Biochemical and Biophysical Research Communications **290,** 73–80 (2002)
doi:10.1006/bbrc.2001.6149, available online at http://www.idealibrary.com on IDEAL®

Differential Expression of the Isoforms for the Monocyte Chemoattractant Protein-1 Receptor, CCR2, in Monocytes

Seiya Tanaka, Simone R. Green, and Oswald Quehenberger[1]
Department of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0682

Received November 19, 2001

Two isoforms of human CCR2, the receptor for monocyte chemoattractant protein-1 (MCP-1), have been identified but their relative expression in monocytes and contribution to inflammatory responses mediated by MCP-1 remain uncertain. All available information on CCR2 expression is based on mRNA data because isoform-specific antibodies were not available until now. To analyze the relative expression of each isoform, we made two antibodies that specifically recognized CCR2A and CCR2B. Examination of receptor protein with these isoform-specific antibodies showed that the total expression of CCR2B in monocytes was about 10-fold higher than that of CCR2A with an equal distribution between the cell surface and intracellular pools. A detailed analysis using purified plasma membranes demonstrated that about 90% of all CCR2 on the cell surface were composed of CCR2B. The relatively abundant expression of CCR2B on the cell surface suggests a principal role of this isoform as a mediator of monocyte responses to MCP-1 in inflammation.

Key Words: **chemokine; monocyte chemoattractant protein-1; receptor; CCR2; MCP-1; inflammation.**

The directed migration of leukocytes from the blood to sites of inflammation is a crucial feature of the immune system and is mediated by chemotactic cytokines, also called chemokines (1–3). Monocyte chemoattractant protein 1 (MCP-1) is the most prominent member of the CC subfamily of chemokines (4, 5). It very effectively induces immune responses in monocytes (6), lymphocytes (7), and basophils (8). MCP-1 is secreted by a variety of cells in response to inflammatory stimuli and has been implicated in a number of pathological disorders including atherosclerosis (9–12) and rheumatoid arthritis (13, 14).

The receptor for MCP-1, CCR2, belongs to the family of G protein-coupled receptors. Two isoforms with alternatively-spliced intracellular carboxyl tails, CCR2A and CCR2B, were isolated from the human THP-1 and MonoMac 6 leukemia cell lines (15, 16). The two receptor forms differ only in their cytoplasmic carboxyl tails; the extracellular ligand binding domain that involves several receptor segments is identical between the two isoforms (17). Several of the extracellular domains are involved in the functional activation of the receptor by MCP-1 (18, 19). The nonredundant role of the receptor in monocyte recruitment and host defense was demonstrated in mice deficient in CCR2 (20, 21).

In the absence of suitable reagents no data is available on the expression of the two isoforms at the protein level and their relative contribution to the host defense. Transcripts for both isoforms are present in freshly isolated human monocytes, however, higher levels were found for CCR2B (16). For this reason it has received preferential scientific attention. The expression of CCR2B on monocytes is dynamic, and depends on the microenvironment. Regulation of receptor expression in addition to chemokine secretion is believed to be crucial for a controlled immune response. Any disruption of this delicate balance in the chemokine system may result in an ill-regulated immune response. Abnormal levels of certain plasma components including lipoproteins and homocysteine have been shown to induce monocyte CCR2B expression (22–24). This may intensify the inflammatory response to MCP-1 and contribute to the pathophysiology of chronic inflammation. Consistent with this hypothesis, we have recently shown that CCR2 expression in circulating monocytes may represent a potential therapeutic target for intervention of pathologic conditions that are characterized by persistent monocyte infiltration such as atherosclerosis (25). While a reduction of

[1] To whom correspondence and reprint requests should be addressed at Department of Medicine, 0682, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0682. Fax: (858) 534 2005. E-mail: oquehenberger@ucsd.edu.

0006-291X/02 $35.00

CCR2 expression in circulating monocytes may blunt the inflammatory response, a reduction of CCR2 expression after their recruitment from the circulation help retain monocytes at sites of inflammation. Proinflammatory cytokines are such modulators of the inflammatory response and are very potent inhibitors of CCR2 gene expression (26–28).

Interestingly, CCR2A is differentially regulated and appears less affected by proinflammatory cytokines and differentiation, suggesting a potentially distinct role for this isoform in macrophage biology. Little is known about the specific contribution of each CCR2 isoform to monocyte responses evoked by MCP-1 because reagents such as specific antibodies that would allow distinguishing between the isoforms were not available. The focus of the present work is to determine at the protein level the expression of monocyte CCR2A and CCR2B and to examine their cellular distribution.

EXPERIMENTAL PROCEDURES

Materials. All cell cultures were obtained from American Tissue Culture Collection (Manassas, VA). Cell culture medium was purchased from Life Technologies Inc. (Gaithersburg, MD) and low-endotoxin fetal calf serum was from Hyclone Laboratories, Inc. (Logan, UT). [^{35}S]GTP[S] (1250 Ci/mmol) was from NEN Life Science Products, Inc. (Boston, MA). Phycoerythrin-conjugated mouse anti-human CCR2 IgG and nonspecific IgG were obtained from R & D Systems Inc. (Minneapolis, MN) and fluorescein isothiocyanate-conjugated rabbit anti-guinea pig IgG was from Sigma (St. Louis, MO).

Cell culture, transfection, and isolation of human monocytes. COS-7 cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 2 mM L-glutamine. The coding sequence of CCR2A was obtained by RT-PCR using mRNA from THP-1 monocytes. The consensus sequence for initiation of translation was added and the cDNA was subcloned into the expression vector pSG5 (Stratagene, La Jolla, CA). Cells were transfected with Fugene 6 according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN) and assayed 2 days after transfection. THP-1 monocytes were maintained in supplemented RPMI 1640 medium below a cell density of 0.5×10^6 cells/ml as described (22).

