THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 7, Issue of February 18, pp. 4746–4753, 1994
Printed in U.S.A.

Rapid Induction of Arachidonic Acid Release by Monocyte Chemotactic Protein-1 and Related Chemokines

ROLE OF Ca^{2+} INFLUX, SYNERGISM WITH PLATELET-ACTIVATING FACTOR AND SIGNIFICANCE FOR CHEMOTAXIS*

(Received for publication, August 3, 1993, and in revised form, October 25, 1993)

Massimo Locati, Dan Zhou, Walter Luini, Virgilio Evangelista‡, Alberto Mantovani, and Silvano Sozzani§

From the Istituto di Ricerche Farmacologiche Mario Negri, via Eritrea 62, 20157 Milan and the ‡Istituto di Ricerche Farmacologiche Mario Negri, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, Italy

Monocyte Chemotactic Protein-1 (MCP-1), a member of the Cys-Cys branch of the chemokine superfamily, induced a mepacrine- and manoalide-sensitive increase in the release of [^{3}H]arachidonic acid from prelabeled human monocytes and monocytic THP-1 leukemic cells. The effect was rapid (<30 s), reached maximum at optimal chemotactic concentrations, and was completely blocked by pretreatment of monocytes with *Bordetella* pertussis toxin. A specific antiserum and heat inactivation blocked the induction of arachidonic release by MCP-1. No [^{3}H]arachidonic acid release was observed in the absence of Ca^{2+} influx (5 mM EGTA or 5 mM Ni^{2+}) or in monocytes loaded with a Ca^{2+}-buffering agent. However, using ionophore-permeabilized monocytes and controlled intracellular Ca^{2+} concentration it was possible to dissociate MCP-1-induced Ca^{2+} influx from [^{3}H]arachidonic acid release. Thus, the MCP-1-induced increase in $[Ca^{2+}]_i$ is necessary but not sufficient for arachidonic acid accumulation. Phospholipase A_2 inhibitors (mepacrine, *p*-bromophenacyl bromide, and manoalide) blocked monocyte polarization and chemotaxis induced by MCP-1. The related Cys-Cys chemokines RANTES and LD78/MIP1α also induced a rapid release of [^{3}H]arachidonic acid, and their chemotactic activity was blocked by phospholipase A_2 inhibitors. Brief (5 min) pretreatment of monocytes with platelet-activating factor amplified MCP-1-induced arachidonic acid release and, at MCP-1 suboptimal concentrations, synergized in inducing monocyte migration. Since MCP-1 and platelet-activating factor are induced concomitantly by inflammatory cytokines in monocytes and endothelial cells, we speculate that the observed synergism may have *in vivo* relevance. The results presented here show that the Cys-Cys chemokines MCP-1, LD78/MIP1α, and RANTES cause rapid release of arachidonic acid in monocytes and that this may be important in inducing monocyte chemotaxis.

The recruitment of leukocytes from the blood compartment into tissues is a highly regulated process that involves receptor and counterreceptor interactions and secretion of chemotactic factors (for reviews, see Refs. 1–3). Upon exposure to inflammatory signals (*e.g.* interleukin (IL)-1[1] and tumor necrosis factor), endothelial cells produce phospholipid metabolites, such as platelet-activating factor (PAF) (4), and chemotactic factors (3) that attract and activate different leukocyte populations. Recently, a new superfamily of low molecular weight chemotactic proteins, named chemokines, active on different leukocyte populations, has been identified (5–9). The structural hallmark of these mediators is represented by 4 cysteine residues, the first 2 of which are in tandem. According to the position of the first 2 cysteines, it is possible to distinguish two subfamilies: the first one, in which the 2 cysteines are interrupted by an intervening amino acid (Cys-*X*-Cys), and the second one, in which they are directly linked (Cys-Cys) (5, 6, 9). In endothelial cells, IL-1, tumor necrosis factor, and lipopolysaccharide induce the expression of IL-8 and groα, two members of the Cys-*X*-Cys branch of the chemokine family, and monocyte chemotactic protein (MCP)-1, a member of the Cys-Cys subfamily (3). The local production of specific chemotactic factors may play an important role in the regulation of leukocyte infiltration present in a number of clinical situations, such as acute lung injury, atherosclerosis, vasculitis, and tumors (2, 8, 9).

