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Molecular Analysis of CCR-3 Events in Eosinophilic Cells

Nives Zimmermann,* Bruce L. Daugherty, † James M. Stark,* and Marc E. Rothenberg**

CCR-3 is a major receptor involved in regulating eosinophil trafficking. Initial analysis of chemokine receptors has demonstrated unique receptor events in different cell types, indicating the importance of investigating CCR-3 events in eosinophilic cell lines. We now report that the eosinophilic cell line, acute myelogenous leukemia (AML) 14.3D10, expresses eosinophil granule proteins and eotaxin, but has no detectable expression of eosinophil chemokine receptors. Treatment of the cell line with butyric acid and IL-5 results in a dose-dependent synergistic induction of CCR-3 and, to a lesser extent, CCR-1 and CCR-5. Interestingly, using a luciferase reporter construct under the control of the hCCR-3 promoter, the uninduced and induced cells display high, but comparable, levels of promoter activity. Differentiated AML cells developed enhanced functional activation, as indicated by adhesion to respiratory epithelial cells and chemokine-induced transepithelial migration. Chemokine signaling did not inhibit adenylate cyclase activity even though calcium transients were blocked by pertussis toxin. Additionally, chemokine-induced calcium transients were inhibited by pretreatment with PMA, but not forskolin. Eotaxin treatment of differentiated AML cells resulted in marked down-modulation of CCR-3 expression for at least 18 h. Receptor internalization was not dependent upon chronic ligand exposure and was not accompanied by receptor degradation. Thus, CCR-3 is a late differentiation marker on AML cells and uses a signal transduction pathway involving rapid and prolonged receptor internalization, calcium transients inhibitable by protein kinase C but not protein kinase A, and the paradoxical lack of inhibition of adenylate cyclase activity. The Journal of Immunology, 2000, 164: 1055–1064.

Eosinophils have an important proinflammatory role in numerous diseases, including allergic disorders, parasitic infections, and malignancies (1–3). It has been increasingly appreciated that eosinophils are mainly tissue-dwelling cells, and their levels in tissues are orchestrated by chemokines, especially eotaxin, the most selective eosinophil chemoattractant identified to date. Chemokines are a large superfamily of chemotactic cytokines implicated in both homeostatic migration of leukocytes and the accumulation of inflammatory cells at sites of inflammation (4, 5). In addition, selected chemokine receptors (e.g., CXCR-4,3 CCR-2, CCR-3, and CCR-5) serve as coreceptors for the entry of HIV-1 into cells (6). Eosinophils respond to a wide range of chemokines as determined by their expression of chemokine receptors, CCR-1 and CCR-3. Chemokine receptors belong to a family of seven-transmembrane-spanning, G protein-coupled receptors (GPCR) (7). The major chemokine receptor operational in eosinophils is CCR-3. This receptor has a central role in allergic responses, because it is expressed not only by eosinophils, but also by basophils and selected Th2 lymphocytes, other cells central in allergic responses (8–13). In addition, the major ligand for CCR-3, eotaxin, has been shown to be responsible for eosinophil trafficking during baseline and inflammatory processes (14–19).

After CCR-3 is engaged by the ligand eotaxin, a series of events is triggered, including calcium transients, mitogen-activated protein kinase activation, actin polymerization, and rapid shape changes associated with chemotactic responses and granule release (20–23). The respiratory burst following CCR-3 activation is inhibited by staurosporin, genistein, and wortmannin, implicating the involvement of protein kinase C (PKC), tyrosine kinase, and phosphatidylinositol 3-kinase, respectively (21). Additionally, CCR-3 signaling (calcium transients and respiratory burst) is inhibited by pertussis toxin (20, 21), suggesting that the receptor is coupled to Gα-type G proteins. Lastly, CCR-3 undergoes prolonged ligand-induced internalization via clathrin-coated pits. Receptor internalization is not dependent on G protein coupling, calcium transients, or PKC (24). Thus, the signal transduction pathways used by CCR-3 in eosinophils have not been fully elucidated.

Although much progress has been made in the study of eosinophils, eosinophil research has been hindered by the small number of cells that can be obtained from peripheral blood of healthy donors, the inability to amplify eosinophils in vitro, and the difficulty in transfecting DNA into eosinophils. Therefore, biochemical analysis of eosinophil receptors, such as those for chemokines, has been based at least in part on analysis of the receptors expressed by cells other than eosinophils. However, extrapolation from transfected heterologous cells to primary cells can be misleading (25). Establishment of eosinophilic cell lines has potential importance for the study of chemokine receptor signal transduction events in eosinophils.

The acute myelogenous leukemia (AML) 14 cell line was established from the mononuclear fraction of a patient with M2 AML (26). Only rare (<1%) cells exhibited eosinophilic differentiation, but supplementation with IL-3, IL-5, and GM-CSF resulted in eosinophilic differentiation in 70% of the cells. In this report we describe further differentiation and maturation of AML14.3D10, a
cytokine-independent eosinophil myelocyte subline (27), by treatment with butyric acid and IL-5. Among numerous induced differentiation pathways, these cells undergo induction of functional CCR-1 and CCR-3 pathways. This system has allowed molecular analysis of CCR-3 events in eosinophilic cells.

