Immunofluorescence analysis of cytokine and granule protein expression during eosinophil maturation from cord blood–derived CD34+ progenitors

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Background: In allergic inflammation and asthma, eosinophils are major effector cells. They have been shown to synthesize at least 23 cytokines, some of which are stored intracellularly in their unique crystalloid granules together with cationic granule protein. Little is known about the synthesis and storage of cytokines relative to cationic granule proteins in maturing eosinophils during eosinophilopoiesis.
Objective: Our purpose was to analyze the expression of eosinophil-derived mediators, major basic protein (MBP), eosinophil cationic protein (ECP), IL-6, and RANTES, during early stages of eosinophil maturation in CD34+ cell-derived colonies.
Methods: Purified human cord blood CD34+ cells were grown in methylcellulose cultures in the presence of recombinant human IL-3 and IL-5. By confocal laser scanning microscopy, the coexpression of eosinophil granular proteins MBP and ECP was determined concurrently with IL-6 and RANTES during eosinophil maturation on days 16, 19, 23, and 28 of culture.
Results: Immunoreactivity against MBP, ECP, IL-6, and RANTES was not detectable in freshly purified CD34+ cells. Maturing eosinophils (>95%) exhibited positive immunostaining for all these proteins between days 16 and 28 of culture. At early stages of culture, discrete immunostaining was observed around the periphery but not in the center of granular structures. By day 28 cultured eosinophil-like cells showed evidence of the acquisition of crystalloid granule-like structures, analogous to those observed in mature peripheral blood eosinophils.
Conclusions: Eosinophils express and store cytokines simultaneously with cationic granule proteins during the process of maturation. We propose that the storage of cytokines during the development of eosinophils is an early event and it may be integral to inflammatory responses involving these cells. The results of this study suggest a potential immunoregulatory function for maturing eosinophils. (J Allergy Clin Immunol 2000;105:1178-84.)

Key words: *Eosinophil, CD34+ progenitor, cytokine, chemokine, cord blood, IL-3, IL-5, IL-6, RANTES, confocal microscopy, crystalloid granule*

Eosinophils are prominent cells in allergic inflammation, asthma, and host defense against parasitic diseases.[1-4] These inflammatory leukocytes are apparently derived from myelocytic progenitors in the bone marrow,[5,6] which have been shown to be CD34+.[7,8] Eosinophils store and release, on appropriate activation, a wide spectrum of proinflammatory mediators including cationic granule proteins, major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN).[1,2] They have also been shown to synthesize up to 23 cytokines, chemokines, and growth factors, many of which are stored in their crystalloid granules.[9,10] Ultrastructurally, the crystalloid granule is composed of two compartments, namely, an electron-dense crystalline core (internum) and an electron-lucent matrix. Mediators have been shown to be differentially stored within these two compartments; thus, although MBP,[11,12] GM-CSF,[13] and IL-2,[14] have been detected in the internum, ECP, EDN, EPO,[1,11] together with a number of other cytokines and chemokines including IL-5,[15] IL-6,[16] RANTES,[17] and TNF-α[18] appear to be stored within the granule matrix.

Recombinant human (rh) IL-3 and rhIL-5 are known to induce the differentiation of maturing eosinophils from isolated progenitors in in vitro culture systems.[19,20] Indeed, the morphologic characteristics of eosinophilopoiesis and the ontogeny of the formation of crystalloid granule have been partially investigated by analysis of eosinophil progenitors with use of electron microscopy and in vitro culture systems.[21] This included the description of early commitment to the eosinophil phenotype by the appearance of numerous large immature granules in the cytoplasm.[21] The observation that immature granules appear during early eosinophil development was confirmed in later studies showing pro-MBP

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Abbreviations used

CLSM: Confocal laser scanning microscopy
ECP: Eosinophil cationic protein
EDN: Eosinophil-derived neurotoxin
EPO: Eosinophil peroxidase
MBP: Major basic protein
rh: Recombinant human
TBS: TRIS-buffered saline solution

messenger RNA and protein expression in developing eosinophils in vitro, which localized to large immature granules containing "hollow" electron-lucent cores.[22] None of these studies, however, has examined the expression and pattern of granule storage of cytokines, in parallel with cationic proteins, in differentiating eosinophils.

