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Intracellular Localization of Migration Inhibitory Factor-Related Protein (MRP) and Detection of Cell Surface MRP Binding Sites on Human Leukemia Cell Lines[1]

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The migration inhibitory factor-related proteins (MRPs) MRP-8 and MRP-14 were detected in differentiated human leukemia cell lines (THP-1 and HL-60) by immunocytochemical analysis. They were induced and colocalized in the cytoplasm and in lesser amounts in the nucleus when THP-1 and HL-60 cells were induced to differentiate by 1α,25-dihydroxyvitamin D_3 or retinoic acid. In a search for a protein capable of binding MRPs, both MRPs were individually produced in insect cells (Sf21) infected with recombinant baculovirus. The purified recombinant MRPs were electrophoretically and antigenically indistinguishable from the native proteins, and their ability to form the MRP8/14 complex was retained. The presence of MRP binding sites was investigated by a binding assay using recombinant MRPs and specific monoclonal antibodies. MRP binding sites were detected on the cell membrane of the human leukemia cell lines THP-1, Raji, and MOLT-4. HL-60 cells treated with 1α,25-dihydroxyvitamin D_3 did not express MRP binding sites on the cell membrane, but a high level of MRPs accumulated in the cells. The occurrence of MRP binding sites on the cell surface of leukemia cell lines of monocyte and lymphocyte origin suggests that MRPs, released from neutrophils under certain conditions, may contribute to the activation and recruitment of effector cells to inflammatory lesions.

Key words: calcium binding protein, cellular localization, human leukemia cells, MRP, MRP binding site.

The migration inhibitory factor-related proteins (MRPs) MRP-8 and MRP-14 belong to the large family of S100 proteins, whose members have EF-hand calcium-binding structures (*1, 2*). They are present at high concentrations in cells of myeloid origin, such as neutrophils and monocytes, but are not present in lymphocytes (*3, 4*). Human neutrophils contain an MRP complex that comprises up to 30-45% of their cytosolic protein and 5% of their total protein (*5, 6*). MRP-8 and MRP-14 have a strong tendency to form a complex non-covalently (*7*), and they were isolated as a complex variously referred to as L1 antigen (*8, 9*), cystic fibrosis antigen (*10*), calgranulin (*11*), and calprotectin (*12, 13*).

We previously reported that two human leukemia cell lines of myeloid origin, HL-60 and THP-1, expressed MRP-8 and MRP-14 during granulocytic and monocytic differentiation induced by Me_2SO_4, all-*trans*-retinoic acid (RA), or 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], but not by 12-*O*-tetradecanoylphorbol-13-acetate (PMA) (*14*). Our results also suggested that the increase of protein kinase C activity during induction might be involved in a stimulatory pathway of MRP synthesis and that protein phosphorylation reactions might play important roles in MRP expression during myelocytic differentiation (*14*).

The biological function of MRP complex is still unclear. MRPs have been found to translocate from the cytosol to the membrane upon neutrophil activation with opsonized zymosan and other agonists (*15*). MRPs purified from peripheral blood monocytes inhibit casein kinases I and II and the protein kinase-mediated stimulation of RNA polymerase activity (*16*). These findings suggest that MRPs may be involved in the regulatory mechanism in the activation of neutrophils and monocytes. In addition to their possible intracellular function, MRPs were reported to have extracellular functions, *i.e.*, antimicrobial, cytostatic, and chemotactic activities (*12, 13, 17, 18*). MRPs were also detected in the serum of patients with chronic inflammatory diseases, *i.e.*, rheumatoid arthritis, Sjogren's syndrome, and systemic lupus erythematosus (*19*). Secreted MRPs were thus suggested to have an important function in immunity against infection and in chronic inflammatory disease (*20, 21*). However, the excretion mechanism of

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Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; NP-40, Nonidet P-40; PEG 20000, polyethyleneglycol 20000; 2-ME, 2-mercaptoethanol; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; FBS, fetal bovine serum.

MRPs is as yet unknown; MRP-8 and MRP-14 were reported to lack a signal sequence, a membrane anchor sequence, or any consensus sequence for *N*-linked glycosylation (*22*).

To clarify the intracellular and extracellular functions of MRPs, the presence of cell components bound to MRPs or MRP binding sites was investigated in this study. The MRP binding sites were found on the cell membrane of the human leukemia cell lines THP-1, Raji, and MOLT-4, and were sensitive to proteolytic treatment. The binding of MRPs to the cell surface of these leukemia cell lines of monocyte and lymphocyte origin suggests that MRPs, released from neutrophils under certain conditions, may contribute to the activation and recruitment of effector cells to inflammatory lesions.

MATERIALS AND METHODS

Reagents—CHAPS, PMSF, and Nonidet P-40 (NP-40) were obtained from Sigma (St. Louis, MO). RPMI 1640, TC-100 medium, and Pluronic F68 were obtained from GIBCO BRL (Grand Island, NY) and ASF104 serum-free medium was obtained from Ajinomoto (Tokyo). The BacPAK baculovirus expression system was obtained from Clontech Laboratories (Palo Alto, CA). Protein G-Sepharose and ProRPC15μmHR columns were obtained from Pharmacia (Uppsala, Sweden). SuperQ Toyopearl, Phenyl Toyopearl, and Toyopearl GC500F were obtained from Tosoh (Ayase). The anti-MRP-8 monoclonal antibody, 8-5C2, was obtained from BMA Biomedicals (August, Switzerland). Fluorescein isothiocyanate (FITC)- and horseradish peroxidase (HRP)-labeled goat anti-mouse Ig antibody $F(ab)_2$ fragments were obtained from Tago (Burlingame, CA). Goat anti-mouse IgG+IgM linked to colloidal gold particles was purchased from British BioCell (UK), and Lowicryl K4M was purchased from Polyscience (Warrington, PA).

Cells—The human leukemia cell lines HL-60, THP-1, K-562, Raji, and MOLT-4 were supplied by the Japanese Cancer Research Resources Bank of the National Institute of Health (Tokyo). The cell lines were maintained in either ASF104 serum-free medium or RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C under 5% CO_2 in a humidified incubator. The Sf21 insect (*Spodoptera frugiperda*) cells were obtained from Clontech and maintained as a spinner culture in TC-100 medium supplemented with 10% inactivated FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1% Pluronic F68. The Sf21 cells were routinely maintained at 27°C at densities from 0.2 to 1×10^6 cells/ml.