Materials and Methods

Cell culture

The AML143D10 cell line (provided by C. C. Paul and M. A. Baumann, Wright State University, Dayton, OH) was grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FCS (Life Technologies), 50 μM 2-ME (Sigma, St. Louis, MO), 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (Sigma), and penicillin-streptomycin (Life Technologies). Initially, the pH of the medium was titrated to 7.8, but after it was established that CCR-3 expression does not depend on the pH of the medium (data not shown) this practice was discontinued. A stock of 50 mM butyric acid (Sigma) in PBS was prepared and stored at 4°C before use. The IL-5 (R&D Systems, Minneapolis, MN) was prepared as a stock of 100 μg/ml and stored at −80°C before use. Induction experiments were performed by growing cells in flasks at a starting density of 3–5 × 10^6 cells/ml. Alternatively, cells were grown in six-well plates starting with a concentration of ∼10^5 cells in 3 ml/well. Medium was changed during the induction period. For the cloning analysis experiment, the cell line was subcloned by starting with 1000 cells/well and performing serial dilutions (1/2) in a 96-well plate. Clones were isolated after 2 wk and expanded in the same medium as the parental cell line.

RNA analysis

Total RNA was prepared using Trizol reagent (Life Technologies) according to the manufacturer’s instructions. The RNA (10 μg) was separated by electrophoresis in a 1.5% agarose gel, transferred to Gene Screen transfer membranes (NEN, Boston, MA) in 10× SSC, and cross-linked by UV radiation. Chemokine receptor probes were labeled with ^32P using the Klenow reaction with random priming. The probes for CCR-1 and CCR-5 included the complete open reading frame and were generated from the pc.CCR-1 and pc.CCR-5 plasmids (AIDS Research and Reference Research Program, Rockville, MD) (28, 29). The open reading frame encoding for CCR-5 was PCR-amplified from human genomic DNA (30) and subcloned into pCR2.1 Invitrogen, Carlsbad, CA), and the CCR-3 fragment was liberated by EcoRI digestion. The cDNA probe for eosinophil cationic protein (ECP) was provided by H. Rosenberg (National Institutes of Health, Bethesda, MD) (31). The eosinogin probe (1.1-kb HindIII fragment) contained exon 2 and the coding portion of exon 3 (32). Blots were hybridized under standard conditions and washed under high stringency (0.1× SSC and 1% SDS at 65°C). Autoradiography was performed for 1–11 days.

Intracellular Ca^{2+} measurement

Cells (2 × 10^6/ml) were loaded with 5 μM fura-2/AM (Molecular Probes, Eugene, OR) in HBSS with 1% FCS for 60 min at 37°C in the dark. After two washes in flux buffer (145 mM NaCl, 4 mM KCl, 1 mM NaHPO_4, 0.8 mM MgCl_2, 1.8 mM CaCl_2, 25 mM HEPES, and 22 mM glucose, pH 7.6), cells were resuspended at 2 × 10^6 cells/ml and maintained on ice. Cells (2 ml) were warmed to 35°C and stimulated in a cuvette with a continuously stirring magnetic bar in a RatioMaster fluorometer (Photon Technology, South Brunswick, NJ). Data were recorded as the relative ratio of fluorescence emitted at 510 nm after excitation at 340 and 380 nm (y-axis) over time (x-axis). For desensitization assays, cells were prewarmed as described above and treated with desensitizing agent (forskolin, isoprotenerol, or PMA); all from Sigma) or diluent (ethanol or buffer, respectively) and 3 min later with chemokine. Stauroporin was used at 100 ng/ml for 5 min before addition of PMA. The ATP (10 μM, Sigma) was used as a positive control.