We have recently demonstrated the transcription of cytokines (IL-4 and RANTES) in maturing eosinophils cultured from $CD34^+$ progenitors (Velazquez et al, unpublished data). In this study, with an in vitro culture system and confocal laser scanning microscopy (CLSM), we aimed to investigate the production of IL-6 and RANTES at the protein level during rhIL-3– and rhIL-5–induced eosinophil maturation from $CD34^+$ progenitors and their ultimate storage in crystalloid granule-like structures. Our previous studies have shown that CLSM is a powerful technique in detecting cytokine expression and storage in peripheral blood eosinophils.[16,17] MBP and ECP were chosen as markers of eosinophil crystalloid granules, whereas IL-6 and RANTES were representative of eosinophil-derived cytokines and chemokines, respectively.

Our data describe the relationship between the storage patterns of cytokines juxtaposed with cationic granule proteins during the course of eosinophil maturation. This study provides new insights on mediator expression during in vitro eosinophilopoiesis. In addition, for the first time our study shows that cytokine storage is an early event in the natural history of the eosinophil and is not only confined to mature or activated cells.

METHODS

Isolation and purification of $CD34^+$ cells from cord blood

Human cord blood samples were provided by Dr B. H. Mitchell, Prenatal Research Center, Royal Alexandra Hospital, Edmonton, Alberta, Canada. A total of 25 mL of cord blood was diluted 1:4 in PBS and loaded onto a 45-mL Ficoll-Paque (Pharmacia Biotech, Inc, Uppsala, Sweden) followed by centrifugation at 400*g* at room temperature for 25 minutes. The upper layer containing mononuclear cells was collected and washed twice in 5 mmol/L PBS EDTA. The isolated mononuclear layer was incubated with micromagnetic beads coupled to anti-CD34 mAb (QBEND-10) (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 minutes and subjected to positive selection by loading the cell suspension on a Mini-MACS separation column (Miltenyi Biotec). $CD34^+$ cells were eluted by positive selection after removing the column from the magnetic field. The purity of isolated cells (>95%) was determined with use of immunofluorescent labeling with phycoerythrin-conjugated antibody (anti-HPCA-2, Becton Dickinson, Mississauga, Ontario, Canada) and fluorescence-activated cell sorter analysis. The number and viability (>99%) of purified $CD34^+$ cells was determined by trypan blue exclusion.

Semisolid culture of $CD34^+$ cells

Purified $CD34^+$ cells were cultured in 35-mm Petri dishes (Falcon Plastic, Oxnard, Calif) at a concentration of 4×10^4 cells per milliliter in RPMI supplemented with Iscove's modified Dulbecco's medium (BioWhittaker, Walkersville, Md), β-mercaptoethanol (1×10^{-5} mol/L), penicillin (10,000 units/mL), streptomycin (10 mg/mL), 0.3% sodium bicarbonate, 20% heat-inactivated FCS (Gibco BRL Life Technologies, Burlington, Ontario, Canada), 5 ng/mL rhIL-3 (Genzyme, Markham, Ontario, Canada), 2 ng/mL rhIL-5 (Pharmingen, Mississauga, Ontario, Canada), and 0.84% methylcellulose (StemCell Technologies, Vancouver, British Columbia, Canada). Cultured cells were maintained at 37°C in 5% carbon dioxide and examined at weekly intervals and the cytokine concentrations adjusted.

Cell collection and cytospin preparation

Cytospins of freshly purified $CD34^+$ cells and in vitro cultured cells at days 16, 19, 23, and 28 were prepared by spinning 2×10^4 cells suspended in 100 μL of 20% FCS in RPMI 1640 in a Cytospin 2 centrifuge (Shandon, Runcorn, UK) at 800 revolutions/min for 2 minutes. Cytospins were air dried and stored at –20°C until used.