Immunocytochemical Analysis—The THP-1 and HL-60 cells were each differentiated with 3×10^{-7} M RA or 5×10^{-8} M $1\alpha,25(OH)_2D_3$ at a cell density of 1×10^6/10 ml/90-mm dish for 3 days. In typical experiments, RA or $1\alpha,25(OH)_2D_3$ induced the differentiation of over 90% of the cells into granulocytes or monocytes under the conditions used, respectively. For controls, cells were incubated without these treatments. The THP-1 and HL-60 cells were fixed for 1 h in 2% paraformaldehyde, 0.05% glutaraldehyde, and 4 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature. The cells were washed with phosphate-buffered saline (PBS) containing 0.1 M lysine, dehydrated in ethanol and embedded in Lowicryl K4M. Polymerization was performed under UV light at −20°C.

Ultrathin sections of the embedded cells were mounted on nickel grids and incubated in a PBS solution containing 10% normal goat serum, 1% bovine serum albumin (BSA), and 0.1% Tween 20 (blocking buffer) for 1 h, then incubated with a mouse monoclonal anti-MRP-8 antibody diluted 1/40 with PBS containing 1% BSA and 0.1% Tween 20 (PBST-BSA) for 3-4 h. After having being washed with blocking buffer, each section was labeled with goat anti-mouse IgG+IgM linked to 5-nm colloidal gold particles diluted 1/50 with PBST-BSA for 90 min. The sections were then washed with PBST-BSA, followed by a rinse with distilled water and drying. The unstained sides of the grids were incubated in blocking buffer followed by mouse anti-MRP-14 antibody diluted 1/40 with PBST-BSA. The sections were washed with blocking buffer, and labeled with goat anti-mouse IgG+IgM linked to 10-nm colloidal gold particles diluted 1/50 with PBST-BSA for 90 min. The sections were then washed with blocking buffer, followed by distilled water. All immunolabeling steps were carried out at room temperature. The sections were then stained with uranyl acetate and lead citrate.

For controls, the sections were reacted only with secondary antibody or with the non-immune mouse serum instead of the primary antibody.

Construction and Infection of the Recombinant Baculovirus—Full-length cDNAs of MRP-8 and MRP-14 (*14*) were cleaved from pBlueScript SK(+) by *Hin*dIII digestion, blunt-ended with the Klenow fragment, ligated with the *Bam*HI linker, and cleaved by *Bam*HI. The resulting fragments were cloned into the *Bam*HI site of the transfer vector pBacPAK1 (Clontech). The polymerase chain reaction (PCR) products of the vectors were directly sequenced to confirm the orientation and sequence of the insert. Recombinant viruses encoding MRP cDNA were obtained by cotransfecting Sf21 cells with a mixture of BacPAK6 viral DNA and the pBacPAK1 transfer vector encoding the MRP cDNA, and were identified as occlusion-negative plaques (*23*). Each recombinant virus was purified by two rounds of plaque purification, evaluated by Western blotting of virus-infected cell proteins, and passaged twice prior to use in the experiments. Typically, 1,000 ml of Sf21 cell culture at an initial density of 1×10^6 cells/ml was infected with the purified viruses at a multiplicity of infection of 5 in a spinner flask (at 50 rpm) and grown under a natural atmosphere at 27°C. The cells used for protein purification were harvested 3 days post-infection and then stored at −80°C.

Purification of Recombinant MRPs from Sf21 Cells—Recombinant MRP-8 and MRP-14 were purified separately, and all steps were carried out at 4°C unless otherwise indicated. All chromatography columns were mounted in the Pharmacia FPLC system. Frozen infected Sf21 cells (about 50 ml) from a total of 10 liters of culture were thawed and homogenized in 50 ml of ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10 mM CHAPS, 10 mM benzamidine, 0.1 mM 2-ME, and 2 mM PMSF, with a Teflon-glass homogenizer at 0°C. The homogenate was centrifuged at $10,000\times g$ for 30 min, and the precipitate was homogenized again in 25 ml of the same buffer and centrifuged. The resulting combined supernatant was saturated with 40% ammonium sulfate for 30

min at 0°C and then centrifuged at 10,000×*g* for 30 min. The supernatant was dialyzed using Spectrapor 3 (MW 3,500) dialysis tubing against equilibration buffer containing 20 mM Tris-HCl (pH 9.0), 1 mM EDTA, 0.1% glycerol, 0.1 mM 2-ME, and 0.1 mM PMSF. The dialysate was applied to a SuperQ Toyopearl anion exchange chromatography column (ϕ 2.5 cm×8 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient of 0 to 1 M NaCl in the equilibration buffer. The amount of MRPs in each fraction was monitored by immunostaining of the Western blot and Coomassie Blue staining of the SDS-PAGE gel (*24*). The recombinant MRP-8 and MRP-14 were eluted as a peak at 0.3–0.8 M NaCl. The pooled SuperQ fractions were dialyzed against the dialysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% glycerol, 0.1 mM 2-ME, and 0.1 mM PMSF, and then trifluoroacetic acid (TFA) was added at a final concentration of 1% (v/v). The dialysates were applied to a Phenyl Toyopearl column (ϕ 2.5 cm×6 cm) equilibrated with 0.1% (v/v) TFA. The proteins were eluted with a linear gradient of 28 to 56% (v/v) acetonitrile in 0.1% TFA. The recombinant MRP-8 and MRP-14 were eluted as peaks at 36–42% and 38–44% acetonitrile in 0.1% TFA, respectively. The pooled fractions were lyophilized and dissolved in the buffer containing 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, 6 M guanidine-HCl, 0.1 mM 2-ME, 0.1% glycerol, and 0.1 mM PMSF, and applied to a Toyopearl GC500F gel filtration column (ϕ 1.6 cm×60 cm) equilibrated with the same buffer. The eluted fractions containing the proteins were pooled, dialyzed against the dialysis buffer and lyophilized. The lyophilized fractions were dissolved in 1% TFA and applied to a ProRPC15μmHR reversed-phase column equilibrated with 0.1% TFA. The proteins were eluted with a linear gradient of 32 to 48% acetonitrile in 0.1% TFA. The recombinant MRP-8 and MRP-14 were eluted as a peak at 36–39% and 38–41% acetonitrile in 0.1% TFA, respectively. The pooled fractions were lyophilized and dialyzed against the buffer containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA and then stored at −80°C. The amino-terminal sequences of the purified recombinant MRPs were confirmed by a protein sequencer (Applied Biosystems, Foster, CA).

The protein concentrations were determined by the Bradford method (*25*) using a commercial dye preparation (Bio-Rad, Hercules, CA).

Western Blot and Immunoprecipitation Analysis of Recombinant MRPs—The recombinant MRPs were analyzed by 15% SDS-PAGE and transferred to nitrocellulose membrane (S&S, Keene, NH). After blocking with 3% (w/v) non-fat dry milk in T-TBS containing 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20, the membrane was incubated with 8-5C2 monoclonal antibody (1:50) or 5C12 culture supernatant for 1 h and then washed with T-TBS. The membrane was incubated for 1 h with goat anti-mouse Ig antibody $F(ab)_2$-HRP (1:200) and then washed with T-TBS. The proteins were detected by $NiCl_2$-enhanced DAB staining (*26*).

