

Identification of cytokine-regulated genes in human leukocytes in vivo

Cornelieke E. G. M. Pals, BSc, Sandra A. B. W. Verploegen, MSc, Jan A. M. Raaijmakers, PhD, Jan-Willem J. Lammers, MD, PhD, Leo Koenderman, PhD, and Paul J. Coffer, PhD *Utrecht, The Netherlands*

Background: Human polymorphic nuclear granulocytes (PMNs) such as neutrophils and eosinophils play a critical role in mediating inflammatory responses to microbial and parasitic infections. Exposure of these leukocytes to cytokines leads to an amplification of granulocyte effector functions by a mechanism termed "priming." Although many studies have investigated the effects of granulocyte priming, little is known concerning the molecular mechanisms that lead to this phenomenon.

Objective: The purpose of this study was to identify potential markers for granulocyte priming and thus also to gain further insight into the pathogenesis of inflammatory responses.

Methods: We used a modified differential display technique, random arbitrary primed-PCR to identify genes regulated during the priming of human polymorphic nuclear granulocytes by GM-CSF in vitro. Genes identified were validated by Northern blot analysis of in vitro and in vivo primed leukocytes.

Results: Several genes were identified and their expression characterized in vitro. One of these genes, 5-lipoxygenase-activating protein, was also found to be up-regulated in leukocytes isolated after allergen challenge of allergic asthmatic patients.

Conclusion: The use of differential display technology is a rapid and effective means of identifying genes whose expression is regulated by priming in vitro and in vivo. Further analysis will lead to a better understanding of the priming phenotype and may provide further insight into the pathologic mechanisms of inflammatory processes. (*J Allergy Clin Immunol* 2000;105:760-8.)

Key words: *Differential display, leukocyte, neutrophil, allergy, priming, GM-CSF, 5-lipoxygenase-activating protein, PCR*

Human polymorphic nuclear granulocytes (PMNs) such as neutrophils and eosinophils play a critical role in mediating inflammatory responses to microbial and parasitic infections.¹ At the site of infection granulocytes participate in the inflammatory reaction by phagocytosis, intracellular killing of bacteria, production of inflamma-

Abbreviations used

cDNA:	Complementary DNA
CO ₂ :	Carbon dioxide
EST:	Expressed sequence tag
FLAP:	5-Lipoxygenase-activating protein
ILT-2:	Ig-like transcript-2
mRNA:	Messenger RNA
oligo-dT:	Oligodeoxythymidine
PAF:	Platelet-activating factor
PC ₂₀ :	Provocative concentration causing a 20% fall in FEV ₁
PMN:	Polymorphic granulocyte
RAP-PCR:	Random arbitrary primed-PCR

tory mediators, and release of preformed cytotoxic enzymes and proteins. Environmental factors can quickly amplify these granulocyte functions by a mechanism termed "priming," which is independent of protein synthesis.²⁻⁴ Priming refers to a process by which the response of cells to a subsequent (activating) stimulus is amplified if these cells were previously exposed to a (priming) stimulus. This process is an important regulatory mechanism whereby phagocytes can be controlled while responding to the local conditions of their environment. For example, prior exposure of human eosinophils to either the cytokine GM-CSF or the inflammatory mediator platelet-activating factor (PAF) will greatly enhance the ability of these cells to be activated by subsequent exposure to opsonized particles.^{5,6} Although many studies have analyzed the effects of granulocyte priming, little is known concerning the molecular mechanisms that lead to this phenomenon. Recent studies have demonstrated the cytokine-mediated activation of various intracellular signaling pathways in human granulocytes, whereas the use of specific pharmacologic inhibitors has implicated some of these events in the regulation of cell effector functions.⁷⁻¹² It is, however, unclear whether cytokine stimulation may also result in changes in granulocyte gene expression that might correlate with this phenomenon. Indeed, cytokine-induced regulation of gene expression in human granulocytes and the functional consequences thereof have not been studied in any detail.

In an attempt to gain further insight into the pathogenesis of inflammatory responses and also to identify potential markers for granulocyte priming, we used the technique of differential display.¹³ This PCR-based technique allows the

From the Department of Pulmonary Diseases, University Medical Center, Utrecht, The Netherlands.

Received for publication June 21, 1999; revised Nov 4, 1999; accepted for publication Nov 12, 1999.

Reprint requests: Paul J. Coffer, PhD, Department of Pulmonary Diseases, G03.550, University Medical Center, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands.

Copyright © 2000 by Mosby, Inc.

0091-6749/2000 \$12.00 + 0 1/1104382

doi: 10.1067/mai.2000.104382

identification and cloning of genes that are differentially expressed in cells under predetermined conditions. The advantage of this technique lies in its ease of operation and the ability to process many samples at one time, unlike previous methods such as subtractive hybridization. With use of a modified differential display technique, random arbitrary primed-PCR (RAP-PCR),¹⁴ we have analyzed the expression of genes either up- or down-regulated in human PMNs after treatment with physiologic concentrations of GM-CSF. Several known genes were identified *in vitro* and their regulation by GM-CSF was confirmed by Northern blotting. Furthermore, we demonstrate that one of the genes identified, 5-lipoxygenase-activating protein (FLAP), was also up-regulated *in vivo* in peripheral blood leukocytes after allergen challenge.

These data confirm that differential-display technology can indeed be used to gain further insight into the mechanisms of granulocyte priming and the pathogenesis of inflammatory reactions.

MATERIAL AND METHODS

Isolation of human granulocytes

Blood was obtained from a healthy donor with no history of allergy or asthma. There was no relevant clinical history (no allergy, no parasitic infection, no indications of other eosinophilic diseases). However, a persistent (>10 years) finding in blood of slight leukocytosis ($\sim 8 \times 10^9$ leukocytes/L) and 30% eosinophils in the granulocyte fraction was observed. Apart from the elevated amount of eosinophils the remainder of the leukocyte population was within the normal range. Total leukocytes numbered 7.3×10^9 /L: 2% basophils, 18% eosinophils, 34% neutrophils, 35% lymphocytes, and 11% monocytes.