Human monocytes were isolated from fasting subjects by centrifugation through Histopaque 1.077 (Sigma) as described (29). The purity of the monocytes was determined by flow cytometry using anti-CD14 antibody (Pharmingen, San Diego, CA) and was >90%. This isolation procedure did not affect CCR2 expression determined by flow cytometry and the expression level was comparable to that of monocytes that were not subjected to any purification procedure but were identified by the expression of CD14 in whole blood.

Preparation of plasma membranes. THP-1 monocytes were collected by centrifugation and washed twice with PBS. The cells were suspended in 20 mM Tris–HCl, pH 8.0, containing a cocktail of protease inhibitors (50 units/ml aprotinin, 5 mM benzamide, 14.5 μM pepstatin, 0.1 mM leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride), allowed to swell on ice for 20 min and homogenized using a Dounce homogenizer. Cell nuclei were removed by centrifugation at 1500 g for 10 min at 4°C. Crude membranes were then isolated by centrifugation at 100,000*g* for 1 h at 4°C and suspended in 1 mM sodium bicarbonate for further purification.

The plasma membranes were purified by an aqueous two-phase partition system in the presence of protease inhibitors (30). A mixture of 1.36 g of 20% dextran T500, 0.578 g of 40% polyethylene glycol 3350, 0.1 ml of 0.2 M potassium phosphate, pH 7.2, 0.4 ml of 1 M sucrose was adjusted to 3.5 g with water, and crude membranes (6 mg of protein) suspended in 0.5 ml of 1 mM sodium bicarbonate were added. The content of the two-phase system was mixed thoroughly and the phases were separated by centrifugation at 750*g* for 10 min at 4°C. The plasma membranes in the upper phase were re-purified using the lower phase from the balance tube that contained the identical partition system except that 0.5 ml of PBS was used instead of membranes. The upper phase of the final spin was diluted with 2 vol of sodium bicarbonate and the plasma membranes were collected by centrifugation at 100,000*g* for 30 min, washed with sodium bicarbonate and suspended in 150 μl PBS for subsequent analyses.

Analysis of CCR2 transcripts. Total RNA was isolated from THP-1 cells and human monocytes by guanidinium thiocyanate-phenol–chloroform extraction, and CCR2A and CCR2B gene expression was estimated by semiquantitative PCR (25). A common 5′-sense primer (5′-ATGCTGTCCACATCTCGTTCTCG) was used in conjunction with 2 isoform-specific 3′-antisense primers (CCR2A, 5′-CTTTCCTTTTCCACGACCATC; CCR2B, 5′-TTATAAACCAGC-CGAGACTTCCTGC) to yield CCR2A or CCR2B. All amplifications were carried out for 30 cycles at an annealing temperature of 68°C. The concentration of reverse-transcribed cDNA in the reaction mixture was adjusted to obtain a linear correlation between template and product. The specificity was confirmed by DNA sequencing. The PCR products were analyzed by agarose gel electrophoresis and densitometric scanning of the DNA bands (Image Quant, Molecular Dynamics, Sunnyvale, CA) and normalized to the expression level of GAPDH.

Generation of antibody and flow cytometry. Two isoform specific antisera directed against the intracellular carboxyl-terminal tails of CCR2A (AbCCR2-A) and CCR2B (AbCCR2-B) were generated in guinea pigs and IgG fractions were prepared. The anti-CCR2B antibody AbCCR2-B has been used in our laboratory for some time (19). It is specific for CCR2B and blocks the interaction between receptor and G-protein, thus preventing transmembrane signaling events. The anti-CCR2A antiserum, AbCCR2-A, was generated using a similar approach. The carboxyl tail of CCR2A between Ser^{327} and Ala^{387} was expressed as fusion protein in *Escherichia coli* using the pGEX-2T vector (Amersham–Pharmacia Biotech, Piscataway, NJ). The antigen was cleaved from the carrier protein with thrombin and purified with glutathione–Sepharose 4B beads (Amersham–Pharmacia Biotech). Antiserum was generated in guinea pigs and the IgG fraction was prepared. For flow cytometry 1×10^6 cells were suspended in 100 μl PBS containing 0.1% BSA and 0.01% NaN_3 (buffer A) and incubated for 30 min at 4°C with 5 μg of anti-CCR2A or anti-CCR2B IgG. After 2 washes with buffer A, the cells were incubated for another 30 min with fluorescein isothiocyanate-conjugated secondary antibody at a 1:200 dilution, washed twice with buffer A and analyzed by flow cytometry (Becton–Dickinson Biosciences, San Jose, CA). In some experiments, phycoerythrin-labeled AbCCR2 (1:20) was used that recognized both CCR2A and CCR2B.

