Interleukin-6 Expression in Human Neutrophil and Eosinophil Peripheral Blood Granulocytes

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Human peripheral blood granulocytes were analyzed for expression of interleukin-6 (IL-6) using reverse-transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization. Neutrophil granulocytes from healthy donors were shown to express variable levels of IL-6. This expression was rapidly down-regulated after the removal of the cells from the circulating blood. In vitro culture of neutrophils abolished IL-6 expression, which could be reactivated by addition of GM-CSF to the culture medium. Constitutive expression of IL-6 was instead demonstrated in eosinophil granulocytes purified from normal donors and from a hypereosinophilic patient. In situ hybridization of unstimulated granulocytes confirmed that IL-6 expression occurs both in eosinophils and in neutrophils from peripheral blood. These findings show that granulocytes can actively contribute to cytokine expression in the peripheral blood and suggest their role in the afferent limb of the immune response, since by IL-6 production they might modulate T- and B-lymphocyte functions, granulocyte self-priming, and endothelial interaction.

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MATERIALS AND METHODS

Approval was obtained from the Institutional Review Board for these studies. Volunteers were informed that blood samples were obtained for research purpose, and that their privacy would be protected.

Cell purification and culture. Polymorphonuclear granulocytes (PMN) were isolated from the buffy-coat cells of healthy donors or from heparinized peripheral blood collected by venipuncture from healthy volunteers. Mononucleated cells were isolated by Ficoll-Hypaque density gradient (Pharmacia, Uppsala, Sweden); neutrophils were recovered from the cell pellet by further sedimentation through dextran (2% final concentration). Erythrocytes were removed by hypotonic lysis. Mononucleated cells were enriched in monocytes by adherence to culture dishes for 30 minutes and removal of nonadherent cells by washing with cold phosphate-buffered saline (PBS); adherent cells were gently detached with rubber scrapers. Eosinophils were obtained for research purpose, and that their privacy would be protected.

Neutrophils and eosinophils were mainly documented by their ability to produce platelet-activating factor (PAF) and cytokines such as IL-1, IL-8, tumor necrosis factor α (TNFα), G-CSF, macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and transforming growth factor α (TGFα), TGFβ, IL-1, IL-3, and GM-CSF, respectively.

IL-6 is a pleiotropic lymphokine with the ability to stimulate cell proliferation and cytotoxic function in T lymphocytes.17 IL-6 gene expression has not been reported in eosinophils and its expression in neutrophils is controversial because of possible monocyte contamination of neutrophil preparations. We investigated whether IL-6 RNA is detectable in granulocytes either by RT-PCR, to detect low level of RNA, or by in situ hybridization, to confirm morphologically which cells express the gene. Here we present evidence for IL-6 expression in both eosinophils and neutrophils.

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chlordode gradient and reverse-transcribed using 0.5 μg of oligo-dT primer (Clontech, Palo Alto, CA) and Moloney murine leukemia virus-reverse transcriptase (MMLV-RT; BRL, Gaithersburg, MD) for 2 hours at 42°C. cDNA, 2 μL, was used for amplification of β2-microglobulin (to check for effective cDNA synthesis) or IL-6 using specific primers (Clontech) according to the protocol provided by the manufacturer. After 30 cycles of amplification, 8 of 50 μL of the PCR product was run on a 1.5% agarose gel and transferred to nylon filters by alkaline blotting. Semi-quantitative PCR was performed for IL-6 according to the method of Dallman et al. The densitometric scanning of the β2-microglobulin–amplified band was used to normalize the cDNA among the samples. Filters were hybridized as described with 0.5 to 1.0 × 10⁶ cpm/mL of 3²P-labeled probe, which was the 587-bp Cfo I internal fragment obtained from the IL-6 amplified product of human phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes.

In situ hybridization. Freshly isolated cells were centrifuged on poly-(l-lysine)–coated slides, fixed in 4% paraformaldehyde (10 minutes at 4°C), and dehydrated through ethanol solution. The slides were soaked in 0.25% acetic anhydride in 0.1 mol/L triethanolamine solution pH 8 for 10 minutes to minimize nonspecific background binding.

A 628-bp cDNA was amplified by PCR from PHA-stimulated peripheral blood lymphocytes and cloned in pGEM-3 vector. Antisense and sense RNA probes were transcribed according to standard procedures and labeled with 3²S-uridine triphosphate. Hybridization was performed at 42°C/50°C in a humid chamber for 18 to 20 hours, in 50% formamide, 20 mmol/L Tris-HCl pH 8.0, 0.3 mol/L NaCl, 5 mmol/L EDTA, 10% Dextran sulphate, 1 × Denhardt solution, and 3 mg/mL salmon sperm DNA, containing a probe concentration of 5 × 10⁵ cpm/mL (50,000 cpm/slide).