Mononuclear cells were removed by centrifugation over Ficoll-Paque (density 1077/cm³), 1000g, 20 minutes at room temperature. The lower cell layer was collected and the remaining erythrocytes were lysed by incubation in isotonic 115 mmol/L ammonium chloride solution (pH 7.4) on ice for 20 minutes. After centrifugation the granulocytes were incubated in RPMI 1640 (Life Technologies, Paisley, UK) containing 0.5% horse serum albumin (wt/vol) for 30 minutes at 37°C. Cells were washed with PBS and half the cells were stimulated with GM-CSF (10^{-10} mol/L) for 3 hours in RPMI medium supplemented with 25 mmol/L HEPES, 8% FCS (Hyclone), 1 mg/mL penicillin, and 1 mg/mL streptomycin in a carbon dioxide (CO₂) incubator (5% CO₂, 37°C).

RNA isolation

Total RNA was isolated as follows: 10^8 cells were resuspended in 500 μ L of sterile PBS, and 10 μ L of RNAsin (Promega, Madison, Wis) was added. The cells were lysed in 20 mL of GIT solution (6 mol/L guanidine thiocyanate, 25 mmol/L sodium citrate, 0.5% N⁻-lauroyl sarcosine, 100 mmol/L β -mercaptoethanol). One volume of sodium acetate (2 mol/L, pH 4.0) was added and an equal volume of phenol, followed by vortexing. Half the total volume of chloroform/isoamylalcohol was added to the solution and vortexed. The solution was incubated on ice for 15 minutes and centrifuged at 4°C for 20 minutes at 10,000g. The upper phase was extracted with a half volume of chloroform/isoamylalcohol and centrifuged for 2 minutes. RNA was precipitated with an equal volume of 2-propanol and 200 mg of carrier glycogen at -20°C overnight. Samples were centrifuged at 81,000 revolutions/min for 1 hour and resuspended in a small volume, and RNA was reprecipitated with ethanol. Poly A⁺ messenger RNA (mRNA) was extracted by oligodeoxythymidine

(oligo-dT) affinity purification with use of Dynabeads Oligo(dT)25 (Dynal, Oslo, Norway). Complementary DNA (cDNA) was synthesized with use of a ProStar First Strand reverse transcription-PCR kit as described by the manufacturer's protocol (Stratagene).

RAP-PCR

The differential display PCR reactions were performed with 1 μ L of cDNA, 1 $\times$ Goldstar reaction buffer, 1.5 mmol/L magnesium chloride, 200 ng of primer, 2 mmol/L deoxyribonucleoside triphosphates, 1 μ Ci of phosphorus 33-labeled deoxyadenosine triphosphate and 4 units of Goldstar Taq polymerase (Eurogentec, Seraing, Belgium) per 20- μ L reaction. Cycles were performed as follows: 3 minutes at 96°C; two cycles: 1 minute at 96°C, 5 minutes at 37°C, 5 minutes at 37°C to 72°C; thirty cycles: 1 minute at 96°C, 2 minutes at 50°C, 5 minutes at 72°C; and 10 minutes at 72°C. The annealing temperature was increased stepwise from 37°C to 50°C, allowing the arbitrary primer to bind over multiple homologous regions. From the PCR reaction, 5 μ L was mixed with 5 μ L of 96% formamide loading buffer (0.05% [wt/vol] xylene cyanol, 0.05% [wt/vol] bromophenol blue) and boiled for 2 minutes. The amplified cDNAs were loaded on a 6% sequencing gel and subjected to electrophoresis. Gels were vacuum dried and autoradiographed with Biomax MR film (Eastman Kodak, Rochester, NY) at -70°C. The dried gel and autoradiogram were orientated and cDNA bands of interest were excised and immersed in 100 μ L of TE (10 mmol/L TRIS-chloride, pH 8.0, 1 mmol/L EDTA). DNA was eluted by twice freezing and thawing in liquid nitrogen and vortexing. Samples were extracted twice with phenol/chloroform/isoamylalcohol (25:24:1) and once with chloroform/isoamylalcohol. cDNA was reamplified by use of 5 to 10 μ L in a standard 50 μ L PCR reaction and with use of the primer used for the original differential display (annealing temperature 50°C). The mixture was separated by agarose gel electrophoresis, bands were excised, purified with Glassmax (Life Technologies, Paisley, UK), and cloned in pGEM-T (Promega, Madison, Wis) or PCR-script (Stratagene, La Jolla, Calif). Plasmid DNA was isolated from 5 to 8 colonies per excised band and inserts were sequenced.

Northern blotting analysis

PMNs (97% neutrophils, <3% eosinophils) were isolated from blood obtained from healthy volunteers from the Red Cross Blood Bank, Utrecht, The Netherlands, as described above. Cells were incubated in the presence or absence of GM-CSF (10^{-10} mol/L) or TNF- α (100 U/mL) in RPMI medium (Life Technologies) supplemented with 25 mmol/L HEPES, 8% FCS, 1 mg/mL penicillin, and 1 mg/mL streptomycin in a CO₂ incubator (5% CO₂, 37°C). Cells were washed twice with PBS and total RNA was isolated with use of the acid-phenol method.¹⁵

Twenty micrograms of RNA was lyophilized and separated on a 0.8% agarose formaldehyde gel. RNA was transferred to Hybond-N membranes (Amersham, Rainham, UK) by capillary transfer with 10 $\times$ SSC (1.5 mol/L sodium chloride, 0.15 mol/L sodium citrate) overnight. The blots were washed and baked for 2 hours at 80°C. Differential display fragments were used as probes and were labeled with α -[³²P]-deoxycytidine triphosphate by Rediprime random priming according to the manufacturer's protocol (Amersham). Blots were hybridized overnight at 42°C, washed subsequently with 2 $\times$ SSC/0.1% SDS, 1 $\times$ SSC/0.1% SDS, 0.1 $\times$ SSC/0.1% SDS and exposed to film (Kodak, Rochester, NY). A 1.4-kb cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase gene was used to control for equal loading.

Allergen challenge

Patients used for this study had a history of periodic wheeze and demonstrated a reversibility of more than 15% in FEV₁ 15 minutes

TABLE I. PC₂₀ and effects of allergen inhalation challenge on lung function

Allergen	PC ₂₀ histamine (mg · mL ⁻¹)	Specific IgE (kU · L ⁻¹)	Fall in FEV ₁ from baseline <20 min (%)	Fall in PEF from baseline (%)	
				<20 min	3-8 h
Der p	0.21	57	29	28	30
Cat	0.16	59	27	18	13

Allergen inhalation challenge was performed with house dust mite (*Dermatophagoides pteronyssinus* [Der p]) or cat allergen. After PC₂₀ allergen challenge, percentage fall in FEV₁ from baseline within 20 minutes and percentage fall in peak expiratory flow from baseline within 20 minutes and 3 to 8 hours were measured. PEF, Peak expiratory flow.

