 3 *A*), and efficiently blocked by the anti-CD11b mAb



**Figure 1.** Polyacrylamide gels of purified CD11b/CD18, CD11c/CD18, fibrinogen, and iC3b. Purified CD11b/CD18 (*A a*) and CD11c/CD18 (*A b*) were run on 8% polyacrylamide gels in the presence of SDS and stained with Coomassie blue. The heterodimeric forms of CD11b/CD18 (*A c*) and CD11c/CD18 (*A d*) were checked on 6% polyacrylamide gels. The positions of the molecular weight markers are indicated to the right. *B* shows the purified fibrinogen and iC3b preparations.

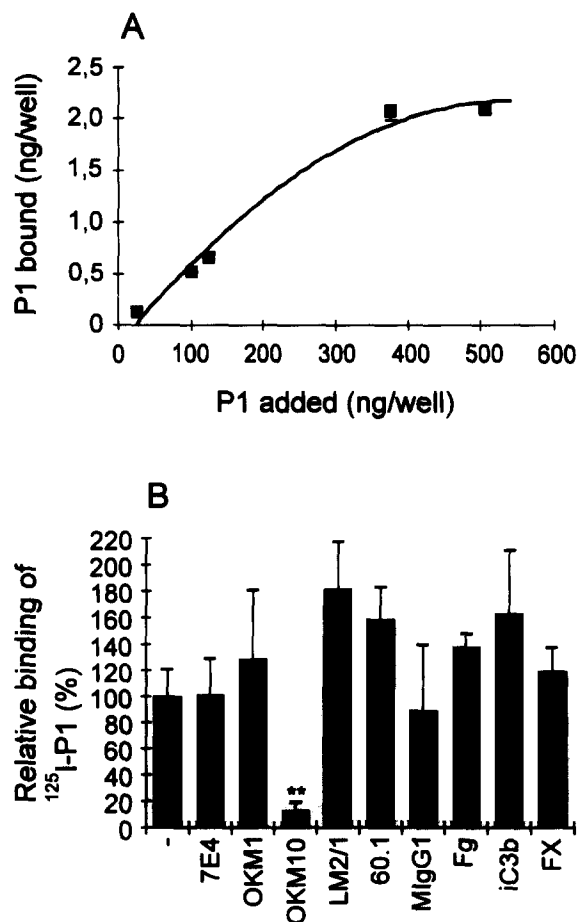


**Figure 2.** Binding of <sup>125</sup>I-labeled P1 peptide to purified CD11b/CD18 and CD11c/CD18, and binding of Eahy926 cells to CD11c/CD18. 12 pmol of <sup>125</sup>I-labeled P1 was incubated in CD11b/CD18 or CD11c/CD18-coated wells in the absence or presence of 100 μM of nonradioactive P1 or P8 peptides (A). 50 μg/ml of mAb 3.9 and control antibody MiIgG was used to block the adhesion of Eahy926 cells to CD11c/CD18 coated on plastic (B). The standard deviations are shown. The inhibitory effects of competing peptide P1 and mAb 3.9 were significant. \*\*\*, *p* < 0.001.

OKM10, but not by the other CD11b antibodies OKM1, LM2/1, or 60.1, nor by the anti-CD18 mAb 7E4. The soluble CD11b/CD18 ligands fibrinogen, iC3b, and factor X were not inhibitory (Fig. 3 B).

#### ICAM-2 Peptide P1-induced CD11b/CD18-ICAM-1-dependent Cell Aggregation

The kinetics of THP-1 and U937 cell aggregation induced by the peptide P1 was similar (Fig. 4 A and Fig. 5 A). After 0.5–1 h incubation with P1, the cells clearly aggregated as compared to cells treated with the control peptide P8, or cells left without any treatment. Maximal aggregation was observed after 3–4 h incubation, and the cells remained in clusters during the experiments. Peptide P1 induced cell aggregation in a concentration-dependent manner, and a 50% aggregation was obtained with ~30 or 40 μM of P1 for THP-1 or U937 cells, respectively (Fig. 4 B and Fig. 5 B). The expression of leukocyte adhesion molecules on THP-1