The immunoprecipitation of the recombinant MRPs was carried out by incubating 50 μl of 8-5C2 or 5C12 monoclonal antibody-conjugated Protein G-Sepharose with each recombinant MRP for 3 h by rotation at 4°C. The immunoprecipitates were washed 8 times with washing buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 0.1% NP-40, and once with 20 mM Tris-HCl (pH 7.5) and 0.1% NP-40. The immunoprecipitates were analyzed by 15% SDS-PAGE, and the proteins were detected with Coomassie Blue.

MRP Binding Assay by Flow Cytometry—K562, HL-60, THP-1, Raji, and MOLT-4 cells were washed three times with PBS and resuspended in PBS containing 0.3% BSA and 0.05% NaN_3 (B-PBS). A total of 2×10^6 cells was incubated in 100 μl of B-PBS with 10 μg of recombinant MRP-8, 10 μg of recombinant MRP-14 or both of them on ice for 1 h, and then washed three times with B-PBS. In the experiments regarding the effects of calcium ion, the B-PBS used in this washing step also contained either 1 mM $CaCl_2$ or 1 mM EGTA. The cells were incubated with 200 μl of the appropriate monoclonal antibody on ice for 30 min and washed three times. The monoclonal antibodies used in these experiments were 8-5C2 (1:50) for recombinant MRP-8, and 5C12 hybridoma culture supernatant for recombinant MRP-14 and MRP-8/14 complex. The cells were stained on ice for 30 min with 200 μl of FITC-conjugated goat anti-mouse Ig antibody $F(ab)_2$ (1:200) containing 20 μg/ml propidium iodide in order to gate out dead cells, and then washed three times. The stained cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Ten thousand cells per sample were recorded, and results are expressed in histograms of the gating. In the experiments regarding the effects of serum proteins, MOLT-4 cells grown in ASF104 serum-free medium were used and PBS containing 0.1% polyethylene glycol 20000 and 0.05% NaN_3 (PEG-PBS) was used as a washing buffer instead of B-PBS. In the experiment regarding the effects of trypsin treatment, the cells were incubated with 0.25% trypsin for 5 min at 37°C and then washed three times with RPMI 1640 supplemented with 10% FBS, followed by the MRP binding assay.

RESULTS

Intracellular Distribution of MRPs—Intracellular MRP-8 and MRP-14 were discriminated by the size of gold particles. MRP-8 molecules were labeled with 5-nm colloidal gold particles mediated by the specific monoclonal antibody, and MRP-14 molecules were labeled with 10-nm colloidal gold particles. As shown in Fig. 1, both MRP-8 (labeled with arrows) and MRP-14 molecules (labeled with arrowheads) were colocalized in the cytoplasm and in lesser amounts in the nucleus (the dense region at the lower right corner in Fig. 1) of THP-1 cells induced to differentiate with RA; some of the small and large particles were observed close to each other. The colocalization of intracellular MRP-8 and MRP-14 was also observed in THP-1 cells differentiated with 1α,25$(OH)_2D_3$ (data not shown). Similar results were obtained using differentiated HL-60 cells, but much more MRP was distributed in the THP-1 cells than in the HL-60 cells (data not shown), a finding which is consistent with previous observations (*16*). Taken together with the strong tendency for MRP8/14 complex formation in the presence of calcium ion as described below, these results suggest that MRP-8 and MRP-14 are associated *in vivo*.

Expression of MRP-8 and MRP-14 in Sf21 Cells—Although MRP-8 and MRP-14 are the most abundant proteins in both human neutrophils and 1α,25$(OH)_2D_3$-in-

duced leukemia cells such as HL-60 and THP-1, the purification of the individual proteins is difficult because they are usually isolated together in the cell lysate and have similar biochemical properties. To characterize the biochemical properties of each protein, MRP-8 and MRP-14 were independently expressed in baculovirus-infected Sf21 cells and purified. The recombinant MRP-14 in Sf21 cell lysate was identified as one band on a 15% SDS-polyacrylamide gel by the subsequent Coomassie Blue staining (Fig. 2B) and immunoblot analysis using the monoclonal antibody 5C12, a monoclonal antibody reactive with MRP-14 (Fig. 2C). Although the Sf21 cells gradually underwent lysis after infection (Fig. 2A), the MRP-14 was retained and accumulated in the cells, and the total amount of synthesized protein reached a maximum at 3 days after infection. The amount of MRP-14 accumulated in the Sf21 cells at 3 days after infection was found to be 6.4% of the total cellular protein by measuring the densitometric tracing of the Coomassie Blue-stained SDS-PAGE gel. Similar results were obtained for the expression of MRP-8 in Sf21 cells (data not shown).

Purification of Recombinant MRPs—The data on the purification of recombinant MRP-8 and MRP-14 are given in Table I, starting from about 50 ml (packed cell volume) of Sf21 cells at day 3 after infection with recombinant baculovirus. Recombinant MRP-8 and MRP-14 showed similar behavior during chromatography. In a typical experiment, 11.5 mg of MRP-8 and 7.4 mg of MRP-14 were purified by repeated chromatography to a high degree of

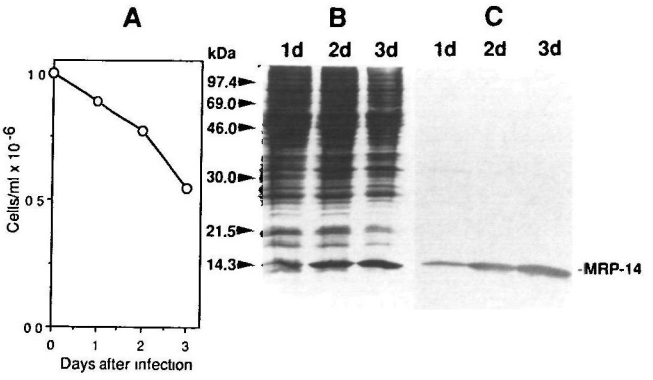

Fig. 2. **Time course of the expression of MRP-14 in baculovirus-infected insect (Sf21) cells.** Sf21 cells at an initial density of 1×10^6 cells/ml were infected with the virus containing MRP-14 cDNA at a multiplicity of infection of 5 in a spinner flask. Panel A, the viability of virus-infected Sf21 cells after infection. Sf21 cells were harvested at 1, 2, and 3 days after infection. The proteins in whole cell extracts from harvested cells were analyzed by 15% SDS-PAGE, followed by staining with Coomassie Blue (panel B) and an immunoblot analysis using anti-MRP-14 monoclonal antibody 5C12 (panel C).