Flow cytometry

Cells (5 × 10^6) were washed with FACS buffer (2% BSA and 0.1% sodium azide in PBS) and incubated with 0.5 μg of anti-hCCR-3 (clone 7B111) Ab (provided by Dr. Paul Ponath, LeukoSite, Cambridge, MA), 0.5 μg of anti-hCCR-1 Ab (clone 53504.111, R&D Systems), anti-hCCR-5 (clone 2D7, PharMingen, San Diego, CA), or the mouse isotype-matched control (PharMingen) for 30 min at 4°C. Other Abs included anti-CD18 (clone TS1/18, American Type Culture Collection, Manassas, VA), anti-CD11b (clone LM2, American Type Culture Collection), anti-MLC-4 (clone 9F10, PharMingen), and anti-CD69 (Becton Dickinson). After two washes in FACS buffer, cells were incubated with 0.5 μg of FITC-conjugated isotype-specific secondary Ab (PharMingen) for 30 min at 4°C in the dark. After two washes, labeled cells were subjected to flow cytometry on FACScan flow cytometer (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson). Down-regulation of surface CCR-3 was asayed by incubating induced cells at 37°C for the indicated lengths of time with 0–1000 ng/ml human eotaxin or RANTES (PeproTech, Rocky Hill, NJ). Following chemokine exposure, cells were immediately placed on ice and washed with at least a 2× volume of FACS buffer. To assess the effect of acute exposure to the chemokine, cells were washed four times after 15-min exposure to the chemokine before being placed back into the culture medium for the indicated lengths of time. Receptor density (percentage) was calculated as 100 × (mean channel fluorescence [chemokine] – mean channel fluorescence [isotype-matched control])/mean channel fluorescence [medium] – mean channel fluorescence [isotype-matched control]. In other experiments, the level of CCR-3 on differentiated AML cells was titrated to 7.8, but after it was established that CCR-3 expression does not depend on the pH of the medium (data not shown) this practice was discontinued. A stock of 50 mM butyric acid (Sigma) in PBS was prepared and stored at 4°C before use. The IL-5 (R&D Systems, Minneapolis, MN) was prepared as a stock of 100 μg/ml and stored at −80°C before use. Induction experiments were performed by growing cells in flasks at a starting density of 3–5 × 10^6 cells/ml. Alternatively, cells were grown in six-well plates starting with a concentration of ∼10^5 cells in 3 ml/well. Medium was changed during the induction period. For the cloning analysis experiment, the cell line was subcloned by starting with 1000 cells/well and performing serial dilutions (1/2) in a 96-well plate. Clones were isolated after 2 wk and expanded in the same medium as the parental cell line.

Adhesion assay

Adhesion was assessed by an assay that uses eosinophil peroxidase as a marker for cell number as previously described (33, 34). A549 type II-like respiratory epithelial cells, American Type Culture Collection (cells were grown in 96-well culture dishes. Cells (dAML, 20,000 cells/well) were added to TNF-α-treated A549 cells in complete HBSS/gelatin in a volume of 0.1 ml/well. Following a 30-min incubation, the wells were washed three times to remove nonadherent AML cells. Titration was performed (0–2000 cells/well) to define the standard activity concentration curve. The substrate was prepared with Tris-HCl (55 mM, pH 8), hydrogen peroxide (1 mM), 0.1% Triton X-100, and o-phenylene-diamine (1 mM) and then added to all wells. Following a 20-min incubation at room temperature, the reaction was stopped by the addition of 4 M HSO_4, and the OD was read at 490 nm. The percent adhesion was determined based on the standard curve generated for each experiment.

Adenylate cyclase activity

Membrane fractions were prepared by washing the cells twice in cold PBS and lysing in a hypotonic buffer (5 mM Tris, 2 mM EDTA (pH 7.4), and 10 μg/ml trypsin inhibitor). Following centrifugation at 36,000 × g for 10 min, the crude membrane pellets were resuspended in 75 mM Tris, 12 mM MgCl_2, 2 mM EDTA (pH 7.4), and 10 μg/ml trypsin inhibitor. The CPR production was measured as previously described (35). Briefly, membranes (20–40 μg) were incubated with 2.7 mM phosphoenolpyruvate, 50 μM GTP, 0.1 mM cAMP, 0.12 mM ATP, 50 μg/ml myokinase, and 1 μCi of α[^32P]ATP for 15 min at 37°C in the presence of 10 μM forskolin with or without 100 ng/ml eotaxin. Basal activity was assayed in the absence of both forskolin and eotaxin. CR-3 receptors were stimulated with the addition of 1 ml of an ice-cold solution containing [^3H]cAMP and excess ATP and cAMP. [^32P]cAMP and [^3H]cAMP were isolated by alumina chromatography, with [^3H]cAMP used to quantitate individual column recovery. The flow-through was subjected to scintillation counting. The α_c10 adrenergic receptor (AR) overexpressed in CHW cells (α_c10-AR, provided by Dr. S. Liggett, University of Cincinnati), was used as a control cell line (35). The inhibition of forskolin-activated cyclase activity in this cell line was obtained with 100 μM epinephrine, which acts exclusively through the transfected α_2C10 receptor coupled to G_{i}.
measured in a beta counter (data not shown). Of cycloheximide. Protein was precipitated by TCA, and radioactivity was incorporation throughout the indicated time in the presence or the absence 90%) was determined by [35S]methionine normalized by dividing relative light units (luciferase assay) by OD (sodium carbonate, and OD was measured at 405 nm. All data were nor-