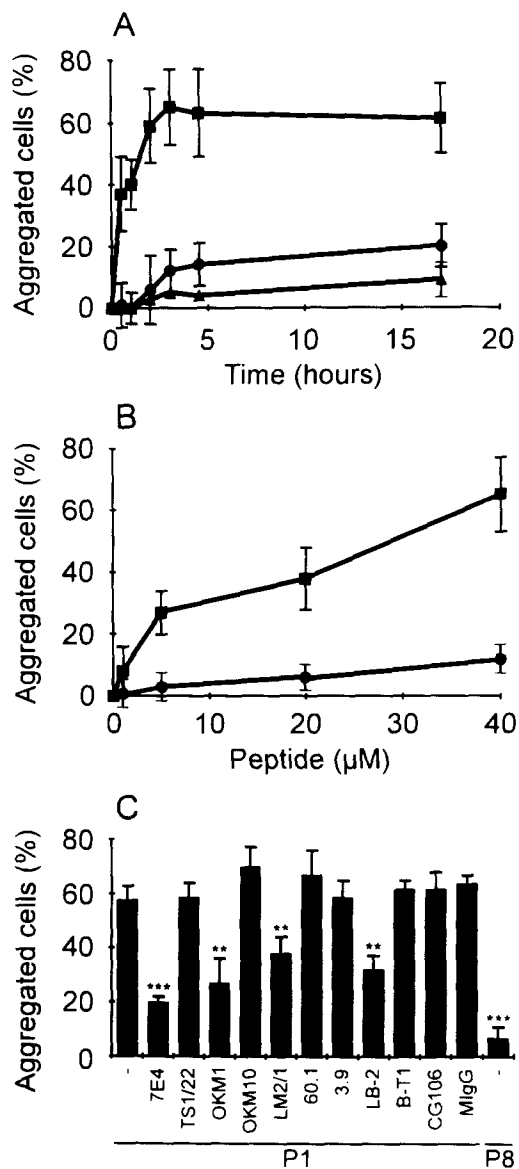


**Figure 3.** Saturation curve of the binding of <sup>125</sup>I-labeled P1 to purified CD11b/CD18, and inhibition of the interaction with monoclonal antibodies. 12 pmol of <sup>125</sup>I-P1 was incubated in CD11b/CD18-coated wells in the presence of increasing amounts of unlabeled P1 (A), or 50 μg/ml of monoclonal antibodies to CD18 (7E4), CD11b (OKM1, OKM10, LM2/1, and 60.1), and control antibody MiIgG1, or 50 μg/ml of fibrinogen (Fg), iC3b, or factor X (FX) (B). The inhibitory effect of mAb OKM10 was significant. \*\*, *p* < 0.01.

and U937 cells was studied by flow cytometry. CD18, CD11b, CD11c, ICAM-1, ICAM-2, and ICAM-3 were well expressed on both THP-1 and U937 cells, while little expression of CD11a was found (Fig. 6). Monoclonal antibodies against CD18 and ICAM-1 efficiently blocked the homotypic adhesion of both THP-1 and U937 cells, whereas the anti-CD11b antibodies OKM1, LM2/1, and 60.1 (for U937) blocked to a smaller, but significant extent (Fig. 4 C and Fig. 5 C). The CD11b antibody OKM10 and antibodies against CD11a, CD11c, ICAM-2, and ICAM-3 did not inhibit the aggregation.

#### Temperature, Energy, Microfilament, and Divalent Cation Requirements for ICAM-2 Peptide P1-induced Cell Aggregation

No P1-stimulated homotypic adhesion of THP-1 and U937 cells was detected at 4°C (Fig. 7, A and B). The aggregation was partially blocked by NaN<sub>3</sub>, but completely inhibited by using NaN<sub>3</sub> in combination with 2-deoxy-D-glucose. Cytochalasin B, which prevents the formation of microfilaments, partially inhibited the aggregation, and EDTA blocked effi-



**Figure 4.** Peptide P1-induced THP-1 cell aggregation. (A) THP-1 cells were treated with 40  $\mu$ M of peptides P1 (■) or P8 (●), or left without any treatment (▲), for indicated time periods. (B) The cells were treated with different amounts of P1 or P8. (C) 50  $\mu$ g/ml of mAbs 7E4 (CD18), TS1/22 (CD11a), OKM1 (CD11b), OKM10 (CD11b), LM2/1 (CD11b), 60.1 (CD11b), 3.9 (CD11c), LB-2 (ICAM-1), B-T1 (ICAM-2), CG106 (ICAM-3), and MiGg1 control antibody were used in the blocking assays. The cell aggregations in B and C were evaluated after a 3-h incubation. The standard deviations and statistic significances are shown.

ciently. Treatment of cells with dibutyryl cAMP, a cell-permeable cAMP analogue, slightly blocked the homotypic cell adhesion of THP-1 cells, but had no effect on U937 cells.

#### ICAM-2 Peptide P1-induced Adhesion of THP-1 Cells to Fibrinogen

The purified fibrinogen was checked by polyacrylamide gel electrophoresis in the presence of SDS and found to be essentially pure (Fig. 1 B). THP-1 cells stimulated with the peptide P1 bound much more efficiently to fibrinogen coated

on plastic (Fig. 8, A, B, and C a) than cells pretreated with the control peptide P8 (Fig. 8, A, B, and C b) or cells left without any treatment (Fig. 8, A and B). ADP treatment also increased the binding of THP-1 cells to fibrinogen, but to a smaller extent (Fig. 8 A). 1  $\mu$ M of P1 induced a substantial binding of THP-1 cells to fibrinogen, and the adhesion increased with increasing amounts of the P1 peptide (Fig. 8 B). The P1-induced adhesion of THP-1 cells to fibrinogen was efficiently blocked by monoclonal antibodies to CD18 (7E4) and CD11b (OKM10), partially blocked by the CD11c antibodies 3.9 and 2E1, and totally inhibited when antibodies OKM10 and 3.9 were used together (Fig. 9). The epitopes on CD11b/CD18 recognized by the monoclonal antibodies OKM1, OKM10, LM2/1, and 60.1 were expressed at similar levels on THP-1 cells (Fig. 6, data not shown). However, with the exception of OKM10, the other CD11b antibodies OKM1, LM2/1, and 60.1 did not block the binding. Another integrin receptor for fibrinogen,  $\alpha_v\beta_3$ , is also expressed on THP-1 cells (data not shown), but the antibody LM609 reacting with this receptor was not inhibitory. The antibody to CD11a (TS1/22), and the control antibody had no effect. EDTA abolished the adhesion completely.

