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Peptide Mimics of Monocyte Chemoattractant Protein–1 (MCP-1) with an Antagonistic Activity

Masatomo Kaji,*,† Masaomi Ikari,* Shuhei Hashiguchi,* Yuji Ito,* Ryo Matsumoto,* Teizo Yoshimura,‡ Jun-ichi Kuratsu,† and Kazuhisa Sugimura*,1

*Department of Bioengineering, Faculty of Engineering, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890-0065; †Department of Neurosurgery, Faculty of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544; and ‡The Immunopathology Section, Laboratory of Immunobiology, National Cancer Institute, Frederick, MD 21701, USA

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In this study, we attempted to analyze the peptide motifs recognized by 24822.111 and F9, monoclonal antibodies (mAbs) that inhibit the chemotactic activity of monocyte chemoattractant protein–1 (MCP-1), a member of the CC subfamily of chemokines. We isolated phage clones from a phage display library and identified six peptide motifs. One of these clones, C27, was strongly and specifically recognized by 24822.111 mAb, while another, G25, was similarly recognized by F9 mAb. Both the C27 motif and the G25 motif contain two cysteines in their sequences and have little homology to the primary amino acid sequence of MCP-1. These clones, however, bound to THP-1 cells, and the binding was competitively inhibited by MCP-1. The clones strongly inhibited the MCP-1–induced chemotaxis of human monocytes. The synthetic and intramolecularly disulfide-linked peptides of C27 and G25 (sC27 and sG25) also inhibited the chemotaxis induced by MCP-1, while their derivatives with serine in place of cysteine did not, suggesting the importance of the loop structure for the inhibition. These results suggest that sC27 and sG25 may mimic the MCP-1-binding domain to the MCP-1 receptor.

Key words: inhibition, MCP-1, molecular design, peptide mimic, phage library.

Random-peptide-displaying phage library clones selected by a conformational epitope-recognizing and inhibitory monoclonal antibody (mAb) may display moieties that mimic a receptor/ligand-like three-dimensional structure. This pseudoreceptor/ligand should be able to bind to natural ligand/receptor molecules. We have tested this approach employing rather complex structural molecules such as CTLA4, a negative regulator of T cell function, which belongs to the immunoglobulin supergene family molecules (*1, 2*), and a chemokine receptor with seven transmembrane-domains as target molecules (*3*). In these two cases, we demonstrated that the HPLC-purified gene-3 protein (g3p) displaying the selected motif exhibited particularly antagonistic activity, *i.e.*, a strong augmenting activity on the T cell proliferation in the case of CTLA4 (*1, 2*) and an inhibitory activity on the M-tropic HIV-1 infection in the case of CCR5 (*3*). However, in both cases, synthetic peptides of these motifs failed to reproduce the antagonistic activities of the motif-bearing g3ps.

To examine further the feasibility of the method to copy the three-dimensional image of the binding site by the mAb recognizing the binding epitope, we tested the idea using a human chemokine, monocyte chemoattractant protein–1 (MCP-1), to design MCP-1-mimic peptides recognized by anti–MCP-1 mAbs.

MCP-1 was identified from the culture supernatant of the human glioma cell line U105 MG (*4, 5*) and is a 76–amino acid polypeptide belonging to the CC subfamily of the chemokine family based on the adjacent position of the two cysteines. Its main biological activity is its chemotactic activity for monocytes, and the CC chemokine receptor 2 (CCR2) is a high-affinity receptor for MCP-1 (*6–8*). The three-dimensional structure of the MCP-1 homodimer was determined by heteronuclear multidimensional NMR at a full high resolution (*9*). MCP-1 is involved in the development of asthma, glomerulonephritis, rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, and meningitis as well as in the accumulation of macrophages in tumor sites (*10–16*).

In this study, we employed the anti–MCP-1 mAbs 24822.111 and F9, which inhibit the chemotactic activity of MCP-1, and identified six distinct phage clones, each bearing a different motif. We characterized here two types of phage clones, C27 isolated from 24822.111 mAb and G25 isolated from F9 mAb, in terms of their binding ability to the MCP-1 receptors and their inhibitory activity against the MCP-1–induced chemotaxis. Furthermore, we demonstrated that the synthetic peptides of the C27 or the G25 motif (cC27 or sG25) inhibited the MCP-1–induced chemotaxis of human monocytes.

These results suggested that sC27 and sG25 might mimic the MCP-1–binding domain to the MCP-1 receptor and thus supported the usefulness of the molecular design employing a conformation-epitope-recognizing mAb and a phage-display library.

[1] To whom correspondence should be addressed. Phone: +81-99-285-8345, Fax: +81-99-258-4706, E-mail: kazu@be.kagoshima-u.ac.jp

Abbreviations: PBMC, peripheral blood mononuclear cells; MCP-1, monocyte chemoattractant protein–1; FBS, fetal bovine serum; g3p, gene 3 protein; mAb, monoclonal antibody.

