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This information is current as of October 17, 2021.

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*J Immunol* 1998; 161:1493-1500; ;  
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Platelet-Activating Factor Synthesized by IL-12-Stimulated Polymorphonuclear Neutrophils and NK Cells Mediates Chemotaxis<sup>1</sup>

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IL-12 is chemotactic for NK cells and polymorphonuclear neutrophils (PMN), but not for monocytes. In the present study, we evaluated whether the chemotactic effect of IL-12 is a direct phenomenon or is dependent on the generation of secondary mediators. The results obtained indicate that IL-12 induces a dose- and time-dependent synthesis of platelet-activating factor (PAF) from PMN and NK cells and of reactive oxygen radicals (ROS) from PMN. Monocytes and CD56-negative PBMC cells did not synthesize PAF or ROS after challenge with IL-12. The production of ROS by PMN was significantly inhibited by two chemically different PAF receptor antagonists (WEB 2170 and CV 3988), suggesting an autocrine stimulation of PMN by PAF newly synthesized after the challenge with IL-12. Moreover, the IL-12-induced chemotaxis of PMN and NK cells was significantly reduced by both WEB 2170 and CV 3988, suggesting that synthesized PAF mediates the chemotactic effect of IL-12. Preincubation with superoxide dismutase, which blocks the formation of superoxide anions, also reduced the chemotactic effect of IL-12 on PMN, but not on NK cells, suggesting that superoxide anion generation is relevant only for the IL-12-induced chemotaxis of PMN. In conclusion, the results of the present study indicate that IL-12-induced PAF synthesis plays a critical role in triggering the events involved in the motogenic response of PMN and NK to IL-12. *The Journal of Immunology*, 1998, 161: 1493–1500.

Polymorphonuclear neutrophils (PMN),<sup>3</sup> monocytes and NK cells belong to the cellular effectors of innate resistance against pathogens. The activation and migration of phagocytic and NK cells during the early inflammatory response are regulated by a complex network of cytokines and proinflammatory mediators. Among these, a relevant role has been ascribed to a phospholipid mediator showing diverse and potent biologic actions, known as platelet-activating factor (PAF) (1, 2). PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) belongs to a family of acetylated phosphoglycerides and is produced from a large range of cells, including PMN, monocytes, and NK cells (3–5). PAF, which acts through a specific receptor (6), is considered a mediator of cell-to-cell communication and may function either as an intercellular or an intracellular messenger (7–9). It has been shown that PAF modulates the mechanisms of cytotoxicity of NK cells (10, 11) and induces chemotaxis and activation of PMN (3, 12, 13). It is at present unknown whether PAF also stimulates chemotaxis on NK cells. It has been reported that PAF mediates the effect of different proinflammatory cytokines such as TNF- $\alpha$

and IL-8 (14–16). Recently, another cytokine, IL-12, has been described to have a key role in the initiation of both innate and Ag-specific proinflammatory immunity (17). IL-12 is mostly produced by phagocytic cells and B lymphocytes in response to LPS and other bacterial products (17, 18). IL-12 enhances the cytotoxic activity of NK cells and favors the generation of CTL (19, 20). Moreover, a chemotactic role of IL-12 has been demonstrated for NK cells and PMN (21, 22). This effect has been demonstrated not to be due to a secondary cytokine or chemokine production (22). However, the roles of other mediators in IL-12 induced chemotaxis have not been explored.

The aim of the present study was to evaluate whether the chemotactic effect of IL-12 is a direct phenomenon or is dependent on the generation of secondary mediators such as PAF or reactive oxygen radicals (ROS). The results obtained indicate that IL-12 induces the synthesis of PAF from PMN and NK cells, but not from monocytes, and that this mediator is involved in IL-12-induced chemotaxis of PMN and NK cells. The generation of ROS triggered by the newly synthesized PAF was shown to significantly contribute to IL-12-induced chemotaxis of PMN, but not of NK cells.

## Materials and Methods

### Materials

Polymyxin B, phospholipase A2, phospholipase A1, BSA fraction V (tested for not more than 1 ng endotoxin/mg), FMLP, sphingomyelin, and lyso-2-phosphatidylcholine, indomethacin, cytochrome *c*, superoxide dismutase (SOD), and FITC-conjugated goat anti-mouse IgG were purchased from Sigma (St. Louis, MO). IL-12 was a gift from G. Trinchieri, Genetics Institute (Cambridge, MA). The anti-IL-12 neutralizing mAb C.8.6 and the anti-IL-12 nonneutralizing mAb C.11.5 (18) were gifts from G. Trinchieri. Anti-IL-12R 12R $\beta$ .44 mAb (23) was a gift from J. Ritz (Dana-Farber Cancer Institute, Boston, MA). All mouse anti-IL-12 mAb and anti-IL-12R mAb were of the IgG1 isotype. The corresponding irrelevant isotypic control (mouse IgG1) was purchased from Cedarlane (Hornby, Ontario, Canada). CD56 mAb was obtained from Becton Dickinson (Mountain View,

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Received for publication November 7, 1997. Accepted for publication April 6, 1998.

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<sup>1</sup> This work was supported by the Associazione Italiana per la Ricerca sul Cancro (to G.C. and R.F.) and Istituto Superiore di Sanità and MURST 40 and 60% (to G.C. and G.P.).

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<sup>3</sup> Abbreviations used in this paper: PMN, polymorphonuclear neutrophils; PAF, platelet-activating factor; ROS, reactive oxygen radicals; SOD, superoxide dismutase; PE, phycoerythrin.

CA). FITC-conjugated mAb to CD3 and PE-conjugated mAb to CD56 were purchased from Caltag Laboratories (Burlingame, CA). Synthetic PAF (1-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) was obtained from Bachem Feinchemikalien (Bubendorf, Switzerland); the stock solution in chloroform was stored at  $-20^{\circ}\text{C}$  until use. The chloroform was evaporated, and saline containing 0.25% BSA (fraction V) and low endotoxin (Sigma) was added immediately before use. WEB 2170 was obtained from Boehringer Ingelheim (Ingelheim, Germany). CV 3988 was purchased from Takeda (Kyoto, Japan).

TNF- $\alpha$  and LPS from *Escherichia coli* (0111:B4) were purchased from Sigma. The stock solution of LPS was prepared by suspending 10 mg of LPS in 2 ml of 20 mM EDTA and by sonicating until clarification (three- to fivefold 20-s burst at maximum intensity using a W375 sonicator with a no. 419 microtip, Heat Systems-Ultrasonics, Farmingdale, NY). Aliquots of LPS stocks (200  $\mu\text{l}$ ) were stored at  $-20^{\circ}\text{C}$  and when thawed for use were sonicated for 15 s using a microsonicator (Microson, Heat Systems-Ultrasonics). LPS working dilutions were prepared in 10 mM HEPES saline formulated using 1 M HEPES stock (Life Technologies, Grand Island, NY) and sterile, nonpyrogenic saline. C3b-coated Baker's yeast particles (C3b-BY), used as substrate for phagocytosis, were prepared as previously described (24). Polycarbonate filters (5- $\mu\text{m}$  diameter, polyvinylpyrrolidone free) were obtained from Nucleopore (Pleasanton, CA).