MCP-1 is a chemotactic and activating factor for monocytes *in vitro* and *in vivo* (10–12), and there is evidence that this protein is indeed an important determinant for monocyte recruitment *in vivo* (8). With the aim of understanding better the mechanisms responsible for monocyte recruitment, we started to investigate the molecular basis for monocyte activation by MCP-1. In previous work we and others have found that MCP-1 activates a *Bordetella* pertussis toxin (PT)-sensitive influx of extracellular Ca^{2+} which resulted in a rapid and transient increase of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (12, 13). This effect was not associated with the breakdown of phosphatidylinositol bisphosphate evaluated as inositol phosphates accumulation and appeared to be important for monocyte migration (14). In an effort to identify Ca^{2+}-sensitive steps crucial for MCP-1-activated monocyte chemotaxis we have investigated the possible role of phospholipase A_2 activation. Monocytes and macrophages have been shown to be rich sources of arachidonate and its metabolites (15). However, the physiologic role of arachidonic acid metabolism in human monocytes has been only partly elucidated. A close association between neu-

* This work was supported by the National Research Council (CNR), Italy project ACRO and Convenzione CNR-Consorzio Mario Negri Sud, by NATO Grant CRG 910243, and by the Italian Association for Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 2-390-141; Telex: 331268 NEGRI I; Fax: 2-354-6277.

[1] The abbreviations used are: IL, interleukin; PAF, platelet-activating factor; MCP-1, monocyte chemotactic protein-1; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PT, *Bordetella* pertussis toxin; GM-CSF, granulocyte macrophage colony-stimulating factor; fMLP, formyl-methionyl-leucyl-phenylalanine; BPB, *p*-bromophenacyl bromide; FCS, fetal calf serum; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBMC, peripheral blood mononuclear cells.

trophil chemotaxis and degradation of phospholipids by phospholipase A_2 was reported (16, 17), and a direct role for arachidonic acid in leukocyte adhesion and spreading was recently shown (18–20).

In the present paper we report that MCP-1, as well as other members of the Cys-Cys chemokine family, induce a rapid accumulation of arachidonic acid in human monocytes and THP-1 promonocytic cell line. This effect is dependent on the influx of extracellular Ca^{2+}, is PT-sensitive, and is relevant for monocyte migration.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant MCP-1 (rMCP-1) was from PeproTech Inc. (Rocky Hill, NJ), and human recombinant IL-8 was from Dainippon (Osaka, Japan). Human natural MG-63-derived MCP-1, purified to homogeneity as described (21), was a kind gift from Dr. J. Van Damme, Rega Institute, University of Leuven, Belgium. Human recombinant RANTES and LD78 were a kind gift from Drs. T. Schall, Genentech Inc., San Francisco, and L. Czaplewski, British Bio-technology Limited, Cowley, U. K., respectively. Human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) and C5a were a generous gift from Drs. Seiler, Behring, Marburg, F. R. G. and H. S. Showell, Pfizer Central Res., Groton, CT, respectively. Recombinant products were endotoxin-free as assessed by Limulus Amebocyte assay. PT was a kind gift from Dr. Rappuoli, IRIS, Siena, Italy. [^{3}H]Arachidonic acid (210 Ci/mmol) was from Amersham International (Amersham, U. K.). Manoalide was from Calbiochem. fMLP, A23187, mepacrine (quinacrine), *p*-bromophenacyl bromide (BPB), standard lipids, and all other reagents were from Sigma. A rabbit anti-MCP-1 antiserum prepared in this laboratory was used to adsorb rMCP-1 specifically as described previously (22). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; *NIH Guide for the Care and Use of Laboratory Animals*, NIH Publication 85-23, 1985).

Cell Culture—THP-1 and HL-60 cells were cultured in RPMI 1640 (Biochrom, Berlin, F. R. G.) supplemented with 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT) in a humidified environment of 5% CO_2 at 37 °C.

Preparation of Monocytes—Human monocytes were obtained from buffy coats of normal blood donors through the courtesy of Centro Trasfusionale, Ospedale Sacco, Milan, Italy. To reduce platelet contamination, monocytes were isolated according to the procedure described by Pawlowski *et al.* (23) with minor modifications. Briefly, anticoagulated whole blood was diluted with cold phosphate-buffered isotonic saline without Ca^{2+} and Mg^{2+} (Life Technologies, Inc.) and centrifuged at 150 × *g* at 4 °C for 20 min. The supernatant was discarded, and the cell pellet was washed in phosphate-buffered saline in the same conditions. Cells were resuspended in phosphate-buffered saline containing 0.3 mM EDTA (Merck, Darmstadt, F. R.G.), layered on top of Ficoll (Biochrom), and centrifuged at 800 × *g* at room temperature for 25 min. Mononuclear cells (PBMC) were recovered, diluted, and washed twice in phosphate-buffered saline at 4 °C. To remove platelets specifically adherent to monocytes, PBMC were resuspended in FCS containing 5 mM EDTA and subjected to two sequential incubations (15 min) at 37 °C. Platelet-free PBMC were recovered by centrifugation at 400 × *g* at room temperature for 15 min. Monocytes were further purified (>90% pure) from PBMC by centrifugation at 600 × *g* on a 46% isoosmotic Percoll (Pharmacia, Uppsala, Sweden) gradient, as described previously (24). The monocyte preparation obtained did not release [^{3}H]arachidonic acid when challenged with 10 units/ml thrombin.