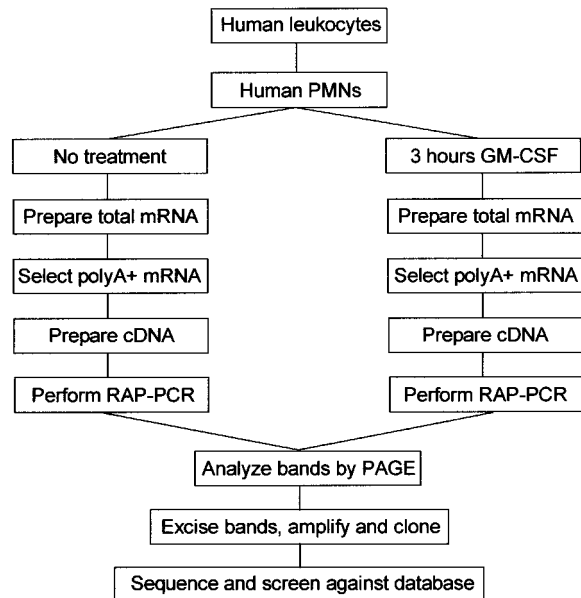


FIG 1. Flow diagram of RAP-PCR method. Human PMNs are isolated from 500 mL of whole blood and cultured for in presence or absence of GM-CSF. Total RNA is isolated, and poly-A⁺ mRNA and cDNA are prepared and subjected to RAP-PCR analysis. Up- or down-regulated bands were excised, cloned, and sequenced. See Material and methods for full details.

after inhalation of 400 µg of salbutamol from a pressurized metered-dose inhaler attached to an aerosol chamber (Volumatic, Allen and Hanburys, Greenford, UK). None of them had used oral corticosteroids in maintenance doses during the 12 months before the study. All subjects were clinically stable at the time of the study and had no evidence of systemic or respiratory disease. On the day of the experiment the FEV₁ value was >60% predicted. Furthermore, patients were required to show an increased bronchial hyperresponsiveness to histamine with a provocative concentration causing a 20% fall in FEV₁ of less than 4.0 mg · mL⁻¹ (PC₂₀) and a fall in FEV₁ within 1 hour after allergen inhalation challenge of at least 20%. All patients had blood eosinophilia (>5%) and raised levels of total serum IgE and specific IgE antibodies to either house dust mite or cat. Patient data are summarized in Table I.

The bronchial provocation with allergen was performed by 2-minute inhalations (tidal breathing) with 10-minute intervals. Allergen provocation was preceded by an initial aerosol of buffer (2.5 mg/mL sodium bicarbonate with 5 mg/mL sodium chloride, 0.03% human albumin solution and 0.5% phenol) to which none of the patients reacted with a fall in FEV₁. Increasing concentrations of antigen aerosol were delivered through a nebulizer (model 646, de Vilbiss, Somerset, Pa). House dust mite antigen (10,000 biological

units/mL, Allergy Laboratory, Copenhagen, Denmark) and cat (10,000 biological units/mL, Allergy Laboratory) were diluted from stock solution, and the dilution used for the first inhalation was calculated from the skin-prick test and the preantigen PC₂₀ for histamine according to Cockcroft et al.¹⁶ Inhalations were performed by stepwise doubling the dose of antigen until the FEV₁ fell at least 20% from baseline values. Blood was collected at various time points after allergen challenge as indicated through an intravenous catheter and was anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) and kept at 4°C. Total leukocytes were isolated after isotonic lysis of the erythrocytes in ice-cold ammonium chloride solution containing 155 mmol/L ammonium chloride, 10 mmol/L potassium carbonate, and 0.1 mmol/L EDTA (pH 7.2). Total RNA isolation, blotting, and hybridization were performed as previously described.

RESULTS

Method of differential screening for cytokine-induced genes

Little is currently known concerning the regulation of genes expressed in response to cytokine challenge of human granulocytes. Genes that are regulated by cytokines may be used as potential markers for preactivation or "priming" of these phagocytes. Furthermore, these genes may provide greater insight into the pathogenesis of inflammatory disorders. To identify differentially expressed genes, several approaches have been previously used. For example, subtractive hybridization or differential hybridization is an approach that is based on removing the common transcripts between different cell types or tissues, leaving the specific transcripts for further manipulation and analysis. These are mainly qualitative methods and they are extremely time consuming. Second, nuclear run-on transcription has been applied to analyze changes in the level of expression of mRNAs but this method can only be applied to the detection of changes in the expression of known genes.

More recently, a novel method of analyzing gene expression, termed differential display, has been described.¹³ This technique provides a sensitive and flexible approach to the identification of genes that are differentially expressed at the mRNA level. Differential display is based on selective reverse transcription of expressed mRNAs and subsequent amplification with PCR in which a radioactive label is included. For the PCR reaction an anchored oligo-dT primer was used to selectively amplify a subset of mRNAs. The amplified, labeled PCR fragments were then separated and compared on a polyacrylamide gel with cell-specific band patterns. A modifica-

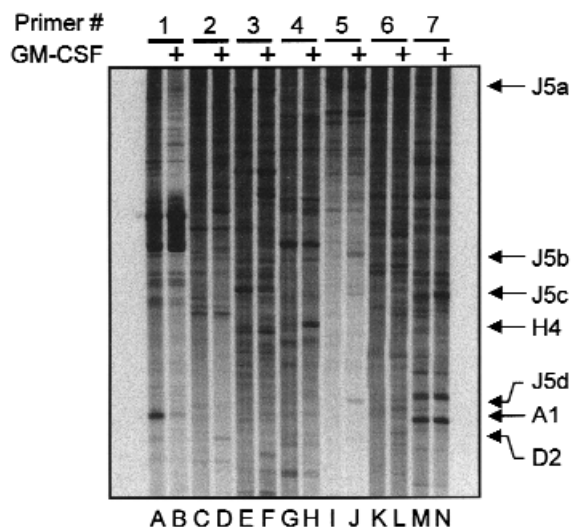


FIG 2. Identification of differentially regulated transcripts in PMNs after GM-CSF treatment. Human PMNs were isolated as indicated in Material and methods. Cells were incubated with or without GM-CSF (10^{-10} mol/L) for 3 hours. Poly A⁺ mRNA was isolated and cDNA prepared for RAP-PCR analysis. Seven different arbitrary primers were used as indicated. Lanes are labeled A to N to aid description. Indicated are bands that were isolated, amplified, purified, cloned, and sequenced (Table III).

