Peptides Derived from the Complementarity-determining Regions of Anti-Mac-1 Antibodies Block Intercellular Adhesion Molecule-1 Interaction with Mac-1*

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Peptides or small molecules that can block the interaction of the integrin Mac-1 with its receptor, intercellular adhesion molecule-1 (ICAM-1), have not previously been developed. We studied this interaction by measuring the adherence of ICAM-1-expressing Chinese hamster ovary (CHO) cells to immobilized, purified Mac-1. Nucleotide sequence information was obtained for the complementarity determining regions (CDRs) of three antibodies (44aacb, MY904, and 118.1) shown to block Mac-1-mediated cell adherence. Peptides were synthesized based on the predicted amino acid sequences of the CDRs and tested for the ability to block cell adherence to Mac-1. Peptides derived from CDR1 of 44aacb, CDR2 of 118.1, and CDR1 and 3 of MY904 heavy chains were found to possess blocking activity at 10–100 μM. This may indicate that one or two CDRs contribute disproportionately to the antibody binding affinity. The binding of ligands to Mac-1 has been shown to require a region of the α-chain known as the I- or A-domain. We have recombinantly produced Mac-1 I-domain, and show that it is also capable of supporting the adherence of ICAM-1-expressing CHO cells. The adherence of ICAM-1-CHO cells to the I-domain is inhibited by 44aacb and 118.1 and by the CDR peptides from 44aacb and 118.1. By using phage display of peptide libraries based on the 118.1 CDR peptide with five residues randomized, we were able to identify a novel peptide inhibitor of Mac-1 with substitutions at all five positions. These peptides provide lead structures for development of Mac-1 antagonists.

The extravasation of white blood cells to sites of inflammation and the phagocytosis of opsinized microorganisms by these cells is clearly crucial to host defense. However, the mounting of an inappropriately large mobilization of phagocytic cells is thought to contribute to organ damage in sepsis, in adult respiratory distress syndrome, and following reperfusion of ischemic tissue (1, 2). Activated neutrophils are recruited into tissues or are sequestered in the microcirculation of the lung and liver; in either case tissue is damaged upon their degranulation and release of enzymes and activated oxygen species. Interruption of neutrophil extravasation or oxidative burst may be an effective means of damage control in these situations.

Mac-1 is a cell surface glycoprotein contributing to several myeloid cell functions including adherence to and transmigration across the endothelium, binding and phagocytosis of opsinized particles, and the oxidative burst (3–5). It is a heterodimer of two transmembrane proteins, CD11b (αM) and CD18 (β2), the latter also being part of the related integrins LFA-1, p150/95, and α5β2. The major adhesion partner for Mac-1 is ICAM-1 (6), which is a member of the Ig supergene family and contains five extracellular Ig domains of the C2 type, characteristic of Fc receptors and proteins involved in cell adhesion (7). However, the complexity of the functions involving Mac-1 results in part from the fact that an array of ligands besides ICAM-1 is also recognized by this molecule, including iC3b, fibrinogen, and factor X (3).

The precise residues of Mac-1 and ICAM-1 mediating their interaction are not known, although the domains responsible have been elucidated. Ig domain 3 of ICAM-1 has clearly been implicated in binding to Mac-1, whereas domain 1 mediates binding to the related adhesion molecule, LFA-1 (8). CD11b contains a 200-amino acid “inserted domain” or “I domain,” so called due to its presence only in the other β2 integrins and in the VLA α1 and α2 subunits and its absence in most other integrins. Antibodies to this domain can block ICAM-1 binding, as well as that of iC3b and fibrinogen (3). Mutations within the I-domain of Mac-1 have been shown to prevent binding of ICAM-1 and iC3b (9–11). The Mac-1 I-domain has been expressed recombinantly, and there are some data suggesting that it interacts with fibrinogen, iC3b, and soluble ICAM-1 (4, 12). Whether the I-domain can support the adherence of ICAM-1-expressing cells independent of other domains of Mac-1 has not been previously demonstrated.

The lack of information on the precise sequences within ICAM-1 and Mac-1 that interact has precluded modeling of small molecule inhibitors. One approach to overcoming this problem that has proven successful in other cases has been to use CDR sequences from antibodies directed at the active site as lead structures. Antibodies useful in this regard have been either developed as anti-idiotypic to the ligand (13) or simply chosen by virtue of their ligand blocking activity (14). As well as providing lead inhibitors, in some cases CDRs have shared sequence similarity with a portion of the known ligand, implying that sequence in receptor binding (14–16).