#### Preparation of human PMN

Human PMN were isolated from venous blood of healthy donors by sequential centrifugation and gelatin sedimentation (2.5% gelatin in PBS, pH 7.2, for 30 min at  $37^{\circ}\text{C}$ ), as previously described (25). Contaminating erythrocytes were removed by hypotonic lysis, and the cells were resuspended in RPMI 1640 medium at a final concentration of  $3 \times 10^6/\text{ml}$ . The percentage of PMN in the cell preparation used in this study was 95 to 97%. Cell viability, as determined by trypan blue exclusion, was 96 to 98%. The percentage of contaminating NK cells was evaluated, using a FACScan flow cytometer (Becton Dickinson), as cells positive for CD56 PE-conjugated mAb. The purified fraction always stained  $<1\%$  positive cells for CD56.

#### Preparation of human NK cells

Buffy coats from healthy donors were obtained from the Blood Bank of the Ospedale S. Giovanni Battista (Torino, Italy). PBMC were isolated from the buffy coats by density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). NK cells were separated on the basis of CD56 expression by magnetic cell sorting using the MACS system (Miltenyi Biotec, Auburn, CA). Briefly, PBMC were labeled with the CD56 mAb for 20 min, then washed twice and resuspended in MACS buffer (PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , supplemented with 1% BSA and 5 mmol/L EDTA) at a concentration of  $2 \times 10^7$  cells/80  $\mu\text{l}$ . MACS superparamagnetic microbeads conjugated with a rat antimurine IgG1 were added to the cell suspension (20  $\mu\text{l}/2 \times 10^7$  cells) and incubated for 15 min at  $6$  to  $12^{\circ}\text{C}$ . After washing twice in MACS buffer, cells were separated on a magnetic stainless steel wool column (Miltenyi Biotec) according to the manufacturer's recommendations. CD56-negative cells were collected from the column eluate, whereas CD56-positive cells remained attached to the magnetized matrix. To obtain CD56-positive cells, the column was removed from the magnet and flushed with MACS buffer into another tube. Small aliquots were taken from each population (total, CD56 positive, and CD56 negative) for cytometric analysis. To determine the purity of the recovered cells, each fraction was double stained with a FITC-conjugated mAb to CD3 and a PE-conjugated mAb to CD56 and analyzed using a FACScan flow cytometer (Becton Dickinson). The CD56-positive fraction always stained  $>93\%$  positive for CD56, while the CD56-negative fraction contained a proportion of contaminating CD56-positive cells ranging from 6 to 11%.

#### Preparation of human monocytes

Monocytes were isolated from PBMC by adhesion to plastic dishes, as described by Valone and Epstein (14). Nonadherent cells were removed by vigorous washing, and 1 ml of RPMI 1640 (Life Technologies, Paisley, Scotland, UK) containing 0.25% BSA was added to each well. Adherent cells in representative wells were removed by scraping with a rubber policeman and counted. The number of cells recovered per well was  $2.5 \pm 0.2 \times 10^6$  (mean  $\pm$  1 SD for 10 consecutive studies). Adherent cells were  $>90\%$  monocytes, as detected by nonspecific esterase staining and immunofluorescence positivity with the anti-CD14 Leu M3 mAb. Less than 1 platelet/10 monocytes was detected. Monocyte viability was  $>90\%$ , as assessed by trypan blue dye exclusion test. Monocytes were also separated on the basis of CD14 expression by magnetic cell sorting, using the MACS system (Miltenyi Biotec). Briefly, PBMC in MACS buffer ( $10^7$  cells/80  $\mu\text{l}$ ) were labeled with the CD14 mAb conjugated with MACS superparamag-

netic microbeads (Miltenyi Biotec) for 20 min at a concentration of 20  $\mu\text{l}/10^7$  cells at  $6$  to  $12^{\circ}\text{C}$ . After washing twice in MACS buffer, cells were separated on a magnetic stainless steel wool column (Miltenyi Biotec), according to the manufacturer's recommendations. CD14-positive cells attached to the magnetized matrix were obtained after removal of the column from the magnet and were flushed with MACS buffer into another tube. Cytometric analysis of the collected cells showed  $>98\%$  CD14 positivity.

#### IL-12R analysis

The presence of the IL-12R on PMN was evaluated by cytofluorometric analysis both by using the murine 12R $\beta$ .44 mAb, specific for the human low affinity IL-12R  $\beta$ 1-chain (23) and by assessing IL-12 binding to the putative receptor using a technique previously described (26, 27).

To evaluate the expression of the human low affinity IL-12R  $\beta$ 1-chain, cells ( $2 \times 10^6$ ) were fixed with paraformaldehyde (0.1%); then, after washings in PBS containing 2% heat-inactivated human serum and incubation for another 15 min with whole heat-inactivated human serum to block remaining nonspecific sites, cells were incubated for 30 min with 1  $\mu\text{g}$  of purified 12R $\beta$ .44 mAb or with the irrelevant isotypic control in PBS containing 2% heat-inactivated human serum. After appropriate washings, cells were stained by the addition of FITC-conjugated goat anti-mouse IgG. To evaluate IL-12 binding to the putative receptor, following blockade of nonspecific sites with heat-inactivated human serum,  $2 \times 10^6$  cells in 100  $\mu\text{l}$  of staining buffer (PBS containing 2% heat-inactivated human serum and 0.1% sodium azide) were sequentially incubated with IL-12 (10 ng/ml) for 1 h, followed by incubation with the anti-IL-12 mAb C.11.5, the neutralizing anti-IL-12 mAb C.8.6, or the irrelevant isotypic control for 30 min and finally with FITC goat anti-mouse IgG for 20 min. All incubations were performed in staining buffer at  $4^{\circ}\text{C}$ , and cells were washed twice between incubations. PHA-activated PBMC, prepared as described previously (26), were used as a positive control. The stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson).

#### Immunoprecipitation and Western blot analysis studies

PMN ( $80 \times 10^6$ ) were extracted with cold DIM buffer (50 mmol/L PIPES (pH 6.8), 100 mmol/L NaCl, 5 mM  $\text{MgCl}_2$ , 300 mmol/L sucrose, 5 mmol/L EGTA, and 2 mmol/L sodium orthovanadate) plus 1% Triton X-100 and a mixture of protease inhibitors (1 mmol/L PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, 0.15 U/ml aprotinin, and 1  $\mu\text{g}/\text{ml}$  pepstatin A) for 20 min at  $4^{\circ}\text{C}$  and centrifuged at  $15,000 \times g$  for 20 min. The clarified supernatant was precleared for 1 h with 50  $\mu\text{l}$  of Sepharose-protein A (3 mg/sample). The protein concentration of PMN lysates was determined by the Bradford's technique, and the protein content of the samples was normalized to 250 mg/samples by appropriate dilution with the cold DIM buffer. The samples were then incubated with 2  $\mu\text{g}$  of 12R $\beta$ .44 mAb or the isotypic control and adsorbed by anti-mouse IgG coupled to Sepharose-protein A. Bound proteins were washed several times in DIM buffer and eluted in boiling Laemmli buffer. Thirty microliters of eluted proteins were subjected to 8% SDS-PAGE.