Chemiluminescence immunoassay for CCR2A and CCR2B. Membranes of THP-1 cells (20 μg of total membranes or 5 μg of plasma membranes) suspended in PBS were plated into 96-well MicroFluor microtiter plates (Dynex Technologies, Inc., Chantilly, VA) and kept overnight at 4°C. The wells were washed three times with PBS, blocked for 10 min with PBS containing 1% BSA and incubated with AbCCR2-A or AbCCR2-B for 1 h at room temperature at 10 μg/ml in a total volume of 50 μl. After three washes with PBS, alkaline phosphatase-conjugated goat anti-guinea pig IgG (Sigma) was added in 50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 0.3 mM EDTA and 1% BSA and the plates were incubated for 1 h at room temperature. After three additional washes with PBS, the plates were rinsed with distilled water, 25 μl of a 50% solution of LumiPhos 530 (Lumigen Inc., Southfield, MI) was added to each well and the plates were incubated for 1.5 h at room temperature in the dark. Lumines-

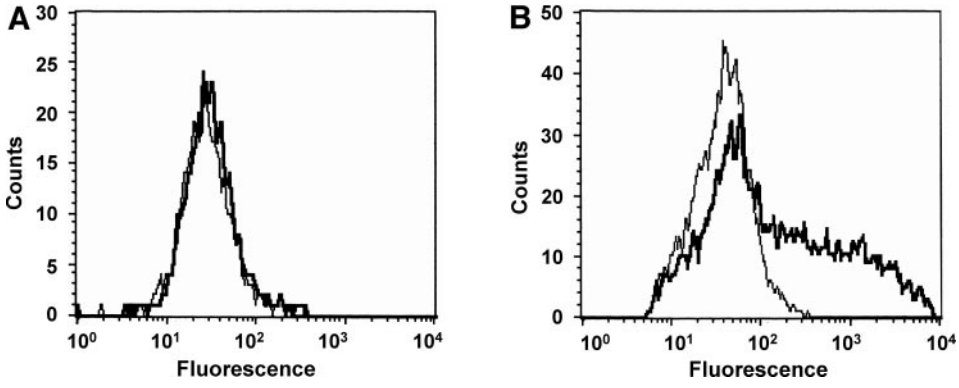

FIG. 1. CCR2A expression in transfected COS-7 cells. COS-7 cells were transiently transfected with CCR2A cDNA and protein expression was analyzed by flow cytometry using phycoerythrin-labeled AbCCR2 IgG (bold line). This antibody is directed against extracellular domains that are common to both CCR2A and CCR2B. Nonspecific fluorescence was determined with phycoerythrin-labeled mouse isotype control IgG (fine line). To distinguish cell surface expression from total expression, the cells were analyzed before (A) and after (B) permeabilization with 0.3% saponin for 5 min at ambient temperature. The antibodies were used at a 1:20 dilution.

cence was determined using a MLX Microtiter Plate Luminometer (Dynex Technologies, Inc.).

GTP[S] binding assay. GTP[S] binding assay was essentially performed as described earlier (19). Briefly, membranes from THP-1 cells were suspended at a protein concentration of 20 μg/ml in 200 μl of 20 mM Hepes, pH 7.4, 3 μM GDP, 10 mM $MgCl_2$ and 100 mM NaCl. The membranes were incubated with the various antibodies at 10 μg/ml for 30 min at 4°C, 5 nM [^{35}S]GTP[S] was added and the reaction was started with 30 nM MCP-1. After 30 min at 37°C the incubation mix was cooled to 4°C and 10 μM unlabeled GTP[S] (Sigma) was added. The membranes were kept on ice for 1 h to reduce nonspecific binding and then filtered through GF/B filters (Whatman Inc., Clifton, NJ). After three washes, the radioactivity associated with the filters was determined by scintillation counting.

Other analytical analyses. Protein was determined by the method of Lowry *et al.* (31). Data are expressed as means ± SD.

RESULTS

Localization of CCR2A in Transfected COS-7 Cells

To examine the role of CCR2A in the immune response of monocytes, we determined if CCR2A is functionally expressed on the cell surface. COS-7 cells were transiently transfected with CCR2A cDNA, and CCR2A protein expression was analyzed by flow cytometry using the anti-human CCR2 IgG, AbCCR2, which recognizes extracellular domains that are common to both isoforms. To differentiate between intracellular and surface expression, we permeabilized one set of transfected cells prior to the antibody treatment. Little surface expression of CCR2A was detected in intact cells by AbCCR2 (Fig. 1A). In contrast, the antibody gave a strong staining for CCR2A in permeabilized cells suggesting that CCR2A remained mainly in intracellular compartments (Fig. 1B).

Differential Expression CCR2A and CCR2B in THP-1 Cells and Circulating Human Monocytes

To determine if the expression pattern was similar for the endogenous receptor, we first examined the mRNA for CCR2A and CCR2B in cultured THP-1 cells and freshly isolated human monocytes by semiquantitative RT-PCR. As shown in Fig. 2, CCR2B was strongly expressed in both THP-1 cells and circulating human monocytes. CCR2A mRNA was also detectable in both cell types, although at much lower (five- to sixfold) levels.

The expression and distribution of CCR2 between the plasma membrane and intracellular pools was examined by flow cytometry on intact and permeabilized THP-1 cells. As expected, THP-1 cells displayed CCR2 on the cell surface (Fig. 3A). However, a substantial amount of receptor protein was also found in the cytoplasm and was accessible to the antibody only after permeabilization of the cells (Fig. 3B). The lack of isoform-specific antibodies has thwarted efforts to examine the relative expression and localization of endogenous CCR2A and CCR2B in human monocytes. Recently, we reported the generation of a polyclonal antibody AbCCR2-B that specifically rec-

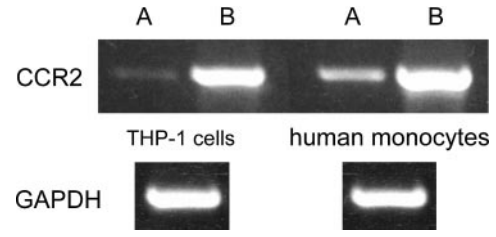

FIG. 2. Analysis of transcripts for CCR2A and CCR2B in THP-1 cells and circulating human monocytes. The differential expression of CCR2A (A) and CCR2B (B) in THP-1 cells and freshly isolated human monocytes was estimated by semi-quantitative RT-PCR. GAPDH was analyzed under identical conditions and served as internal standard. Results shown are representative of three individual experiments. To calculate relative ratios of CCR2B/CCR2A transcripts, the PCR products were analyzed using agarose gel electrophoresis and densitometric scanning of the DNA bands. The mean values for the ratios of CCR2B/CCR2A transcripts after normalization to GAPDH were 6.9 ± 2.1 for THP-1 cells and 5.5 ± 0.8 for human monocytes (n = 3).