Western blotting

Whole cell lysates were prepared from AML cells by washing twice in cold PBS and lysing in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxy-
colate, and 0.1% SDS in PBS) with 10 μg/ml aprotinin, 10 μg/ml antipain, 10 μg/ml chymostatin, 10 μg/ml leupeptin, 10 μg/ml peptatin A (all from Roche, Indianapolis, IN) and 2 mM PMSE (Sigma). Additional shearing was accomplished by passing the lysates through a 26-gauge needle, and detergent-insoluble materials were removed by centrifugation at 12,000 × g for 15 min at 4°C. Supernatants were stored in siliconized tubes and either used immediately or stored at −80°C. The protein concentration was determined using bicinchonic acid assay (BCA; Pierce, Rockford, IL), and 50 μg was separated by electrophoresis on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Equal loading was verified by staining with Ponceau S (Sigma). After blocking the membrane for 1 h at room temperature in Tris-buffered saline with 0.2% Tween 20 (TBST) with 5% dry milk, the CCR3-specific polyclonal rabbit Ab (Ab) (10) was added for 1 h at room temperature (1/5,000 in TBST), followed by goat anti-rabbit HRP-conjugated secondary Ab (1/10,000 in TBST; Calbiochem, San Diego, CA). Signal was developed using enhanced chemiluminescence (Am-

tersham, Arlington Heights, IL) according to the manufacturer’s instruc-
tions. The Ab specificity was determined on lysates obtained from HOS.CD4 cells transfected with CCR3 (AIDS Research and Reference Reagent Program) (29, 37). Cycloheximide was used at 10 μM for 3 h. Inhibition of protein synthesis (>90%) was determined by [35S]methionine incorporation throughout the indicated time in the presence or the absence of cycloheximide. Protein was precipitated by TCA, and radioactivity was measured in a beta counter (data not shown).

CCR3 promoter activity measurement

The human CCR3 promoter (a 1.6-kb fragment proximal to the transcription initiation site at position −1544 to +60 of exon 1) was subcloned into promoterless pGL3Basic (N. Zimmermann, B. L. Daugherty, and M. E. Rothenberg, manuscript in preparation). AML14.3D10 cells were transfected by electroporation as previously described (38). Briefly, 15 × 10^6 cells were electroporated in RPMI with 15 μg of reporter construct and 5 μg of control construct (pCDNA3, Bgal) at 960 μF and 350 V. Cells were incubated for 7 h in RPMI with FCS, and lysates were made with Reporter Lysis Buffer (Promega). Luciferase assay was performed according to the manufacturer’s instructions (Promega), and data were recorded on a Mono-

light 3010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) as relative light units. β-Galactosidase activity was measured using o-nitrophenyl β-D-galacto-pyranoside (Sigma) as a substrate in sodium phosphate buffer for 2 h at 37°C. The reaction was stopped by addition of sodium carbonate, and OD was measured at 405 nm. All data were nor-

malized by dividing relative light units (luciferase assay) by OD (β-galac-
tosidase assay).

Results

Chemokine receptor induction by butyric acid and IL-5 in AML cells

The AML14.3D10 cell line is an eosinophil myelocyte cell line, a potentially valuable cell type to examine eosinophil chemokine receptors. However, even though these cells have multiple features of eosinophils (27), they have no detectable expression of the eosinophil chemokine receptors CCR-1 and CCR-3. Because butyric acid and IL-5, an eosinophil growth and differentiation factor, have been shown to induce the maturation of other cell lines (39), we examined their effect on the differentiation of AML cells. In the first set of experiments, varying concentrations of butyric acid (0–2.5 mM) were added, and 2 days later IL-5 was added at a concentration of 10 ng/ml. After 7 days from initial culture, the expression of both chemokine receptors in a dose-dependent manner, with peak expression seen at 10 ng/ml. In the absence of IL-5 but at the optimal dose of butyric acid, low levels of CCR3 were detected. In contrast, CCR-1 expression was not detected in the absence of IL-5 (Fig. 1A, right panel; 0 ng/ml IL-5).

In contrast to the induction of CCR-1 and CCR-3, the uninduced AML cells expressed abundant mRNA for eosinophil cationic protein, a granule protein highly specific for eosinophils, and low
levels of eotaxin mRNA (Fig. 1A). The normalized level of eosinophil cationic protein and eotaxin mRNA did not vary with IL-5. Additionally, low levels of CCR-5 mRNA were induced with butyric acid and IL-5 (Fig. 1A). These results indicated that butyric acid and IL-5 synergistically induced the selective expression of CCR-1 and CCR-3 mRNA.

Chemokine receptor expression on the cell surface was examined by FACS analysis during induction. Staining with anti-CCR-1 and anti-CCR-3 Abs showed no detectable cell surface expression on uninduced AML cells (data not shown). However, CCR-1 and CCR-3 were strongly induced in dAML (Fig. 1B). When performed with replicate cells, CCR-3 staining was stronger than CCR-1 staining at saturating Ab concentrations (n = 2). To compare the level of CCR-3 on the surface of dAML to eosinophils, we compiled the FACS data from 11 experiments with dAML and three eosinophil donors performed in a 2-mo time frame using the same lot of anti-CCR-3 Ab. Results show that the average CCR-3 fluorescence was 19.8 ± 8.3 and 36.9 ± 5.3 on dAML and eosinophils, respectively. This estimate suggests that dAML cells express about 55% CCR-3 on their surface compared with eosinophils. In contrast to the expression of mRNA for CCR-5, no CCR-5 cell surface staining was detected (data not shown).