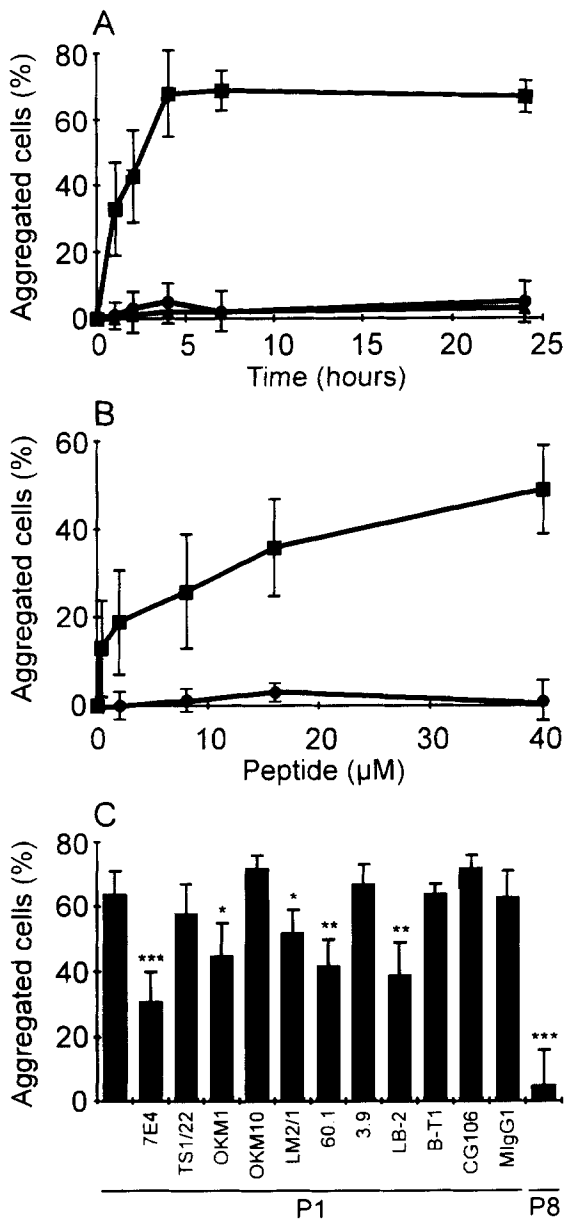
#### ICAM-2 Peptide P1-induced Adhesion of THP-1 Cells to Complement Fragment iC3b

The purified iC3b preparation was checked by polyacrylamide gel electrophoresis in the presence of SDS. It contained the  $\beta$  chain and the typical  $\alpha$  chain fragments, and no major impurities were observed (Fig. 1 B). Nonstimulated THP-1 cells bound to iC3b coated on plastic, but ADP and P1 peptide increased the binding to a small but significant extent, whereas the control peptide P8 had no effect (Fig. 10 A). The binding was efficiently inhibited by mAb 7E4, partially by OKM10 and 3.9, and an additive effect was observed when OKM10 and 3.9 were used together (Fig. 10 B). Antibodies TS1/22, OKM1, LM2/1, and 60.1 were not inhibitory, whereas EDTA blocked the interaction. Little binding occurred to C3b.

#### Discussion

CD11b/CD18 is the predominant myeloid cell integrin (Springer, 1990; Arnaout, 1990; Patarroyo et al., 1990). It is involved in cell-cell interactions, and has been shown to bind to the third immunoglobulin domain of ICAM-1 (Diamond et al., 1991). Furthermore, it binds to several soluble proteins like fibrinogen (Wright et al., 1988; Altieri et al., 1988), iC3b (Beller et al., 1982; Wright et al., 1983), and factor X (Altieri and Edgington, 1988a), and to various complex carbohydrates (Ross et al., 1985; Wright and Jong, 1986). The binding specificity of CD11c/CD18, which is mainly found on macrophages, is less understood, but it binds to fibrinogen (Loike et al., 1991; Postigo et al., 1991), iC3b (Micklem et al., 1985; Myones et al., 1988), and a counter-receptor on the surface of endothelial cells (Stacker and Springer, 1991), which was confirmed in the present work.