Immunofluorescent labeling (CD34, MBP, ECP, IL-6, and RANTES)

Slides of purified $CD34^+$ and cultured cells (days 16, 19, 23, 28) were fixed for 8 minutes in 2% paraformaldehyde in PBS (room temperature) and washed five times in TRIS-buffered saline solution (TBS, pH 7.4). After fixation, cells were blocked with 3% FCS in a humidified container for 30 minutes. After a second washing step, slides were incubated overnight with TBS containing 1% mouse monoclonal antihuman MBP (BMK-13, generated inhouse) at 4°C. Immunoreactivity to MBP was detected by incubating slides with 15 μg/mL Rhodamine (TRITC)-labeled goat antimouse antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa) for 2 hours. After another washing step, slides were blocked again for 2 hours with 50 μg/mL goat antimouse IgG Fab fragment (Molecular Probes, Eugene, Ore) and double-labeled with one of the following: primary mouse monoclonal antihuman ECP (EG2) (2 μg/mL, Kabi Pharmacia, Newington, NH), antihuman IL-6 (2 μg/mL, R&D Systems, Minneapolis, Minn), antihuman RANTES (5 μg/mL, R&D Systems) overnight at 4°C. Immunoreactivity against ECP, IL-6, and RANTES was visualized with use of 20 μg/mL BODIPY FL-conjugated goat antimouse antibody (Molecular Probes). In double immunofluorescent staining of cytospins of purified $CD34^+$ cells, phycoerythrin-conjugated anti-CD34 (10 μg/mL) (Becton Dickinson) was used to detect CD34 expression, and BODIPY-FL conjugated secondary antibody (20 μg/mL) was used to detect immunoreactivity for MBP, ECP, IL-6, and RANTES. Mouse IgG1 (R&D Systems) at equivalent concentrations was used as the isotype control. After a final washing step, 10 μL of the antibleaching agent, 0.4% *n*-propyl gallate (Sigma, Oakville, Ontario, Canada) in 3:1 glycerol/10 × TBS) was applied to the slides before coverslip attachment.

CLSM

Immunofluorescent staining of freshly purified $CD34^+$ and developing eosinophils was examined with a Leica CLSM as indicated in earlier reports.[16,17]