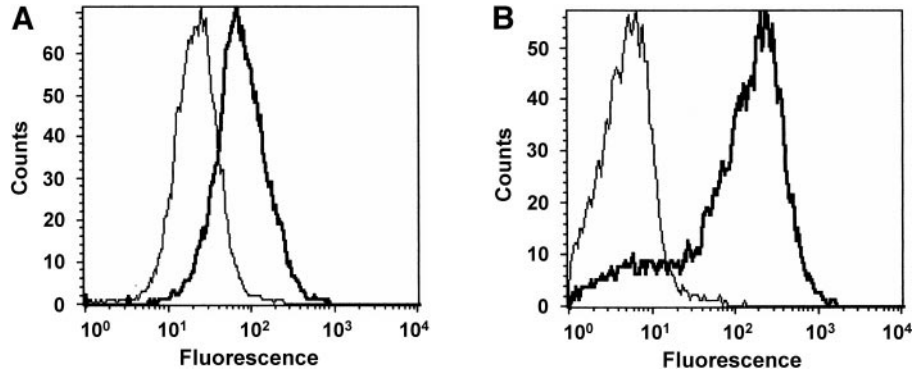

FIG. 3. Analysis of CCR2 cell surface and total cellular expression in THP-1 cells. The binding of AbCCR2 recognizing both CCR2A and CCR2B (bold line), and mouse isotype control IgG (fine line) to THP-1 cells was determined by flow cytometry. Surface expression was estimated on intact cells (A) and compared to total cellular expression in permeabilized cells (B) after treatment with saponin as described in the legend to Fig. 1.

ognized CCR2B (19). We have employed a similar approach to produce the antibody AbCCR2-A against the cytoplasmic carboxyl-terminal tail of CCR2A. The specificity of these antibodies was tested by flow cytometry with transfected cells expressing CCR2A and CCR2B. Both antibodies were specific for their respective isoform and did not bind to control transfected cells (Fig. 4A). AbCCR2-A specifically recognized CCR2A and did not stain for recombinant CCR2B (Fig. 4B). Similarly, AbCCR2-B only recognized CCR2B and did not react with CCR2A (Fig. 4C). The absence of any cross-reactivity provided us with the tools to examine CCR2A and CCR2B expression at the protein level in human monocytes.

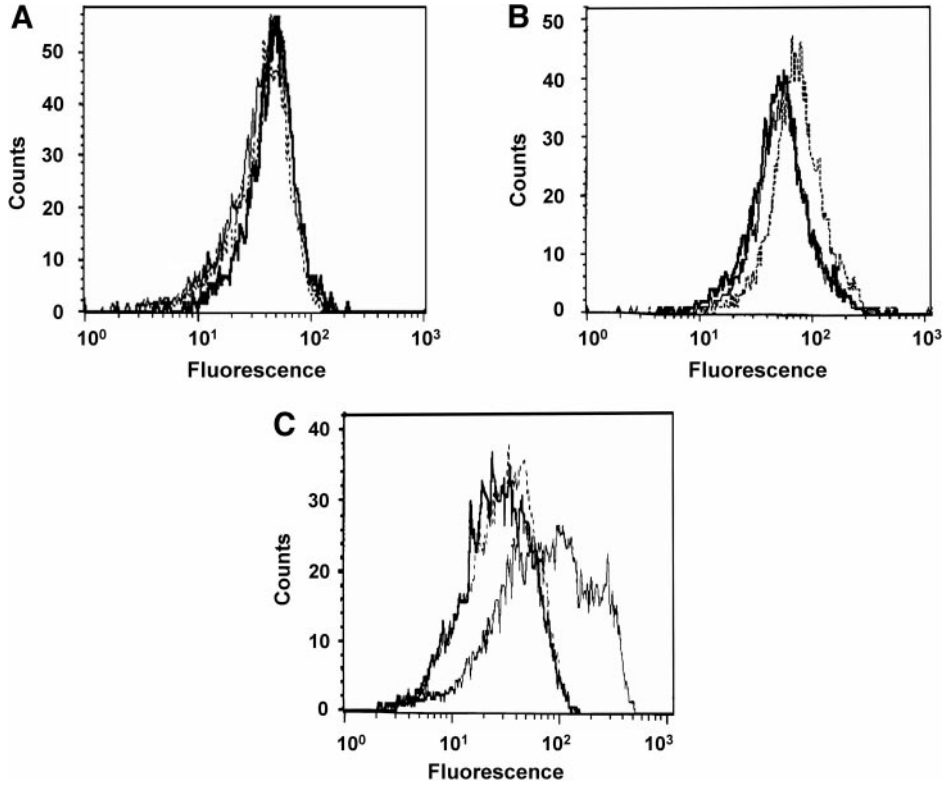

FIG. 4. Specificity of AbCCR2-A and AbCCR2-B. The specificity of AbCCR2-A and AbCCR2-B for their respective isoforms was determined on permeabilized (0.3% saponin) nontransfected control COS-7 cells (A), and transfected COS-7 cells expressing CCR2A (B) or CCR2B (C). Nonspecific fluorescence was determined with preimmune guinea pig IgG (bold line). The dashed line represents the specific staining mediated by AbCCR2-A IgG, and the fine line represents the staining mediated by AbCCR2-B IgB. The antibodies were used at 5 μg/ml. The binding of the primary antibody was detected with fluorescein isothiocyanate-conjugated secondary antibody at a 1:200 dilution.