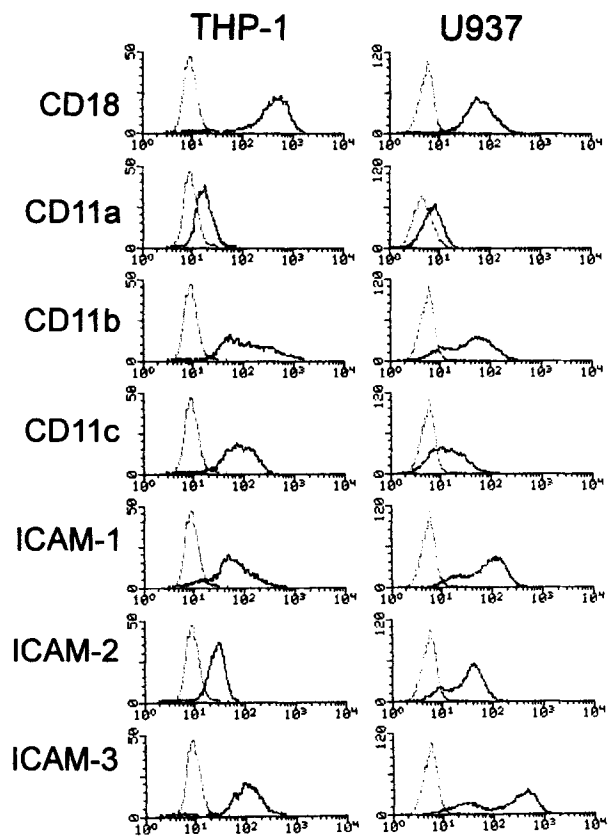
Obviously, it is important to define the leukocyte integrin ligands in detail, determine the binding sites, and establish the mode of activation. ICAM-2 is a relatively simple integrin ligand, and therefore we thought that it should be an ex-



**Figure 5.** Peptide P1-induced U937 cell aggregation. (A) U937 cells were treated with 40 µM of peptides P1 (■) or P8 (●), or left without any treatment (▲), for indicated time periods. (B) The cells were treated with different amounts of P1 or P8. (C) The antibody blocking assays were performed as shown in Fig. 4. The aggregations of cells in B and C were evaluated after a 4-h incubation. The standard deviations are shown. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

cellent model for detailed studies on integrin-ligand interactions.

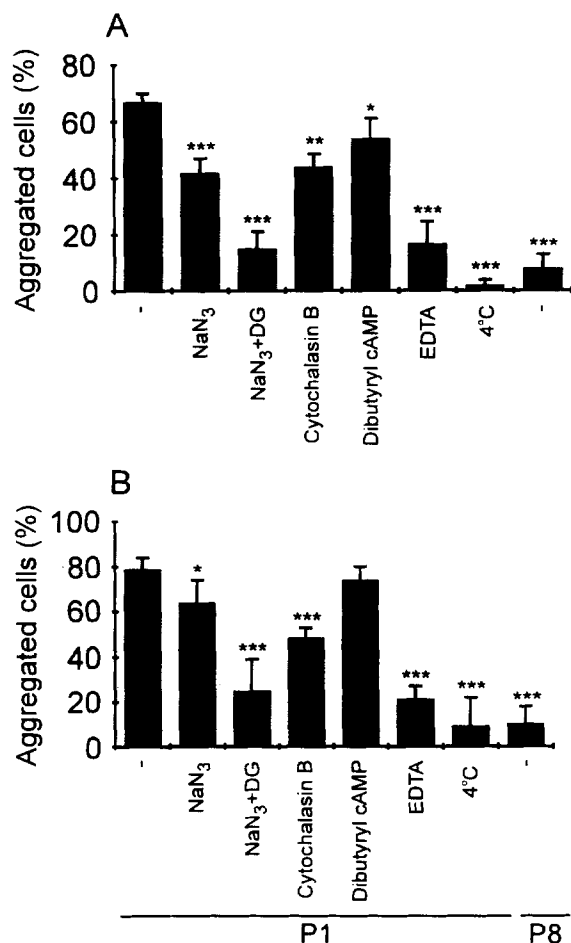
We previously showed that the 22-amino acid residue peptide P1, derived from the first immunoglobulin domain of ICAM-2, specifically binds to CD11a/CD18 (Li et al., 1993b). However, we did not find any CD11a antibody that blocked the interaction. The P1 peptide inhibits the binding of endothelial cells to purified CD11a/CD18, and the binding of lymphoblastoid cells to endothelial cells (Li et al., 1993b). In the present study, we show that the same peptide also specifically binds to purified CD11b/CD18, but not to



**Figure 6.** Expression of CD11/CD18 integrins and ICAMs on the monocytic cell lines THP-1 and U937. The monoclonal antibodies 7E4 (CD18), TS1/22 (CD11a), OKM10 (CD11b), 3.9 (CD11c), LB-2 (ICAM-1), B-T1 (ICAM-2), CG106 (ICAM-3) (dark lines), and mouse IgG1 (control) (gray lines) were used. The expression of the epitopes recognized by the CD11b antibodies OKM1, LM2/1, and 60.1 was similar to that of the OKM10 epitope (data not shown).