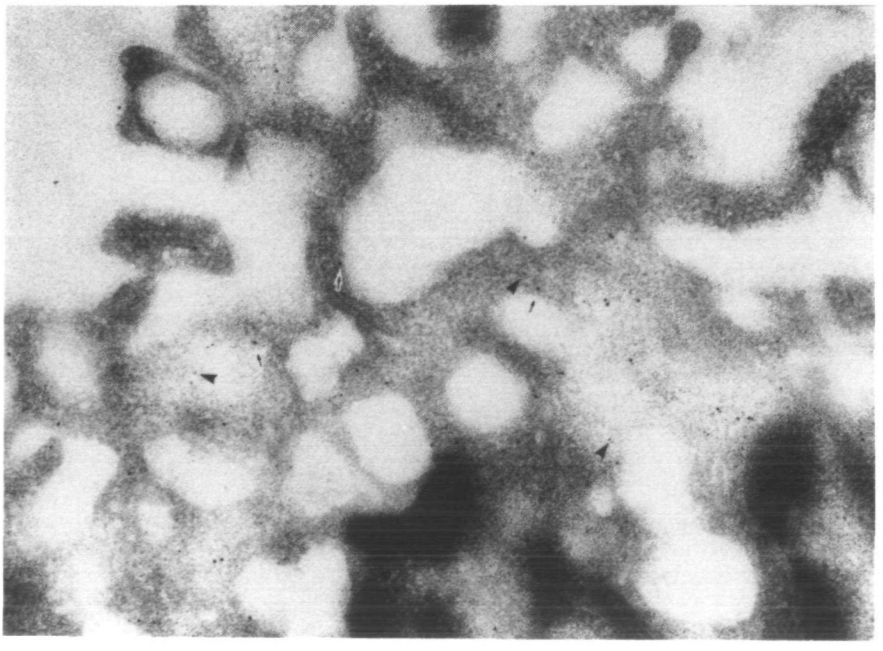

Fig. 1. **Intracellular colocalization of MRP-4 and MRP-14 in THP-1 cells induced to differentiate with RA.** THP-1 cells were treated with RA, and ultrathin sections were prepared and stained with monoclonal antibodies against MRPs and colloidal gold particle-linked anti-mouse IgG+IgM as described in "MATERIALS AND METHODS." MRP-8 (arrows) was labeled with 5-nm colloidal gold particles, and MRP-14 (arrowheads) was labeled with 10-nm colloidal gold particles. The dense region at the lower right corner of the figure represents the nucleus region.

TABLE I. **Purification of recombinant MRP-8 and MRP-14 from recombinant baculovirus infected Sf21 cells.** Starting from 50 ml (packed cells) of baculovirus infected Sf21 cells, purification of recombinant MRP-8 and MRP-14 proteins was performed as described in "MATERIALS AND METHODS."

Steps	RecMRP-8			RecMRP-14		
	Total protein (mg)	recMRP-8 (mg)	Yield (%)	Total protein (mg)	recMRP-8 (mg)	Yield (%)
Cell lysate	2,154	121.5	100	2,047	181.1	100
40% $(NH_4)_2SO_4$ sup	820.8	58.8	48.3	802.6	143.9	79.5
SuperQ Toyopearl	368.2	38.1	31.4	276.5	60.8	33.6
Phenyl Toyopearl	81.2	N.D.	N.D.	66.6	N.D.	N.D.
Gel filtration	25.2	N.D.	N.D.	14.1	N.D.	N.D.
Proprep RPC	11.8	11.4	9.4	7.4	7.2	4.1

homogeneity, with yields of 9.4 and 4.1%, respectively. Purified recombinant MRP-8 and MRP-14 were analyzed by 15% SDS-PAGE and subjected to Coomassie Blue staining and an immunoblot analysis by using 8-5C2 and 5C12, monoclonal antibodies reactive with human MRP-8 and MRP-14, respectively (Fig. 3). The antigenicities of both recombinant proteins were retained during the purification procedures. The monoclonal antibody 5C12, established in our laboratory, reacted specifically with MRP-14 in the presence of calcium ions, but not without calcium ions (Fig. 4B, lanes 4 and 5), indicating that calcium ions may play a crucial role in epitope recognition. Native MRP-8 and MRP-14 exist mainly in a complex form in cells, as shown in Fig. 1; 5C2 monoclonal antibody was consequently able

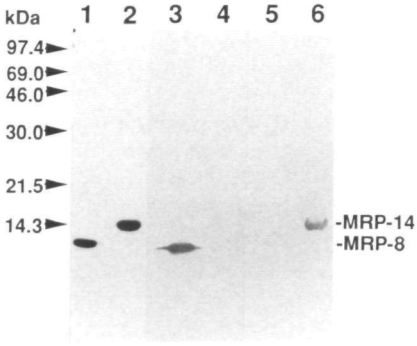

Fig. 3. **Immunoblot analysis of recombinant MRPs.** Purified MRP-8 and MRP-14 were analyzed by 15% SDS-PAGE, followed by staining with Coomassie Blue (lanes 1 and 2) or immunoblotting using anti-MRP-8 monoclonal antibody 8-5C2 (lanes 3 and 4), and anti-MRP-14 monoclonal antibody 5C12 (lanes 5 and 6). One microgram of MRP-8 was loaded on lanes 1, 3, and 5, and 1 μg of MRP-14 was loaded on lanes 2, 4, and 6.

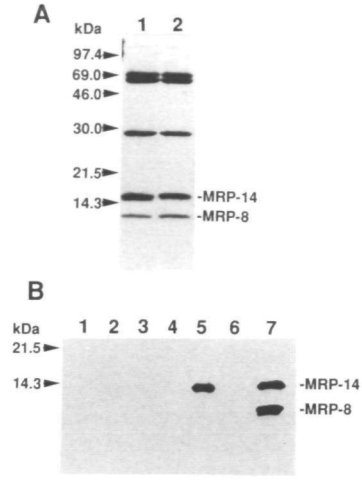

Fig. 4. **Immunoprecipitation and immunoblot analyses of recombinant MRPs.** Panel A, the immunoprecipitation of 1α,25-$(OH)_2D_3$-treated HL-60 cell lysate and the mixture of purified MRP-8 and MRP-14. Lane 1, HL-60 cells were cultured at a density of 10^5 cells/ml in 10 ml of RPMI 1640 medium containing 5×10^{-8} M 1α,25$(OH)_2D_3$ for 3 days. The cell lysate was immunoprecipitated with 5C12 monoclonal antibody and analyzed by 15% SDS-PAGE, followed by staining with Coomassie Blue. Lane 2, the mixture of 5 μg each of purified recombinant MRP-8 and MRP-14 was immunoprecipitated with 5C12 monoclonal antibody. Panel B, the effect of calcium ions on the reactivity of 5C12 monoclonal antibody with MRPs determined by immunoprecipitation. MRP-8, MRP-14, and a mixture of them were immunoprecipitated with monoclonal antibody 5C12: lane 1, none; lanes 2 and 3, 1 μg of MRP-8; lanes 4 and 5, 1 μg of MRP-14; lanes 6 and 7, the mixture of 1 μg of each MRP. In lanes 1, 3, 5, and 7, the immunoprecipitation buffer contained 1 mM $CaCl_2$, and in lanes 2, 4, and 6, the buffer contained 1 mM EGTA instead of $CaCl_2$.