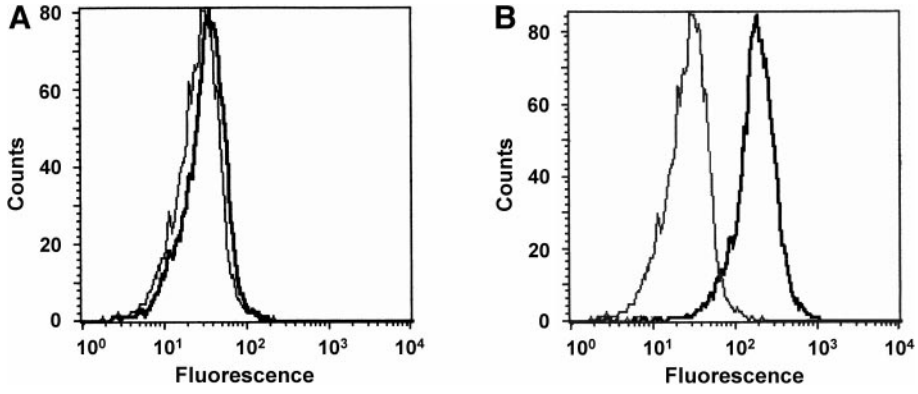

FIG. 5. Differential expression of CCR2A and CCR2B in monocytic THP-1 cells. To determine the relative expression of CCR2A and CCR2B, THP-1 cells were permeabilized with 0.3% saponin as described in the legend to Fig. 1 and incubated with 5 μg/ml of AbCCR2-A (A) or AbCCR2-B (B) IgG, respectively. The binding of isoform-specific IgG was analyzed by flow cytometry with fluorescein isothiocyanate-conjugated secondary antibody at a 1:200 dilution (bold line). Nonspecific binding was estimated with 5 μg/ml of control guinea pig preimmune IgG (fine line).

These two antibodies were used to determine the differential expression of CCR2A and CCR2B in permeabilized THP-1 cells and in freshly isolated human monocytes. As shown in Fig. 5A, THP-1 cells expressed CCR2A at marginal levels. In comparison, CCR2B was expressed at much higher levels and represented the majority of MCP-1 receptors on THP-1 cells. Essentially identical results for total, i.e., intracellular and cell surface expression of CCR2A and CCR2B, were obtained by immunoassay performed on crude membranes prepared from THP-1 cells (Fig. 6A). Again, CCR2B represented the majority of the receptor population and only little CCR2A expression was observed. More quantitative estimation of the cell surface expression of both isoforms was obtained by immunoassay using highly purified plasma membranes. The results were consistent with the data derived from flow cytometry and indicated that only a small portion (about 10%) of all CCR2 receptor molecules on the cell surface represented CCR2A (Fig. 6B).

THP-1 cells are derived from human monocytic leukemia cells and may display a protein expression that is different from that of circulating human monocytes. Therefore, the CCR2 expression pattern was also established in freshly isolated human monocytes by flow cytometry. As with THP-1 cells, CCR2A was barely detectable in permeabilized monocytes and the B isoform represented the majority of the receptors (Fig. 7).

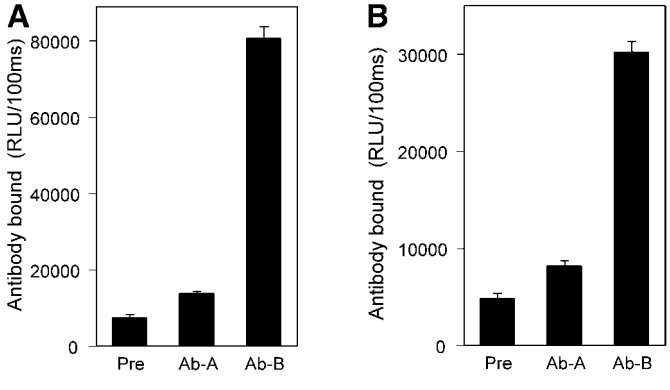

FIG. 6. Quantitative immunoassay of CCR2A and CCR2B expressed on crude and purified plasma membranes from THP-1 cells. Crude membranes and purified plasma membranes were isolated from THP-1 cells as described under Experimental Procedures. The membranes (20 μg of crude membranes and 5 μg of purified plasma membranes) were plated into microtiter plates and incubated with 10 μg/ml of guinea pig preimmune IgG (Pre), AbCCR2-A IgG (Ab-A) and AbCCR2-B IgG (Ab-B). The binding of the antibodies was determined by chemiluminescence immunoassay using alkaline phosphate-conjugated goat anti-guinea pig IgG. (A) CCR2A and CCR2B on crude membranes; (B) expression of the isoforms on purified plasma membranes. Data are expressed as means ± SD of three independent experiments. RLU, relative light units.