tion of this technique, termed RAP-PCR, was used in this study,¹⁴ and the procedure is represented schematically in Fig 1. In this modification a single arbitrary primer is used in the PCR cycles. Such an arbitrary primer can be selected with the criteria that it should be approximately 18 bp long and have roughly a 50% (A/T)/(G/C) ratio and have an annealing temperature of around 50°C¹⁴ (Table II). Primers were also selected that generated between 20 and 50 bands per lane. This primer is allowed to anneal to the reverse-transcribed PMN cDNA at low temperature, allowing it to act as both a forward and reverse primer. It thus tends to select a subset of cDNAs with somewhat palindromic 5' and 3' sequences. A disadvantage of the original anchored oligo-dT method was the identification of many 3' untranslated regions of genes, which are difficult to analyze. The RAP-PCR protocol does not restrict primer annealing to these untranslated regions and thus provides greater opportunities to amplify the coding regions of differentially expressed genes. This leads to a more rapid analysis of the identification of the cloned gene. Primers were tested for their suitability in this assay and were selected by two criteria: the length should be around 18 bp and the annealing temperature should be greater than 50°C. Arbitrary primers were subsequently chosen that met these criteria and are listed in Table II.

Identification of novel GM-CSF induced genes in human PMNs

RAP-PCR was performed on RNA isolated from human granulocytes treated with or without GM-CSF (10^{-10} mol/L) for 3 hours, as described in the Material and methods section. Samples were analyzed by ac-

TABLE II. Sequences of primers used for differential display reactions

Primer	Sequence	Length (bp)
1	GGCCAGATGCATGTTCAA	18
2	CACAAGGTGGCAGGATGT	18
3	CTGTCGCCAGAGAAGATG	18
4	GGAAGCCTTCTCTCTCC	17
5	GGAGTCCTGTCTCTCCG	18
6	GTCTCGACCGGCACCAAG	18
7	CTGCAGCTTCCGTTCTCA	18

TABLE III. cDNAs isolated and cloned from RAP-PCR reactions

Band code	Fragment size (bp)	Identity
A1	500	PECAM-1
D2	500	ADP/ATP carrier protein
H4	900	FLAP
J5a	550	Ferritin heavy chain
J5b	250	ILT-2
J5c	250	c-Fos
J5d	300	Cytochrome oxidase-1

ILT-2, Ig-like transcript-2.

rylamide gel electrophoresis as shown in Fig 2. A total of around 30 fragments was found to be clearly differentially regulated and most of them represented up-regulation in the presence of GM-CSF. Each differentially expressed band was excised from the gel and amplified with the original primer used for differential display reactions. The fragments obtained were cloned and partially sequenced. These sequences were then screened against available nucleotide databases and the results of the sequence comparisons are outlined in Tables III and IV.

Interestingly, FLAP, an important regulator of the leukotriene synthesis, was identified as a gene up-regulated by GM-CSF.¹⁷ Leukotrienes are potent lipid mediators of inflammatory responses and have been implicated in the pathophysiologic mechanisms of both acute and chronic inflammatory diseases including asthma. A second potentially interesting product was the ILT-2. ILT-2 has a large cytoplasmic tail that contains putative immunoreceptor tyrosine-based inhibitory motifs similar to those found in killer cell inhibitory receptors.¹⁸ These tyrosine-based motifs implicate ILT-2 in inhibitory signaling pathways. Furthermore, the transcription factor *c-fos* was identified. *c-Fos* is transiently induced by a wide variety of extracellular stimuli.¹⁹ Ferritin, another gene identified, is the major iron-storage protein in eukaryotes and also has a potential role in the regulation of myelopoiesis and furthermore has been implicated as a tumor marker.²⁰

Although we found several previously described genes, many novel sequences were also identified that, at this time, have no identity to either known genes or human EST sequences (Table IV). The percentage homology of these clones with EST clones is given along with the length of the insert obtained after cloning the

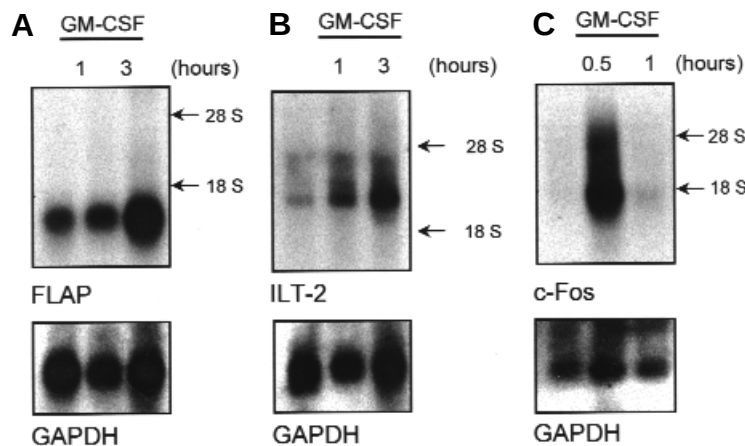


FIG 3. GM-CSF induces expression of RAP-PCR products in human PMNs. Human PMNs were incubated for indicated times with GM-CSF (10^{-10} mol/L). Twenty micrograms of total RNA per lane was used for Northern blotting and blots were hybridized with isolated RAP-PCR fragments (upper panels). **A**, FLAP. **B**, ILT-2. **C**, c-Fos. All blots were reprobed with glyceraldehyde phosphate dehydrogenase (GAPDH) to correct for equal loading (lower panels). Positions of 28S and 18S RNA are indicated.