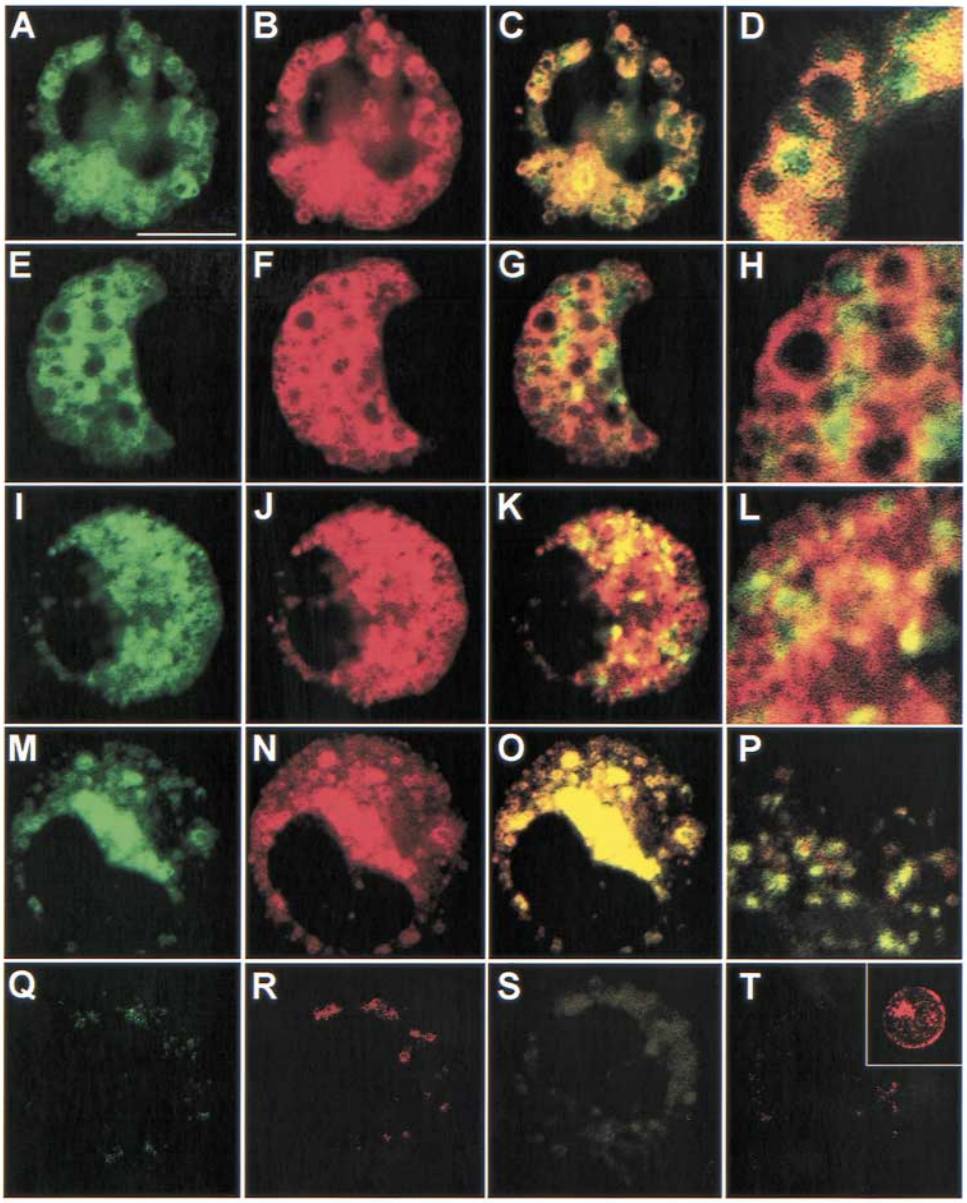

FIG 1. CLSM images of single and double immunofluorescent staining of maturing eosinophils with antibodies for MBP and ECP, freshly isolated cord blood–derived CD34⁺ progenitor, magnified images of granules and isotype control. The BODIPY-FL channel (green) corresponded to ECP immunoreactivity of developing eosinophils at days 16 **(A),** 19 **(E),** 23 **(I),** and 28 **(M)** of culture. The rhodamine (TRITC) staining (red) corresponded to MBP at days 16 **(B),** 19 **(F),** 23 **(J),** and 28 **(N).** Combined images of the same cells are shown to the right **(C, G, K, O)** and yellow color in these images indicates the colocalization of cationic granule proteins. Isotype control images for ECP, MBP, combined (day 19 of culture), and purified CD34⁺ are shown in **Q** through **T**, respectively. Maturing eosinophils from day 16 and after, unlike freshly isolated cord-blood CD34⁺ progenitors, exhibited positive immunoreactivity to MBP and ECP. The inset in **T** shows a freshly isolated CD34⁺ cell, double-labeled with antibodies for MBP (green) and CD34 (red). *Right,* Magnified images of granules at day 16 **(D),** 19 **(H),** 23 **(L),** and 28 **(P).** (Original magnification ×100.) Bar **(A)** indicates 10 μm.

RESULTS

Immunofluorescence of cytokines in purified cord blood–derived CD34⁺ cells

We examined the immunoreactivity of freshly prepared CD34⁺ cells purified from human cord blood within 12 hours of collection. Immunofluorescence studies in double-labeled cells showed that no MBP immunoreactivity was detected in cells positive for CD34 (Fig 1, *T* [inset]). Similar results were obtained for ECP, IL-6, and RANTES (results not shown). Interestingly, although our earlier study (Velazquez et al, unpublished data) indicated that cord blood–derived CD34⁺ cells express messen-

ger RNA for RANTES, our immunofluorescent staining suggested that the translated protein for RANTES was undetectable. This was in contrast to $CD34^+$-derived maturing eosinophils produced in culture from day 16 onward, which exhibited positive immunoreactivity to MBP, ECP, IL-6, and RANTES.

Immunofluorescent staining characteristics of maturing eosinophils

To examine the expression and storage of granule cationic proteins (MBP, ECP) together with cytokines (IL-6, RANTES) and to characterize the immunostaining pattern of colonies collected from selected time points, cytospins of cultured cells on days 16, 19, 23, and 28 were prepared and double-labeled with BMK-13 followed by EG2, antihuman IL-6, or antihuman RANTES. Images taken from isotype controls (Fig 1, *Q* to *T*) demonstrate that there was negligible autofluorescence or nonspecific binding in these samples. In these figures isotype control images were visualized by artificial enhancement of the images.