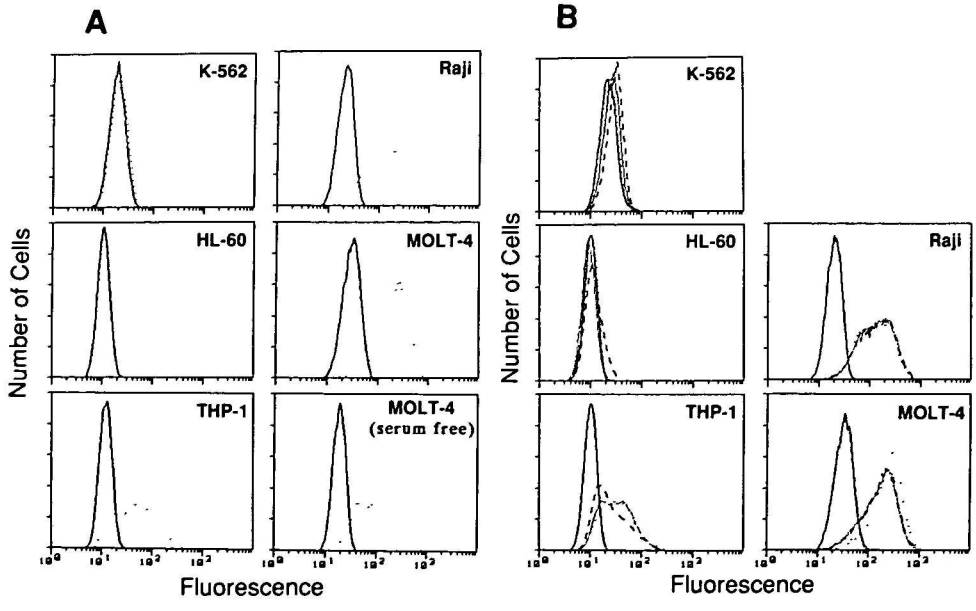

Fig. 5. **Detection of the binding of MRPs to human leukemia cell lines and the effect of differentiation inducers.** Panel A shows the MRP binding to non-treated human leukemia cell lines. A total of 2×10^6 cells was incubated in 100 μl of B-PBS with either a mixture of 10 μg each of recombinant MRP-8 and MRP-14 (dotted line) or B-PBS alone (solid line) as described in "MATERIALS AND METHODS," and then stained for flow cytometric analysis with the monoclonal antibody 5C12 and FITC-conjugated goat anti-mouse Ig antibody. In the experiment using MOLT-4 cells grown under serum-free conditions, PEG-PBS was used as a buffer instead of B-PBS as described in "MATERIALS AND METHODS." Panel B shows the MRP binding to human leukemia cell lines treated with the differentiation factors. Human leukemia cell lines were cultured for 3 days in RPMI 1640 medium with 3×10^{-7} M RA, 5×10^{-8} M 1α,25$(OH)_2$ D_3, or medium alone. A total of 2×10^6 cells was incubated with either a mixture of 10 μg each of recombinant MRP-8 and MRP-14 or B-PBS alone as described in "MATERIALS AND METHODS," and then stained for flow cytometric analysis with 5C12 and FITC-conjugated goat anti-mouse Ig antibody. —, undifferentiated cells incubated with B-PBS alone; ····, undifferentiated cells incubated with MRPs; ·····, RA-treated cells incubated with MRPs; ----, 1α,25$(OH)_2D_3$-treated cells incubated with MRPs.

to immunoprecipitate both human MRP-8 and MRP-14 of the differentiated HL-60 cells in the presence of 1 mM $CaCl_2$ (Fig. 4A, lane 1). Since similar results were obtained in the immunoprecipitation of the mixture of recombinant MRP-8 and MRP-14 with 5C12 monoclonal antibody (Fig. 4A, lane 2), recombinant MRP-8 and MRP-14 seemed to maintain the ability to form a complex. Furthermore, since 5C12 antibody immunoprecipitated recombinant MRP-14 but not MRP-8 in the presence of 1 mM $CaCl_2$ in each experiment, the co-immunoprecipitation of the recombinant MRPs was not the result of a cross-reaction of 5C2 with recombinant MRP-8 (Fig. 4B, lane 3). The native human MRP-14 of $1\alpha,25(OH)_2D_3$-induced HL-60 cells was slightly smaller than the recombinant MRP-14 (Fig. 4A, lane 1); it might be a product of a post-translational proteolytic cleavage of a C-terminus peptide, as reported elsewhere (*7*).

The amino terminus of recombinant MRP-14, as well as human native MRP-14, was blocked. The amino acid sequence of an internal peptide derived by cyanogen bromide (BrCN) cleavage was analyzed and was found to be identical to the deduced amino acid sequence (amino acid #6 to #12) (data not shown) of human MRP-14 (*1*). The amino terminus of recombinant MRP-8 was analyzed and was identical to the deduced amino acid sequence (amino acid #1 to #7) (data not shown) of human MRP-8 (*1*).

Binding of Recombinant MRP Complexes to the Cell Surface of Human Leukemia Cells—We first attempted to detect proteins bound to MRPs by immunoprecipitation using monoclonal antibodies specific to the MRPs, but we failed to obtain any positive results. We, therefore, shifted our focus to the existence of binding sites for MRPs on the cell surface. The possibility of MRP binding sites on the cell membrane is high, since in many patients with an infection (viral, bacterial, or parasitic) or malignancy (*e.g.*, pulmonary or gastrointestinal cancer), the plasma concentration of MRPs is increased and MRPs are released into the plasma from neutrophils (*6, 27, 28*). We used the cell lines K-562, HL-60, THP-1, Raji, and MOLT-4 to analyze the MRP molecules bound on the cell surface by flow cytometry (Fig. 5A). The cells were incubated with recombinant MRP complex and stained indirectly with a monoclonal antibody (8-5C2 or 5C12) and FITC-conjugated anti mouse Ig $F(ab)_2$. The human erythroleukemia cell line (K-562) and myeloblastic leukemia cell line (HL-60) had no binding sites for the MRP complex. The human monocytic leukemia cell line (THP-1), B-cell leukemia cell line (Raji), and T-cell leukemia cell line (MOLT-4) had abundant binding sites for the MRP complex. MOLT-4 cells grown in serum-free medium still had binding sites for the MRP complex. This result suggests that the binding of MRPs on the cell surface might not require any other proteins (such as serum proteins) (Fig. 5A).