Inhibition of Transmembrane Signaling by Neutralizing Anti-CCR2 Antibodies

Activation of CCR2 by MCP-1 induces functional coupling of the receptor to heterotrimeric G protein. The stimulation of G protein requires the interaction with several intracellular domains of CCR2, including the third intracellular loop and carboxyl tail (19, 32). To determine to what extent each isoform contributes to MCP-1-mediated responses of monocytes, we analyzed the functional coupling of each isoform to G protein in THP-1 cells by [^{35}S]GTP[S] binding assay. Recently, we demonstrated that the antibody AbCCR2-B we made against the receptor carboxyl tail blocked GTP[S] binding to G protein in transfected cells expressing CCR2B (19). This antibody blocked transmembrane signaling also in THP-1 cells and decreased [^{35}S]GTP[S] binding by more than 90% (Table 1). In contrast, AbCCR2-A, made against the identical region of CCR2A had little effect on [^{35}S]GTP[S] binding. AbCCR2-A, like AbCCR2-B, was made against the re-

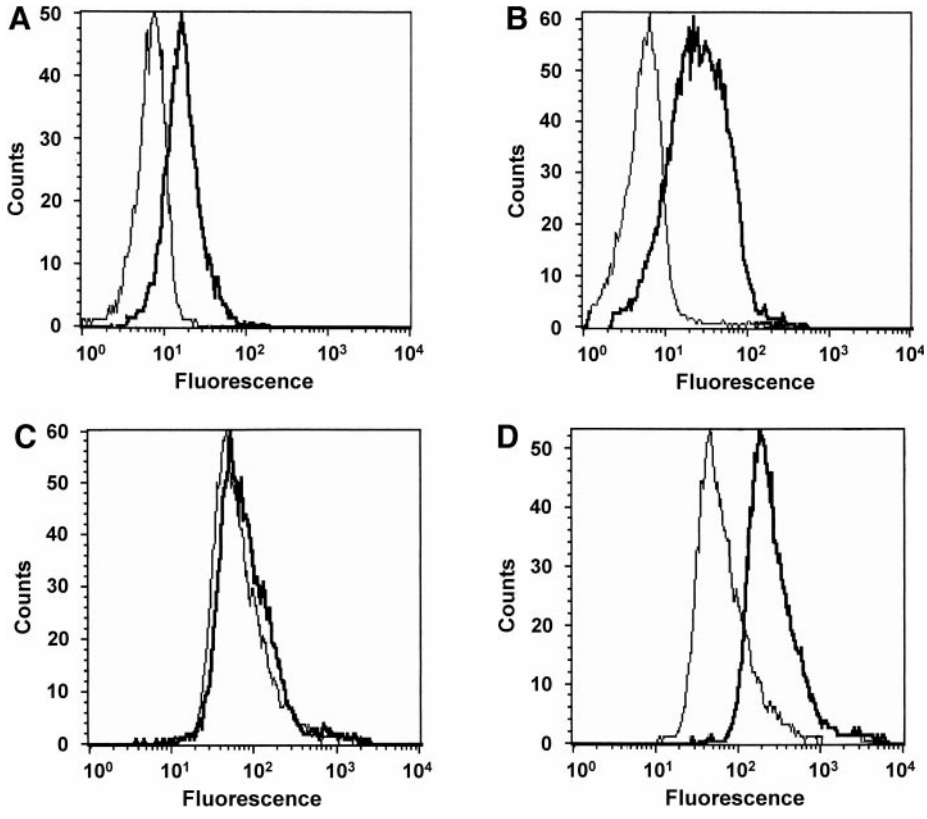

FIG. 7. Expression of CCR2A and CCR2B in circulating human monocytes. The expression of CCR2 (CCR2A and CCR2B) and that of each individual isoform was estimated on freshly isolated human monocytes by flow cytometry. CCR2 cell surface and total cellular expression was estimated on intact monocytes (A) and after permeabilization with 0.3% saponin (B), respectively. For the detection of CCR2, phycoerythrin-labeled AbCCR2 IgG (1:20) was used that does not discriminate between the isoforms and stains for both (bold line). As control, phycoerythrin-labeled nonspecific mouse isotype IgG (1:20) was included (fine line). The isoform-specific expression was determined in permeabilized monocytes with 5 μg/ml of AbCCR2-A or AbCCR2-B IgG, respectively. The specific staining representative for CCR2A (C) and CCR2B (D) is shown in bold. Nonspecific staining was determined with 5 μg/ml of guinea pig preimmune IgG (fine line). The binding of primary antibodies was detected with fluorescein isothiocyanate-conjugated secondary antibody at a 1:200 dilution.

ceptor segment that is implicated in functional G protein-coupling. Although we were not able to independently verify in transfected cells the neutralizing property of the antibody due to low expression of CCR2A, a functional role for CCR2A in monocytes can only be small. More than 90% of transmembrane signaling in the THP-1 cells is attributable to CCR2B, and CCR2A can maximally account for 10% (Table 1). Together, these results indicate that CCR2A is expressed only at low levels on the cell surface and may not be importantly involved in the responses of circulating monocytes to MCP-1.

TABLE 1

Inhibition of MCP-1-Mediated Transmembrane Signaling in THP-1 Cells by AbCCR2-A and AbCCR2-B

Antibody	GTP[S] binding (%)
No	100 ± 9
Preimmune	93 ± 8
AbCCR2-A	92 ± 7
AbCCR2-B	9 ± 8

Note. ^{35}S-GTP[S] binding experiments were carried out with membranes from THP-1 cells either in the absence of antibody (No) or in the presence of guinea pig preimmune IgG, AbCCR2-A, or AbCCR2-B at 10 μg/ml. Data are presented as means ± SD (n = 3).

DISCUSSION

The interaction of MCP-1 with respective receptors mediates monocyte migration and recruitment into tissues during inflammation. Two receptor isoforms, CCR2A and CCR2B, have been identified, but their relative contribution to the inflammatory response of monocytes to MCP-1 remained unclear (15). Our findings are in agreement with a previous study showing poor surface expression of epitope-tagged CCR2A in transfected cells (16). We have now extended these studies to THP-1 cells and freshly isolated human monocytes and provide evidence that the overall expression of CCR2A is very low in these cells, suggesting

a minor role for this isoform in the function of circulating monocytes.