We have produced the Mac-1 I-domain recombinantly and show that it supports the adherence of ICAM-1-expressing cells independent of other domains of Mac-1 has not been previously demonstrated.

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1 The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; CDR, complementarity-determining region; HBSS, Hank’s buffered salt solution; HBSS++, HBSS with calcium and magnesium; HC, heavy chain; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; HPLC, high pressure liquid chromatography; HSA, human serum albumin.
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cells. Several antibodies that block the adherence of ICAM-1 to both Mac-1 and to the I-domain have been sequenced to allow determination of CDR structures. Peptides based on these CDR structures are shown to block the adherence of ICAM-1 to both Mac-1 and the I-domain.

MATERIALS AND METHODS

Purification of Mac-1—Peripheral blood leukocytes were purified from “Buffco coats” (Stanford Medical School Blood Center, Stanford, CA). Buffy coats were diluted 1:1 with HBSS and layered on Histopaque (300 g, 60 min; Ref. 18). The cell layers were collected, and the blood cells were lysed one to two times as necessary. Mac-1 was purified from lysates of the remaining leukocytes by immunoaffinity chromatography essentially as described by Diamond et al. (6) and purity assessed using SDS-polyacrylamide gel electrophoresis (see Fig. IA).

Construction of Mac-1 I-domain—The construction of the I-domain was based on a plasmid construct reported by Michishita et al. (11). The I-domain of human Mac-1 from the glycine residue at position 111 to the alanine at position 318 was generated using synthetic oligonucleotides and PCR. Eight overlapping oligonucleotides were synthesized and combined in a stepwise PCR procedure to generate the final 603-base fragment. The 5′-most oligo included a BamHI site present naturally in the Mac-1 sequence, whereas the 3′-most oligo included an added EcoRI site. The internal EcoRI site in this I-domain sequence was eliminated via a single base change from A to T at the third position in the glutamate codon at position 179 (a silent mutation). Each pair of oligonucleotides sharing partial complementarity was annealed and subjected to PCR (3′ at 94 °C, followed by 10 cycles of 1′ at 94 °C, 2′ at 55 °C, and 3′ at 72 °C) using 250 μM dNTPs and VENT polymerase (New England Biolabs, Beverly, MA). The PCR products were then mixed, melted for 3′ at 94 °C, and subjected to PCR as described above. After assembly of all of the oligos, the resulting BamHI/EcoRI fragment was cloned into the pGEX-2T (Pharmacia Biotech Inc.) vector at the SacI site, forming I-domain-expressing plasmids pGEX-Mac-1 (strain JM101). Overnight cultures of E. coli JM101 were diluted 1:10 in LB broth and incubated overnight. Cells (108 cells each, grown in RPMI 1640, 10% fetal bovine serum, 10 mM HEPES, 2 mM MgCl2, and 0.1% HSA) with a specific activity of 3–5 mg/liter bacterial culture.

Enzymes that cleave the I-domain were prepared using Escherichia coli cells (strain JM101). Overnight cultures of E. coli JM101 were diluted 1:10 in LB broth and incubated overnight. Cells (108 cells each, grown in RPMI 1640, 10% fetal bovine serum, 10 mM HEPES, 2 mM MgCl2, and 0.1% HSA) with a specific activity of 3–5 mg/liter bacterial culture.

Electrospray ionization Mass Spectrometric Analysis of I-domain—The adherence of human neutrophils to wells coated with human serum was carried out exactly as described previously (17). CHO cells were stably transfected with an expression vector encoding human ICAM-1 (20), and their adherence was assessed as follows. ICAM-1-CHO cells were grown in RPMI 1640 with 10% fetal bovine serum and used at 80% confluence. Cells were loaded with calcein-acetoxymethyl ester (Molecular Probes, Eugene, OR) at 5 μM for 30 min at 37 °C. The cells were then detached from the flask with PBS containing 5 mM EDTA (15 min, 37 °C), washed in HBSS (Sigma), and resuspended in HBSS and containing 0.5% HSA at 2 × 106/ml. 96-well plates were coated with purified Mac-1 or I-domain in HBSS and 10 μl/well for 2 h at 37 °C. In the case of Mac-1, β-octylglucoside at a final concentration of 0.15% was present in the coating solution. For each lot of Mac-1, the optimal protein concentration for coating the wells (5–15 μg/ml) was determined as that which best supported the adherence of ICAM-1-CHO cells but not of vector-C10 cells. Wells were washed twice with HBSS and blocked with HBSS and supplemented with 0.5% HSA for 30 min at 37 °C. Test compounds were preincubated in blocked wells for 15 min at 37 °C in 50 μl, and then 50 μl of cells were added for an additional 60-min incubation. Nonadherent cells were removed by gently inverting plates and blotting on paper towels. Wells were washed twice with HBSS and containing 0.5% HSA. Adherent cells were quantitated in a 96-well fluorescence plate reader (IDEXX Labs, Westbrook, ME). HSA lots were tested in this assay to find those that gave a minimal background adherence and a maximal adherence to Mac-1. All incubations in HBSS were carried out without CO2.