Lymphocytes ( $20 \times 10^6$ ) were extracted by sonication. Proteins were then transferred electrophoretically to nitrocellulose; the filters were incubated with blocking solution (10% low fat milk in 20 mmol/L Tris-HCl (pH 7.6) and 17 mmol/L NaCl) for 1 h. The anti-IL-12R 12R $\beta$ .44 mAb (2  $\mu\text{g}$ ) was added at the same solution, and the incubation was conducted overnight at room temperature. For detection, the filters were washed four times (15 min each wash) with PBS/0.5% Tween-20 and reacted for 1 h at room temperature with horseradish peroxidase-conjugated protein A. The enzyme was removed by washing as described above. The filters were reacted for 1 min with a chemiluminescence reagent (ECL, Amersham, Arlington Heights, IL) and exposed to autoradiography film for 1 to 15 min. To reprobe, nitrocellulose filters were first stripped of Ab by 62 mmol/L Tris-HCl (pH 6.7), 2% SDS, and 100 mmol/L  $\beta$ -ME.

#### Purification and quantification of PAF

PAF was quantified after extraction and purification by TLC (silica gel plates 60 F254, Merck, Darmstadt, Germany) and HPLC ( $\mu$ Porasil column, Millipore Chromatographic Division, Waters, Milford, MA) by aggregation of washed rabbit platelets, as previously reported (24, 25). The biologically active material extracted from cells and supernatants in different experiments was characterized by comparison with synthetic PAF according to the following criteria (24, 25, 28, 29): 1) induction of platelet aggregation by a pathway independent from both ADP and arachidonic acid/thromboxane A $_2$ -mediated pathways; 2) specificity of platelet aggregation as inferred from the inhibitory effect of 5  $\mu\text{M}$  WEB 2170 or CV 3988, two different PAF receptor antagonists (30, 31); 3) TLC and HPLC behavior and physico-chemical characteristics, such as inactivation by strong bases and by phospholipase A $_2$  treatment, but resistance to phospholipase A $_1$ , acids, weak bases, and 5-min heating in boiling water (28, 29).

*O<sub>2</sub><sup>-</sup> assay*

Production of O<sub>2</sub><sup>-</sup> was measured as the SOD-inhibitable reduction of ferricytochrome *c* (32). PMN ( $2.5 \times 10^6$  cells) were incubated at 37°C in Tyrode's buffer (2.6 mM KCl, 1 mM MgCl<sub>2</sub>, 137 mM NaCl, 6 mM CaCl<sub>2</sub>, 0.1% glucose, and 1 mM Tris, pH 7.4) containing 80 μM cytochrome *c* with or without SOD (50 U/ml) and appropriately stimulated. Basal O<sub>2</sub><sup>-</sup> production was assessed in the absence of stimulating factors. Supernatants were removed at specified times and centrifuged, and the absorbance was measured in a spectrophotometer at 550 nm. The extinction coefficient of ferricytochrome *c* at 550 nm was taken as  $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . The protein content of PMN was measured according to the Lowry technique. O<sub>2</sub><sup>-</sup> production was expressed as nanomolar concentrations of cytochrome *c* reduced per milligram of protein (32).

*Chemiluminescence*

Luminol (50 μM)-enhanced chemiluminescence was measured on  $2 \times 10^6$  PMN suspended in HEPES-buffered Krebs medium containing 0.05% BSA using a LKB 1250 luminometer (LKB, Rockville, MD). Data are expressed in millivolts (33).

*Chemotaxis*

Chemotaxis of PMN or NK cells in Boyden's chambers across a polycarbonate filter 5 μm in diameter was performed as previously described (12, 22). RPMI medium containing 0.25% BSA (Sigma) and the stimulus or the vehicle alone was placed in the lower compartment of the chamber, and  $1 \times 10^5$  PMN or  $2 \times 10^5$  NK cells, suspended in RPMI medium, were then seeded in the upper compartment of the Boyden's chamber. After incubation at 37°C (2 h for PMN and 4 h for NK cells), the upper surface of the filter was scraped with a rubber policeman. The filters were then fixed and stained with Diff-Quick (Harleco, Gibbstown, NJ), and 10 fields at  $\times 200$  magnification were counted.

*Experimental protocols*

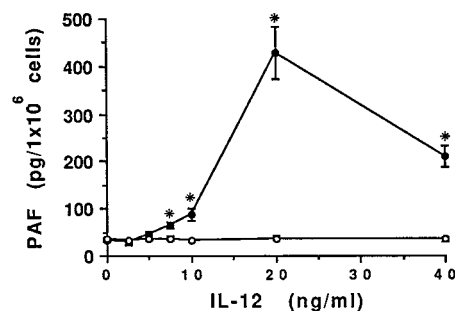
The production of PAF from PMN, monocytes, NK, and CD56-negative cells stimulated with IL-12 was studied. Cells were equilibrated for 15 min in Tris-buffered Tyrode's buffer containing 0.25% delipidized BSA (fraction V) as previously described (24, 25) and incubated at 37°C for the indicated time with IL-12 at different concentrations. To evaluate the priming effect of IL-12, cells were incubated with low doses of IL-12 at 37°C for 30 min and then stimulated with LPS or at nonstimulatory doses. To assess the specificity of the reaction, IL-12 was preincubated for 10 min at 37°C with the neutralizing anti-IL-12 mAb C.8.6 (10 μg/ml) or with the nonneutralizing anti-IL-12 mAb C.11.5 (10 μg/ml) (18). Selected experiments were conducted in the presence of 5 μg/ml polymyxin B for 30 min at 37°C to exclude LPS contamination. The supernatants and the cell pellets were extracted according to a modification of the Bligh and Dyer procedure (34), with formic acid added to lower the pH of the aqueous phase to 3.0. Each individual experiment was performed in duplicate.

To evaluate the production of ROS, PMN were stimulated with IL-12 (20 ng/ml) or with IL-12 preincubated for 10 min at 37°C with anti-IL-12 mAb C.8.6 (10 μg/ml) or C.11.5 (10 μg/ml). Phagocytosis of  $1 \times 10^7$  C3b-BY or FMLP (10 μM) was used as a positive control. To evaluate the priming effect of IL-12, PMN were incubated with IL-12 at 37°C for 10 min and then stimulated with FMLP (1 μM). Moreover, the influence of PAF on IL-12-dependent ROS production was studied using two PAF-specific inhibitors, WEB 2170 (3 μmol/L) and CV 3988 (4 μmol/L), incubated for 10 min at 37°C before the stimulation with IL-12.