Immunofluorescent staining of developing eosinophils with anti-MBP and anti-ECP

Analysis of the developing eosinophil immunoreactivity for MBP and ECP at day 16 indicated that most of the cells were mononuclear in appearance with a large nucleus and diffuse immunostaining. As shown in Fig 1, the pattern of immunoreactivity to MBP and ECP displayed a number of large granule-like structures, suggesting that these may localize to immature granules. The majority of these granule-like structures was spherical and exhibited intense immunoreactivity against MBP and ECP, which mostly localized to the periphery of these structures (Fig 1, *A* to *D*). At this stage of culture, ECP predominantly colocalized with MBP, which resulted in the appearance of yellow color in the combined image (Fig 1, *C* and *D*). At day 19 maturing eosinophils were mononuclear in appearance and contained a heterogeneous population of granule-like structures with various sizes (Fig 1, *E* to *H*). These structures showed heterogeneous immunostaining with intense immunoreactivity against MBP and ECP localizing to their periphery and negligible immunoreactivity in the core regions (Fig 1, *H*). On day 19 MBP and ECP colocalization was not as evident as day 16, and sites of immunoreactivity were observed that roughly coincided with either ECP (green regions in Fig 1, *E*) or MBP (red regions in Fig 1, *F*), indicating distinct localization sites for either of the granule cationic proteins (shown in combined images in Fig 1, *G* and *H*). At day 23 the immunostaining pattern of the developing eosinophils began to approach the immunofluorescent characteristics of mature peripheral blood eosinophils. Developing cells from day 23, immunostained for granular cationic proteins, showed signs of lobular formation in their nuclei (large dark shape) (Fig 1, *I* to *K*). Developing eosinophils on day 23 showed strong immunoreactivity for MBP and ECP; however, the large granular structures, which were previously seen at earlier stages of culture, were less visible because immunoreactivity against MBP and ECP appeared to localize to distinct intracellular sites with very little overlap (Fig 1, *L*). At day 28, the immunoreactivity pattern of cultured cells resembled that of mature peripheral blood eosinophils in that they exhibited bilobed nuclei and highly focused regions of immunoreactivity to MBP and ECP. Interestingly, the immunostaining pattern of some granular structures was similar to that previously described in mature peripheral blood crystalloid granules.[17] Immunoreactivity to ECP appeared to localize to the periphery of granular structures, whereas immunoreactivity to MBP localized to the core of these structures (Fig 1, *P*). Nevertheless, unlike mature peripheral blood eosinophils, which contain a large number of crystalloid granules homogeneously distributed in cytoplasm, the number of granular structures observed in cultured cells was substantially less. The reduced number of granular structures in day 28 cultured cells may be due to the release of the granules under these culture conditions because of increased fragility and cell activation (Fig 1, *M* to *P*).

Immunoreactivity of developing eosinophils to anti-IL-6 and RANTES

Developing eosinophils at day 16 of culture coexpressed MBP and IL-6 as well as MBP and RANTES. The immunostaining pattern of day 16 cells with IL-6 and MBP along with RANTES and MBP was comparable to that of MBP and ECP. Immunoreactivity for both IL-6 (Fig 2) and RANTES (Fig 3) predominantly colocalized with granule proteins, indicating a similar storage pattern for these mediators at early stages of development. At day 19 of culture, immunoreactivity against IL-6 and RANTES was observed to be localized to the granular structures similar to those observed with ECP. However, unlike ECP and MBP, which were distributed in distinct compartments, IL-6 and RANTES colocalized strongly with MBP as indicated by yellow color in the combined images (Figs 2 and 3, *C, F, I,* and *L*). By day 23 of culture, immunoreactivity to IL-6 and RANTES in developing eosinophils localized to a population of smaller granular structures. On day 28, immunoreactivity to IL-6 and MBP in addition to RANTES and MBP showed immunostaining patterns in developing eosinophils that approached that of mature peripheral blood eosinophils. Immunoreactivity to IL-6 and RANTES in day 28 cells localized to a population of granular structures, which exhibited an immunostaining pattern suggestive of the appearance of crystalloid granules. Although immunoreactivity to IL-6 and RANTES localized to the periphery of the granular structures, MBP staining localized to the core regions of these structures (Figs 2 and 3, *J* to *L*).