Release of [^{3}H]Arachidonic Acid—Monocytes (10^7/ml, in RPMI 1640, 10% FCS) were labeled in Petriperm dishes (Haereus, Vienna, Austria) with 1 μCi/ml [^{3}H]arachidonic acid overnight (16–18 hours). At the end of incubation the uptake of [^{3}H]arachidonic acid was 45–50% with the following phopholipid distribution: 59 ± 1.3% phosphatidylcholine, 14 ± 1.1% phosphatidylinositol/phosphatidylethanolamine, 24 ± 1.0% phosphatidylserine, 3 ± 0.6% phosphatidylglycerol (n = 4). The incubation did not affect cell viability (>95%, by trypan blue dye exclusion) or the ability of monocyte to migrate in response to rMCP-1. In some experiments, monocytes were labeled for 42 h. In these conditions phospholipid distribution of the label was slightly different (50% phosphatidylcholine, 13% phosphatidylinositol/phosphatidylethanolamine, 33% phosphatidylserine, and 4% phosphatidylglycerol). No change was observed in the magnitude of rMCP-1-induced [^{3}H]arachidonic acid release (data not shown). At the end of the incubation, cells were washed twice and resuspended in RPMI 1640 supplemented with 0.2% fatty acid-free bovine serum albumin (Sigma). Monocytes (10^7/ml) were prewarmed for 5 min at 37 °C and then stimulated for different times. The reaction was terminated by the addition of 3 ml of chloroform/methanol/formic acid (1:2:0.2, v/v) followed by agitation. Cell extracts were transferred to centrifuge tubes and 1 ml of water and 2 ml of chloroform added. Chromatographic separation of lipids was performed by evaporating the organic phase under a stream of nitrogen, redissolving the residue in chloroform, and loading the extract on Silica Gel G plates (Merck). Arachidonic acid was separated by solvent system I (hexane/ethyl ether/formic acid, 15:10:1, v/v) for 30 min as reported previously (25). The arachidonic acid position on TLC plates was determined as comigration with commercially available standards after exposure to iodine vapors. Quantitative determinations were obtained by scraping portions of the silica gel into scintillation vials followed by liquid scintillation spectrometry. Results are expressed as a percent of radioactivity in the fatty acid band on the total radioactivity recovered from each lane. For phospholipid analysis, TLC plates (Silica Gel G) were resolved with solvent system II (chloroform/methanol/acetic acid/water, 50:25:8:2, v/v) for 15 cm. Phospholipids were identified based on comigration with commercially available standards, and quantitative evaluation was performed by liquid scintillation spectrometry.

Measurement of $[Ca^{2+}]_i$—Changes in $[Ca^{2+}]_i$ were monitored using the fluorescent probe FURA-2 as described previously (12, 14) according to the technique reported by Grynkiewicz *et al.* (26).

Migration Assay—Cell migration was evaluated using a microchamber technique (27) as described previously (12, 14). Twenty-seven ± 1 μl of chemoattractant diluted in RPMI with 1% FCS was seeded in the lower compartment of the chemotaxis chamber, and 50 μl of cell suspension (1.5 × 10^6/ml monocytes in PBMC) was seeded in the upper compartment. The two compartments were separated by a 5-μm pore size polyvinylpyrrolidone membrane (Nucleopore Corp., Pleasanton, CA). Chambers were incubated at 37 °C in air with 5% CO_2 for 90 min. At the end of the incubation, filters were removed, fixed, and stained with Diff-Quik (Baxter s.p.a., Rome, Italy), and five high power oil-immersion fields were counted.