CD11c/CD18. The anti-CD11b mAb, OKM10, which recognizes a discontinuous epitope that requires the presence of both the NH<sub>2</sub>-terminal and divalent cation-binding regions (Diamond et al., 1993), efficiently blocked this interaction. The results suggest that the epitope recognized by mAb OKM10 on the  $\alpha$  chain is in the vicinity of the binding site for the P1 peptide. However, the soluble ligands for CD11b/CD18 fibrinogen, iC3b, and factor X, were not able to interfere with this interaction, indicating that different regions on CD11b/CD18 are involved in binding of P1 and these ligands. The binding of the ICAM-2 derived peptide to CD11b/CD18 raises the interesting possibility that ICAM-2 could bind to this integrin. It has been shown that there exists a second CD11b/CD18 ligand on vascular endothelium, besides ICAM-1 (Carlos and Harlan, 1994). Our preliminary results indicate that ICAM-2 binds to CD11b/CD18 (Xie, J., R. Li, P. Kotou, C. Kantor, C. Vermot-Desroches, J. Wijdenes, M. A. Arnaout, P. Natamo, and C. G. Gahmberg, manuscript in preparation).

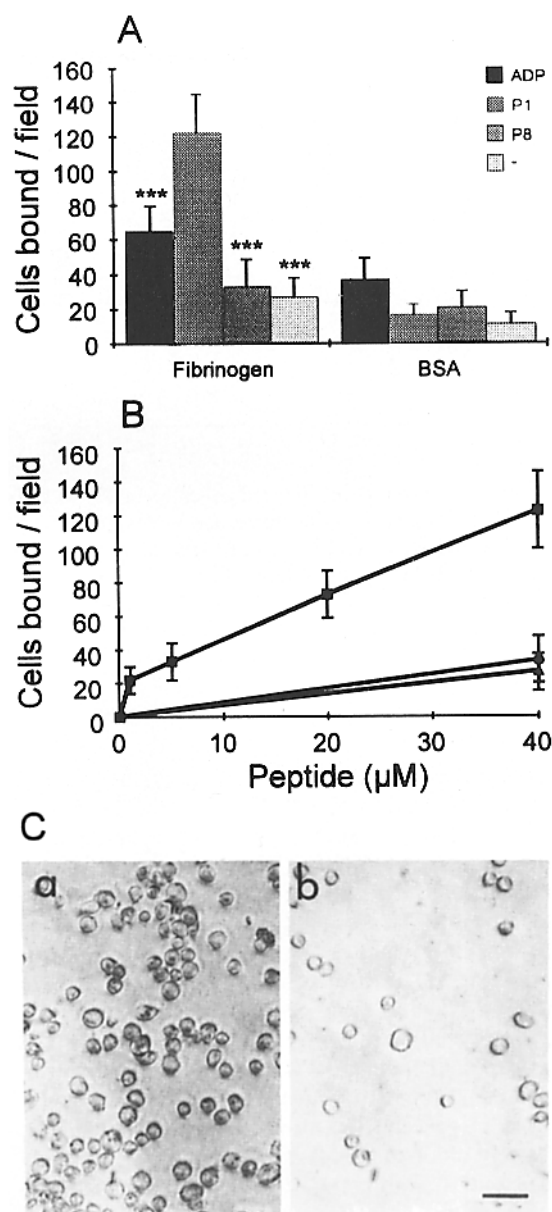
The peptide P1 is relatively hydrophobic, and most part of it is probably buried in the first domain of ICAM-2, except that both of its NH<sub>2</sub>- and COOH-terminals seem to be exposed (Li et al., 1993b). It is essential that the peptide is in reduced form. We have found that when preserved without the presence of reducing agents, the peptide was easily oxi-



**Figure 7.** Inhibition of peptide P1-induced THP-1 and U-937 cell aggregations. THP-1 (A) and U-937 (B) cells were kept at 4°C or pretreated with 0.2% of NaN<sub>3</sub>, 0.2% NaN<sub>3</sub> and 50 mM 2-deoxy-D-glucose, 20 μM cytochalasin B, 2 mM dibutyryl cAMP or 5 mM EDTA for 15 min at room temperature, before the addition of 40 μM of P1 peptide. The aggregations were determined after 3-h or 4-h incubations for THP-1 and U937, respectively, at 37°C, with the exception of the assay marked 4°C.

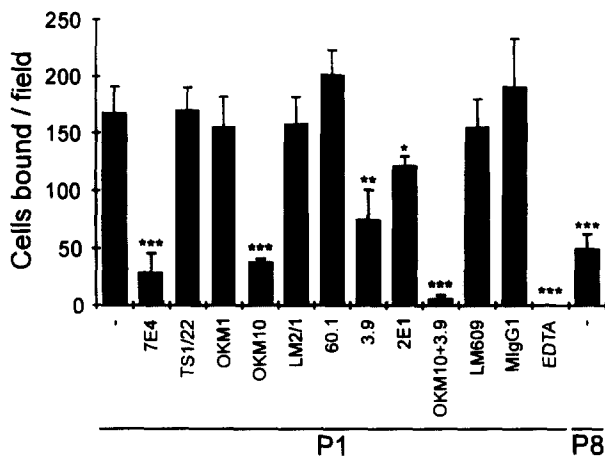
dized and lost activity. On the other hand, alkylated peptide did not work either (data not shown). Therefore, the two cysteines in the peptide seem to be essential for its activity.