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MATERIALS AND METHODS

Antibodies, Chemokines, and Synthetic Peptides—Anti-human MCP-1 mAb 24822.111 and anti-human CCR2 mAb 48607.121 were purchased from R&D Systems, Inc. (Minneapolis, MN). F9 was prepared by T. Yoshimura (*17*). MCP-1 and IL-8 were purchased from Pepro Tech, Inc. (Rocky Hill, NJ). The chemically synthesized synthetic peptides (19mers) of C27 (sC27) and G25 (sG25) bearing the motif sequence of 15 amino acids with the flanking sequences of 4 amino acids of g3p at the C-terminal end were prepared by use of a peptide synthesizer (9050 plus, Perseptive; Millipore, Bedford, MA). The lyophilized peptides of sC27 and sG25 were dissolved in a 0.5 M Tris-HCl buffer (pH 8.6) containing 4 M urea, then oxidized by bubbling air to form the intramolecular disulfide bond. The oxidation of the peptides was confirmed by reversed phase HPLC (μ-Bondasphere, 5 μ, C18-100A, 3.9 mm × 150 mm; Millipore Waters, Milford, MA). The oxidized form was eluted just in front of the reduced form on the chromatogram in both peptides. After bubbling for 24 h, the oxidized form was purified on the reversed phase HPLC. The monomeric form of the isolated peptide preparation was confirmed by MALDI-TOF mass spectrometry on Voyager DE-RP (Applied Biosystems, Foster City, CA). The peptide was lyophilized for storage. For use in cell culture experiments, sG25 (1 mg) was dissolved in 5 μl of dimethylformamide and diluted with phosphate-buffered saline (PBS; pH 7.3) to an appropriate concentration. The pH was adjusted with 1 M HEPES (pH 7.3; Dojin, Kumamoto). sC27 was dissolved in PBS. The amino acid sequences of the peptides were confirmed by use of a peptide sequencer (Model 473A; Applied Biosystems).

Immunoblotting—SDS-PAGE was performed as described (*1, 2*). Blotting was carried out with a semidry electroblotter (Sartorius, Tokyo) according to the manufacturer's instructions. After blocking with 5% skim milk, the PVDF membrane (Applied Biosystems) with blotted peptides was incubated with each anti-MCP-1 mAb at a dilution of 1:1,000 and alkaline phosphatase (AP)-labeled rat anti-mouse IgG at 1:1,000 (KPL, Gaithersburg, MD). The NTB and BCIP development system (Promega, Madison, WI) was used for the development.

Panning of Phage Display Library—A phage random display library was constructed, and micropanning was performed as previously described (*1*). The 15-amino acid sequence motif was inserted at the fifth position from the N-terminal of the g3p molecule in the fUSE5 vector. In this case, anti-MCP-1 mAb (24822.111 or F9) was used as a template to select phages. Briefly, phages (1.2×10^{12} transducing unit [TU]) expressing random peptide motifs were panned with an anti-MCP-1 mAb (24822.111 or F9; 10 μg)-coated 35-mm plastic plate (Iwaki Glass, Tokyo). The binding phages were eluted with a 0.1 N HCl-glycine buffer (pH 2.2). The eluate was immediately neutralized with 1.0 M Tris-HCl (pH 9.1) and amplified by infecting into starved K91kan cells. To select more specific phage clones, the amplified eluate was incubated with 5 μg of 24822.111 or F9 for the second round and 1 μg for the third round of panning at 4°C for 16 h. The reaction mixture was incubated with an anti-mouse IgG (5 μg)-coated 35-mm plastic plate at 4°C for 1 h to select specific binding phage clones.

DNA Sequencing—The DNA inserted in the phage clones was sequenced using a primer (5'-TGAATTTTCTGTATGAGG-3') by use of an ABI DNA sequencer 373A-36S, as described (*1*).

Cell Preparations—Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors and isolated by the Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) technique. THP-1 (human monocytic cell line) was donated by Dr. Hideo Takeshima (Kumamoto University Medical School, Kumamoto) and maintained in an RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

ELISA—ELISA was performed as described (*1*). Briefly, microtiter plates (Nunc, Denmark) were coated with the monoclonal antibody (80 ng/40 μl) or the isotype-matched control antibody (mouse IgG1) and blocked with 1% BSA. Phage clones (8×10^9 TU/40 μl) were added into wells and incubated with biotinylated anti-M13 mAb (40 ng/40 μl, Pharmacia, CA). The bound phages were detected by AP-conjugated streptavidin (Vector Laboratories, CA) in the presence of 1 mg/ml *p*-nitrophenylphosphate (Wako, Tokyo) with 10% diethanolamine (Wako). Absorbances were measured at 405 nm by use of a microplate reader (NJ-2300, Nunc, Tokyo).