Polarization Assay—Monocyte polarization assay was performed as described previously (22) with minor modifications. Briefly, monocytes (10^6/ml) were prewarmed in polypropylene tubes at 37 °C for 5 min, exposed to different concentrations of inhibitors or control medium for 15–30 min, and then stimulated with rMCP-1 for 10 min. The reaction was stopped by adding an equal volume (0.5 ml) of ice-cold phosphate-buffered formaldehyde (10% v/v; pH 7.2). The percentage of cells with bipolar configuration (front-tail) was determined in at least 200 cells for each tube by phase-contrast microscopy at × 400 magnification. Each experiment was performed in duplicate.

Statistical Analysis—Data were analyzed by Student's *t* test. Synergism between PAF and rMCP-1 was analyzed by two-way analysis of variance for repeated measures with treatment as between subjects factor and PAF concentrations as within subjects factor. *Post hoc* comparisons were made with Tukey's test.

RESULTS

rMCP-1-induced Release of [^{3}H]Arachidonic Acid—Fig. 1 shows that rMCP-1 induced the release of [^{3}H]arachidonic acid in human monocytes in a concentration- and time-dependent manner. The effect was present at 10 ng/ml rMCP-1, reached the maximum at 50–100 ng/ml, and decreased at 300 ng/ml. [^{3}H]Arachidonic acid accumulation started 15 s after monocyte stimulation with the peak of the activity observed at 2–3 min (Fig. 2*A*). Fifteen min after stimulation the concentration of [^{3}H]arachidonic acid returned to base-line level (Fig. 2*A*). A similar kinetic was observed in THP-1 cells stimulated with 50 ng/ml rMCP-1 (Fig. 2*B*). These cells are known to express specific binding sites and to migrate in response to rMCP-1 (28). On the other hand, undifferentiated HL-60 cells, which do not possess binding sites for this cytokine,[2] did not respond to rMCP-1 activation (Fig. 2*B*).

rMCP-1 is known to activate human monocytes through a PT-sensitive GTP-binding protein (12). Fig. 3 shows that rMCP-1-induced [^{3}H]arachidonic acid release is inhibited when monocytes are pretreated with PT. Complete inhibition of

[2] J. M. Wang, personal communication.

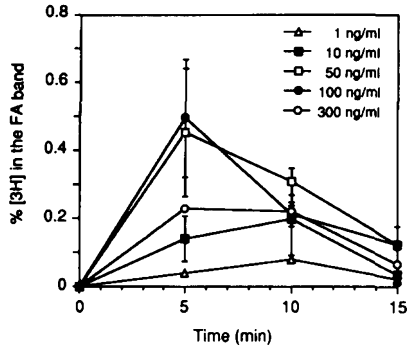

FIG. 1. **Effect of rMCP-1 on the release of [^{3}H]arachidonic acid from labeled human monocytes.** Human monocytes were separated and labeled as detailed under "Experimental Procedures." Cells (10^7/ml) were stimulated with different concentrations of rMCP-1 for different times. The reaction was stopped by the addition of 3 ml of chloroform/methanol/formic acid (1:2:0.2, v/v) followed by extraction. The accumulation of [^{3}H]arachidonic acid was evaluated in the extracted organic phase by TLC on Silica Gel G plates using solvent system I. The results are expressed as percentage of [^{3}H] in the fatty acid (*FA*) band at the net of control values. Each point represents the average value of 3–10 (10–300 ng/ml) or 2 (1 ng/ml) different experiments.