Monoclonal Antibodies—Hybridoma cells producing the anti-Mac-1 monoclonal antibodies LM2/1 (murine IgG1), 44aacb (murine IgG2a κ), and MY904 (murine IgG1 κ) were from ATCC (21, 22). The hybridoma cell line secreting mAb 118.1 (murine IgG1 κ) was generated from a Balb/C mouse immunized according to Diamond et al. (3). Spleen cells were mixed in a 5:1 ratio with FOX-NY murine myeloma cells (ATCC 1732 CRL) and fused by slow addition of polyethylene glycol 1500 (0.5 ml/108 cells, Boehringer Mannheim). After 1 min at 37 °C, the cell suspension was slowly diluted in RPMI, centrifuged at 1000 g for 7 min, and resuspended in selection medium (RPMI 1640, 20% fetal bovine serum, Pen/Strep, AAT media supplement (Sigma), and Stm Mitogen (RIBI ImmunoChem Research Inc.). After 9 days of growth in 96-well culture plates, the hybridoma supernatants were screened for antibodies binding to purified Mac-1 in an enzyme-linked immunosorbent assay adapted from Diamond and Springer (23). Serum-free conditioned media from the hybridoma cell line secreting 118.1 was tested in this assay to find those that gave a minimal background adherence and a maximal adherence to Mac-1. All incubations in HBSS were carried out without CO2.
The GY region, three or four positive clones were picked, DNA was prepared using the Wizard miniprep system (Promega, Madison, WI), and the PCR product carried in the plasmid DNA was sequenced.

**RESULTS**

Production of Recombinant Mac-1 I-domain—Purified Mac-1 has been shown to support the adherence of ICAM-1-expressing L-cells (6). To determine whether the I-domain of Mac-1 could similarly support the adherence of cells expressing ICAM-1, this domain was expressed recombinantly in E. coli. Analysis of the purified protein by SDS-polyacrylamide gel electrophoresis showed a major band migrating as expected and in some cases a minor band with slightly slower migration (Fig. 1B). Analysis of the purified protein by mass spectrometry showed a major peak of 24,103 Da, in agreement with the molecular mass predicted for this construct. Also revealed was a minor peak of 25,102 Da, present at approximately 10–20% the level of the major peak in different preparations. To determine the source of the additional 1000 Da, the proteins in the I-domain preparation were separated by SDS-polyacrylamide gel electrophoresis and subjected to both N-terminal sequence analysis and tryptic peptide mapping. Both the 24- and 25-kDa proteins had the expected N-terminal sequence of GSNLRQQP. The 24- and 25-kDa bands showed a similar tryptic map pattern with the exception of the C-terminal peptide (IFANSS; mass, 638 Da), which disappeared from the digest of the 25-kDa band. A new peptide (mass, 1634 Da) was evident in the map of the 25-kDa protein. The mass of the new peptide was 997 Da larger than the predicted C-terminal peptide, similar to the mass difference observed in the intact molecules, and thus we conclude that the added mass is contained at the C terminus. The 1634-Da peptide could be accounted for by translation of 10 additional vector residues with the exception of the C-terminal peptide (IFANSS; mass, 638 Da), which disappeared from the digest of the 25-kDa protein. The mass of the new peptide was 997 Da larger than the predicted C-terminal peptide, similar to the mass difference observed in the intact molecules, and thus we conclude that the added mass is contained at the C terminus.

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**FIG. 1. Characterization of purified Mac-1 and I-domain.** Purified Mac-1 (A) or Mac-1 I-domain (B) were subjected to SDS-polyacrylamide gel electrophoresis using 8 and 16% acrylamide, respectively. Prestained proteins used as standards were from Bethesda Research Technologies, Inc. (Gaithersburg, MD). Gels were stained with Comassie Brilliant Blue.