Flow Cytometry Analysis—The experiment was performed as described (*1*). THP-1 cells (5×10^5 cells/50 μl) were pre-incubated with human IgG (400 μg/ml) for 1 h to block Fc receptors. After washing cells with PBS containing 2% FBS and 0.1% NaN_3, they were incubated with or without 10 μg/ml of MCP-1, MIP-1α, or IL-8. After washing, the cells were incubated in the presence or absence of phage clones (2×10^{11} TU/ml) for 1 h. They were then incubated with 20 μg/ml of biotinylated anti-M13 mAb or 20 μg/ml of biotinylated anti-CCR2 mAb (R&D Systems) for 30 min, followed by the addition of fluorescein isothiocyanate (FITC)-conjugated streptavidin (Vecter, Buringama, CA). All reactions were carried out on ice, and analysis was performed using a Coulter EPICS XL (Coulter, Miami, FL).

Chemotaxis Assay—Monocyte chemoattractant activity toward human PBMCs was determined using a 48-well micro chemotaxis chamber (Neuroprove, Cabin John, MD) with a 5-μm pore size polycarbonate filter membrane as described (*17*). Briefly, MCP-1 (5 nM: 43 ng/ml) or IL-8 (5 nM: 40 ng/ml) was added into the lower chamber, while human PBMCs (4×10^4 cells/40 μl/well) were added into the upper chamber with or without varying concentrations of antagonist (phage clones or peptides). The incubation time was 90 min for MCP-1 and 180 min for IL-8. After the incubation at 37°C in humidified air-5% CO_2, the filters were removed. Nonmigrating cells on the upside of the filters were wiped away; the filters were air-dried and stained with Diff-Quik. The migrating cells were counted under the microscope (magnification: 200). Data were calculated as % of control response induced with 5 nM MCP-1 or IL-8 alone. Each experiment was repeated at least three times.

RESULTS

Characterization of Anti-MCP-1 mAb—We characterized two anti-MCP-1 mAbs, 24822.111 and F9. MCP-1 was treated with or without 2-mercaptoethanol (2ME) and heating and then electrophoresed on SDS-PAGE (15%). The gel was immunoblotted with each mAb as described in

"MATERIALS AND METHODS." The 24822.111 mAb reacted with both the non-treated and treated MCP-1, while F9 mAb bound to the non-treated preparation only (Fig. 1). These results suggested that 24822.111 recognized the primary structure, whereas F9 mainly reacted with the disulfide-linked conformational structure of the MCP-1 molecule. Both mAbs inhibited the MCP-1–induced chemotaxis of PBMC (87% inhibition by 24822.111, 44% inhibition by F9 mAb and 0% by control IgG at 3 μg/ml) in our experimental system.

Selection of Phage Clones—Panning of the phage display library was performed using the anti–MCP-1 mAbs. The isolated phage clones were tested for reactivity to each anti–MCP-1 mAb by ELISA (Fig. 2). When the 24822.111 mAb was used as a probe, we selected 25 clones which specifically bound to this mAb. In the case of F9, 42 clones were selected as specific binding phage clones. These clones showed no binding activities to isotype-matched control IgG1. The DNA sequencing of the g3p gene revealed three types of peptide motifs for each mAb (Fig. 2). The C27 or G25 was the clone exhibiting the strongest binding activity in the case of 24822.111 or F9, respectively. The 24822.111 mAb did not recognize G25, and F9 did not recognize C27. It is of note that two cysteines were found in all motifs, suggesting the important role of an intramolecular disulfide bond in these motifs. A homology search of all the motifs showed little similarity to the primary sequence of MCP-1. There is, however, a significant similarity among the selected motifs, particularly in the case of F9, whose consensus sequence was P(F)WYS(P)C at the side of COOH. The motifs selected by F9 may be related in the conformational structure of MCP-1. In the case of 24822.111, there is an ambiguous similarity between C27 and A3.

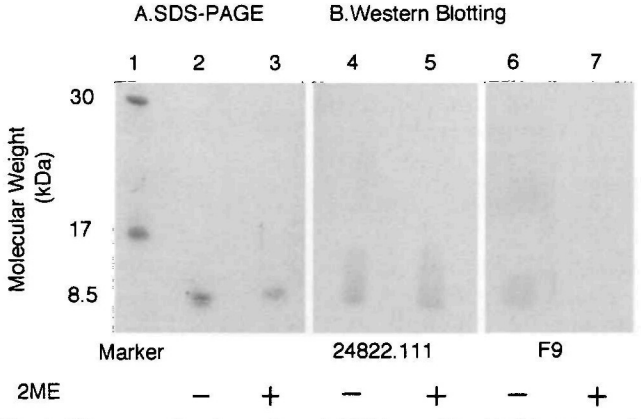

Fig. 1. **Characterization of anti–MCP-1 mAbs (24822.111 and F9).** (A) MCP-1 was treated with (+) or without (–) 2ME and electrophoresed on 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. The protein marker was in lane 1. (B) Immunoblot analysis of MCP-1 with 24822.111 and F9 mAbs under reducing (lanes 5 and 7) and non-reducing (lanes 4 and 6) conditions.