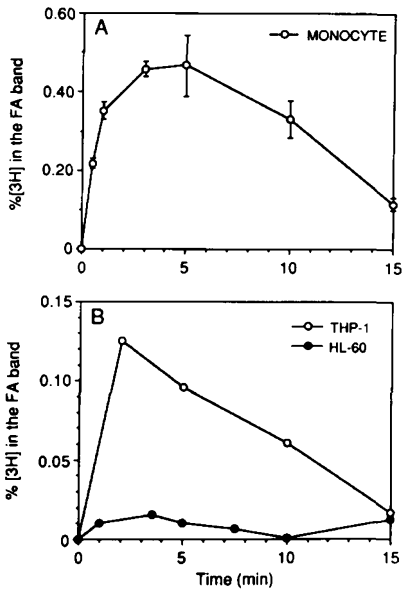

FIG. 2. **Time course of rMCP-1-induced release of [^{3}H]arachidonic acid.** Human monocytes (*panel A*), monocytic THP-1 cells and promyelocytic HL-60 cells (*panel B*) prelabeled with [^{3}H]arachidonic acid for 16–18 h were stimulated with 50 ng/ml rMCP-1 for the indicated periods of time. Samples were processed as described in the Fig. 1 legend. Results are expressed as percentage of ^{3}H in the fatty acid (*FA*) band at the net of control values (0.572, 0.446, and 0.421% for monocytes, THP-1, and HL-60 cells, respectively). *Panel A*, mean ± S.D. of three experiments. *Panel B*, mean of two experiments. The variation between the two experiments was less than 15%. Release of [^{3}H]arachidonic acid after stimulation with 1 μM A23187 for 3 min was: 4.476% (monocytes), 0.602% (THP-1), and 0.505% (HL-60). Note the different *y* axes between *panels A* and *B* presumably because of the low levels of phospholipase A_2 activity in undifferentiated monocytic cell lines (29).

[^{3}H]arachidonic acid release was not caused by toxicity of the PT treatment since monocytes maintained, unaltered, their ability to respond to 1 μM A23187 (Fig. 3).

The effect of rMCP-1 was not present if the cytokine was preadsorbed with a specific antiserum or if the cytokine was boiled for 10 min (Fig. 4). Pretreatment of monocytes with mepacrine (100 μM) or with manoalide (5 μM), two phospholipase A_2 inhibitors, strongly decreased the effect of 50 ng/ml rMCP-1 (116 ± 20%, n = 4, and 86%, n = 2, inhibition, respectively). A similar level of inhibition (80%, n = 2) was observed in

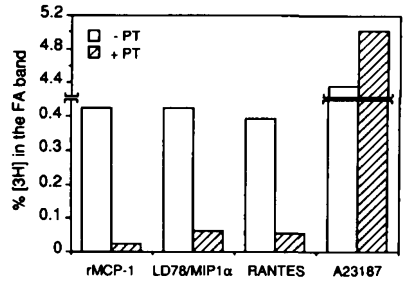

FIG. 3. **Effect of PT pretreatment on [^{3}H]arachidonic acid release in human monocytes.** Human monocytes were prepared and labeled as detailed under "Experimental Procedures." Labeled monocytes (10^7/ml) were incubated at 37 °C in RPMI, 10% FCS with 1 μg/ml PT for 90 min. At the end of the incubation (viability > 95% by trypan blue dye exclusion), cells were washed twice; resuspended in RPMI, 0.2% bovine serum albumin; and stimulated (10^7/ml) with 50 ng/ml rMCP-1, 100 ng/ml LD78/MIP1α, 100 ng/ml RANTES, or 1 μM A23187 for 5 min. The reaction was blocked and the extracted organic phase loaded onto Silica Gel G TLC plates. Results are the average of two similar experiments and are expressed as the percentage of ^{3}H in the fatty acid (*FA*) band at the net of control value (0.430% and 0.550%, without and with PT, respectively). The two experiments differed by less than 10%.

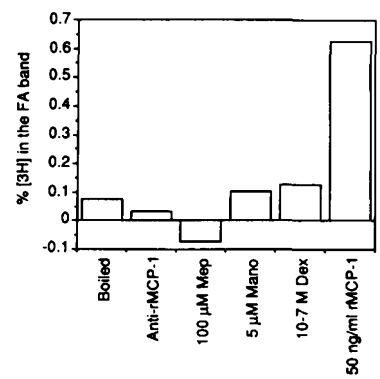

FIG. 4. **Regulation of rMCP-1-induced [^{3}H]arachidonic acid release.** [^{3}H]Arachidonic acid-prelabeled monocytes (10^7/ml) were stimulated with 50 ng/ml rMCP-1 for 3 min. The reaction was stopped and the samples processed as described in the Fig. 1 legend. In the appropriate group, 50 ng/ml rMCP-1 boiled for 10 min was used. Anti-rMCP-1 was used as detailed under "Experimental Procedures." When the effect of inhibitors was tested, monocytes were exposed to the drugs for 30 min in buffer, (mepacrine (*Mep*)), or for 15 min in Me_2SO, or manoalide (*Mano*), and then stimulated with rMCP-1. Dexamethasone (*Dex*) was added to monocytes during the labeling time (18 h). Dexamethasone, at the concentration used, did not change the uptake of [^{3}H]arachidonic acid, the distribution of the label, or the viability of the cells (>95%). The results are the means of two to four experiments and are expressed as the percent of ^{3}H in the fatty acid (*FA*) band at the net of respective control values. All of the experimental groups are statistically different from their control group ($p < 0.001$).

monocytes pretreated with 10^{-7} M dexamethasone overnight.