Recently, we found that the ICAM-2 peptide strongly induces blood T cell aggregation, which is mainly mediated by CD11a/CD18-ICAM-1 interaction, and an increase in the binding and cytotoxicity of natural killer cells (Li et al., 1993a). Here we show that the stimulatory effect is not restricted to T lymphocytes and natural killer cells. Myelomonocytic THP-1 and U937 cells became aggregated after treatment with the P1 peptide. Blood neutrophils were also strongly aggregated by P1 stimulation (data not shown). Unlike T lymphocytes, on which CD11a/CD18 is the main leukocyte integrin expressed, CD11b/CD18 and CD11c/CD18 are well expressed on THP-1 and U937 cells, while there is little expression of CD11a/CD18. The THP-1 and U937 cell aggregations were efficiently blocked by CD18 and ICAM-1 antibodies. The CD11b antibodies OKM1, LM2/1, and 60.1 (for U937) blocked to a smaller, but significant extent, while CD11a and CD11c antibodies had no effect. These results in-



**Figure 8.** Peptide P1-induced adhesion of THP-1 cells to fibrinogen coated on plastic. (A) THP-1 cells were preincubated with 40 μM of peptides P1 or P8 for 2 h at 37°C, washed, and resuspended by pipetting, before being added to fibrinogen or BSA-coated wells. 10 μM of ADP was added at the same time when the cell suspension was added to the wells. (B) THP-1 cells were stimulated with different concentrations of P1 (■) or P8 (●), or left without any treatment (▲), before being added to fibrinogen-coated wells. (C) Binding of peptide P1 (C a) or P8 (C b)-stimulated THP-1 cells to immobilized fibrinogen. The photomicrographs were taken after the nonadherent cells had been removed by washing three times. A 200× magnification was used. The bar indicates 50 μm.

dicates that CD11b/CD18 becomes activated, and mediates the cell aggregation by interacting with ICAM-1. Since the CD11b antibody OKM10, which blocked the binding of P1 peptide to purified CD11b/CD18, did not inhibit the THP-1 and U937 cell aggregation, it is probable that CD11b/CD18 is activated by signals resulting from the interaction of P1 with the small amount of CD11a/CD18 expressed on the

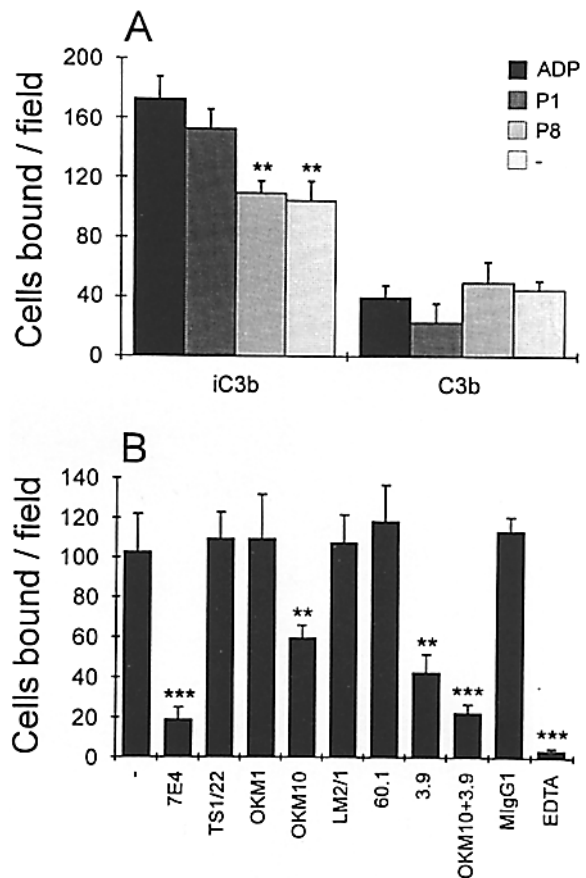


**Figure 9.** Inhibition of peptide P1-induced adhesion of THP-1 cells to immobilized fibrinogen by monoclonal antibodies and EDTA. Aliquots of P1-stimulated THP-1 cells were separately pretreated with 50  $\mu\text{g/ml}$  of mAbs against CD18 (7E4), CD11a (TS1/22), CD11b (OKM1, OKM10, LM2/1, and 60.1), CD11c (3.9, 2E1),  $\alpha_v\beta_3$  (LM609) and control MigG1, before being added to fibrinogen-coated wells. 5 mM EDTA was used. The effect of P1 is significant as indicated.

cells. Alternatively, during the incubation of the cells at 37°C, the OKM10-engaged CD11b/CD18 molecules are no longer available on the cell surface, because of the rapid internalization of occupied CD11b/CD18 (Rab et al., 1993). It has been shown that translocation of neutrophil CD11b/CD18 complex to the cell surface can be induced at 37°C (Todd et al., 1984). We think that a minor possibility could be that these OKM10-free CD11b/CD18 derived from the intracellular pool bound P1 and became activated.