TABLE IV. Novel cDNAs isolated from RAP-PCR

Clone	Size	Primer	Sequence	EST	Identity	Strand
13	500	1	caagggacgtttgcatgagtcctatggtatgttcagggtgccaagagtga	AA470449	71/104 (68%)	Minus/plus
20	750	1	aagactgagtggtgggtgagtggtgctcacactgtaattcagatttt	THC83029	130/158 (82%)	Minus/plus
36	600	1	gcaaggtttgacaagcggttacaaaattcctaattctcacaatcacc	THC280785	93/147 (63%)	Plus/plus
42	450	1	ggcagacacacactggggcaatggatggaaggagactggccaaggaac	W23538	46/60 (76%)	Plus/plus
79	350	4	gctgctctgtctcttctgctcaaggcttaaggccgtgtctccca	THC287677	53/73 (72%)	Minus/plus
90	500	5	agaggctcaaaaagcagccggcctgaatttcattctgtttccaagaga	THC266464	61/92 (72%)	Minus/plus
93	600	5	cctgctgtccccagcaccgctggctcacgtcacctcgtaattgctcc	THC101232	98/158 (62%)	Minus/plus
99	350	5	ttgaggtgcgtctgttagtagtagtgatgagccagcagtaggactggg	THC197030	101/141 (78%)	Minus/plus
101	500	5	ggacagacaggaaatcgtgtgcaggccctgctgtgccagcaccgc	THC254052	54/73 (73%)	Plus/plus
103	400	5	gtcctcatggcctaggcagctgcagccgcagacagtgagcccgacaca	R81083	50/67 (74%)	Plus/plus
113	450	5	gatccattcgtccatggatgatacttaagttgattccatttcagtta	AA084052	94/124 (75%)	Minus/plus
122	650	2	cagattggcttcttagtaatacattgagtttcttcattgcttaacatc	AA205247	65/85 (76%)	Minus/plus
129	650	5	tgattggccctgggattcttctcctgctcctctcctcctgggtcatc	THC292692	97/148 (65%)	Plus/minus
139	600	5	gtcctcaggccctaggcacctgcagccgcagacagtgagcccgacaca	AI05922	97/152 (63%)	Minus/plus
148	500	6	tcaatacaggtagctgatgactaacgagagggaacataaggaccctatg	AA558722	65/91 (71%)	Minus/plus
238	300	2	gtcatcggtgaggtcaggtcggatcagcccaactggaacacctg	EST185621	62/87 (71%)	Minus/plus
244	650	2	aggatgtccagcccatagaggagctgttgtaattgactgctggagac	EST68741	49/70 (70%)	Plus/plus
247	550	2	ggatgtgggacaaaagtaagattatttaagctcaaggttttaagt	THC267228	131/176 (74%)	Minus/plus
249	600	2	aggatgtgtgtagcaacaggagctctcgttcacggctgctgggaacgaa	THC150192	90/127 (70%)	Minus/plus
255	650	2	gaccctcagaggacacagaaaagctgtccctcagtcctcttgaggca	THC269356	57/84 (67%)	Plus/plus
256	700	2	gtgccaggccttaggtataccagacttgccagggttgaggatcagggg	THC203964	68/94 (72%)	Minus/plus
262	400	3	ctgtgccagagaagatgcccactttatgaataagcagaattaactacat	THC270838	120/123 (97%)	Plus/plus
273	400	4	ctgtgccagagaagatgtttattgatgaacaaaacatacagttagt	THC254502	120/125 (96%)	Minus/plus
286	600	5	ggacagacaggaaatcgtgtgcaggccctgctgtccccagcaccgc	AA502694	46/64 (71%)	Minus/plus
294	350	5	gggcatgttctccactcaccagcttgccccatcacagagcttctccc	THC282228	71/104 (68%)	Plus/plus
311	650	7	atattgattcagataggcataatctattcattttcataatgaatgtat	THC212219	137/157 (87%)	Minus/plus
314	540	7	gggagctaaggagaaccatcatatgtgtccatcttccgatttacatg	THC232302	114/154 (74%)	Minus/plus
320	400	7	cttcgcttcaggagttggccctgtgtgtttgtgaaaactctgcctt	THC271399	73/112 (65%)	Minus/plus

Data were obtained by screening the Institute for Genome Research's expressed sequence tag (EST) databases, which can be found at <http://www.tigr.com>.

RAP-PCR product. Extensive work is now required to determine the identity of these coding sequences and to correlate their expression with cytokine priming.

Confirmation of GM-CSF-mediated induction of genes in human PMNs

To confirm that the genes isolated were indeed differ-

entially expressed, Northern blots of total RNA from human PMNs (97% neutrophils, <3% eosinophils) incubated with GM-CSF were probed with selected cDNAs. Fig 3, A, shows a strong up-regulation of the FLAP gene after 3 hours of treatment with GM-CSF. Similarly, ILT-2 mRNA was also rapidly induced after 3 hours. Because c-fos mRNA is known to be very rapidly induced by

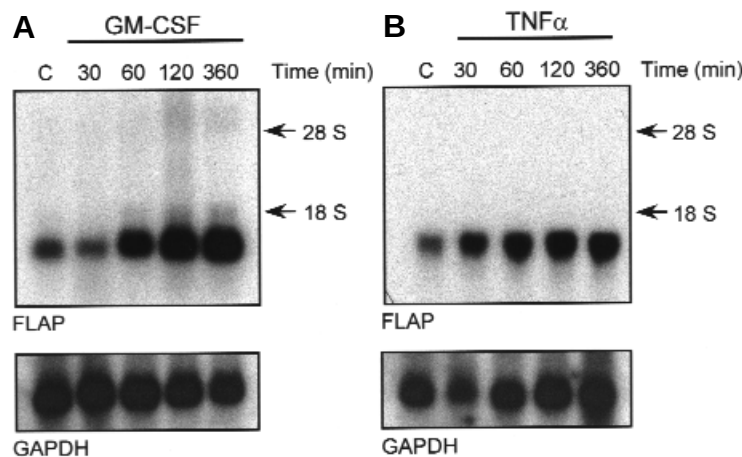


FIG 4. FLAP mRNA expression is induced in GM-CSF and TNF- α -stimulated PMNs. Northern blots were prepared from RNA of PMNs stimulated with **(A)** GM-CSF (10^{-10} mol/L) and **(B)** TNF- α (100 U/mL) for indicated time points. Twenty micrograms of total RNA per lane was loaded and blots were probed with RAP-PCR fragment of FLAP. Positions of 28S and 18S RNA are indicated.

extracellular stimuli,²¹ we prepared a Northern blot including a 30-minute GM-CSF time point. Indeed, *c-fos* expression was extremely rapidly and transiently up-regulated, being optimally expressed after 30 minutes. Interestingly, although *c-fos* appears to be both rapidly and transiently up-regulated, as is characteristic of immediate early genes, both FLAP and ILT-2 are slower, and levels remain high for at least 3 hours. Data were confirmed in at least 3 independent donors. These data demonstrate that the genes identified as being differentially expressed by the RAP-PCR technique are indeed up-regulated in human PMNs stimulated with GM-CSF.