Binding Activity of C27 and G25 Phage Clones to MCP-1 Receptors—THP-1 cells express an MCP-1 receptor, CCR2 (Fig. 3A). Therefore, flow cytometry analysis was performed on THP-1 cells to determine whether the phage clones had the ability to bind to the MCP-1 receptors. As shown in Fig. 3, C and D, respectively, C27 and G25 exhibited remarkable binding activity to THP-1 cells, while a control phage clone L4 showed no binding activity (Fig. 3B). The binding activities of C27 and G25 were completely blocked by the preincubation of THP-1 cells with MCP-1, and partially blocked by preincubation with MIP-1α (Fig. 3, C and D). This result suggested that C27 and G25 bound to the other MIP-1α-receptor molecule expressed on THP-1. The preincubation of THP-1 cells with IL-8 showed no blocking effect (data not shown).

Migration Inhibitory Activity of C27 and G25 Phage Clones on MCP-1–Induced Chemotaxis—To examine the effects of C27 and G25 on the chemotactic activity of MCP-1, we assayed the migration of PBMC using 5 nM MCP-1 in the presence or absence of these phage clones (Fig. 4). Both C27 and G25 clones showed marked inhibitory activities at a similar level. The inhibition was observed at 1×10^5 TU/ml, and at 1×10^8 TU/ml it was almost complete in the case of G25 and 75% complete in the case of C27. The

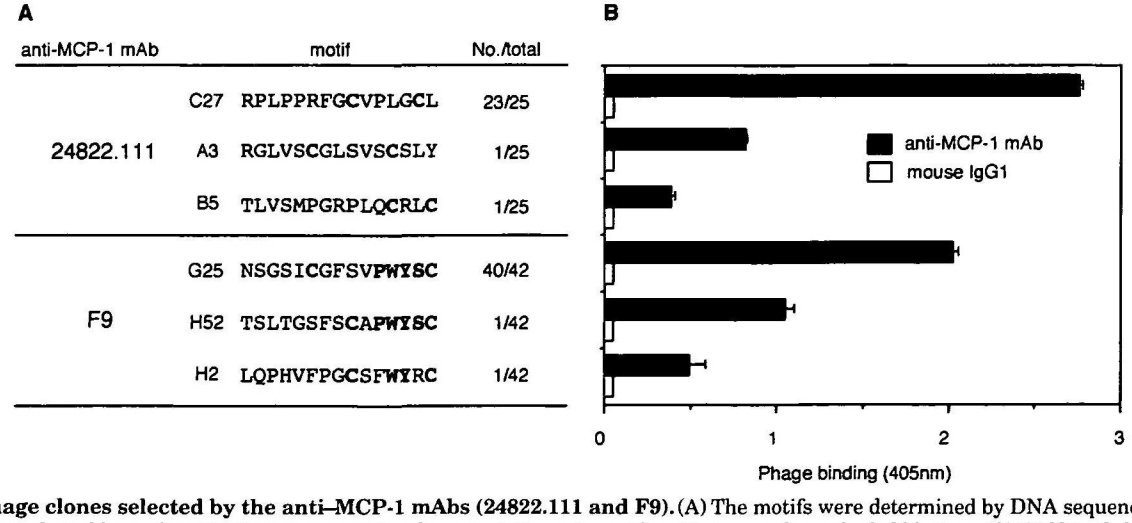

Fig. 2. **Phage clones selected by the anti–MCP-1 mAbs (24822.111 and F9).** (A) The motifs were determined by DNA sequencing. Three clones were selected by each mAb. Cysteines and the characteristic amino acid residues are shown by bold letters. (B) ELISA of phage clones recognized by anti–MCP-1 mAbs. The isotype-matched antibody was used as a control.