**FIG. 2. Characterization of adherence assays based on purified Mac-1 or Mac-1 I-domain.** The I-domain (A and B) or Mac-1 (B) was plated at the indicated concentrations, and the adherence of calcine-loaded ICAM-1-CHO cells or vector-CHO cells was quantitated as described under "Materials and Methods." Where indicated, plates were preincubated for 15 min with 10 µg/ml of the blocking Mac-1 antibody, 44aacb. In some cases, cells were preincubated for 15 min with the anti-ICAM-1 antibody, WS02025. These data are the means of duplicate determinations (± individual values) and are representative of numerous experiments.
CHO cells. The adherence of ICAM-1-CHO cells was blocked by the anti-Mac-1 antibody 44aacb. However, at high levels of I-domain, vector-transfected CHO cells were also adherent, and this adherence was blocked by 44aacb. These results indicate that the I-domain of Mac-1 interacts not only with ICAM-1 but also with an unknown receptor on CHO cells. This interaction with a CHO cell receptor is not unique to the recombinant I-domain, because vector-CHO cells also adhered to high levels of purified Mac-1, and this adherence was similarly blocked by 44aacb (data not shown).

To verify that under typical assay conditions with low substrate level, the observed adherence of ICAM-1-CHO cells was due to ICAM-1 rather than endogenous CHO receptors for Mac-1, a blocking ICAM-1 antibody was preincubated with cells prior to addition to the plate. This antibody completely blocked the adherence of ICAM-1-CHO cells to both the I-domain and to Mac-1 (Fig. 2B). The number of fluorescently labeled ICAM-1-CHO cells adhering to the I-domain under optimal conditions was typically 3–4-fold less than that adhering to Mac-1.

Characterization of Anti-Mac-1 Antibodies—The anti-Mac-1 antibodies 44aacb and MY904 block the Mac-1-mediated adherence of neutrophils (17, 22). The antibody 118.1 was generated as described under “Materials and Methods” and also shown to block Mac-1-mediated neutrophil adherence (data not shown). 44aacb, MY904, and 118.1 were found to bind to Mac-1 in an enzyme-linked immunosorbent assay format with IC50 values of 12.0, 9.3, and 10.7 nM, respectively. Preincubation of these antibodies with Mac-1 (Fig. 3A) resulted in inhibition of the adherence of ICAM-1-CHO cells with IC50 values of 50–500 μM in four assays. The three antibodies also blocked I-domain-mediated adherence (Fig. 3B) with IC50 values of 1–3 nM in four assays. LM 2/1, which does not block neutrophil adherence, does not block ICAM-1-CHO adherence to Mac-1 (Fig. 3A).

Characterization of CDRs of Anti-Mac-1 Antibodies—mRNA isolated from hybridoma cells producing 44aacb, MY904, and 118.1 was used to determine the nucleotide sequence encoding the CDRs of each of the antibodies. The deduced amino acid sequences are shown in Table I. Peptides were synthesized corresponding to the underlined portions of the sequences in Table I. These peptides were tested for inhibition of ICAM-1-CHO cell adherence to Mac-1 (Table I). One or two HC CDRs from each antibody blocked Mac-1-mediated adherence with an IC50 at or below 106 μM. Of the light chain CDR peptides made for antibodies 44aacb and MY904, none possessed blocking activity. Two of the active HC CDR peptides were also tested for blocking activity in the adherence assay using the I-domain as a substrate. HC CDR1 from 44aacb and HC CDR2 from 118.1 were found to possess comparable blocking activity in this assay (data not shown).

Identification of Additional Blocking Peptides through Phage Display—Variants of the 118.1 HC CDR2 blocking peptide were selected to block ICAM-1-CHO adherence to Mac-1. The phage library used in this assay expressed the variable region of the antibody on the surface oflambda phage and selecting for those phage that bound to Mac-1. To select high affinity peptides, a monovalent phage display system was utilized in which one copy or less of the gene III protein is utilized in which one copy or less of the gene III protein is expressed as N-terminal fusions with M13 gene III. To select for high affinity peptides, a monovalent phage display system was utilized in which one copy or less of the gene III protein is expressed as a fusion peptide on each phage (24). Phage expressing these libraries were panned on Mac-1 for four rounds with increasingly stringent wash conditions. Sequencing of phage eluted after four rounds revealed a consensus from one of the libraries, in which all five randomized residues differ from

![Table I](image)