Peptide P1-induced homotypic adhesion of THP-1 and U937 cells does not happen at 4°C, and requires energy, intact microfilaments, and divalent cations. However, cytochalasin B, which prevents the formation of microfilaments, could not totally block the aggregation; and CD18 antibody 7E4 was not able to completely inhibit the aggregation either. These findings suggested that besides  $\beta_2$  integrins, some other integrins or adhesion systems were also activated.

We previously reported that cross-linking of CD11b/CD18 on neutrophils with monoclonal antibodies causes a rise in cytosolic free  $\text{Ca}^{2+}$ , which is functionally coupled to a transient activation state of CD11b/CD18 (Altieri et al., 1992). In addition, engagement of the divalent ion binding site(s) on CD11b/CD18 induces the expression of activation-dependent neoantigenic epitopes on CD11b/CD18 and leukocyte adhesion (Altieri, 1991). Furthermore, binding of eosinophils to endothelial cells causes a considerable upregulation of CD11b and an increased capacity to generate an oxidative burst (Walker et al., 1993). It is possible that binding of P1 to CD11b/CD18 has a similar effect as CD11b-specific activating antibodies. The complexity of integrin-activating antibodies is illustrated by the opposite effects of CD11a and CD18 antibodies on T cell activation (van Noesel et al., 1988). In the presence of CD18 antibodies, CD11a antibodies increased T cell proliferation, whereas CD18 antibodies were inhibitory. These findings indicate that integrin-ligand



**Figure 10.** Peptide P1-induced adhesion of THP-1 cells to iC3b or C3b coated on plastic. (A) THP-1 cells were preincubated with 40  $\mu\text{M}$  of peptides P1 or P8 for 2 h at 37°C, washed, resuspended by pipetting, and added to iC3b- or C3b-coated wells. 10  $\mu\text{M}$  of ADP was added at the same time when the cell suspension was added to the wells. (B) Aliquots of P1-stimulated THP-1 cells were separately pretreated with 50  $\mu\text{g/ml}$  of mAbs against CD18 (7E4), CD11a (TS1/22), CD11b (OKM1, OKM10, LM2/1, and 60.1), CD11c (3.9), and control MigG1, before being added to iC3b-coated wells. 5 mM EDTA was used.

binding may have different effects on integrin functions depending on the site of interaction.

Although several stimuli such as phorbol esters, the peptide formyl-methionyl-leucyl-phenylalanine and complement fragment C5a, can increase the cell surface expression of CD11b/CD18 (Lo et al., 1989; Detmers et al., 1990; Diamond and Springer, 1993), no detectable increase in CD11b/CD18 expression on granulocytes and THP-1 cells was observed after treatment with P1 (data not shown). Phorbol ester or ADP-induced high avidity state of CD11b/CD18 is characterized by the formation of activation-dependent neoantigenic epitopes recognized by the  $\alpha$  chain antibodies CBRM1/5 (Diamond and Springer, 1993) or 7E3 (Altieri and Edgington, 1988b). However, the P1 peptide-stimulated functional modulation of CD11b/CD18 was not accompanied by an increased expression of these epitopes (data not shown). Recent work has shown by using CD18 antibodies, that the peptide P1 was able to induce the aggregation of the  $\beta_2$  integrins in the plane of cell membrane on natural killer cells (Somersalo et al., 1995), which is supposed to be



tightly correlated with high ligand-binding capacity of CD11b/CD18 (Detmers et al., 1987).

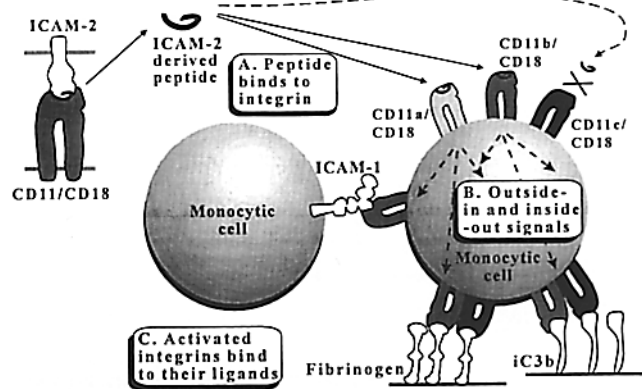
The adhesion of THP-1 cells to fibrinogen and iC3b, was increased by stimulating the cells with peptide P1. The interactions were efficiently blocked by CD18, CD11b, and CD11c antibodies, and an additive effect was observed when CD11b and CD11c antibodies were used together. The P1-stimulated adhesion of THP-1 cells to immobilized fibrinogen and iC3b was blocked by the antibody OKM10, which was found previously to be able to block the interactions between leukocytes and immobilized fibrinogen and iC3b (Wright et al., 1983, 1988; Anderson et al., 1986). On the other hand, some reports have shown that OKM1, rather than OKM10, inhibits the interaction between the leukocytes and soluble fibrinogen (Altieri et al., 1988, 1990). It has been found that conformational changes occur in fibrinogen when the protein is adsorbed onto a plastic surface (Ugarova et al., 1993). This could be the reason for the fact that soluble fibrinogen did not inhibit the interaction between P1 and CD11b/CD18, in which the OKM10 epitope was involved. The background binding of resting THP-1 cells to iC3b coated on plastic was quite high, because iC3b constitutes a ligand for nonactivated CD11b/CD18 (Ross and Vetvicka, 1993).