Although RA and $1\alpha,25(OH)_2D_3$ are known to induce differentiation (over 90%) of leukemia cell lines (*14, 27*), these inducers did not change the amount of binding sites for the MRP complex in each cell line (Fig. 5B). On the contrary, HL-60 cells expressed no binding sites for the MRP complex, although they expressed an abundant amount of MRPs under these conditions. Although leukemia cell lines of lymphocyte origin (Raji and MOLT-4) did not express MRPs (*4, 14*), they had abundant binding sites for the MRP complex.

In addition, interleukin (IL)-1, IL-3, IL-6, IL-8, and interferon (IFN)γ did not change the amount of MRP binding sites in the RA- or $1\alpha,25(OH)_2D_3$-treated cell lines (data not shown).

Effects of Calcium Ion on the Binding of MRPs to MOLT-4 Cells—Since MRP-8 and MRP-14 are calcium-binding proteins with two EF-hand-type calcium binding sites and calcium ions were essential for the recognition of MRP-14 by the specific monoclonal antibody 5C12 (as shown in Fig. 4), the effects of calcium ions on the binding of MRPs on the cell membrane were examined (Fig. 6). The MOLT-4 cells had many binding sites for MRP-8 on the cell membrane, and their binding of MRP-8 was independent of calcium ions (Fig. 6A). In contrast, MOLT-4 cells had few binding sites for MRP-14 on the membrane, and the binding of MRP-14 was dependent on calcium ions (Fig. 6B). The binding of MRP-14 to the membrane in the presence of MRP-8 was increased by more than 4-fold over that of MRP-14 alone, and was almost independent of calcium ions (Fig. 6C). These results show that MRP-8 plays a main role in the binding of the MRP complex to the membrane in the absence of calcium ions, and that most of the MRP-14 binds to the membrane *via* MRP-8 by forming a complex with MRP-8 in the absence or presence of calcium ions.

Effects of Trypsin Treatment on the Binding of Recom-

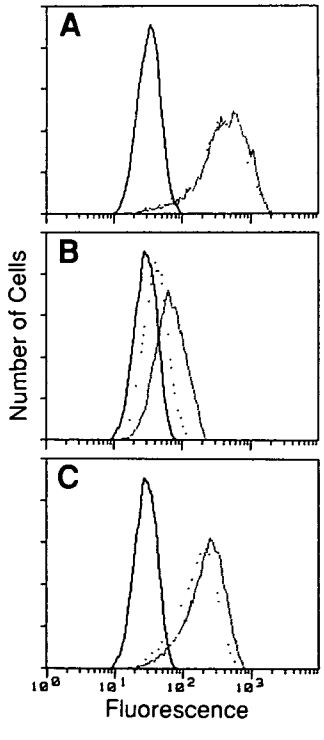

Fig. 6. **Effects of calcium ions on the binding of MRPs to MOLT-4 cells.** A total of 2×10^6 MOLT-4 cells was incubated in 100 μl of B-PBS with either 10 μg of MRP-8 (panel A) or MRP-14 (panel B) or a mixture of them (panel C) in the presence or absence of calcium ions as described in "MATERIALS AND METHODS." The cells were stained for flow cytometric analysis with 8-5C2 antibody (panel A) or 5C12 antibody (panels B and C) in the presence of FITC-conjugated goat anti-mouse Ig antibody. The cells were incubated with B-PBS containing 1 mM $CaCl_2$ (—); B-PBS containing the MRPs and 1 mM EGTA (····); B-PBS containing the MRPs and 1 mM $CaCl_2$ (· ·).

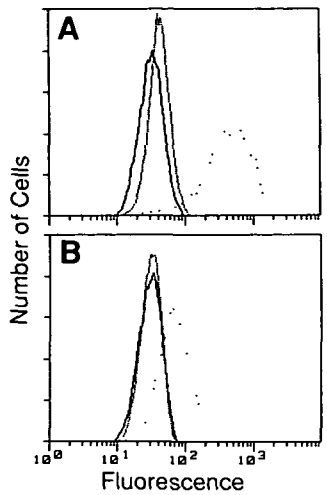

Fig. 7. **Effects of the trypsin treatment of MOLT-4 cells on the binding of MRPs.** MOLT-4 cells were incubated with 0.25% trypsin for 5 min at 37°C and then washed 3 times with RPMI 1640 supplemented with 10% FBS, followed by an MRP binding assay. A total of 2×10^6 cells was incubated in 100 μl of B-PBS containing 1 mM $CaCl_2$ with 10 μg of either MRP-8 (panel A) or MRP-14 (panel B) as described in "MATERIALS AND METHODS" and then stained for flow cytometric analysis with 8-5C2 or 5C12 antibody in the presence of FITC-conjugated anti-mouse Ig antibody. —, untreated cells stained with B-PBS alone; ····, untreated cells stained with the indicated monoclonal antibody; ··· , trypsin-treated cells stained with the indicated monoclonal antibody.

binant MRPs to MOLT-4 Cells—To characterize the MRP binding molecule(s) on the membrane, the effects of the protease digestion of MOLT-4 cells on the binding of MRPs were examined (Fig. 7). The binding of MRP-8 (Fig. 7A) and MRP-14 (Fig. 7B) on the membrane was markedly decreased in the trypsin-treated MOLT-4 cells. These results suggest that the MRP binding sites may be composed of protein(s) or contain protein portions in their components.

DISCUSSION

The two cytosolic Ca-binding proteins of myeloid cell origin, MRP-8 and MRP-14, are members of a large family of S-100 proteins. These MRPs are expressed in circulating neutrophils and monocytes as well as infiltrated macrophages in chronic inflammatory tissues (*4*). Our previous study (*14*) showed that leukemia cell lines of myeloid cell origin, HL-60 and THP-1, expressed MRPs during the granulocytic and monocytic differentiation induced by several inducers such as $MeSO_4$, RA, and $1\alpha,25(OH)_2D_3$. Human neutrophils contain abundant MRPs comprising up to 30-45% of their cytosolic protein and 5% of their total protein (*5*). In addition, the serum levels of MRPs were found to be highly elevated in patients with rheumatoid arthritis, Sjorgren's syndrome, and progressive systemic sclerosis, and moderately elevated in patients with mixed connective tissue disease and polymyositis or dermatomyositis, whereas the antigen was not detectable in the sera of healthy volunteers (*6, 20, 27*). MRPs were always present in the sera of some patients with connective tissue diseases, indicating the release of the antigen from infiltrated monocytes in the chronically inflamed tissue of these patients (*19*).