In chemotactic experiments, the implication of different mediators in IL-12-induced chemotaxis was studied using specific inhibitors. The involvement of PAF was studied using WEB 2170 (3 μmol/L) or CV 3988 (4 μmol/L) (30, 31), and the involvement of oxygen radicals was studied using SOD (50 U/ml), an enzyme scavenging O<sub>2</sub><sup>-</sup> anions. Finally, the involvement of cyclo-oxygenase was studied using indomethacin ( $10^{-7}$  M), a cyclo-oxygenase inhibitor. Cells were preincubated for 30 min at 37°C with the different inhibitors and then added to the upper compartment of the chamber. IL-12 (10 ng/ml), FMLP (10 μM), or the vehicle alone was placed in the lower compartment.

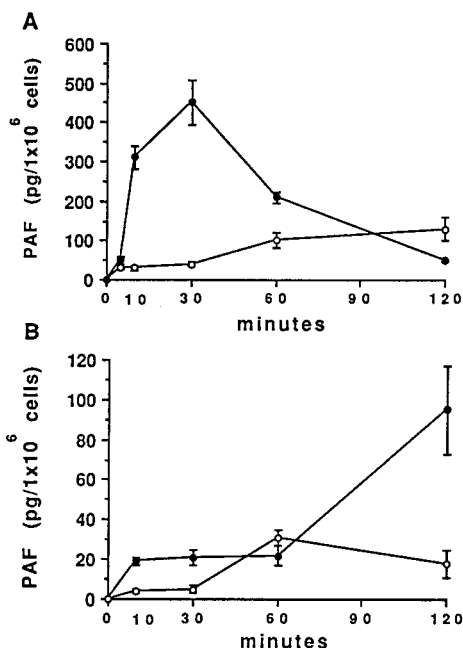
**Results***Synthesis of PAF from PMN, monocytes, and NK cells*

As shown in Figure 1, IL-12 induced a dose-dependent production of PAF from PMN. Inactivation of the cytokine by boiling prevented the stimulatory effect (Fig. 1). Time-course studies showed a peak of PAF production 30 min after stimulation with IL-12 (Fig.



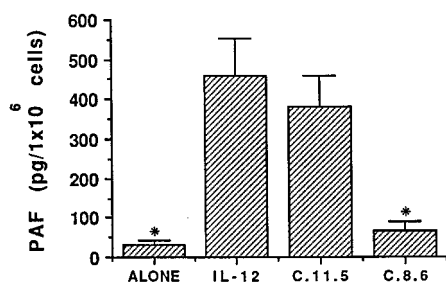
**FIGURE 1.** Total amount of PAF synthesized by  $1 \times 10^6$  PMN 30 min after stimulation with different doses of IL-12 (●) or of IL-12 boiled for 10 min (○) as a control. Data represent the mean  $\pm$  SE of four individual experiments. Analysis of variance with Dunnett's multicomparison test was performed among PMN stimulated with IL-12 vs PMN stimulated with boiled IL-12 (\* indicates  $p < 0.05$ ).

2A). PAF detected at that time was all cell associated, whereas 120 min after stimulation PAF was detected released in the supernatant (Fig. 2B). Cell viability tested at the end of each experiment by trypan blue dye exclusion test was  $>90\%$ . The contamination of PMN preparations with CD56<sup>+</sup> cells was  $<1\%$ . To rule out the possibility that the very few contaminating NK cells may release mediators in response to IL-12 and activate PMN, we added to PMN 10% purified NK cells, a concentration of CD56<sup>+</sup> cells that in PBMC was found insufficient to synthesize detectable amounts of PAF after direct IL-12 stimulation. In this experimental condition, an enhanced response of PMN preparations to IL-12 was not observed (data not shown). To evaluate the specificity of the effect induced by IL-12, the cytokine preparation was incubated for 30 min at 37°C with a neutralizing (C.8.6) and a nonneutralizing (C.11.5) anti-IL-12 mAb. PAF production was almost completely abrogated in the presence of the C.8.6 mAb, but not in the presence of the C.11.5 mAb, which lacks neutralizing activity (Fig. 3).



**FIGURE 2.** Time course of PAF synthesis from  $1 \times 10^6$  PMN unstimulated (○) or stimulated with 20 ng/ml IL-12 (●). A, PAF was detected as cell associated. B, PAF was detected as released in the supernatant. Data represent the mean  $\pm$  SE of six individual experiments.

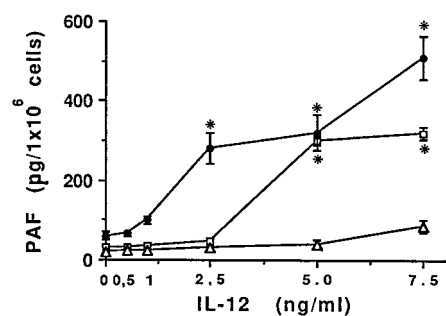




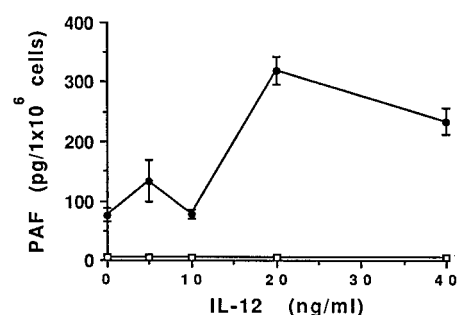
**FIGURE 3.** Total amount of PAF synthesized after 30-min incubation by  $1 \times 10^6$  PMN unstimulated (ALONE) or stimulated with 20 ng/ml IL-12 or with 20 ng/ml IL-12 preincubated (30 min at 37°C) with the nonneutralizing anti-IL-12 C.11.5 mAb (10 µg/ml; C.11.5) or with the neutralizing anti-IL-12 C.8.6 mAb (10 µg/ml; C.8.6). Data are the mean  $\pm$  SE of four individual experiments. Analysis of variance with Dunnett's multicomparison test was performed among PMN stimulated with IL-12 vs PMN unstimulated or stimulated with anti-IL12 mAb C.11.5 or with anti-IL-12 C.8.6 mAb (\* indicates  $p < 0.05$ ).

To evaluate whether IL-12 primes the synthesis of PAF induced by TNF- $\alpha$  and LPS, PMN were preincubated with various doses of IL-12 and then challenged with a nonstimulatory dose of TNF- $\alpha$  or LPS. As shown in Figure 4, the production of PAF was enhanced when PMN were costimulated with IL-12 and TNF- $\alpha$  or LPS.