The slides were washed twice in 50% formamide-2× SSC (1× SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate) for 30 minutes at 45°C. RNAse A treatment (20 μg/mL) to remove nonspecifically bound single-stranded probe was performed at 37°C for 30 minutes in appropriate buffer. Slides were then washed twice in 0.1× SSC (10 minutes each at room temperature) and finally dehydrated in graded ethanol solutions and air-dried. Autoradiography was performed at 42°C using nuclear track emulsion (Kodak type NTB-2; Eastman Kodak, Rochester, NY) diluted 1:1 with water and performed for 10 days at 4°C in light-tight boxes containing a drying agent. The slides were developed at 18°C in Kodak D-19 (diluted 1:1) for 2 minutes, fixed with Kodak fixer (Unifix diluted 1:2), and stained with May–Grunwald Giemsa.

RESULTS

RNA from peripheral blood–purified granulocytes was reverse-transcribed and the resulting cDNA amplified with β2-microglobulin and IL-6 primers. As a control, we used total RNA as a template in PCR, to exclude DNA contamination, and endonuclease digestion with Taq I, Mbo I, and Cfo I to confirm IL-6 specificity (Fig 1). Southern blots were hybridized with the IL-6 cDNA probe and the hybridization signal was stronger in neutrophil granulocytes from immediately processed blood withdrawals than in neutrophils from donors’ buffy-coat cells provided by the blood bank (Fig 2A). However, IL-6 was inducible in low-expressing samples by a 1-hour incubation with 50 ng/mL recombinant huGM-CSF (Fig 2A, lanes 6 and 7). When granulocytes expressing IL-6 were left in DMEM for 1 hour in ice or at 37°C, IL-6 transcript was no longer detectable, but was still inducible by addition of GM-CSF (Fig 2B).

To evaluate the amount of IL-6 transcribed by cells contaminating our preparations, we performed a semi-quantitative PCR on cDNA from (1) a preparation of more than 98% pure neutrophils with less than 2% contaminating eosinophils and lymphocytes, (2) a mononuclear cell population enriched in monocytes, and (3) eosinophil granulocytes obtained from a normal donor (>82% pure) and from a patient with IL-2–induced hypereosinophilia (>96% pure). The results shown in Fig 3 indicate that the low number of contaminating cells of each population examined cannot by itself account for the amount of IL-6 RNA detected. In fact, the eosinophil preparations (lanes 2 and 3) displayed a signal quantitatively similar to that shown by the neutrophil granulocyte preparation (lane 1). As expected, the monocyte-enriched population expressed the highest level of IL-6 (Fig 3, lane 4) and the positive signal was already detectable after 20 cycles of amplification; however, both unstimulated neutrophil and eosinophil granulocytes from normal donors (lanes 1 and 2) and the eosinophils of the hypereosinophilic patient (lane 3) also expressed IL-6 (Fig 3). This suggests that the signal of neutrophils (lane 1) cannot be due to the weak eosinophil contamination.

These results were confirmed by in situ hybridization. IL-6 antisense probe positively hybridized 50% of the neutrophils immediately purified from whole peripheral blood (Fig 4C through E), but not the neutrophils isolated from buffy coats.
Fig 2. Expression and modulation of IL-6 in neutrophil granulocytes. (A) Hybridization of RT-PCR product shows a strong expression of IL-6 in unstimulated PMN from fresh peripheral blood (lanes 1-3), and a weaker expression in PMN from buffy-coat bags of a blood bank (lanes 4, 5), which can be induced by treatment with GM-CSF (lanes 6, 7, respectively). Negative (no cDNA, lane 8) and positive (cDNA from PHA-stimulated peripheral blood lymphocytes, lane 9) controls are included. At the bottom, β2-microglobulin amplification is shown that has been used to normalize the amount of cDNA among the samples. (B) Down-regulation and induction of IL-6 expression in three samples of neutrophil granulocytes from fresh peripheral blood. Lanes 1, 4, 7: unstimulated PMN; lanes 2, 5, 8: PMN in culture with DMEM for 1 hour; lanes 3, 6, 9: PMN treated with GM-CSF for 1 hour after 1 hour of culture in DMEM; lanes 10, 11: negative and positive controls, respectively, as in (A). At the bottom, the ethidium bromide staining of the gel with the β2-microglobulin amplification from the corresponding cDNAs confirms the efficacy of the reverse transcription.

(Fig 4G). We hypothesized that a rapid down-regulation of IL-6 occurred in neutrophils during the time elapsing between the venipuncture and the slide preparation, which is necessarily longer for blood samples taken from a blood bank; therefore, the low mRNA level could remain detectable after PCR amplification (Fig 1, lanes 4 and 5), but not after in situ hybridization (Fig 4G). Eosinophils were all positively hybridized (Fig 4F), even when purified from buffy coats (Fig 4G).