In the present study, we determined the intracellular colocalization of MRP-8 and MRP-14 and the specific binding sites of MRPs on the cell membrane of leukemia cell lines of lymphocyte and monocyte origin, and the results suggest that plasma MRPs may be responsible for the activation and recruitment of effector cells to inflammatory lesions.

The purification of MRP-8 and MRP-14 is difficult because they exist as a complex and have similar biochemical properties. In order to examine the biochemical characters of these proteins, each was expressed separately in baculovirus-infected Sf21 cells, followed by purification. The purified preparations of the recombinant MRPs preserved the differential antigenicity against the monoclonal antibody 5C12 and the ability to form a complex, and the amino-terminal sequences of the two recombinant MRPs were identical to the amino acid sequences predicted from their cDNA sequences.

Both MRP-8 and MRP-14 have a strong tendency to form a complex together, and our immunocytochemical analysis showed the colocalization of these proteins in the cells (Fig. 1). With the use of a chemical cross-linker, the *in vivo* formation of three different complexes was also demonstrated: a 48.5 kDa tetramer (MRP-8×2/MRP-14×2), a 35.0 kDa trimer (MRP-8×2/MRP-14×1) corresponding to the L-1 complex, and a 24.5 kDa heterodimer of MRP-8/MRP-14 (*7*). All complexes were shown to be noncovalently associated *in vivo*, and the association of MRPs was shown to be calcium-dependent (*7*).

The monoclonal antibody 5C12, established in our laboratory, reacted specifically with MRP-14 in the presence of calcium ions, and immunoprecipitated both MRP-14 and MRP complex, but not either in the absence of calcium ions. The monoclonal antibody 8-5C2 reacted specifically with MRP-8 independently of calcium ions and immunoprecipitated MRP-8. Using these monoclonal antibodies, the bindings of MRP-8 and MRP-14 on the MOLT-4 plasma membrane were examined. Figure 6 shows that MRP-8 plays a main role in the binding of the MRP complex to the membrane in the absence of calcium ions, and that most of the MRP-14 molecules bind the membrane *via* MRP-8 by forming a complex with MRP-8 in the absence of calcium ions. These data suggest that MRP-8 bound to MRP binding sites may acquire a high affinity for MRP-14 in the absence of calcium ions. Furthermore, since the binding of MRPs on the plasma membrane was trypsin-sensitive (Fig. 7), the MRP binding sites may involve protein(s) or contain protein moieties. The component(s) of the MRP binding sites were not detected by regular immunoprecipitation, the cross-linking of MRP-8 to MRP binding sites using a cleavable cross-linker (DTSSP) prior to immunoprecipitation (*29*) or West-Western blotting methods. These findings may indicate that the MRP binding sites are detergent-sensitive and easily lose the binding activity.

The human erythroleukemia cell line K-562 and the promyeloblastic leukemia cell line HL-60 had no binding activity for an MRP complex. The human monocytic leukemia cell line THP-1, the B-cell leukemia cell line Raji, and the T-cell leukemia cell line MOLT-4 had abundant binding sites for an MRP complex. Although RA and $1\alpha,25(OH)_2D_3$ are known to induce the differentiation of leukemia cells (*30*), these inducers did not change the amount of binding

sites for an MRP complex in any of these cell lines (Fig. 5A). However, HL-60 cells expressed no binding sites for MRP complexes, although a large amount of MRPs was synthesized and accumulated in the cell under the differentiated conditions. Thus, the expression of the MRP binding sites was not always parallel to the synthesis of the MRPs.

There is a report that MRP binding sites could be stably expressed as membrane proteins in lymphocyte- and monocyte-lineage cell lines. Bhardwaj *et al.* reported that an epitope recognized by a monoclonal antibody (25E10) of monocytes/macrophages in chronic inflammatory disease is non-covalently associated with the two calcium binding proteins MRP-8 and MRP-14 (*31*). They discussed the involvement of the MRP binding sites in the cytosol MRPs transport and their association with the plasma membrane surface by an unknown mechanism. The MRPs, however, do not contain a signal sequence or transmembrane region (*22*). MRP-8, MRP-14, and their complex have not been demonstrated to be released by viable cells (*5*). Since the MRP binding sites were found here in the plasma membrane of leukemia cell lines of lymphocyte and monocyte origin, these membrane-associated MRPs might be released from neutrophils, which are notably short-lived cells, in inflammatory lesions.

The function of MRPs itself is still unknown. S100β proteins were shown to stimulate the proliferation of rat C6 glioma cells and primary astrocytes (*32*). CP-10 is a member of the S100 protein family; it has high amino acid sequence identity (59%) and homology (76%) with MRP-8. CP-10 has chemotactic activity toward inflammatory polymorphonuclear cells *in vitro* and stimulates superoxide production and granular enzyme release in neutrophils (*18, 33*). Taken together, these observations and the present findings suggest that the binding of MRPs to monocytes, macrophages and lymphocytes through the MRP binding sites might be responsible for the activation and recruitment of the effector cells to inflammatory lesions. In addition, MRPs were demonstrated to have fungiostatic and bacteriostatic activities (*12, 13*), and it was suggested that MRP-14 was of key importance in these biological activities. However, the involvement of MRP binding site-related membrane receptors in the action of these fungiostatic and bacteriostatic activities is unknown.

Therefore, the characterization and molecular cloning of MRP binding sites are necessary to elucidate the function and signal transduction mechanism of MRPs in addition to the identification of the cell types which express the binding sites in patients and healthy volunteers and the influence of the cell differentiation stage.