Unstimulated NK cells showed a low basal production of PAF, which was detectable at 15 min both cell associated (76.66 + 12.31 pg/1  $\times 10^6$  cells) and in the supernatant (41.25 + 13.47 pg/1  $\times 10^6$  cells) of NK cells (mean  $\pm$  SE of five individual experiments). IL-12 significantly enhanced the production of PAF, which was detectable mainly in the cell-associated form (Fig. 5). In contrast, an enhancement of PAF release was not observed (data not shown). Maximal stimulation was reached at the dose of 20 ng/ml IL-12 (Fig. 5). Control experiments were performed on the CD56-negative leukocyte fraction. IL-12 did not stimulate PAF synthesis from CD56-negative cell fractions (Fig. 5). Preincubation with the C.8.6 neutralizing anti-IL-12 mAb abrogated the IL-12-dependent synthesis of PAF (Fig. 6). The study of the time course of PAF production showed a sustained synthesis of PAF until 1 h after stimulation with IL-12, with a decrease thereafter (Fig. 6). IL-12, at the lower nonstimulatory doses, failed to prime PAF synthesis from NK stimulated with TNF- $\alpha$  or LPS (data not shown).



**FIGURE 4.** Priming effect of IL-12 on the synthesis of PAF by PMN. PAF synthesis by  $1 \times 10^6$  PMN unstimulated ( $\Delta$ ) or stimulated with 10 ng/ml TNF- $\alpha$  ( $\bullet$ ) or with 1 ng/ml LPS ( $\square$ ) was evaluated after preincubation (30 min at 37°C) with different doses of IL-12. PAF was detected as cell associated 30 min after stimulation. Data are the mean  $\pm$  SE of four individual experiments. Analysis of variance with Dunnett's multicomparison test was performed among PMN stimulated with IL-12 alone vs PMN stimulated with IL-12 plus TNF- $\alpha$  or vs PMN stimulated with LPS (\* indicates  $p < 0.05$ ).

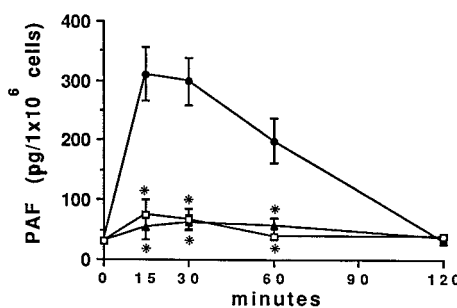


**FIGURE 5.** Total amount of PAF synthesized by  $1 \times 10^6$  CD56-positive ( $\bullet$ ) or CD56-negative ( $\square$ ) cells at 15 min after stimulation with different doses of IL-12. Data are the mean  $\pm$  SE of four individual experiments.

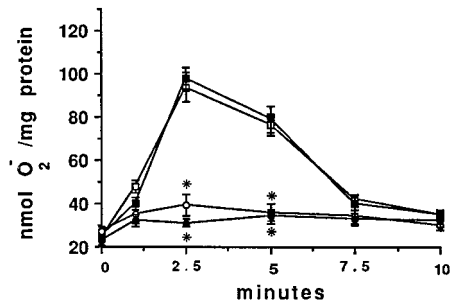
IL-12 did not stimulate the synthesis of PAF from monocytes purified either by plastic adhesion or by CD14 magnetic beads, nor did it prime monocytes to synthesize PAF after stimulation with TNF- $\alpha$  or LPS.

#### Production of ROS from PMN, monocytes, and NK cells

IL-12 induced  $O_2^-$  production, evaluated as a reduction of cytochrome *c*, by PMN. The production of  $O_2^-$  peaked 2.5 min after the addition of IL-12 (Fig. 7). In contrast, IL-12 stimulated only minimal chemiluminescence by PMN (Fig. 8). However, IL-12 acted synergistically with FMLP to stimulate chemiluminescence by PMN. Preincubation of IL-12 with 10 µg/ml of the C.8.6 neutralizing anti-IL-12 mAb, but not with 10 µg/ml of the C.11.5 nonneutralizing anti-IL12 mAb, abrogated the generation of ROS (Fig. 7). IL-12 failed to stimulate  $O_2^-$  production by monocytes and NK cells (data not shown). Since previous studies suggested a role for intracellular PAF in superoxide secretion by PMN (35), we evaluated whether IL-12-induced PAF synthesis could contribute to the generation of ROS. For this purpose, cells were pretreated for 10 min with the PAF receptor antagonists WEB 2170 and CV 3988 before stimulation with IL-12. It was found that both inhibitors significantly reduced  $O_2^-$  production by PMN stimulated with IL-12, suggesting an autocrine stimulation of PMN by newly synthesized PAF after challenge with IL-12 (Fig. 9). In contrast, WEB 2170 and CV 3988 did not significantly reduce  $O_2^-$  production by PMN stimulated with FMLP. This is consistent with the observation that the intracellular signaling pathways of FMLP and PAF



**FIGURE 6.** Time course of PAF synthesis from  $1 \times 10^6$  NK cells alone ( $\blacktriangle$ ) or challenged with IL-12 (20 ng/ml) ( $\bullet$ ) or with IL-12 (20 ng/ml) preincubated (30 min at 37°C) with the neutralizing anti-IL-12 C.8.6 mAb (10 µg/ml;  $\square$ ). PAF was detected as cell associated. Data are the mean  $\pm$  SD of five individual experiments. Analysis of variance with Dunnett's multicomparison test was performed among NK cells stimulated with IL-12 vs NK cells unstimulated or stimulated with anti-IL-12 C.8.6 mAb (\* indicates  $p < 0.05$ ).

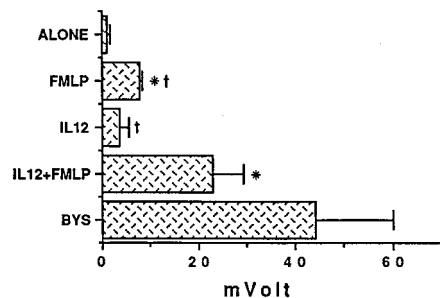


**FIGURE 7.** Time course of the production of  $O_2^-$  from PMN ( $1 \times 10^6$  cells) unstimulated ( $\circ$ ) or stimulated with 20 ng/ml IL-12 ( $\blacksquare$ ) or with 20 ng/ml IL-12 preincubated (30 min at  $37^\circ\text{C}$ ) with the neutralizing anti-IL-12 C.8.6 mAb ( $10 \mu\text{g/ml}$ ) ( $\blacktriangle$ ) or with the nonneutralizing anti-IL-12 C.5.11 ( $\square$ ). Values are given as the mean  $\pm$  SE of six individual experiments. Analysis of variance with Dunnett's multicomparison test was performed among PMN stimulated with IL-12 vs unstimulated PMN or PMN stimulated with IL-12 plus C.8.6 mAb or C.5.11 mAb (\* indicates  $p < 0.05$ ).