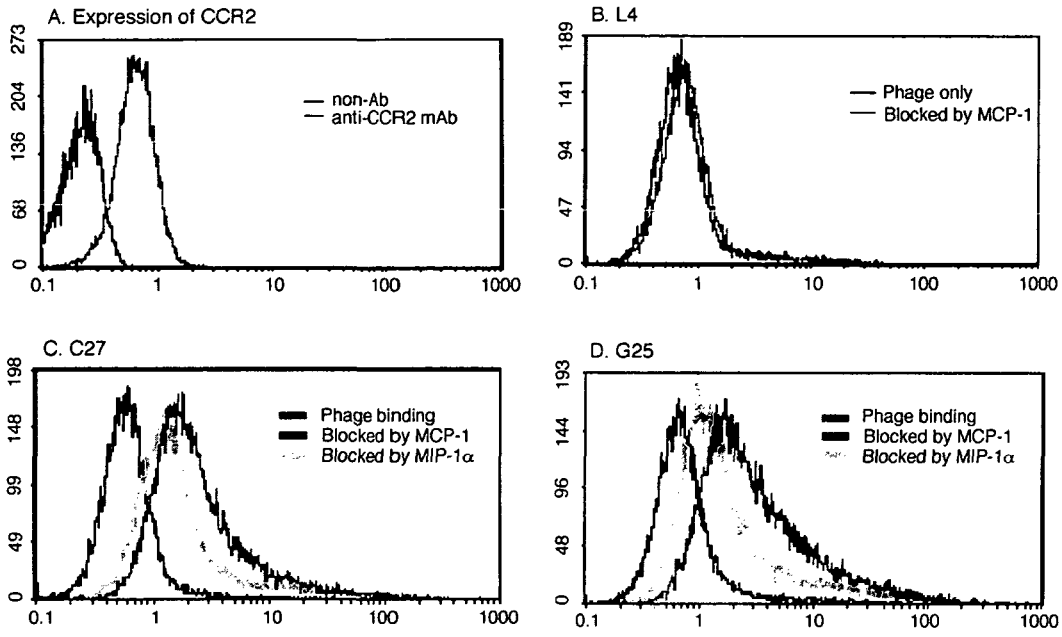

Fig. 3. **The binding of C27 or G25 phage clones to THP-1 cells on flow cytometer analysis.** THP-1 cells were preincubated with MCP-1, MIP-1α, or IL-8 (10 μg/ml). After washing, cells were incubated with or without phage clones (2×10^{11} TU/ml) for 1 h. The binding of phage was detected by the biotinylated anti-M13 mAb in combination with FITC-conjugated streptavidin. (A) Expression of CCR2 on THP-1. Cells were stained with anti-CCR2 (20 μg/mg). (B) No binding of a control phage clone, L4. (C, D) The binding of C27 or G25 to THP-1 cells preincubated with MCP-1, MIP-1α, or IL-8. The preincubation of IL-8 showed no effect on this binding (data not shown).

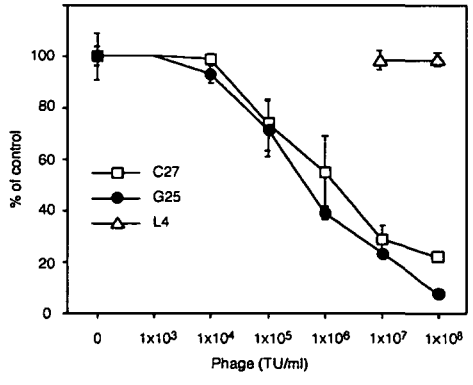

Fig. 4. **The C27 and G25 phage clones inhibit the MCP-1–induced chemotaxis of human monocytes.** The chemotaxis assay was performed as described in "MATERIALS AND METHODS." The representative results are presented as the mean ± SD value. The MCP-I–induced cell count without the inhibitor and the background count without MCP-1 and the inhibitor are 552 and 48 in this experiment. The control phage clone, L4, showed no inhibitory effect at 2×10^8 TU/ml.

control phage clone, L4, showed no inhibitory activity even at 2×10^8 TU/ml.

Synthetic Peptides of the MCP-1–Binding Domain—As C27 and G25 phage clones might display the motifs mimicking the MCP-1–binding domain, we chemically synthesized these peptide-motifs, sC27 and sG25. Since both motifs contain two cysteines in their sequences, we prepared control peptides by replacing cysteines by serines, which cannot form the loop structures. The preparations of sG25 or sC27 were intramolecularly crosslinked by a disulfide bond formed by air oxidation as described in "MATERIALS AND METHODS."

First, we tested the reactivity of these synthetic peptides with the anti–MCP-1 mAbs, 24822.111 and F9, by examining their inhibitory activity on the binding of the mAb to the C27 or G25 phage clone. sC27 inhibited the binding of the 24822.111 mAb to C27, while sG25 inhibited the binding of the F9 mAb to G25 (Fig. 5). The control peptides showed no blocking effects on these interactions. These results suggested that the disulfide formation of these peptides was critical for the binding to the anti–MCP-1 mAbs.

The sC27 and sG25 Inhibit the MCP-1–Induced Chemotaxis—As C27 and G25 phage clones inhibited the migration of PBMC induced by MCP-1, we examined the effects of sC27 and sG25 in the same assay system. sC27 and sG25 inhibited the migration in a dose-dependent fashion and showed 35 and 70% inhibition at a concentration of 1 mM, respectively (Fig. 6). On the other hand, the control peptides, sC27 Ser and sG25 Ser, had no effect (Fig. 6, B and C). These results suggested that the cysteines forming an intramolecular disulfide bond were necessary for binding to the MCP-1 receptors. sC27 and sG25 showed no influence on the chemotactic activity of IL-8. These results suggest that sC27 and sG25 may be MCP-1–binding domain mimics which specifically bind to the MCP-1 receptor.