Taken together, our results are compatible with a model in which the ICAM-2-derived peptide P1 binds to the leukocyte integrins CD11a/CD18 (Li et al., 1993b) and CD11b/CD18, but not to CD11c/CD18. These interactions induce high avidity states of CD11a/CD18 on T lymphocytes (Li et al., 1993a), and CD11b/CD18 and CD11c/CD18 on monocytic cells for their ligands (Fig. 11). Evidently, the activation of CD11b/CD18 and CD11c/CD18 involves both outside-in and inside-out signals, but it is not clear yet whether the signals come from the interaction of P1 peptide with CD11a/CD18, or with CD11b/CD18, or with both. The detailed mechanisms involved in the signaling remain poorly understood.

We tested the ability of peptide P1 to induce the binding of CD11b/CD18 transfected CHO cells to fibrinogen. The CD11b and CD18 are in the expression vector  $\pi$ H3M (Dana et al., 1991), and was obtained from Dr. M. A. Arnaout. Approximately 20–30% of the transfected cells expressed CD11b/CD18 as determined by FACScan analysis. The CD11b/CD18 transfected cells bound to fibrinogen, but no additional activation by P1 was observed (data not shown). Phorbol esters were not either capable of activating the transfected cells. These findings show that the CD11b/CD18 is active in the transfected cells, but further activation is difficult to achieve. Evidently, CHO cells lack the activation system found in leukocytes.

Recent studies have provided evidence of how fibrinogen participates in inflammatory responses and host defense. Fibrinogen is involved in the regulation of leukocyte adhesion to vascular endothelium by interacting with both CD11b/CD18 on leukocytes and ICAM-1 on endothelium (Altieri et al., 1993; Languino et al., 1993). The finding that P1 stimulates the binding of leukocytes to fibrinogen raises the intriguing possibility that ICAM-2, which is constitutively expressed on most endothelia (Nortamo et al., 1991a; de Fougères et al., 1991), could have an important regulatory role in leukocyte binding during physiological conditions.

Besides the proposed triggering role for ICAM-2 peptide



**Figure 11.** Hypothetical model to explain the ability of the ICAM-2-derived peptide P1 to regulate the avidity of leukocyte integrins. Peptide P1 binds to CD11a/CD18 and CD11b/CD18, but not to CD11c/CD18. The binding induces outside-in and inside-out signals, by which CD11a/CD18 (Li et al., 1993a), CD11b/CD18, and CD11c/CD18 are activated to bind to their ligands.

P1 in ligand binding to integrin, it is obvious that this peptide also contributes to the adhesive interaction itself. This is based on the capacity of P1 to bind to purified CD11a/CD18 and CD11b/CD18, as well as the capacity of this peptide to inhibit the adhesion of endothelial cells to purified CD11a/CD18. Furthermore, it partially blocks the binding of B lymphoblastoid cells to endothelial cells (Li et al., 1993b). The ability of P1, on one hand, to inhibit the binding of ICAM-2 containing nonleukocytic cells to CD11a/CD18, but on the other hand, to activate leukocyte binding, is incompletely understood. The main reason may be that as shown here, the three CD11/CD18 cellular integrins become activated by P1, and they bind to several ligands at different binding sites. Therefore, blocking of activated cell-bound integrins is more difficult than interference with the binding of single purified integrin to its ligands.

An explanation for the high avidity ligand binding triggered by peptide P1 could be that additional ligand-binding sites are generated in the integrin, as proposed for the activation of the platelet integrin  $\text{gpIIb/IIIa}$  by arginine-glycine-aspartic acid (RGD)-containing peptides (Ruoslahti and Pierschbacher, 1986, 1987; Du et al., 1991). However, unlike the RGD-containing peptide-induced high affinity state of  $\text{gpIIb/IIIa}$ , which appears to be due to a direct conformational change in the receptor itself (Du et al., 1991), the P1-induced activation of  $\beta_2$  integrins seems to involve signal transduction events (Li et al., 1993a), although a direct effect on CD11/CD18 integrins cannot be excluded.

Our results show that the high avidity state of leukocyte integrins for different ligands can be induced by interaction between the integrins and the ICAM-2-derived P1 peptide. This indicates the possibility of induction of active forms of leukocyte integrins through interaction with agonistic ligands. Such regulatory reagents could be useful in therapeutic maneuvers in the future.

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