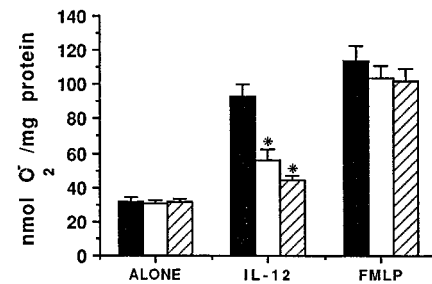
are distinct in human PMN (36). Moreover, previous studies have shown that WEB 2170 does not exhibit an antioxidative action per se (30).

#### Modulation of IL-12-induced chemotaxis

As previously reported, IL-12 induced chemotaxis of PMN and NK cells, but not of monocytes (21, 22). Since we observed that IL-12 induced the synthesis of PAF by PMN and NK, we studied whether the newly synthesized PAF was instrumental in triggering the events involved in the motogenic response to IL-12. As shown in Figure 10, PAF induced, at doses as low as 1 nM PAF, migration of NK cells, which was almost completely inhibited by the PAF receptor antagonist WEB 2170, as previously described for PMN (12). The IL-12-induced chemotaxis on NK cells and PMN was significantly reduced by two structurally different PAF receptor antagonists: WEB 2170 and CV 3988 (Figs. 11 and 12A). FMLP-induced chemotaxis on PMN was not significantly reduced by PAF receptor antagonists (Fig. 12A). Preincubation with SOD, which blocks the formation of superoxide anions, also reduced the chemotactic effect of IL-12 on PMN (Fig. 12B) but not that on NK cells (data not shown). In contrast, indomethacin, which inhibits



**FIGURE 8.** Chemiluminescence of PMN recorded 5 min after stimulation with  $1 \mu\text{mol/L}$  FMLP, 20 ng/ml IL-12, 20 ng/ml IL-12 (preincubated for 10 min at  $37^\circ\text{C}$ ) plus FMLP ( $1 \mu\text{mol/L}$ ), or phagocytosis of  $1 \times 10^7$  C3b-BYS (BYS). ALONE indicates chemiluminescence of unstimulated PMN incubated for the same period of time. Values are given as the mean  $\pm$  SE of six individual experiments. Analysis of variance with Newman-Keuls multicomparison test was performed among unstimulated PMN vs PMN stimulated with FMLP, IL-12, or IL-12 plus FMLP (\* indicates  $p < 0.05$ ) and among PMN stimulated with FMLP plus IL-12 vs PMN stimulated with IL-12 or FMLP ( $\dagger$  indicates  $p < 0.05$ ).

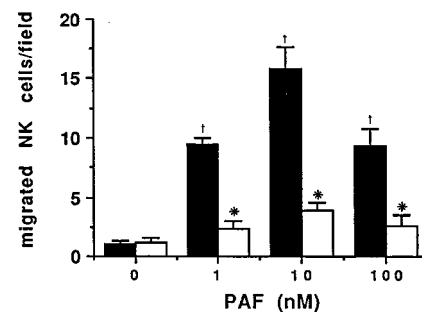


**FIGURE 9.** Production of  $O_2^-$  from PMN ( $1 \times 10^6$  cells) stimulated for 5 min with 20 ng/ml IL-12 or FMLP ( $1 \mu\text{mol/L}$ ). PMN were preincubated with WEB 2170 (blank column) or CV 3988 (striped column) for 10 min at  $37^\circ\text{C}$  or with the vehicle alone (dark column) and then stimulated with IL-12 or FMLP. Values are given as the mean  $\pm$  SE of six individual experiments. Analysis of variance with Dunnett's multicomparison test was performed among PMN stimulated with IL-12 vs PMN stimulated with IL-12 plus WEB 2170 or with IL-12 plus CV 3988 (\* indicates  $p < 0.05$ ) and among PMN stimulated with FMLP vs PMN stimulated with FMLP plus WEB 2170 or with FMLP plus CV 3988 (no statistical significance).

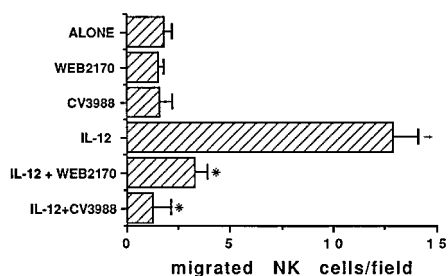
cyclo-oxygenase-derived metabolites of arachidonic acid, did not affect IL-12-dependent chemotaxis on PMN (Fig. 12B).

#### IL-12R expression by PMN

The presence of the IL-12R on PMN was evaluated by cytofluorometric analysis using the murine 12R $\beta$ .44 mAb, specific for the human low affinity IL-12R  $\beta$ 1-chain (23). As shown in Figure 13A, 42% of PMN were stained with the 12R $\beta$ .44 mAb but not with the irrelevant isotypic control IgG. 12R $\beta$ .44 mAb also stained 60% of human PBMC activated for 72 h with PHA and used as a positive control (data not shown) (23). IL-12 binding to the putative receptor was evaluated by incubating PMN with IL-12 and then with the anti-IL-12 mAb C.11.5. As shown in Figure 13B, 35% of PMN expressed low but significant levels of IL-12 binding. PMN unchallenged with IL-12 or incubated with the irrelevant isotypic control were not stained. As previously described by Desai et al. (26), IL-12R detection specificity by this method was confirmed by the diminished staining observed when the C.11.5 mAb was replaced with the neutralizing anti-IL-12 mAb. Because the neutralizing anti-IL-12 C.8.6 mAb inhibits binding of IL-12 to its receptor, it has been previously reported that the reaction of IL-12 with

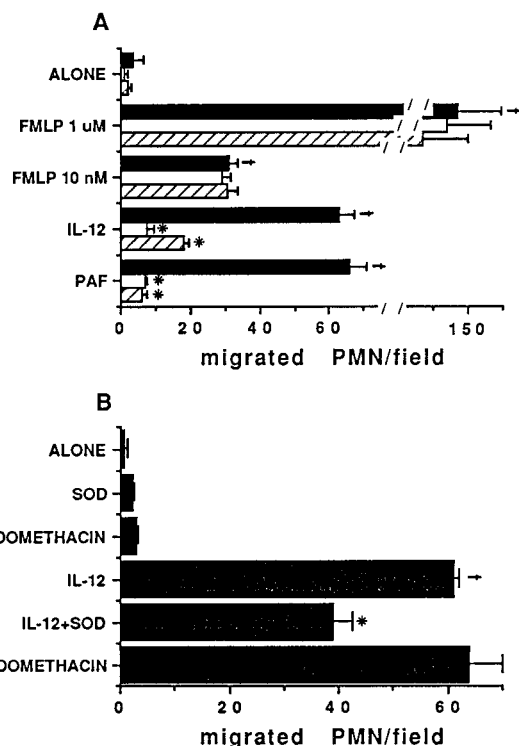


**FIGURE 10.** NK cell migration induced by PAF at different concentrations (dark column) or by PAF plus WEB 2170 ( $3 \mu\text{mol/L}$ ; blank column). The migration assay was performed using the modified Boyden's chamber technique as described in *Materials and Methods*. Cells ( $2 \times 10^5$ ) were seeded in the upper compartment. Cells migrated across the polycarbonate filters after 4-h incubation were counted. The numbers are the mean  $\pm$  SE of cells counted per 10 fields ( $\times 200$ ) in three individual experiments. Analysis of variance with Newman-Keuls multicomparison test was performed: unstimulated NK cells vs NK cells stimulated with PAF,  $p < 0.05$  ( $\dagger$ ); alone vs WEB 2170, not statistically significant; PAF vs PAF in the presence of WEB 2170,  $p < 0.05$  (\*).