FLAP is rapidly induced in human PMNs by both GM-CSF and TNF- α

To analyze the duration and extent of FLAP expression in human PMNs in detail, we prepared RNA from cells stimulated for up to 6 hours with GM-CSF (Fig 4, A). Interestingly, although FLAP expression is rapidly induced, it remains high for at least 6 hours. Because the "life span" of human neutrophils is rather short, even in the presence of cytokines, it appears that once FLAP is up-regulated it remains highly expressed until the granulocyte apoptotic program is induced. Thus FLAP provides a somewhat permanent marker for the activation status of these phagocytes.

To determine whether FLAP may be induced by other inflammatory stimuli and thus is a general marker of inflammation, we also stimulated human PMNs with TNF- α (Fig 4, B). Again, the expression of FLAP in PMNs treated with TNF- α was increased and remained high for at least 6 hours. This suggests that at least some of the GM-CSF-stimulated mRNAs are also markers of a common pool of inflammatory cytokine-induced genes.

Allergen challenge induces FLAP-expression in peripheral blood leukocytes in vivo

We wished to determine whether the increase in FLAP

expression we observed by in vitro stimulation of PMNs could also be observed in vivo. To do this, we analyzed FLAP expression in the total leukocyte population from allergic asthmatic patients after allergen inhalation challenge. Total leukocytes were used and not PMNs for several reasons. (1) It is not possible to isolate a sufficient amount of eosinophils and neutrophils from 10 mL of blood to obtain enough mRNA for Northern blotting. (2) Isolation of cells takes at least 2 hours (neutrophils) or 4 hours (eosinophils). These rather long isolation times can interfere with the detection of fast and transient-induced genes. (3) By using total leukocytes, specific priming caused by the isolation procedures was minimized. Blood was collected after allergen challenge and leukocytes were isolated as indicated in detail in Material and methods. Northern blots of RNA from 2 patients who were allergic to either house dust mite or cat were probed for FLAP mRNA expression. The method used for allergen challenge is detailed in Material and methods. The expression of FLAP in vivo was also very rapidly induced, reaching a maximum level in both patients between 30 minutes and 1 hour, before decreasing to basal levels within 4 to 5 hours (Fig 5). This decrease to basal levels is in contrast to what was observed in isolated PMNs stimulated in vitro with cytokines (Fig 4). This discrepancy is likely to reflect the fact that in these in vivo experiments the primed leukocytes will migrate out of the peripheral blood after several hours. These data demonstrate that gene expression identified in vitro by cytokine challenge of human PMNs reflects the in vivo situation after induction of an inflammatory response.

DISCUSSION

Phagocytes (granulocytes and monocytes) in the peripheral blood are characterized by a nonprimed phenotype that is refractory to activation in the context of cytotoxic responses.²⁻⁴ This is a safeguard for the inappropri-

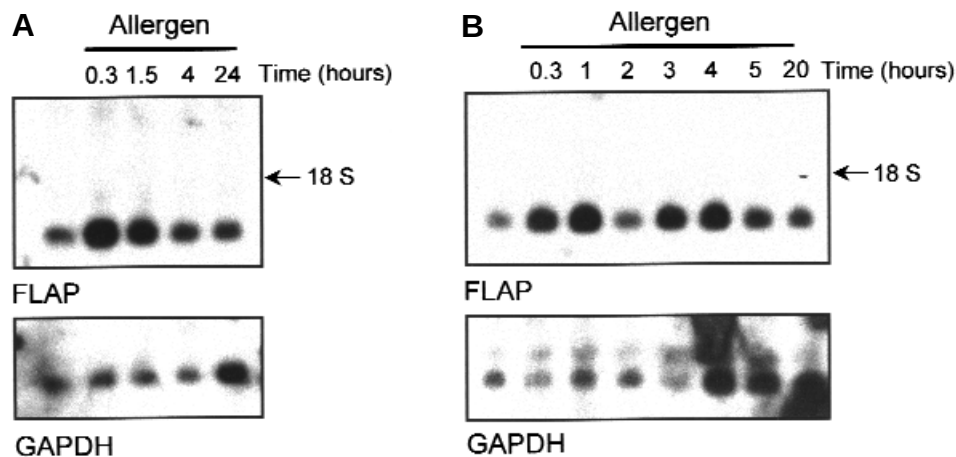


FIG 5. FLAP expression is increased in leukocytes isolated from allergic asthmatic patients after allergen inhalation challenge. Total RNA was prepared from total white blood cell fractions of blood taken from patients before (control) and after allergen challenge at times indicated. House dust mite allergen (10,000 biological units/mL) (**A**) and cat allergen (10,000 biological units/mL) (**B**) were used. Inhalations were performed as previously described and samples were collected after FEV₁ was at least 20% below baseline values. Ten micrograms of total RNA per lane was loaded and blots were probed with differential display fragment of FLAP (upper panels). Blots were subsequently reprobed with glyceraldehyde phosphate dehydrogenase (GAPDH) to correct for equal loading (lower panels).