DISCUSSION

We have attempted to design functional peptide mimics by panning a phage display library using the anti-receptor/ligand mAb (*1–3*). We studied here the case of a chemokine, MCP-1, employing two anti–MCP-1 mAbs (24822.111 and F9) with distinct binding specificities (*17*). The 24822.111 mAb bound to both the 2-ME/heat–treated and the non-treated MCP-1 molecules, while the F9 mAb bound to only

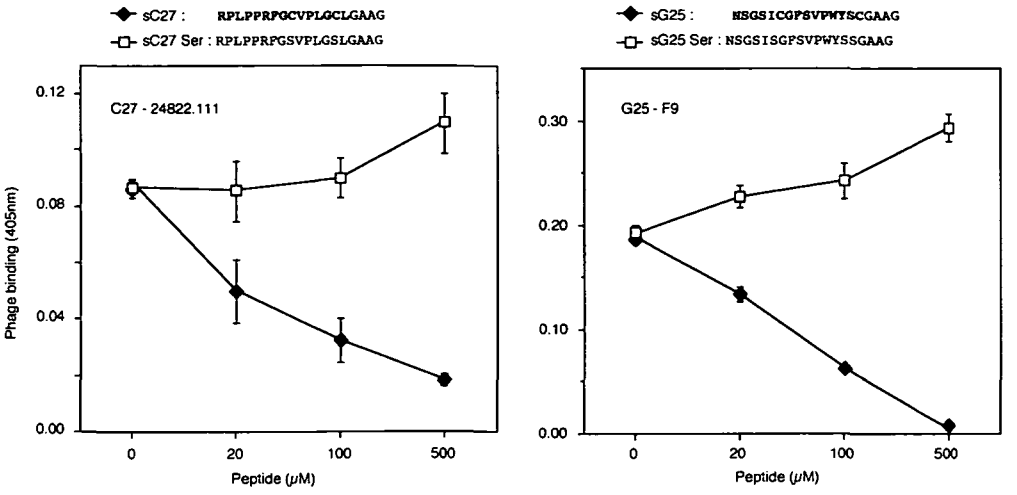

Fig. 5. **The synthetic motif-peptide inhibits the binding of anti-MCP-1 mAb to C27 or G25 phage clones.** The sC27 and sG25 peptides were chemically synthesized. The sC27 Ser or sG25 Ser peptide is a serine derivative in place of cysteine. The MCP-1 mAb (80 ng/40 μl/well: 24822.111 or F9) was coated in ELISA plates, followed by the addition of the C27 or G25 phage clones (1×10^8 TU/50 μl/well) in the presence of varying concentrations of synthetic peptides. The concentration of phage clone was 1:100 lower than that used in the experiments of Fig. 2. The binding of phage clones was detected by biotinylated anti-M13 mAb in combination with AP-conjugated streptavidin. The chemically synthesized peptides (19mers) bear 15 amino acids of the unique motif sequence (bold letter) with the g3p-flanking sequence of 4 amino acids at the C-terminus.

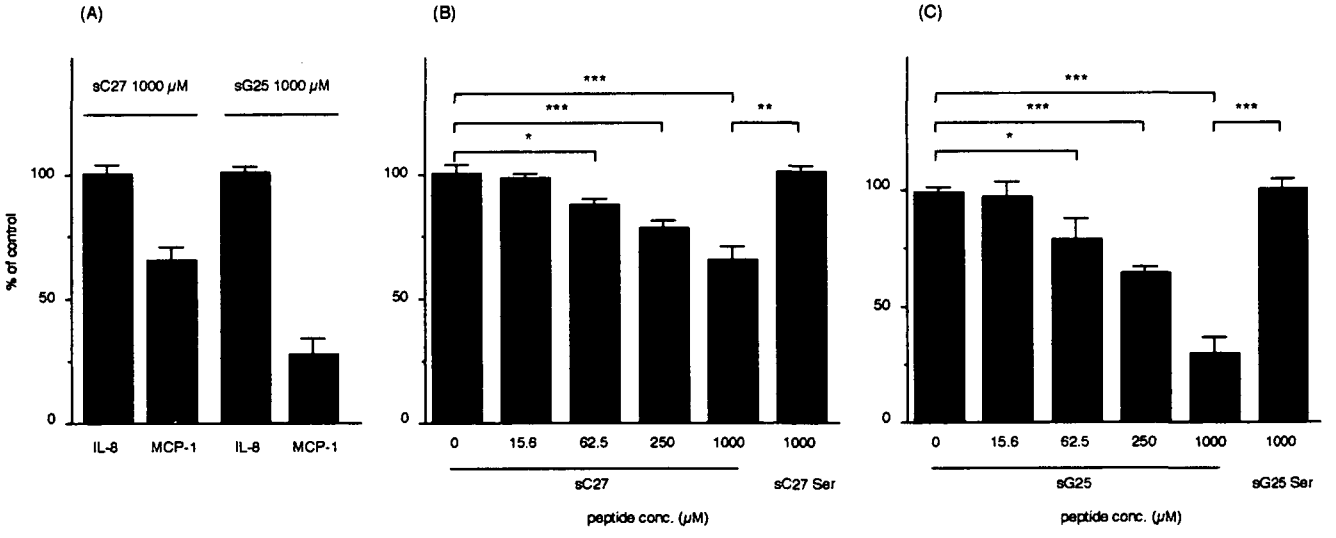

Fig. 6. **sC27 and sG25 inhibit the MCP-1–induced chemotaxis of human monocytes.** The migration of PBMC was induced with MCP-1 or IL-8 in the presence or absence of sC27, sG25, and their serine derivatives. The numbers of migrating cells are represented as % of control response induced with 5 nM MCP-1 (43 ng/ml) or IL-8 (40 ng/ml) alone. The experiments shown in this figure was performed in the same experiment. The migrating cell count with IL-8 was 275, and with MCP-1, 670; background response of cells alone was 56. The identical experiments were repeated three times. The representative results are presented as the mean ± SD. The significance of the difference was analyzed by ANOVA followed by post-hoc Fisher's PLSD test. A level of $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