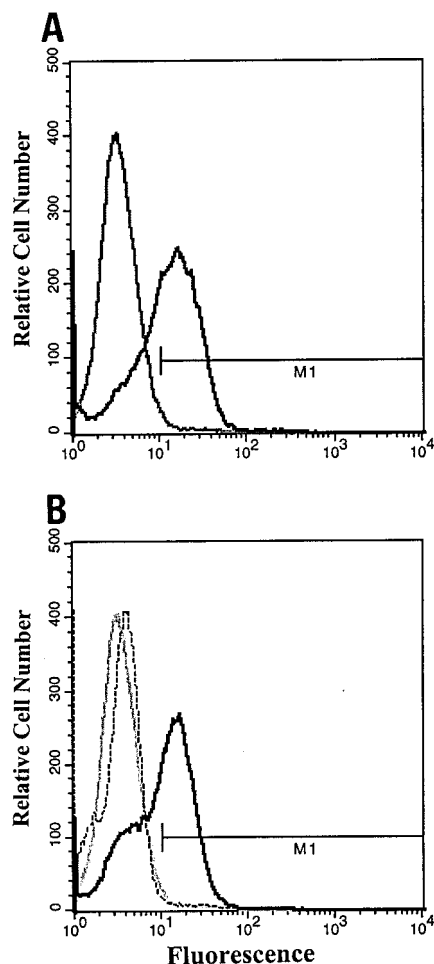


**FIGURE 11.** Effects of WEB 2170 (3  $\mu\text{mol/L}$ ) and CV 3988 (4  $\mu\text{mol/L}$ ) on NK cell migration induced by IL-12 (20 ng/ml). The numbers are the mean  $\pm$  SE of cells counted per 10 fields ( $\times 200$ ) in eight individual experiments. Analysis of variance with Newman-Keuls multicomparison test was performed between alone vs IL-12 ( $\dagger$  indicates  $p < 0.05$ ), alone vs WEB 2170 and CV 3988 (not statistically significant), and IL-12 vs IL-12 in the presence of WEB 2170 and CV 3988 (\* indicates  $p < 0.05$ ).

its receptor prevents binding of neutralizing anti-IL-12 mAb to cell-associated IL-12 (26). Using this technique, IL-12 binding to the putative receptor was detected on 70% PMBC activated



**FIGURE 12.** A, PMN migration of unstimulated cells (ALONE) or of PMN stimulated with FMLP (10 nmol/L and 1  $\mu\text{mol/L}$ ), IL-12 (10 ng/ml), or PAF (10 nmol/L). The effects of WEB 2170 (3  $\mu\text{mol/L}$ ; blank column) and CV 3988 (4  $\mu\text{mol/L}$ ; striped column) were studied. B, Effects of SOD (80 U/ml) and indomethacin (10 $^{-7}$  mol/L) on PMN migration induced by IL-12 (10 ng/ml). The migration assay was performed using the modified Boyden's chamber technique as described in *Materials and Methods*. Cells ( $1 \times 10^5$ ) were seeded in the upper compartment. Cells that migrated across the polycarbonate filters after 2-h incubation were counted. The numbers are the mean  $\pm$  SE of cells counted per 10 fields ( $\times 200$ ) in eight individual experiments. Analysis of variance with Newman-Keuls multicomparison test was performed: control vs WEB 2170, CV 3988 and control vs SOD and indomethacin, not statistically significant; unstimulated NK cells vs stimulated NK cells,  $p < 0.05$  ( $\dagger$ ); stimulated NK cells vs stimulated NK cells in the presence of WEB 2170 and CV 3988 or in the presence of SOD or indomethacin,  $p < 0.05$  (\*).



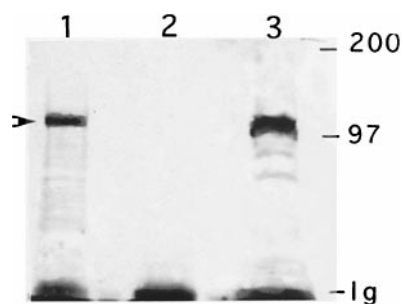
**FIGURE 13.** Detection of IL-12R by flow cytometry on PMN. A, Cells were stained with 12R $\beta$ .44 mAb specific for the human low affinity IL-12R  $\beta$ 1 chain (dark line) or with the isotypic control IgG (gray line), as described in *Materials and Methods*. Two experiments were performed with similar results. In each experiment the Kolmogorov-Smirnov statistic analysis was significant ( $p < 0.001$ ). B, Binding of IL-12 to the putative receptor was evaluated by incubating PMN with IL-12 and then with anti-IL-12 mAb C.11.5 (dark line), with the neutralizing anti-IL-12 mAb C.8.6 (dotted line), or with an irrelevant isotypic control IgG (gray line) as described in *Materials and Methods*. Two experiments were performed with similar results. In each experiment the Kolmogorov-Smirnov statistic analysis between C.11.5 mAb and the isotypic control or between C.5.11 and C.8.6, but not between C.8.6 and the isotypic control, was significant ( $p < 0.001$ ).

for 72 h with PHA and used as a positive control (data not shown).

The expression of IL-12R  $\beta$ 1-chain by PMN was also evaluated by immunoprecipitation followed by Western blot analysis. The anti-IL-12R 12R $\beta$ .44 mAb was capable of recognizing a band of approximately 110 kDa, which corresponds to the  $\beta$ 1 receptor (37), in PMN immunoprecipitated with the 12R $\beta$ .44 mAb, but not with the isotypic control (Fig. 14). The expression of the  $\beta$ 1 receptor in unstimulated and PHA-stimulated lymphocytes was used as control.

## Discussion

The results of the present study indicate that IL-12 induces the synthesis of PAF from PMN and NK cells, but not from monocytes. Moreover, we could demonstrate that this mediator is involved in IL-12-induced chemotaxis of PMN and NK cells.