DISCUSSION

The main objective of our study was to investigate the expression and storage of eosinophil-derived cytokines/chemokines in comparison with granule cationic proteins in cord blood $CD34^+$ cell–derived maturing eosinophils. In vitro culture of $CD34^+$ cells in the presence of appropriate cytokines has provided an excellent tool for researchers in the field of hemopoiesis to study leukocyte

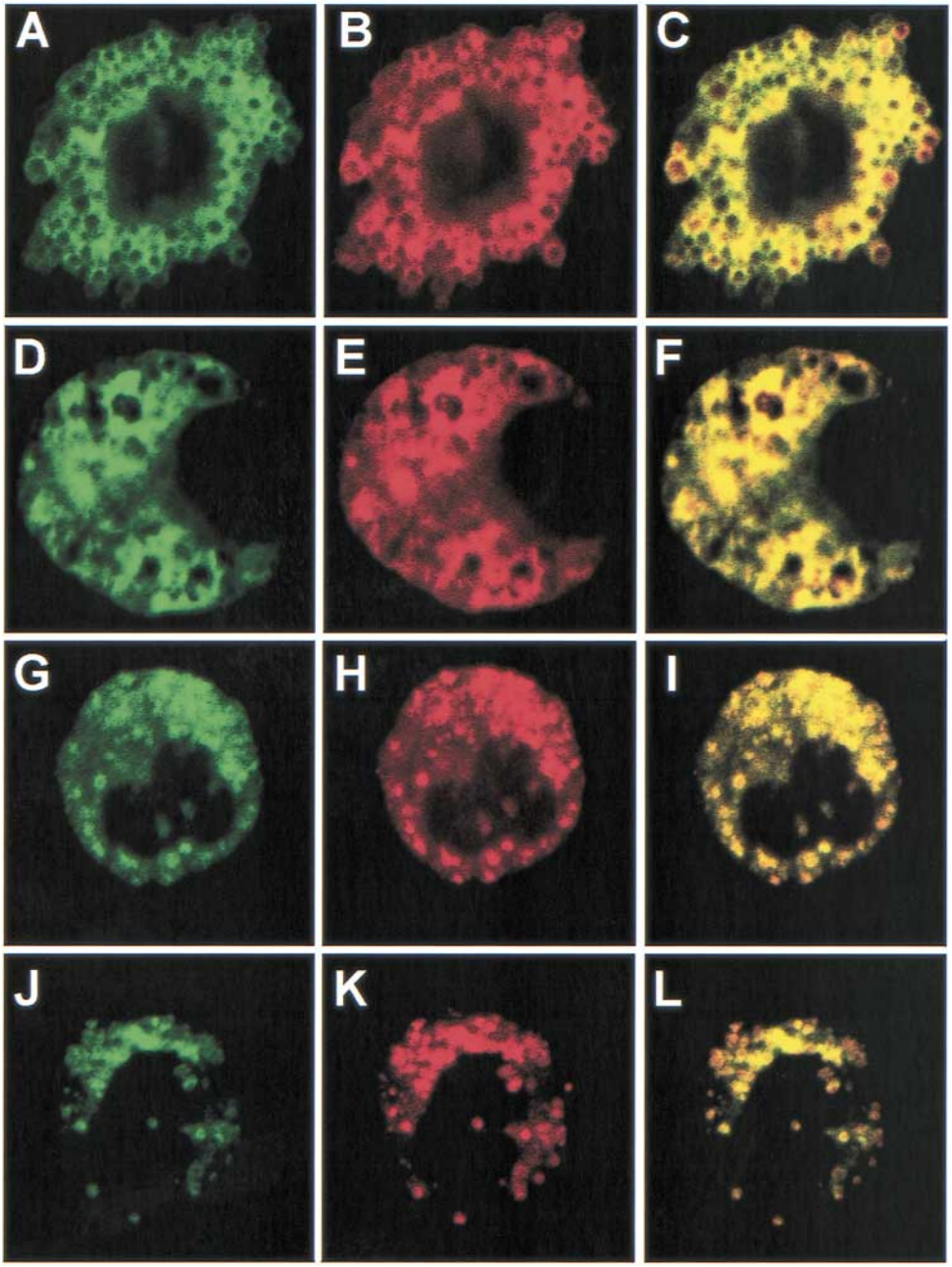

FIG 2. CLSM images of developing eosinophils immunostained for MBP and IL-6. Developing eosinophils were labeled with BODIPY FL (green) representing IL-6 staining at days 16 **(A),** 19 **(D),** 23 **(G),** 28 **(J),** and TRITC (red) demonstrating MBP staining at days 16 **(B),** 19 **(E),** 23 **(H),** and 28 **(K).** *Right,* Combined MBP and RANTES immunoreactivity in developing eosinophils at days 16 **(C),** 19 **(F),** 23 **(I),** and 28 **(L).**

maturation and differentiation. We have combined this method with double immunofluorescent staining together with CLSM to examine the evolution of the expression pattern of cytokines/chemokines and granule cationic proteins during maturation and differentiation.

We chose to examine the expression and storage pattern of four eosinophil-derived mediators, namely, MBP, ECP, IL-6, and RANTES. The intracellular storage patterns of MBP and ECP are well known in circulating and tissue eosinophils.[1,11,12]

Although EG2[23] was originally described as immunoreactive for the extracted form of ECP in activated eosinophils, our studies have shown that it is also immunoreactive with the granular stored form of ECP, similarly to a recent study by Nakajima et al.[24] In addition, we chose to investigate the protein expression for IL-6 and RANTES as representatives of eosinophil-derived cytokines and chemokines, respectively. IL-6[25,26] and RANTES[27,28] are thought to be important in the development and maintenance of the inflammatory response in the airways.