We next determined the kinetics of CCR-1 and CCR-3 induction during the culture period (Fig. 1C). Neither CCR-1 nor CCR-3 was detectable constitutively, but both had detectable expression at 5 days, peak expression at 7 days, and reduced expression at 9 days. CCR-1 returned to near baseline after 9 days, whereas CCR-3 remained detectable (n = 2). In subsequent experiments AML cells were differentiated with 0.5 mM butyric acid and 10 ng/ml of IL-5, and all analyses were performed between 6 and 8 days. These differentiated cells will be referred to as dAML.

Addition of butyric acid resulted in a dose-dependent increase in the percentage of adherent cells. In the absence of butyric acid, none of the cells was adherent; however, in the presence of 0.5 mM butyric acid, about 50% of the cells became adherent. To quantitate adhesion in a more physiological environment, cells were cultured with an optimal dose of IL-5 and butyric acid and were subsequently assessed for their adhesion to respiratory epithelial cells in a brief (30-min) incubation. As shown in Fig. 2A, uninduced cells had only -4.6 ± 3% adhesion, whereas induced cells had 9.8 ± 3.9% adhesion (average ± SD; n = 3). We next examined the expression of adhesion molecules before and after differentiation and compared the expression to freshly isolated eosinophils (Fig. 2B). The FACS analysis indicated that CD18 was present on all cells but was markedly up-regulated on dAML cells (3-fold increase in receptor density as indicated by the mean channel fluorescence). CD11b was negative on uninduced cells, and following induction it was comparable to the level on peripheral blood eosinophils. Very late Ag-4 (CD49d) was present on all three cell populations. In contrast to CD18 and CD11b, CD49d decreased by 35% with induction, consistent with the finding that CD49d expression decreases on cord blood-derived eosinophils with maturation (40). However, the level of CD49d on dAML was...
3-fold higher than that on freshly isolated eosinophils. Additionally, the activation marker CD69 was negative on peripheral blood-derived eosinophils, but was present on uninduced cells and at even higher levels (13-fold) after induction.

Morphological changes were also examined during the culture. The uninduced AML14.3D10 cells were described by others as eosinophilic myelocytes. Consistent with this, cytocentrifuge preparations of the cell line stained with Diff-Quick revealed homogeneous cells with round nuclei, prominent nucleoli, sparse dark blue cytoplasm, and discrete eosinophilic granules. After induction with butyric acid and IL-5, dAML were heterogeneous, with cells containing oval, indented, or occasional segmented nuclei and larger cytoplasm to nuclear proportions, and some cells had prominent eosinophilic granules (data not shown).

**Clonal analysis of AML14.3D10 cells**

The AML cells exhibited heterogeneous expression of CCR-3 following induction. We were interested in whether this was due to the presence of different clones in our parental AML14.3D10 cell line. To test this hypothesis we isolated nine clones by limiting dilutions and subjected them to treatment with butyric acid and IL-5. The clones varied in their CCR-3 expression (data not shown). Most clones (five of nine) exhibited homogeneous staining with strong CCR-3 expression. A minority of clones (four of nine) exhibited a heterogeneous response. These data suggested the existence of different cell populations in the parental AML14.3D10 cell line with variable potentials for CCR-3 induction.

**Transcriptional regulation of the CCR-3 gene promoter with butyrate/IL-5**

Because of the marked up-regulation of CCR-3 mRNA with butyric acid/IL-5 treatment of AML cells, we were interested in determining whether this was related to induction of new transcription factors. We hypothesized that if IL-5 and butyrate induced new transcription factors, then the CCR-3 promoter activity on an exogenous vector should be activated. Therefore, we compared the CCR-3 promoter activity in uninduced and induced cells. Both cell types were transiently transfected with a reporter construct consisting of the human CCR-3 promoter driving transcription of the luciferase gene along with a control construct to account for any differences in transfection efficiency. As shown in Fig. 3, there was strong promoter activity in uninduced undifferentiated AML (uAML) and dAML, with no increase noted in dAML. Interestingly, promoter activity even appeared reduced in dAML. Additionally, the level of CCR-3 promoter activity was comparable to the activity of the SV40 promoter. This indicates that undifferentiated AML cells have all the necessary transcriptional machinery for transcription of the CCR-3 gene.

**Transepithelial migration**

We were next interested in determining whether the induced CCR-3 was functionally active. Eosinophil trafficking events in the inflamed airway involve multiple steps, including migration across the epithelial cell layer. We therefore examined the ability of dAML to migrate across a layer of respiratory epithelial cells in response to eotaxin. Cells (dAML) were placed in the upper chamber of a Transwell coated with confluent A549 cells pretreated with TNF-α. Eotaxin induced a dose-dependent increase in migration through the Transwell. A representative experiment is shown.
Adenylate cyclase activity was measured in the absence of both forskolin and the agonist. Results are representative of four experiments for dAML and two for the α2-AR cell line. Each point is the mean ± SD of three replicates.