**Table I**

| CDR regions from anti-Mac-1 antibodies | Amino acid sequences | IC50* μM |
|----------------------------------------|---------------------|---------|
| Heavy chain CDR1                       | 44aacb              | 33.2 ± 7.9 |
|                                       | MY904               | 77.9 ± 19.2 |
|                                       | 118.1               | 100 (2)   |
| Heavy chain CDR2                       | 44aacb              | >100 (1)  |
|                                       | MY904               | >100 (1)  |
|                                       | 118.1               | 30.4 ± 6.1 |
| Heavy chain CDR3                       | 44aacb              | >100 (2)  |
|                                       | MY904               | 106 ± 9.7 |
|                                       | 118.1               | 200 (1)   |
| Light chain CDR1                       | 44aacb              | >200 (1)  |
|                                       | MY904               | >200 (2)  |
|                                       | 118.1               | NT*       |
| Light chain CDR2                       | 44aacb              | >200 (1)  |
|                                       | MY904               | >100 (1)  |
|                                       | 118.1               | NT        |
| Light chain CDR3                       | 44aacb              | >100 (2)  |
|                                       | MY904               | >200 (1)  |
|                                       | 118.1               | NT        |

* Peptides (underlined portions) representing the CDR regions plus some flanking sequences were synthesized and tested for inhibition of ICAM-1-CHO adherence to Mac-1; data shown are mean ± S.D.E.V.  
* NT, not tested.
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A variant of HC CDR2 from 118.1 was identified using a monovalent display system on the phage M13 and selecting for Mac-1 binding. Interestingly, although all five randomized residues differed from the original 118.1 sequence, this peptide was found to possess similar Mac-1 blocking activity to the parental 118.1 peptide, indicating that there is considerable room for variation, and presumably improvement, within this CDR sequence. The selection of this CDR variant through binding of phage to Mac-1 demonstrates that this peptide does bind to Mac-1 and provides evidence that its blocking activity can be attributed to a specific interaction with Mac-1.

Evidence has suggested that protein-protein interaction involves multiple contact sites, and until recently little progress had been made in disrupting such interactions with small peptides. However, several examples now exist of peptides that can block protein-protein interaction; for example, an 8-amino acid peptide has been identified that can block the activation of FGFR1 by basic fibroblast growth factor (26). Further, the interaction of the two cell surface proteins, CD4 and MHC class II, can be blocked by a heptapeptide based on a protruding loop from one of the immunoglobulin-like domains of CD4. Thus even in this latter example, where many contact sites from two Ig domains of CD4 are thought to interact with MHC class II, binding appears to depend on a single \(\beta\)-turn-containing loop (27). In the case of antibody-antigen interaction, varying numbers of hypervariable regions may act together to provide the net affinity. In each of the three antibodies we have examined, we have identified one or two CDRs as candidates for important binding sites.

Peptides that block the interaction of ICAM-1 with Mac-1 have not previously been reported. Peptides derived from factor X and related peptides derived from filamentous hemagglutinin have been shown to prevent factor X binding to cells, presumably via Mac-1 (28, 29). However, the I-domain is not the primary binding site for factor X (12), and these peptides have not been shown to affect ICAM-1 binding. There is precedent, however, for peptide inhibition of ICAM-1-\(\beta_2\)-integrin interaction. A peptide from ICAM-2 has been shown to bind to LFA-1 and inhibit endothelial cell adhesion (at 100 \(\mu\)g/ml), presumably via ICAM-1 (30). Peptides from ICAM-1, domains 1 and 2, have also been shown to inhibit ICAM-1-mediated adhesion to unknown ligands (at 100 \(\mu\)M) (31).

In conclusion, we have utilized sequence information from anti-Mac-1 antibodies to derive the first peptide Mac-1 antagonists. These findings support a growing body of evidence that protein-protein interactions may depend disproportionately on one site or pocket.

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**Fig. 4. Consensus peptide derived from phage display of 118.1 CDR blocks ICAM-1-CHO cell adherence.** Mac-1 (A) or the I-domain (B) was preincubated with the 118.1 HC CDR2 peptide GYID-PYYGGITYNQIFKG or the phage derived variant GYRGYAGPILYN-QIFKFG for 15 min prior to the addition of ICAM-1-CHO cells. These data are the means of duplicate determinations (\(\pm\) individual values) and are representative of three (A) and two (B) experiments.

**TABLE II**

Alignment of 118 HC CDR2 with ICAM-1

| ICAM-1        | 224HLALGDQRNLNPTVYNGDNSFSAKA*229 |
|---------------|----------------------------------|
| 118.1 CDR2    | EWIGYIDPYYGGITY-NQIFKGA          |

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