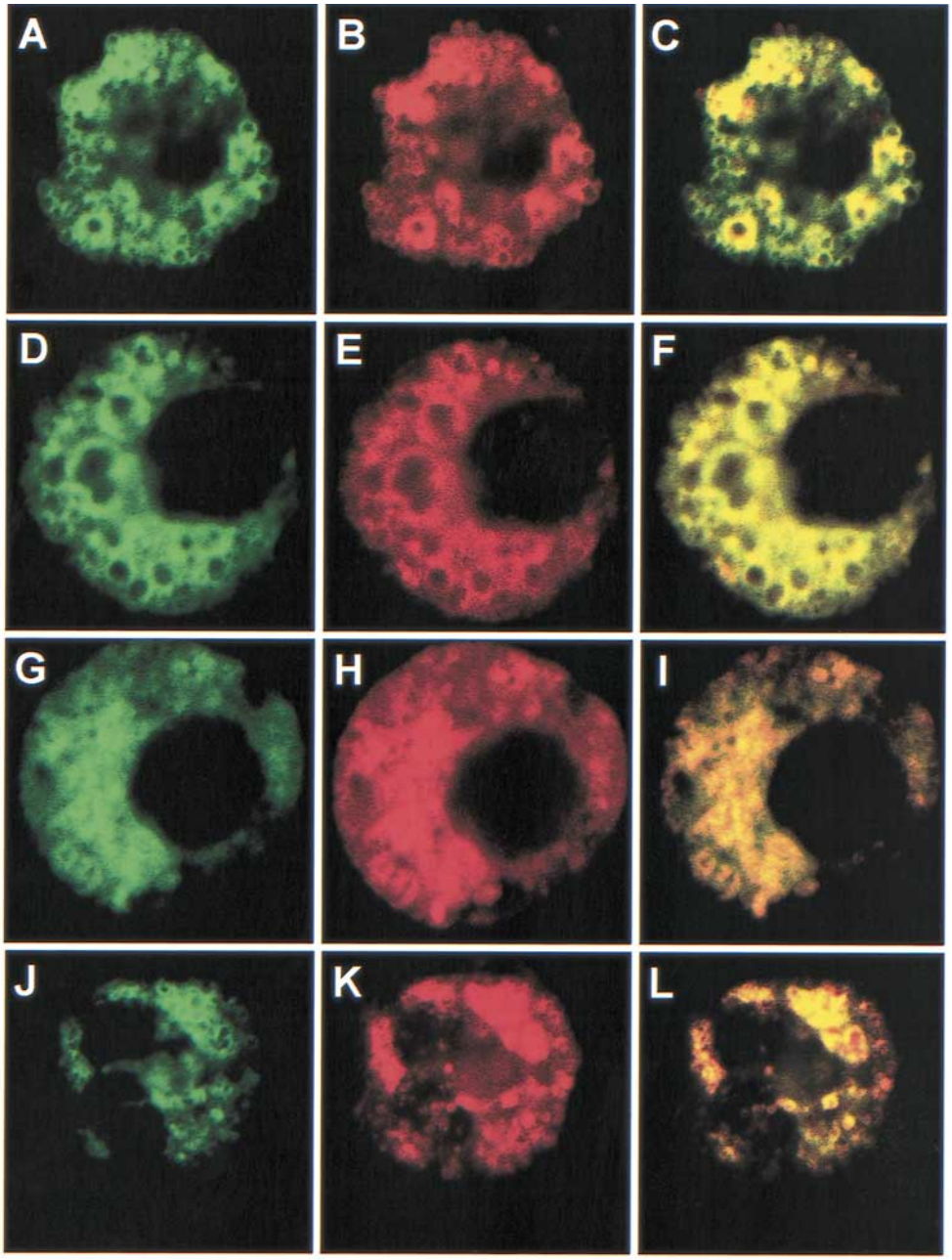

FIG 3. Images of developing eosinophils immunostained for MBP and RANTES. Green color represents RANTES immunostaining in developing eosinophils at days 16 **(A),** 19 **(D),** 23 **(G),** and 28 **(L),** whereas red color is associated with MBP immunostaining at days 16 **(B),** 19 **(E),** 23 **(H),** and 28 **(K).** Combined images for each row are shown in (**C, F, I,** and **L**).

Our observations suggest that MBP, ECP, IL-6, and RANTES are not expressed at detectable levels in freshly purified $CD34^+$ cells. However, in our hands, they appeared to be fully expressed at the protein level in maturing cells at day 16 of culture. At this stage, all eosinophil-derived mediators included in this study appeared to be associated with large, hollow-cored granules, an observation in agreement with earlier study by Scott and Horn.[21] and Popken-Harris et al.[22] Interestingly, on day 16, only a few granules showed exclusive immunostaining for MBP. This may be due to a differential mediator storage pattern among immature granules. At later stages of maturation (day 19), granules appeared more condensed with intensely colocalizing immunoreactivity for MBP and all other mediators studied.

It is tempting to speculate that the large granules present at day 19 in developing eosinophils may have formed by condensation of numerous hollow-cored granules observed at day 16. Interestingly, at days 19 and 23 of culture, ECP showed a slightly distinct pattern of immuno-

staining from that of MBP, IL-6, and RANTES. Yet at more advanced stages of maturation (day 28 of culture), the distribution of immunoreactivity for ECP largely resembled those of IL-6 and RANTES. This may well indicate differential mediator packaging throughout eosinophil maturation under these culture conditions.