Chemokine-induced calcium transients in dAML

Chemokines are also known to induce rapid fluxes in intracellular calcium following receptor binding. We were therefore interested in assessing the induction of functional chemokine receptor pathways in dAML. Eotaxin induced a dose-dependent increase in intracellular calcium transients in dAML between 50 and 500 ng/ml of eotaxin, with a plateau between 200 and 500 ng/ml (data not shown). A representative calcium transient induced by eotaxin at 250 ng/ml is shown in Fig. 5A; RANTES and MIP-1α also induced calcium transients in dAML (Fig. 5, B and C, respectively), consistent with CCR-1 expression. Macrophage inflammatory protein-1β, a CCR-5-specific ligand, did not induce a calcium transient (data not shown) consistent with the absence of CCR-5 cell surface expression.

Pertussis toxin and adenylate cyclase inhibition in dAML

The CCR-3 responses in human eosinophils have been reported to be inhibited by pertussis toxin. We therefore examined pertussis toxin inhibition of eotaxin-induced calcium transients in dAML. Incubation of dAML cells in 20 ng/ml of pertussis toxin for 3 h, resulted in complete inhibition of eotaxin-induced calcium flux. In contrast, the response to ionomycin was not changed (data not shown). Pertussis toxin inhibition of chemokine-induced responses is generally interpreted to indicate that the chemokine signals through a GPCR that is linked to Gi proteins. However, the direct activation of Gi proteins by chemokines in eosinophils has not been previously examined. We therefore examined adenylate cyclase activity in dAML following eotaxin treatment. Adenylate cyclase activity was first induced by forskolin treatment, and then activity was measured 15 min after eotaxin treatment. Even though a control cell line (α2-AR) routinely exhibited inhibition of adenylate cyclase activity by its agonist, epinephrine, no inhibition by a stimulatory dose of eotaxin in dAML was observed (Fig. 6). These data raise the possibility that CCR-3 is coupled to a pertussis-toxin sensitive pathway that does not involve Gi proteins in these cells.

Inhibition of chemokine-induced calcium mobilization by PKC activation

Heterologous desensitization of GPCRs can occur via phosphorylation of the receptor by second-messenger protein kinases such as protein kinase A (PKA) or PKC (41, 42). The effect of PKA activation on CCR-3 desensitization was first assessed by stimulating cells with isoproterenol, a β2-adrenergic agonist that signals through a Gi-coupled receptor and is known to activate PKA (43, 44). First, isoproterenol (10^{-4} M) was shown to stimulate calcium mobilization in dAML cells (data not shown). Isoproterenol treatment (up to 2 × 10^{-4} M) did not diminish calcium transients induced by eotaxin or RANTES (data not shown). Consistent with this, treatment of dAML cells with forskolin (10^{-4} M), a pharmacological activator of adenylate cyclase, did not block chemokine-induced calcium transients. We next tested the ability of PKC to inhibit chemokine-induced calcium transients. Cells were pre-treated with PMA, a pharmacological activator of PKC. PMA induced a dose-dependent inhibition of chemokine-induced calcium transients between 4 and 400 nM (Fig. 7). As a control, the response to ATP was only partially inhibited at the highest dose (400 nM). The inhibition was reversed if cells were pretreated with 100 ng/ml staurosporin for 5 min before PMA addition (data not shown).

Ligand-induced down-modulation of CCR-3

We have recently shown that CCR-3 undergoes prolonged ligand-induced internalization in eosinophils (24). To determine whether ligand-induced internalization occurs in dAML, we examined the surface expression of CCR-3 over 48 h following exposure of dAML to eotaxin. Eotaxin (100 ng/ml) caused receptor loss, which was detectable after 15 min, peaked at 1 h, and remained reduced for at least 18 h. The combined results are shown in Fig. 8A which demonstrate only 68 ± 9%, 34 ± 9%, 36 ± 6%, and 54 ± 10% surface CCR-3 expression at 15 min, 1 h, 3 h, and 18 h, respectively. By 24 and 48 h of eotaxin exposure the receptor density returned to the level in untreated cells (data not shown; n = 2). The down-regulation was also seen with RANTES (100 ng/ml), another CCR-3 ligand. Only 33 ± 7% was expressed at 3 h and 23% at 18 h. To rule out epitope blockade by chemokine binding to the receptor, studies were performed at 4°C, which would allow the binding but prevent receptor internalization (45, 46). There was no down-modulation of surface CCR-3 at this temperature (n = 2; data not shown).

To determine whether continuous exposure to eotaxin was required for CCR-3 internalization, dAML were exposed to eotaxin for only 15 min and then incubated in media without chemokine for the remainder of the time. Alternatively, cells were exposed to chemokine throughout the experiment. As shown in Fig. 8B, the receptor level was inhibited to the same degree in both sets of cells. This indicates that acute chemokine exposure is sufficient for induction of CCR-3 internalization.