non-treated MCP-1 on the immunoblotting analysis (Fig. 1). From the fd phage display library, we isolated the C27 phage clone by probing with 24822.111 and the G25 phage clone by probing with the F9 mAb (Fig. 2). A phage displayed three to five copies of the motif-bearing g3p molecule (*1*). These phage clones showed not only binding activity to THP-1 cells, which was almost completely blocked by preincubation of the cells with MCP-1 (Fig. 3), but also migration-inhibitory activity on the MCP-1–induced chemotaxis (Fig. 4). These features suggested that the C27 and G25 phage clones might display the MCP-1–mimic motif on their g3p molecules. These results confirmed our previous experiments on CTLA4 and CCR5 (*1–3*). The prominent feature of this study is that we successfully synthesized the functional peptides sC27 and sG25, which inhibited the binding of the C27 and G25 phage clones to the corresponding mAbs (Fig. 5) as well as the chemotactic activity of MCP-1 (Fig. 6). However, a high concentration of these peptides was required for their activities, in comparison with the natural MCP-1. The binding affinity seems to decrease markedly when the motif is chemically synthesized. In agreement with these findings, we failed to demonstrate the direct binding of sC27 or sG25 to THP-1 cells using biotinylated peptides by flow cytometry analysis (data not shown). This might have been the result of the steric hindrance caused by the biotinylation at

the N-terminal amino acid or the monovalent feature of the synthetic peptide in comparison with the multivalent display of g3p on the phage, in addition to the conformational alteration of the functional motif structure.

The sequence homology of the G25 and C27 motifs with MCP-1 was analyzed by using PIMA multiple-sequence-alignment software. The binding region of MCP-1 that interacts with its receptor, CCR2b, had been identified by mutation analysis (*18–23*). On searching for homology to this region (residue number 13–35), a sequence with very low similarity was found between a part of the C27 motif and the region of residues 24–29 of MCP-1 comprising the two characteristic arginine residues. It is uncertain why the C27 motif selected by the 24822.111 mAb did not reflect the primary sequences of MCP-1, as the mAb bound to the denatured MCP-1 preparation. The homology search of the G25 motif also showed little similarity to MCP-1.

It is reported that not only CCR2a/b but also CCR1, CCR4, CCR9, or CCR10 function as the MCP-1 receptor (*24, 25*). Regarding the binding-specificity of the C27 and G25 motifs, the preincubation of THP-1 cells with MCP-1 appeared to completely inhibit the binding of the C27 or G25 phage clone to THP-1 cells (Fig. 3). In contrast, MIP-1α (5 nM) exhibited partial inhibition on peptide binding (Fig. 3, C and D), while IL-8 showed no inhibition (data not shown). As receptor molecules for MIP-1α are reported to be CCR1, CCR4, and CCR5 (*24*), it is conceivable that both phage clones may bind to the structure of CCR2 shared with the other MCP-1 receptors. The precise binding specificity of these phage clones remains to be studied. When searching for a CTLA4-binding domain mimic (*1, 2*), we found an F2 motif which bound to CD80 but not CD86, whereas CTLA4 bound equally to CD80 and CD86. Thus, using this molecular-design approach, it is possible to find a serendipitous peptide molecule with binding specificity differing from the natural receptor/ligand-specificity.

In this study, we have not studied the Biacore analysis regarding the binding-affinity of the motifs to the MCP-1 receptors, because we have not conclusively specified the MCP-1 receptors as shown in Fig. 3, and because the purified phage g3p inevitably contained the aggregated form as described previously (*2*).

The results of Refs. *1–3* and this study together demonstrate the feasibility of copying the conformational image of the binding site, even though the binding specificity of the inhibitory mAb profoundly influenced the results. These C27 and G25 peptide sequences may be useful for the development of small molecules mimicking MCP-1.