**FIGURE 14.** PMN express IL-12R  $\beta$ 1-chain. PMN ( $4 \times 10^7$ ) were lysed and immunoprecipitated with 12R $\beta$ .44 mAb (lane 1) or the irrelevant isotypic control IgG (lane 2). The eluted proteins were subjected to 8% SDS-PAGE, and then the filter was immunoblotted with anti-IL-12R  $\beta$ 1-chain 12R $\beta$ .44 mAb. The position of the IL-12R  $\beta$ 1-chain is indicated by the arrow. Unstimulated lymphocytes ( $2 \times 10^7$ ; lane 3) were used as a positive control. Similar results were obtained using PHA-stimulated lymphocytes (not shown).

IL-12 is a key cytokine in both the innate resistance and the adaptive immune response (17). IL-12 is produced early during the response to infectious agents (38), leading to the activation of the phagocytic cell system as the first line of defense against pathogens. This activation of phagocytic cells is due to the IL-12-dependent production of IFN and other cytokines, such as granulocyte macrophage-CSF, IL-8, and TNF- $\alpha$  (17, 39, 40). In the present study, we documented that IL-12 induces the synthesis of PAF from both PMN and NK cells. PAF is a proinflammatory mediator produced early in response to bacterial products and proinflammatory cytokines such as TNF- $\alpha$  and IL-8 (1, 2, 14, 15). The inhibition of the synthesis of proinflammatory cytokines by IL-10, a Th2 cytokine, also inhibits the synthesis of PAF by PMN (41). Moreover, PAF itself mediates some of the biologic effects exerted by proinflammatory cytokines (14–16). Several cell types are capable of synthesizing PAF, including PMN, NK, and monocytes (3–5). In the present study IL-12 failed to stimulate the synthesis of PAF from monocytes. In contrast, IL-12 not only stimulated the synthesis of PAF from PMN, but, at nonstimulatory doses of this cytokine, it primed the synthesis of PAF by PMN stimulated with TNF- $\alpha$  or LPS. The possibility that very few contaminating NK cells may drive the activation of PMN by releasing mediators in response to IL-12 cannot be completely ruled out. However, a direct stimulation of PMN by IL-12 is likely, since an increase in the number of CD56<sup>+</sup> cells from <1% to 10% did not induce an enhanced response of PMN preparations to IL-12. In addition, PMN were shown, by both cytofluorometric analysis and immunoprecipitation, to express the human low affinity IL-12R  $\beta$ 1-chain and to bind IL-12 at a low but significant level, suggesting a direct stimulation of PMN by this cytokine. A similar expression of low affinity IL-12R  $\beta$ 1-chain has been observed in unstimulated T lymphocytes (42), in which, in the absence of significant binding, IL-12 induces an efficient production of IFN- $\gamma$  but no proliferation (17). It has been suggested that, similarly to the IL-2R, the IL-12R may also use different receptor chain combinations, with different affinities, to mediate the signal transduction leading to expression of various biologic activities (17). However, since we observed a low but significant binding of IL-12 to PMN, further studies are needed to characterize such binding and to evaluate the expression of the  $\beta$ 2-subunit receptor of IL-12. Moreover, we observed a direct stimulatory effect on PAF synthesis by NK cells, but not a priming for the stimulation with TNF- $\alpha$  or LPS. In contrast, CD56-negative PBMC stimulated with IL-12 failed to synthesize PAF. IL-12-induced synthesis of PAF by NK

cells was dose dependent, started rapidly, was sustained up to 1 h, and was detected mainly as associated with the cellular fraction.

IL-12 directly potentiates the cytolytic effect of NK cells against target cells (19, 21, 40, 43). The enhancement of NK cell-mediated cytotoxicity is due to an increase in the number of cytotoxic cytoplasmic granules containing perforin, esterases, and other lytic components (40, 43). Moreover, an increase in binding to the target cells due to an increase in adhesion molecules (21) contributes to the IL-12-induced lytic activity of NK cells. Since PAF has been shown to enhance NK cell cytotoxicity, and PAF antagonists are known to reduce NK cell activity (11, 12), it can be speculated that an endogenous synthesis of PAF may be instrumental in the adhesion of NK effectors to their target. In fact, several studies indicate that PAF contributes to the mechanisms of leukocyte adhesion (44). It has been recently shown that NK cells synthesized PAF after cross-linking of CD16 (45), the low affinity receptor for the Fc fragment of IgG that is considered a major receptor capable of triggering cytotoxicity (46, 47). Thereby, we provide direct evidence that NK synthesize PAF after stimulation with IL-12, a specific NK stimulatory cytokine. Moreover, the observation that the two cell types (PMN and NK) that responded to the chemotactic action of IL-12 (21, 22) also produced PAF, whereas the IL-12-insensitive monocytes did not, prompted us to explore whether PAF mediates the action of this cytokine. Indeed, the blockade of the PAF receptor prevented chemotaxis of PMN and NK cells stimulated by IL-12. In addition, exogenous PAF was found to stimulate the chemotactic response not only of PMN (12), but also of NK cells. These results suggest that PAF synthesis may be required for full expression of adhesion molecules that are necessary for cell migration. Indeed, it has been shown that PAF plays a critical role in neutrophil migration across monolayers of cytokine-prestimulated endothelial cells (48). However, the endogenous synthesis of PAF by PMN was not the only mechanism involved in IL-12-induced chemotaxis. Previous studies have excluded a role for cytokine or chemokine production by PMN or NK cells on chemotaxis induced by IL-12 (22). We observed that ROS scavengers were capable of reducing the chemotaxis elicited by IL-12 on PMN by about 50%. Although stimulation of leukocytes with PAF is known to induce a weak ROS production (36), previous studies have demonstrated that PAF present within the cell can mediate the release of superoxide anions, acting as a second messenger (36). In the present study, we observed that the production of ROS by PMN stimulated with IL-12 was significantly reduced by PAF receptor antagonists. These results suggest that the endogenous production of PAF elicited by IL-12 and the PAF-related generation of ROS mediates the chemotaxis of PMN induced by IL-12. In contrast, no production of ROS by IL-12-stimulated NK cells was observed.

In conclusion, the results of our study indicate that the chemotactic effect of IL-12 is not a direct phenomenon, but is dependent on the production of secondary mediators. As inferred from the inhibitory effect of the PAF receptor antagonists, IL-12-induced PAF synthesis plays a critical role in triggering the events involved in the motogenic response of PMN and NK cells to IL-12.

### Acknowledgments

We thank Drs. L. Pegoraro and F. Malavasi for discussions and helpful suggestions, Dr. G. Trincheri and the Genetics Institute (Cambridge, MA) for providing IL-12 and anti-IL-12 mAb (C.8.6 and C.11.5), and Dr. J. Ritz (Dana-Farber Cancer Institute, Boston, MA) for providing the anti-IL-12 receptor 12R $\beta$ .44 mAb. We thank Patrizia Dentelli and Fiorella Sanavio for technical assistance.



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