A large proportion of immunoreactivity to these mediators in early stages of culture appeared to localize to cytoplasmic regions in these cells. It is important to emphasize that these mediators may also be stored in small secretory vesicles, which may result in an immunostaining pattern resembling that of "cytoplasmic" distribution. This has been demonstrated for RANTES immunoreactivity, which appeared to localize to a population of small secretory vesicles in mature peripheral blood eosinophils.[17]

Although the morphologic and biosynthetic properties of maturing human eosinophils have been partially investigated by a number of laboratories,[19-22,29,30] our work is the first to examine the differential expression and storage of cytokines/chemokines during eosinophil development. The early expression of these mediators is potentially important because it may indicate that maturing eosinophils have the potential to exhibit local immunoeffector and immunoregulatory function during differentiation and maturation in the bone marrow as well as local tissues. Indeed, maturing eosinophils generated from bone marrow–derived $CD34^+$ cells are capable of mediator release at early stages of their development.[31] It will be of interest to quantify these mediators, although in the current study this could not be pursued because of practical considerations, such as limited cell numbers and the high viscosity of the methylcellulose used to grow these cells. However, we emphasize that, in this respect at least, cord blood–derived eosinophils in vitro are likely to be phenotypically distinct from those produced within the bone marrow in vivo. The findings of this study should contribute to our understanding of cytokine synthesis in early progenitor cells as well as developing eosinophils.

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