REFERENCES

1. Lagasse, E. and Clerc, R.G. (1988) Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. *Mol. Cell. Biol.* **8**, 2402-2410
2. Hessian, P.A., Edgeworth, J., and Hogg, N. (1993) MRP-8 and MRP-14, two abundant Ca^{2+}-binding proteins of neutrophils and monocytes. *J. Leukoc. Biol.* **53**, 197-204
3. Zwadlo, G., Bruggen, J., Gerhards, G., Schlegel, R., and Sorg, G. (1988) Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin. Exp. Immunol.* **72**, 510-515
4. Hogg, N., Allen, C., and Edgeworth, J. (1989) Monoclonal antibody 5.5 reacts with p8,14, a myeloid molecule associated with some vascular endothelium. *Eur. J. Immunol.* **19**, 1053-1061
5. Edgeworth, J., Gorman, M., Bennett, R., Freemont, P., and Hogg, N. (1991) Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J. Biol. Chem.* **266**, 7706-7713
6. Fagerhol, M.K., Dale, I., and Andersson, T. (1980) Release and quantitation of a leucocyte derived protein (L1). *Scand. J. Haematol.* **24**, 393-398
7. Teigelkamp, S., Bhardwaj, R.S., Roth, J., Meinardus-Hager, G., Karas, M., and Sorg, C. (1991) Calcium-dependent complex assembly of the myeloic differentiation proteins MRP-8 and MRP-14. *J. Biol. Chem.* **266**, 13462-13467
8. Dale, I., Fagerhol, M.K., and Naesgaard, I. (1983) Purification and partial characterization of a highly immunogenic human leukocyte protein, the L1 antigen. *Eur. J. Biochem.* **134**, 1-6
9. Andersson, K.B., Sletten, K., Berntzen, H.B., Dale, I., Brandtzaeg, P., Jellum, E., and Fagerhol, M.K. (1988) The leucocyte L1 protein: identity with the cystic fibrosis antigen and the calcium-binding MRP-8 and MRP-14 macrophage components. *Scand. J. Immunol.* **28**, 241-245
10. Wilkinson, M.M., Busuttil, A., Hayward, C., Brock, D.J.H., Dorin, J.R., and van Heyningen, V. (1988) Expression pattern of two related cystic fibrosis-associated calcium-binding proteins in normal and abnormal tissues. *J. Cell Sci.* **91**, 221-230
11. Clark, B.R., Kelly, S.E., and Fleming, S. (1990) Calgranulin expression and association with the keratinocyte cytoskeleton. *J. Pathol.* **160**, 25-30
12. Steinbakk, M., Naess-Andresen, C.-F., Lingaas, E., Dale, I., Brandtzaeg, P., and Fagerhol, M.K. (1990) Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. *Lancet* **336**, 763-765
13. Murthy, A.R.K., Lehrer, R.I., Harwig, S.S.L., and Miyasaki, T. (1993) In vitro candidastatic properties of the human neutrophil calprotectin complex. *J. Immunol.* **151**, 6291-6301
14. Koike, T., Harada, N., Yoshida, T., and Morikawa, M. (1992) Regulation of myeloid-specific calcium binding protein synthesis by cytosolic protein kinase C. *J. Biochem.* **112**, 624-630
15. Lemarchand, P., Vaglio, M., Manuel, J., and Markert, M. (1992) Translocation of a small cytosolic calcium-binding protein (MRP-8) to plasma membrane correlates with human neutrophil activation. *J. Biol. Chem.* **267**, 19379-19382
16. Murao, S., Collart, F.R., and Huberman, E. (1989) A protein containing the cystic fibrosis antigen is an inhibitor of protein kinases. *J. Biol. Chem.* **254**, 8356-8360
17. Murao, S., Collart, F., and Huberman, E. (1990) A protein complex expressed during terminal differentiation of monomyelocytic cells is an inhibitor of cell growth. *Cell Growth Differ.* **1**, 447-454
18. Lackmann, M., Rajasekariah, P., Iismaa, S.E., Jones, G., Cornish, C.J., Hu, S., Simpson, R.J., Moritz, R.L., and Geczy, C.L. (1993) Identification of a chemotactic domain of the pro-inflammatory S100 protein CP-10. *J. Immunol.* **150**, 2981-2991
19. Kuruto, R., Nozawa, R., Takeishi, K., Arai, K., Yokota, T., and Takasaki, Y. (1990) Myeloid calcium binding proteins: expression in the differentiated HL-60 cells and detection in sera of patients with connective tissue diseases. *J. Biochem.* **108**, 650-653
20. Brandtzaeg, P., Gabrielson, T.O., Dale, I., Muller, F., Steinbakk, M., and Fagerhol, M.K. (1995) The leucocyte protein L1 (calprotectin): a putative nonspecific defence factor at epithelial surfaces. *Adv. Exp. Med. Biol.* **371A**, 201-206
21. Muller, F., Froland, S.S., Aukrust, P., and Fagerhol, M.K. (1994) Elevated serum calprotectin levels in HIV-infected patients: the calprotectin response during ZDV treatment is associated with clinical events. *J. Acquir. Immune Defic. Syndr.* **7**, 931-939
22. Odink, K., Cerletti, N., Bruggen, J., Clerc, R.G., Tarcsay, L., Zwadlo, G., Gerhards, G., Schlegel, R., and Sorg, C. (1987) Two calcium-binding proteins in infiltrate macrophages of rheumatoid

arthritis. *Nature* **330**, 80-82
23. O'Reilly, D.R., Miller, L.K., and Luckow, V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, W.H. Freeman and Company, New York
24. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
25. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
26. Hsu, S.-M. and Soban, E. (1982) Color modification diaminobenzidine (DAB) precipitation by metallic ions and application for double immunohistochemistry. *J. Histchem. Cytochem.* **30**, 1079-1082
27. Sander, J., Fagerhol, M.K., Bakken, J.S., and Dale, I. (1984) Plasma levels of the leucocyte L1 protein in febrile conditions: relation to aetiology, number of leucocytes in blood, blood sedimentation reaction and C-reactive protein. *Scand. J. Clin. Lab. Invest.* **44**, 357-362
28. Stockley, R.A., Dale, I., Hill, S.L., and Fagerhol, M.K. (1984) Relationship of neutrophil cytoplasmic protein (L1) to acute and chronic lung disease. *Scand. J. Clin. Lab. Invest.* **44**, 629-634
29. Altin, J.G. and Pagler, E.B. (1995) A one-step procedure for biotinylation and chemical cross-linking of lymphocyte surface and intracellular membrane-associated molecules. *Anal. Biochem.* **224**, 382-389
30. Collins, S.J. (1987) The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* **70**, 1233-1244
31. Bhardwaj, R.S., Zotz, C., Klarwasser, G.Z., Roth, J., Goebeler, M., Mahnke, K., Falk, M., Hager, G.M., and Sorg, C. (1992) The calcium-binding proteins MRP8 and MRP14 form a membrane-associated heterodimer in a subset of monocytes/macrophages present in acute but absent in chronic inflammatory lesions. *Eur. J. Immunol.* **22**, 1891-1897
32. Selinfreund, R.H., Barger, S.W., Pledger, W.J., and Van Eldik, L.J. (1991) Neurotrophic protein S100β stimulates glial cell proliferation. *Proc. Natl. Acad. Sci. USA* **88**, 3554-3558
33. Lackmann, M., Cornish, C.J., Simpson, R.J., Moritz, R.L., and Geczy, C.L. (1992) Purification and structural analysis of a murine chemotactic cytokine (CP-10) with sequence homology to S100 proteins. *J. Biol. Chem.* **267**, 7499-7504