A detailed analysis of the relative expression of CCR2A and CCR2B in human monocytes at the protein level was not possible until now, because isoform-specific antibodies were unavailable. CCR2A and CCR2B are identical in their amino acid sequence except for their alternatively spliced intracellular carboxyl tails (15). We exploited this difference and generated two isoform-specific antibodies. Using these two antibodies we were able to demonstrate by flow cytometry that THP-1 cells and freshly isolated human monocytes expressed only low levels of CCR2A compared with CCR2B. A more quantitative immunoassay analysis indicated that the expression of CCR2B was at least one order of magnitude higher than that of CCR2A.

Our data do not entirely rule out some expression of CCR2A on the cell surface. Indeed small amounts of CCR2A were detectable by immunoassay. The physiological role of receptors expressed at such low levels is not clear and it remains questionable whether the receptor is functional. Stimulation of G protein-coupled receptors with agonist activates nucleotide exchange on G proteins. Analysis of this exchange activity can be used as a measure of receptor-mediated G protein activation and, consequently, also to estimate expression of functional receptor. MCP-1 induced a robust GTP-binding to G protein in THP-1 cells. The neutralizing antibody, AbCCR2-B, which blocks the interaction between CCR2B and G protein (19), reduced MCP-1-induced transmembrane signaling in THP-1 monocytes by more than 90%. The antibody AbCCR2-A, like AbCCR2-B, is directed against the receptor segment that is believed to be critically involved in G protein coupling. Nevertheless, AbCCR2-A had little effect on transmembrane signaling and CCR2A is responsible for maximal 10% of the signaling activity. Together, these results suggest that MCP-1 functions mainly through CCR2B.

A substantial portion of CCR2B was also found in the cytoplasm, which most likely represents the pool of recycled receptors. Upon agonist treatment many of the G protein coupled receptors undergo endocytosis into early endosomes (33, 34). Receptor internalization may be a possible mechanism of long-term receptor desensitization and may account for the loss of responsiveness due to prolonged exposure to agonist (35). Alternatively, it has been suggested that sequestration plays a role in receptor dephosphorylation and resensitization (36). MCP-1 stimulates a redistribution of chemokine receptors with a polarized expression to the leading edge of migrating cells (37). Although definite data remain elusive, intracellular pools of CCR2, as observed in human monocytes, may serve as a source for rapid receptor translocation to the plasma membrane following stimulation with MCP-1.

CCR2A is predominant localized in intracellular sites and a physiological function for it in circulating monocytes is difficult to discern. However, under conditions that result in a severe reduction of CCR2B expression, CCR2A may become functionally important. CCR2B expression and the chemotactic response of monocytes to MCP-1 are generally lost during differentiation (26, 27). In contrast, CCR2A mRNA was not significantly modulated during monocyte maturation (38). It is intriguing to speculate that under certain conditions, such as in mature macrophages, the receptor isoform A may perform functions that are distinct from those of the B isoform in circulating monocytes. In support of this, data show that CCR2A and CCR2B differ in their G protein specificity (39). This selective and differential coupling to G proteins may result in the activation of pathways that are specific for each isoform, which could be the basis for the diversity of the MCP-1-induced immune response ranging from chemotaxis to respiratory burst.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant HL56989 (Specialized Center of Research in Molecular Medicine and Atherosclerosis, La Jolla, CA) and by an Established Investigator Grant from the American Heart Association (O.Q.).

REFERENCES

1. Baggiolini, M., Dewald, B., and Moser, B. (1997) Human chemokines: An update. *Annu. Rev. Immunol.* **15,** 675–705.
2. Proost, P., Wuyts, A., and Van Damme, J. (1996) The role of chemokines in inflammation. *Int. J. Clin. Lab. Res.* **26,** 211–223.
3. Luster, A. D. (1998) Chemokines—Chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **338,** 436–445.
4. Rollins, B. J., Morrison, E. D., and Stiles, C. D. (1988) Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA* **85,** 3738–3742.
5. Yoshimura, T., Robinson, E. A., Tanaka, S., Appella, E., and Leonard, E. J. (1989) Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J. Immunol.* **142,** 1956–1962.
6. Yoshimura, T., and Leonard, E. J. (1990) Identification of high affinity receptors for human monocyte chemoattractant protein-1 on human monocytes. *J. Immunol.* **145,** 292–297.
7. Carr, M. W., Roth, S. J., Luther, E., Rose, S. S., and Springer, T. A. (1994) Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc. Natl. Acad. Sci. USA* **91,** 3652–3656.
8. Kuna, P., Reddigari, S. R., Rucinski, D., Oppenheim, J. J., and Kaplan, A. P. (1992) Monocyte chemotactic and activating factor is a potent histamine-releasing factor for human basophils. *J. Exp. Med.* **175,** 489–493.
9. 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.