ate activation of these cells in the peripheral blood. In severe clinical conditions, such as septic shock, uncontrolled activation of phagocytes can lead to multiorgan failure and death. At inflammatory loci, such as bronchial tissue in allergic asthma, eosinophils are attracted to the site of inflammation and become activated.¹ The process of extravasation of inflammatory cells to these loci is a tightly controlled multistep process. The first step involves the interaction of phagocytes in the peripheral blood with cytokines liberated from the inflammatory locus. This results in preactivation or priming of cells, which is essential for their subsequent extravasation and the eventual activation of cytotoxic responses on interaction with activating stimuli. Therefore the process of priming is critical for regulation of phagocyte effector functions in vivo. This multistep paradigm of phagocyte activation predicts that cytokine-mediated priming of peripheral blood granulocytes is a result of a local inflammatory reaction in a given organ. Indeed, several studies have shown that inflammatory cells are characterized by a primed phenotype in the peripheral blood of patients with allergic inflammatory conditions.²²⁻²⁵ Understanding the mechanisms of priming might be useful in the development specific approaches to inhibit this phenomenon, thereby preventing inflammatory cells from becoming optimally activated. Moreover, this in vivo primed phenotype of inflammatory cells in the peripheral blood might be used to develop strategies to measure this phenotype as read out for the severity of inflammatory processes localized in tissues. This diagnostic approach has been followed by Venge et al²⁶ and Venge,²⁷ who measured the release of eosinophil cationic protein by eosinophils in the blood of allergic asthmatics ex vivo. This phenomenon occurs during clotting of blood in samples of patients with various inflammatory diseases, in particular allergic asthma.²⁸ The ability

of eosinophils to release eosinophil cationic protein under these conditions can be considered as an indirect measure of eosinophil priming in vivo. We have tried to measure priming of isolated eosinophils as a measure of changes in inflammatory processes in the lung during anti-inflammatory treatment (unpublished data). Although PAF-induced eosinophil chemotaxis is inhibited by this type of treatment, functional eosinophil responses such as chemotaxis and activation of the respiratory burst cannot be routinely used for this type of study. The method is too laborious and the data are too sensitive for variations in processing of the cells.

Despite recognition of the importance of priming, very little is currently known as to the precise molecular mechanisms of this phenomenon. Therefore we set out experiments to find new determinants of cytokine-induced priming with use of the technique of differential display. This powerful PCR-based technique has enabled us to define several mRNAs that were either up- or down-regulated during GM-CSF treatment of PMNs in vitro (Table III). Although use of RAP-PCR will not lead to the identification of *all* genes regulated by cytokine priming, it does result in the identification of a large subset of genes. In this article alone we describe around 25 unknown genes that must now be subsequently characterized (Table IV). Interestingly, 2 of the genes identified, ferritin H and cytochrome oxidase, were also identified as allergen-induced genes in monocytes isolated from mononuclear cells of patients with allergic asthma.²⁹ Ferritin H plays an important role in iron storage and iron sequestration.²⁰ Our data suggest that in human PMNs GM-CSF may play a role in regulating iron homeostasis through regulating the expression of ferritin H. Indeed, it has been reported in primary human myoblasts that TNF- α induces ferritin H mRNA independently of cellular

iron concentrations and known iron-dependent regulatory pathways.³⁰ This suggests that regulation of iron homeostasis by inflammatory cytokines might be a general response of cells. Increased sequestration of iron by increasing the levels of ferritin H might serve the beneficial functions of, (1) making iron unavailable to invading organisms or (2) reducing free-radical tissue damage. The latter may play an important role in phagocyte functioning, protecting cells against damage during the killing of microbial organisms by superoxide production.

We have also identified the transcription factor *c-fos* as a rapidly up-regulated gene in PMNs (Fig 3, C). This transcription factor is a member of a family of immediate early response genes whose expression is regulated by a variety of cytokines, growth factors, and hormones.¹⁹ Once expressed, it is then able to bind and activate the promoters of a diverse array of cellular genes. Thus GM-CSF-induced *c-fos* expression in human neutrophils may be responsible for rapidly "switching on" relevant target genes needed for initiating a correct inflammatory response.

Another GM-CSF-regulated gene identified is the ILT-2 (Fig 3, B), a transmembrane protein that is expressed on many myeloid and lymphoid cells.¹⁸ This receptor binds MHC class I molecules and delivers a negative signal that inhibits killing by natural killer and T cells, as well as Ca⁺⁺ mobilization in B cells triggered through the B-cell antigen receptor.³¹ Cytokine-regulated expression of this gene has not been previously reported, and neither has its expression in neutrophils. Our finding that ILT-2 can be up-regulated by GM-CSF suggests a potentially novel mechanism for regulating cellular activation during an immune response.

FLAP was identified as a gene potentially up-regulated in neutrophils treated with GM-CSF and TNF- α (Fig 4). This protein is a critical component in the synthesis of leukotrienes.¹⁷ Leukotrienes are secreted by neutrophils, eosinophils, mast cells, and macrophages and potentially stimulate bronchoconstriction and mucus secretion and cause vasodilation with increases in postcapillary permeability.³² It is thus interesting that through differential display we were able to identify FLAP as a marker of primed granulocytes in vitro. The consequences of FLAP up-regulation are thus likely to be an increased inflammatory response.

Differential display was used to find determinants of priming in the peripheral blood of allergic asthmatic subjects. The most prominent signal in vitro was found with the FLAP mRNA on Northern blots from cytokine-treated granulocytes. To confirm that genes identified in vitro were also relevant in vivo, mRNA was prepared from total leukocytes isolated from allergic asthmatic patients before and after allergen challenge. Total leukocytes were chosen over isolated PMNs because of the relatively long time of isolation and the sensitivity of these granulocytes for a specific preactivation during isolation.³³ As can be seen from Fig 5, FLAP mRNA is rapidly induced after allergen challenge and this increase is transient. These findings are consistent with a model that

inflammatory blood cells encounter priming signals in the peripheral blood. These cytokines modulate the transcription of several genes, including the induction of the FLAP gene. Moreover, these cells become much more responsive to chemotactic factors.³⁴ These primed cells are destined to leave the bloodstream and extravasate into the tissues, leaving the unprimed cells in the peripheral blood. The observation that FLAP is also up-regulated in vivo validates our chosen method. Further, in vivo analysis of both the known and unknown genes identified in our screen may well lead to the identification of novel markers of inflammation as well as further insight into immune regulation.

In conclusion, differential display enables the characterization of genes that are differentially transcribed in response to cytokines and are relevant for the pathogenesis of inflammatory diseases. This occurs in vitro and in vivo in patients with symptomatic asthma. Characterization of novel genes will enable us to find new markers of inflammatory diseases and will provide valuable information on the processes that occur early in the sequence eventually leading to activation of inflammatory cells in the tissues.