Both RNAse pretreatment of the slides (not shown) and hybridization with the sense probe (Fig 4A and B) failed to show any positive signals. Neutrophil, but not eosinophil, cell integrity was affected by certain hybridization conditions, eg, hybridization temperature of 50°C. A reduced hybridization temperature (42°C) allowed both the recognition of neutrophils for their nuclear morphology and the hybridization with the IL-6 riboprobe (Fig 4E) without affecting hybridization specificity, because no positive signal was detected with the sense probe (Fig 4B).

DISCUSSION

Human granulocytes have been considered for a long time as cells with reduced biosynthetic activity but, more recently, it has been shown that PMN are able to produce many different cytokines upon appropriate stimulation. Unstimulated expression of cytokine mRNA has been described for TNFα in neutrophils and TGFα in eosinophils. Human peripheral blood granulocytes have been shown to express IL-6 in response to stimulation
Fig 4. In situ hybridization of human peripheral blood granulocytes. Bright- (A and C) and dark- (B and D) field visualization of neutrophil granulocytes hybridized to IL-6 sense (A and B) and antisense (C and D) riboprobe; specific hybridization is detected with the antisense IL-6 in the neutrophils immediately processed after blood collection (original magnification ×150). Original magnification of (E) a positively hybridized neutrophil (×1,250) and (F) eosinophils (×1,000). (G) Hybridization of a mixture (1:1) of neutrophil and eosinophil granulocytes prepared from a blood bank bag shows that a rapid down-modulation of IL-6 expression occurs in neutrophils, but not in eosinophils, when a longer time elapses between blood collection and slide preparation (original magnification ×750).
with GM-CSF or TNFα,22 but constitutive expression of IL-6 has not yet been reported.

IL-6 has been detected in murine circulating granulocytes by immunohistochemical analysis,25 but the corresponding mRNA was undetectable at the Northern blot assay, while it was evident in the bone marrow myeloid precursors, thus suggesting that circulating PMN were only able to store IL-6 synthesized in the early stage of their maturation.

Using RT-PCR to increase the sensitivity of mRNA detection, we found IL-6 expression in circulating, unstimulated human granulocytes, both neutrophils and eosinophils, and confirmed this finding by in situ hybridization.8

Bazzoni et al26 were not able to detect IL-6 transcript by Northern blot in neutrophils stimulated with either lipopolysaccharide or IgG-opsonized yeast, and they argued that IL-6 expression reported by Cicco et al27 in GM-CSF- and TNFα-stimulated neutrophils could be due to contaminating monocytes. We used a semiquantitative RT-PCR assay on highly purified cell populations to burst the sensitivity of the detection and minimize the contribution of the contaminating cells to the final amount of the amplified product. Our results confirm that peripheral blood granulocytes are able to transcribe IL-6 mRNA constitutively, even though mononuclear cells express this cytokine at higher levels.26-28

In neutrophils, IL-6 expression was rapidly up-modulated by GM-CSF or down-regulated by unknown starvation factor or handling procedures, since differences were found between neutrophils prepared from heparinized peripheral blood immediately after withdrawal and cells purifed from buffy coats of blood bank donors. We postulate that the time lag between venipuncture and cell separation, longer than 1 hour in the case of buffy-coat cells and less than 15 minutes in the other cases, can affect IL-6 expression. However, differences in the anticoagulant used (heparin for peripheral blood of volunteers, citrate/phosphate/dextrose/adenine in the case of buffy coats from the blood bank) cannot be ruled out.

In eosinophils isolated from subjects with atopy or helminthic infection, IL-6 expression and secretion, as well as its up-regulation by interferon-γ, has been documented in vitro.29 These investigators were concerned whether circulating eosinophils express IL-6 constitutively, and their data were obtained in activated eosinophils cultured for 24 hours. While confirming IL-6 expression, our results indicate that the mRNA is detectable in freshly isolated eosinophils in both normal individuals and hyper eosinophilic patients treated with IL-2.

The role of IL-6 expressed by circulating PMN is currently unknown. The broad spectrum of action of this cytokine includes the ability to “prime” PMN, which enhances superoxide secretion.30 One can speculate that IL-6 expressed by circulating granulocytes could be used for a rapid priming of the other PMN recruited at the same time by a PMN chemotactic factor.

The quantitative dominance of granulocytes in the peripheral blood could compensate for the low amount of IL-6 transcript detected in unstimulated leukocytes and suggests that IL-6 may exert other functions in addition to the acute-phase response and inflammation, by participating to the signals governing cell-cell and cell-endothelium cross-talk. Moreover, it has been found that IL-6 can induce the expression of ICAM-1 in canine myocytes and greatly enhance the CD18-mediated adhesion of neutrophils to these cells31; granulocytes could therefore be able to modulate their own capacity to adhere to mesenchymal cells by expressing IL-6.

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REFERENCES


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