Ligand-induced modulation of CCR-3 protein level

Re-expression of CCR-3 may result from receptor recycling or alternatively may involve new protein synthesis if a significant amount of the internalized receptor is degraded. Indeed, our results in eosinophils indicate that CCR-3 is partially degraded following

FIGURE 6. Adenylate cyclase activity in dAML cells. Cyclase was activated by forskolin and inhibited by the GPCR ligand: epinephrine for the α2-AR cell line and eotaxin for the dAML cell line. Basal cyclase activity was measured in the absence of both forskolin and the agonist. Results are representative of four experiments for dAML and two for the α2-AR cell line. Each point is the mean ± SD of three replicates.

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Therefore, we were interested in CCR-3 trafficking in dAMLs. Total cell protein was isolated and assessed for the level of CCR-3 protein by Western blot analysis. The CCR-3 protein was detected using a polyclonal rabbit anti-human CCR-3. Whole cell lysates from dAML showed the specific induction of a band at 55–60 kDa (Fig. 8C). The steady state level of CCR-3 protein did not change with eotaxin treatment despite surface modulation in the replicate cells (Fig. 8C; n = 4). To further assess whether receptor down-modulation only involved receptor recycling, protein synthesis in dAML cells was inhibited by cycloheximide for 3 h throughout the time cells were exposed to eotaxin. Induced AML cells treated with cycloheximide had no reduction in CCR-3 expression compared with nontreated cells (Fig. 8D; n = 2). Upon exposure to eotaxin, dAML continued to have receptor down-modulation at a level comparable to noncycloheximide-treated cells, indicating that re-expression of CCR-3 was independent of protein synthesis.

**Discussion**

In this study we have demonstrated that the AML14.3D10 cell line undergoes further differentiation along the eosinophil lineage with butyric acid and IL-5 treatment. The cells become activated, adhere avidly to respiratory epithelial cells, and express functional CCR-1 and CCR-3 on their cell surface. In addition to establishing a valuable model system for the study of eosinophils, this investigation provides novel insight into the molecular processes triggered by chemokine receptors in eosinophilic cells. First, CCR-3 is identified as a late differentiation marker in eosinophils. The CCR-3 expression on eosinophilic cells occurs after the expression of granule proteins and eotaxin itself. Although CCR-3 has been shown to be present on eosinophilic IL-5Rα<sup>+</sup> precursors in the bone marrow (47–50), the kinetics of CCR-3 expression have not been previously examined. It is interesting that eotaxin expression occurs before its receptor, suggesting a possible positive feedback...
loop. Alternatively, the expression of CCR-3 on IL-5Rα+ , CD34+ cells, but not on uninduced AML cells, indicates a possible distinction between leukemic eosinophils and normal eosinophil precursors. Second, eosinophilic cells are shown to be capable of expressing multiple chemokine receptors that are regulatable at least in part by IL-5. Although CCR-1 and CCR-3 are known to be expressed by eosinophils, CCR-5 has not been previously demonstrated on eosinophilic cells. Third, we demonstrate that activation of CCR-3 does not lead to inhibition of adenylate cyclase in eosinophilic cells. CCR-3-mediated events have been reported to be inhibited by pertussis toxin (20), suggesting the involvement of G protein coupling of the receptor. However, direct inhibition of adenylate cyclase by eotaxin in eosinophils has not been previously examined. We therefore measured adenylate cyclase activity in dAML cells treated with a stimulating dose of eotaxin. Surprisingly, we were unable to demonstrate inhibition of forskolin-induced adenylate cyclase. However, previous results suggest that G protein coupling in primary granulocytes may be different from other cells. For example, one study demonstrated that FMLP cannot inhibit adenylate cyclase in neutrophils, even though it does in FMLP-transfected heterologous cells (25). These findings raise the possibility that pertussis toxin mediates its inhibitory effect on CCR-3 events through an alternative mechanism. Recently, we found that pertussis toxin, in doses commonly used in previous studies, causes marked removal of CCR-3 from the surface of eosinophils (24). This may be a possible mechanism of its action on eosinophils.