REFERENCES

1. Busse WW, Sedgwick JB. Eosinophils in asthma. *Ann Allergy* 1992;68:286-90.
2. Hallett MB, Lloyds D. Neutrophil priming: the cellular signals that say "amber" but not "green." *Immunol Today* 1995;16:264-8.
3. Koenderman L, van der Bruggen T, Schweizer RC, Warringa RA, Coffey P, Caldenhoven E, et al. Eosinophil priming by cytokines: from cellular signal to in vivo modulation. *Eur Respir J Suppl* 1996;22:119-25.
4. Coffey PJ, Koenderman L. Granulocyte signal transduction and priming: cause without effect? *Immunol Lett* 1997;57:27-31.
5. van der Bruggen T, Kok PT, Raaijmakers JA, Verhoeven AJ, Kessels RG, Lammers JW, et al. Cytokine priming of the respiratory burst in human eosinophils is Ca²⁺ independent and accompanied by induction of tyrosine kinase activity. *J Leukoc Biol* 1993;53:347-53.
6. Nagata M, Sedgwick JB, Busse WW. Differential effects of granulocyte-macrophage colony-stimulating factor on eosinophil and neutrophil superoxide anion generation. *J Immunol* 1995;155:4948-54.
7. Knall C, Worthen GS, Johnson GL. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc Natl Acad Sci U S A* 1997;94:3052-7.
8. Kuroki M, O'Flaherty JT. Differential effects of a mitogen-activated protein kinase inhibitor on human neutrophil responses to chemotactic factors. *Biochem Biophys Res Commun* 1997;232:474-7.
9. Bracke M, Coffey PJ, Lammers JW, Koenderman L. Analysis of signal transduction pathways regulating cytokine-mediated Fc receptor activation on human eosinophils. *J Immunol* 1998;161:6768-74.
10. Coffey PJ, Geijsen N, M'rabet L, Schweizer RC, Maikoe T, Raaijmakers JA, et al. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *Biochem J* 1998;329:121-30.
11. Coffey PJ, Schweizer RC, Dubois GR, Maikoe T, Lammers JW, Koenderman L. Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper 2-derived cytokines interleukin-4 and interleukin-5. *Blood* 1998;91:2547-57.
12. Miike S, Nakao A, Hiraguri M, Kurasawa K, Saito Y, Iwamoto I. Involvement of JAK2, but not PI-3-kinase/Akt and MAP kinase pathways, in anti-apoptotic signals of GM-CSF in human eosinophils. *J Leukoc Biol* 1999;65:700-6.
13. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 1992;257:967-71.

14. Kester HA, van der Leede BM, van der Saag PT, van der Burg B. Novel progesterone target genes identified by an improved differential display technique suggest that progestin-induced growth inhibition of breast cancer cells coincides with enhancement of differentiation. *J Biol Chem* 1997;272:16637-43.
15. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
16. Cockcroft DW, Murdock KY, Kirby J, Hargreave F. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. *Am Rev Respir Dis* 1987;135:264-7.
17. Dixon RA, Diehl RE, Opas E, Rands E, Vickers PJ, Evans JF, et al. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* 1990;343:282-4.
18. Samaridis J, Colonna M. Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur J Immunol* 1997;27:660-5.
19. Cahill MA, Janknecht R, Nordheim A. Signalling pathways: jack of all cascades. *Curr Biol* 1996;6:16-9.
20. Theil EC. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu Rev Biochem* 1987;56:289-315.
21. Kruijer W, Cooper JA, Hunter T, Verma IM. Platelet-derived growth factor induces rapid but transient expression of the c-fos gene and protein. *Nature* 1984;312:711-6.
22. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L. In vivo priming of plateletactivating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. *Blood* 1992;79:1836-41.
23. Warringa RA, Mengelers HJ, Raaijmakers JA, Bruijnzeel PL, Koenderman L. Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma. *J Allergy Clin Immunol* 1993;91:1198-205.
24. Evans DJ, Lindsay MA, O'Connor BJ, Barnes PJ. Priming of circulating human eosinophils following late response to allergen challenge. *Eur Respir J* 1996;9:703-8.
25. Moser R, Fehr J, Olgiate L, Bruijnzeel PL. Migration of primed human eosinophils across cytokine-activated endothelial cell monolayers. *Blood* 1992;79:2937-45.
26. Venge P, Dahl R, Peterson CG. Eosinophil granule proteins in serum after allergen challenge of asthmatic patients and the effects of anti-asthmatic medication. *Int Arch Allergy Appl Immunol* 1988;87:306-12.
27. Venge P. Soluble markers of allergic inflammation. *Allergy* 1994;49:1-8.
28. Reimert CM, Poulsen LK, Bindslev-Jensen C, Kharazmi A, Bendtsen K. Measurement of eosinophil cationic protein (ECP) and eosinophil protein X/eosinophil derived neurotoxin (EPX/EDN). Time and temperature dependent spontaneous release in vitro demands standardized sample processing. *J Immunol Methods* 1993;166:183-190.
29. Fukuda T, Mochida S, Fukushima Y, Makino S. Detection of allergen-induced genes in peripheral blood mononuclear cells of patients with allergic asthma using subtractive hybridization. *J Allergy Clin Immunol* 1995;96:1076-82.
30. Miller LL, Miller SC, Torti SV, Tsuji Y, Torti FM. Iron-independent induction of ferritin H chain by tumor necrosis factor. *Proc Natl Acad Sci U S A* 1991;88:4946-4950.
31. Colonna M, Navarro F, Bellón T, Llano M, García P, Samaridis J, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med* 1997;186:1809-18.
32. Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human diseases. *N Engl J Med* 1990;323:645-55.
33. Kuijpers TW, Tool AT, van der Schoot CE, Ginsel LA, Onderwater JJ, Roos D, et al. Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* 1991;78:1105-11.
34. Warringa RA, Koenderman L, Kok PT, Kreukniet J, Bruijnzeel PL. Modulation and induction of eosinophil chemotaxis by granulocyte-macrophage colony-stimulating factor and interleukin-3. *Blood* 1991;77:2694-700.

Receive tables of contents by e-mail

To receive the tables of contents by e-mail, send an e-mail message to
majordomo@mosby.com

Leave the subject line blank and type the following as the body of your message:
subscribe jaci_toc

You can also sign up through our website at
<http://www.mosby.com/jaci>

You will receive an e-mail to confirm that you have been added to the mailing list.
Note that TOC e-mails will be sent out when a new issue is posted to the website.