10. 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.
11. 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.
12. Gu, L., Okada, Y., Clinton, S. K., Gerard, C., Sukhova, G. K., Libby, P., and Rollins, B. J. (1998) Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol. Cell* **2,** 275–281.
13. Koch, A. E., Kunkel, S. L., Harlow, L. A., Johnson, B., Evanoff, H. L., Haines, G. K., Burdick, M. D., Pope, R. M., and Strieter, R. M. (1992) Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. *J. Clin. Invest.* **90,** 772–779.
14. Gong, J. H., Ratkay, L. G., Waterfield, J. D., and Clark-Lewis, I. (1997) An antagonist of monocyte chemoattractant protein 1 (MCP- 1) inhibits arthritis in the MRL-*lpr* mouse model. *J. Exp. Med.* **186,** 131–137.
15. Charo, I. F., Myers, S. J., Herman, A., Franci, C., Connolly, A. J., and Coughlin, S. R. (1994) Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc. Natl. Acad. Sci. USA* **91,** 2752–2756.
16. Wong, L. M., Myers, S. J., Tsou, C. L., Gosling, J., Arai, H., and Charo, I. F. (1997) Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene—Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *J. Biol. Chem.* **272,** 1038–1045.
17. 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.
18. Monteclaro, F. S., and Charo, I. F. (1996) The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1α receptor, confers chemokine selectivity. *J. Biol. Chem.* **271,** 19084–19092.
19. Han, K. H., Green, S. R., Tangirala, R. K., Tanaka, S., and Quehenberger, O. (1999) Role of the first extracellular loop in the functional activation of CCR2—The first extracellular loop contains distinct domains necessary for both agonist binding and transmembrane signaling. *J. Biol. Chem.* **274,** 32055–32062.
20. Boring, L., Gosling, J., Chensue, S. W., Kunkel, S. L., Farese, R. V. J., Broxmeyer, H. E., and Charo, I. F. (1997) Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C–C chemokine receptor 2 knockout mice. *J. Clin. Invest.* **100,** 2552–2561.
21. Kuziel, W. A., Morgan, S. J., Dawson, T. C., Griffin, S., Smithies, O., Ley, K., and Maeda, N. (1997) Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc. Natl. Acad. Sci. USA* **94,** 12053–12058.
22. Han, K. H., Tangirala, R. K., Green, S. R., and Quehenberger, O. (1998) Chemokine receptor CCR2 expression and monocyte chemoattractant protein-1-mediated chemotaxis in human monocytes. A regulatory role for plasma LDL. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1983–1991.
23. Han, K. H., and Quehenberger, O. (2000) Ligands for peroxisome proliferator-activated receptor inhibit monocyte CCR2 expression stimulated by plasma lipoproteins. *Trends Cardiovasc. Med.* **10,** 209–216.
24. Wang, G., and O, K. (2001) Homocysteine stimulates the expression of monocyte chemoattractant protein-1 receptor (CCR2) in human monocytes: Possible involvement of oxygen free radicals. *Biochem. J.* **357,** 233–240.
25. Han, K. H., Chang, M. K., Boullier, A., Green, S. R., Li, A., Glass, C. K., and Quehenberger, O. (2000) Oxidized LDL reduces monocyte CCR2 expression through pathways involving peroxisome proliferator-activated receptor γ. *J. Clin. Invest.* **106,** 793–802.
26. Tangirala, R. K., Murao, K., and Quehenberger, O. (1997) Regulation of expression of the human monocyte chemotactic protein-1 receptor (hCCR2) by cytokines. *J. Biol. Chem.* **272,** 8050–8056.
27. Sica, A., Saccani, A., Borsatti, A., Power, C. A., Wells, T. N., Luini, W., Polentarutti, N., Sozzani, S., and Mantovani, A. (1997) Bacterial lipopolysaccharide rapidly inhibits expression of C–C chemokine receptors in human monocytes. *J. Exp. Med.* **185,** 969–974.
28. Weber, C., Draude, G., Weber, K. S., Weubert, J., Lorenz, R. L., and Weber, P. C. (1999) Downregulation by tumor necrosis factor-alpha of monocyte CCR2 expression and monocyte chemotactic protein-1-induced transendothelial migration is antagonized by oxidized low-density lipoprotein: A potential mechanism of monocyte retention in atherosclerotic lesions. *Atherosclerosis* **145,** 115–123.
29. Han, K. H., Han, K. O., Green, S. R., and Quehenberger, O. (1999) Expression of the monocyte chemoattractant protein-1 receptor CCR2 is increased in hypercholesterolemia: Differential effects of plasma lipoproteins on monocyte function. *J. Lipid Res.* **40,** 1053–1063.
30. Morre, D. J., and Morre, D. M. (1989) Preparation of mammalian plasma membranes by aqueous two-phase partition. *Biotechniques* **7,** 946–958.
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.
32. Arai, H., and Charo, I. F. (1996) Differential regulation of G-protein-mediated signaling by chemokine receptors. *J. Biol. Chem.* **271,** 21814–21819.
33. Tolbert, L. M., and Lameh, J. (1996) Human muscarinic cholinergic receptor Hm1 internalizes via clathrin-coated vesicles. *J. Biol. Chem.* **271,** 17335–17342.
34. Von Zastrow, M., and Kobilka, B. K. (1994) Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J. Biol. Chem.* **269,** 18448–18452.
35. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) Turning off the signal: Desensitization of beta-adrenergic receptor function. *FASEB J.* **4,** 2881–2889.
36. Pippig, S., Andexinger, S., and Lohse, M. J. (1995) Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization. *Mol. Pharmacol.* **47,** 666–676.
37. Nieto, M., Frade, J. M. R., Sancho, D., Mellado, M., Martinez, C., and Sánchez-Madrid, F. (1997) Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis. *J. Exp. Med.* **186,** 153–158.
38. Fantuzzi, L., Borghi, P., Ciolli, V., Pavlakis, G., Belardelli, F., and Gessani, S. (1999) Loss of CCR2 expression and functional response to monocyte chemotactic protein (MCP-1) during the differentiation of human monocytes: Role of secreted MCP-1 in the regulation of the chemotactic response. *Blood* **94,** 875–883.
39. Kuang, Y., Wu, Y., Jiang, H., and Wu, D. (1996) Selective G protein coupling by C–C chemokine receptors. *J. Biol. Chem.* **271,** 3975–3978.