REFERENCES

1. Fukumoto, T., Torigoe, N., Kawabata, S., Murakami, M., Uede, T., Nishi, T., Ito, Y., and Sugimura, K. (1998) Peptide mimics of the CTLA4-binding domain stimulate T-cell proliferation. *Nat. Biotechnol.* **16**, 267–270
2. Fukumoto, T., Torigoe, N., Ito, Y., Kajiwara, Y., and Sugimura, K. (1998) T cell proliferation-augmenting activities of the gene 3 protein derived from a phage library clone with CD80-binding activity. *J. Immunol.* **161**, 6622–6628
3. Meta, A., Torigoe, N., Ito, Y., Arakaki, R., Nakashima, H., and Sugimura, K. (1999) Inhibition of M-tropic HIV-1 infection by the fd phage-gene 3 protein with MIP-1α-binding activity. *Mol. Immunol.* **36**, 1249–1254
4. Kuratsu, J., Leonard, E.J., and Yoshimura, T. (1989) Production and characterization of human glioma cell-derived monocyte chemotactic factor. *J. Natl. Cancer Inst.* **81**, 347–351
5. Yoshimura, T., Robinson, E.A., Tanaka, S., Appella, E., Kuratsu, J., and Leonard, E.J. (1989) Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J. Exp. Med.* **169**, 1449–1459
6. Zlotnik, A. and Yoshie, O. (2000) Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121–127
7. Yoshimura, T. and Leonard, E.J. (1992) Human monocyte chemoattractant protein-1: structure and function. *Cytokines* **4**, 131–152
8. Luster, A.D. (1998) Chemokines—chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **338**, 436–445
9. Handel, T.M. and Domaille, P.J. (1996) Heteronuclear (^{1}H, ^{13}C, ^{15}N) NMR assignments and solution structure of the monocyte chemoattractant protein-1 (MCP-1) dimer. *Biochemistry* **35**, 6569–6584
10. Boring, L., Gosling, J., Cleary, M., and Charo, I.F. (1998) Decreased lesion formation in CCR2–/– mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* **394**, 894–897
11. Nelken, N.A., Coughlin, S.R., Gordon, D., and Wilcox, J.N. (1991) Monocyte chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest.* **88**, 1121–1127
12. Peng, L., Shu, S., and Krauss, J.C. (1997) Monocyte chemoattractant protein inhibits the generation of tumor-reactive T cells. *Cancer Res.* **57**, 4849–4854
13. Rollins, B.J. (1997) Chemokines. *Blood* **90**, 909–928
14. Takeya, M., Yoshimura, T., Leonard, E.J., and Takahashi, K. (1993) Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal antibody. *Hum. Pathol.* **24**, 534–539
15. Yla-Herttuala, S., Lipton, B.A., Rosenfeld, M.E., Sarkioja, T., Yoshimura, T., Leonard, E.J., Witztum, J.L., and Steinberg, D. (1991) Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* **88**, 5252–5256
16. Harigai, M., Hara, M., Yoshimura, T., Leonard, E.J., Inoue, K., and Kashiwazaki, S. (1993) Monocyte chemoattractant protein-1 (MCP-1) in inflammatory joint diseases and its involvement in the cytokine network of rheumatoid synovium. *Clin. Immunol. Immunopathol.* **69**, 83–91
17. Yoshimura, T., Takeya, M., Takahashi, K., Kuratsu, J., and Leonard, E.J. (1991) Production and characterization of mouse monoclonal antibodies against human monocyte chemoattractant protein-1. *J. Immunol.* **147**, 2229–2233
18. Beall, C.J., Mahajan, S., Kuhn, D.E., and Kolattukudy, P.E. (1996) Site-directed mutagenesis of monocyte chemoattractant protein-1 identifies two regions of the polypeptide essential for biological activity. *Biochem. J.* **313**, 633–640
19. Gong, J.H. and Clark-Lewis, I. (1995) Antagonists of monocyte chemoattractant protein 1 identified by modification of functionally critical NH_2-terminal residues. *J. Exp. Med.* **181**, 631–640
20. Monteclaro, F.S. and Charo, I.F. (1996) The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1alpha receptor, confers chemokine selectivity. Evidence for a two-step mechanism for MCP-1 receptor activation. *J. Biol. Chem.* **271**, 19084–19092
21. Monteclaro, F.S. and Charo, I.F. (1997) The amino-terminal domain of CCR2 is both necessary and sufficient for high affinity binding of monocyte chemoattractant protein 1. Receptor activation by a pseudo-tethered ligand. *J. Biol. Chem.* **272**, 23186–23190
22. Steitz, S.A., Hasegawa, K., Chiang, S.L., Cobb, R.R., Castro, M.A., Lobl, T.J., Yamada, M., Lazarides, E., and Cardarelli, P.M. (1998) Mapping of MCP-1 functional domains by peptide analysis and site-directed mutagenesis. *FEBS Lett.* **430**, 158–164
23. Zhang, Y.J., Rutledge, B.J., and Rollins, B.J. (1994) Structure/activity analysis of human monocyte chemoattractant protein-1 (MCP-1) by mutagenesis. Identification of a mutated protein

that inhibits MCP-1–mediated monocyte chemotaxis. *J. Biol. Chem.* **269**, 15918–15924
24. Pharmingen/Cytokine/Chemokine Manual. Application Manual, 3rd ed., June 1999, Pharmingen
25. Tsou, C.L., Gladue, R.P., Carroll, L.A., Paradis, T., Boyd, J.G., Nelson, R.T., Neote, K., and Charo, I.F. (1998) Identification of C-C chemokine receptor 1 (CCR1) as the monocyte hemofiltrate C-C chemokine (HCC)-1 receptor. *J. Exp. Med.* **188**, 603–608