FIGURE 8. Internalization of CCR-3 from the cell surface induced by eotaxin. A, Cells were incubated with eotaxin for the indicated lengths of time, and cell surface expression of the receptor was assessed by FACS analysis. Data are expressed as the mean ± SEM (n = 3–8 for each time point). B, Cells (dAML) were incubated with eotaxin continuously for 3 h (a) or 18 h (b). Alternatively, cells were incubated with eotaxin for 15 min, washed extensively to remove unbound ligand, and incubated in media without eotaxin for the remainder of 3 h (c) or 18 h (d). The cell surface expression of CCR-3 was assessed by FACS. The isotype-matched control is depicted as the filled histogram; the CCR-3 expression without chemokine is shown as a dashed line, and that with chemokine is shown as the solid line. The results are representative of three experiments. C, Cells (dAML) were incubated with eotaxin for the indicated lengths of time, whole cell lysates were prepared, and 50 μg was electrophoresed in a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with the polyclonal anti-CCR-3 Ab. Uninduced AML are also shown. The results are representative of three separate experiments. In D, the effect of cycloheximide (CHX) was assessed by incubating dAML cells with eotaxin and CHX for 3 h. The results are representative of three separate experiments. Molecular weight standards are shown to the left of each panel.
CCR-3-mediated events. Alternatively, pertussis toxin may non-specifically inhibit other downstream G proteins. Lastly, we have not ruled out the possibility that adenylyl cyclase activity is inhibited at a different dose of eotaxin than that employed in this study. Next, we demonstrate that CCR-3 undergoes distinct ligand-induced internalization pathway in different eosinophilic cell types. We have recently determined that CCR-3 undergoes ligand-induced internalization in freshly isolated human eosinophils (24). In this report we demonstrate conserved and distinct features of the internalization pathway between dAML and normal human eosinophils. Whereas only RANTES induces prolonged CCR-3 internalization in fresh eosinophils, RANTES and eotaxin both induce sustained CCR-3 internalization in dAML. Additionally, chronic chemokine exposure in freshly isolated human eosinophils is necessary for sustaining CCR-3 internalization. In contrast, dAML undergoes prolonged internalization following only 15 min of chemokine exposure. Another difference between dAML and primary eosinophils is the lack of CCR-3 degradation following internalization in dAML cells. Taken together, it appears that the initial receptor endocytosis events between dAML and fresh eosinophils are similar, whereas the receptor recycling events may differ. This highlights the occurrence of unique biochemical events even in related cells.

Additionally, we provide insight into the mechanism of butyrate/IL-5 induction of CCR-3. Because our induction regimen appeared to induce multiple eosinophil gene products, we hypothesized CCR-3 induction might involve the induction of new transcription factors. This would, in turn, drive the transcription of eosinophil-specific genes, thereby inducing differentiation. However, our results indicate that uninduced AML14.3D10 cells have all the transcriptional machinery necessary for efficient transcription of the CCR-3 gene on an exogenous plasmid, comparable to induced cells. This is similar to previous findings on the HL60 c15 cell line, in which the Charcot-Leyden crystal protein promoter is equally efficient with and without butyrate induction (51), but in contrast to the eosinophil peroxidase gene promoter, which was found to be 2- to 3-fold more active in butyrate-induced cells (38). Butyrate has been shown previously to lead to histone hyperacetylation (52), which, in turn, makes genes more accessible to the transcriptional machinery. This appears to be the more likely mechanism, because butyric acid and IL-5 act as differentiation factors in our system. Alternatively, the induction protocol may induce CCR-3 expression by stabilizing its mRNA.

Lastly, we demonstrate that chemokine-induced calcium mobilization is inhibited by phorbol esters. In contrast, stimulation of PKA, by either forskolin or isoproterenol, did not affect CCR-3 signaling. This is consistent with the presence of PKC phosphorylation sites in the cytoplasmic portion of CCR-3 (S231 in the third intracellular loop and S333 in the C-terminal tail) and the lack of PKA sites. We have previously demonstrated that PMA induces internalization of CCR-3 (24) that may be responsible at least in part for the observed inhibition of chemokine responses on PMA-pretreated cells; PMA has been shown to lead to phosphorylation of other chemokine receptors (i.e., CXCR-2 and CXCR-4) (53, 54), but this has not been addressed for CCR-3 yet. Alternatively, PMA may be regulating other downstream signal transduction molecules such as phospholipase C. Heterologous desensitization by PMA has been demonstrated previously for several G_q (FMLP-R, C5a-R) as well as G_q (α1-AR)-coupled receptors (55–57).

Conclusions concerning eosinophil chemokine receptor events have often been drawn from investigation of heterologous cell lines transfected with individual receptors. Because individual cell types often use distinct signaling events, it is important to examine chemokine-triggered biochemical events in eosinophils. We have therefore performed our experiments in an eosinophilic cell line. The eosinophil cell line, AML14.3D10, expressed numerous eosinophil markers, but did not express CCR-1 or CCR-3. However, butyric acid and IL-5 synergistically induced these chemokine receptors. Induced cells had relative receptor levels and functional capabilities comparable to those of normal human eosinophils based on comparative FACS analysis. Butyric acid and IL-5 have been shown to induce eosinophil differentiation in several myeloid progenitor cell lines, such as HL-60, HL-60 clone 15, and EoL-3 (39, 58–61). These cell lines did not appear as similar to human eosinophils as the induced AML cells. For example, the induced EoL-3 cell line expresses functional CCR-1 and CCR-2 (61); this is not a characteristic pattern of eosinophil chemokine receptors. Induced HL-60 clone 15 cells express CCR-1 and CCR-3, but at a ratio (CCR-1 > CCR-3) inverse of that in normal human eosinophils (39). Future studies using this cell line will provide a valuable opportunity to evaluate CCR-3-related processes.

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