In the presence of 50 ng/ml rMCP-1, the release of [^{3}H]arachidonic acid in human monocytes was stimulated about 2-fold over the control value. In the same experimental conditions 10 ng/ml GM-CSF, 10^{-7} M fMLP, and 1 μM A23187 were 1.1-, 3.6-, and 5.2-fold more active then rMCP-1, respectively (Table I). Analysis of phospholipid classes showed that the major site of [^{3}H]arachidonic acid release by rMCP-1 was phosphatidylcholine with a breakdown of 1.4 ± 0.5% after 50 ng/ml rMCP-1 for 3 min (n = 4). rMCP-1 did not induce significant changes in any other labeled phospholipid class (data not shown).

Role of Extracellular Ca^{2+} in rMCP-1-induced [^{3}H]Arachidonic Acid Release—rMCP-1 induces a rapid and transient increase in $[Ca^{2+}]_i$ in human monocytes (12, 13). The receptor-mediated opening of plasma membrane calcium channels appears to be the main mechanism responsible for this effect

TABLE I

[^{3}H]Arachidonic acid release in human monocytes

Monocytes were separated and labeled as described under "Experimental Procedures." Cells (10^7/ml) were prewarmed at 37 °C for 5 min and then exposed to the agonists for an additional 3 min (rMCP-1, fMLP, A23187) or 10 min (GM-CSF). The reaction was stopped by the addition of 3 ml of chloroform/methanol/formic acid (1:2:0.2, v/v), and the extracted lipids were separated by TLC on a Silica Gel G plate using solvent system I. Results are expressed as mean values ± S.E.

Agonist	[^{3}H]Arachidonic acid release		
	^{3}H in the FA band[a]	Increase[b]	
	%	*-fold*	
Control	0.415 ± 0.032		(n = 6)
50 ng/ml rMCP-1	0.821 ± 0.055	1.98 ± 0.16	(n = 6; $p < 0.001$[c]
10 ng/ml GM-CSF	0.907 ± 0.041	2.18 ± 0.21	(n = 3; $p < 0.001$)
10^{-7} M fMLP	2.981 ± 0.443	7.18 ± 2.16	(n = 6; $p < 0.002$)
1 μM A23187	4.281 ± 0.673	10.31 ± 1.11	(n = 3; $p < 0.001$)

[a] Results are expressed as percent of ^{3}H present in the fatty acid (FA) band on the total radioactivity recovered from the TLC lane.
[b] Fold increase was calculated as percent stimulated/percent control.
[c] Statistical analysis was performed by Student's t test.

(14). To elucidate the possible relationship between rMCP-1-induced Ca^{2+} influx and [^{3}H]arachidonic acid mobilization, labeled monocytes were stimulated in conditions in which Ca^{2+} influx is blocked (12, 14). As reported in Table II, incubation with 5 mM EGTA or 5 mM Ni^{2+} in the extracellular medium did not change the spontaneous release of [^{3}H]arachidonic acid. On the other hand, rMCP-1-stimulated release was greatly reduced in the presence of both EGTA and Ni^{2+} (75 ± 8% and 99 ± 4% of inhibition, respectively). In the same conditions the response to 10^{-6} M fMLP was inhibited (90 and 95% in the presence of EGTA and Ni^{2+}, respectively; n = 2). Similar results were obtained with monocytes loaded with BAPTA/AM (30). BAPTA-loaded cells showed a lower basal concentration of $[Ca^{2+}]_i$ than normal cells and did not respond, in terms of $[Ca^{2+}]_i$ transients, to 50 ng/ml rMCP-1 or 10^{-6} M fMLP, indicating that the intracellular concentration of BAPTA was high enough to chelate intracellular Ca^{2+} and to buffer the influx/release of Ca^{2+} after receptor stimulation (data not shown). As reported in Table II, BAPTA-loaded monocytes failed to release [^{3}H]arachidonic acid in response to 50 ng/ml rMCP-1. In the same experimental conditions the response to 1 μM A23187 was also inhibited (92% inhibition; n = 2). These results strongly suggest that rMCP-1-induced [^{3}H]arachidonic acid release is dependent on the influx of extracellular Ca^{2+}.

To evaluate whether rMCP-1-induced accumulation of ^{3}Harachidonic acid was dependent on extracellular Ca^{2+} or was caused by the receptor-activated influx of extracellular Ca^{2+}, experiments with permeabilized monocytes were performed. In these experiments it was possible to dissociate receptor stimulation from changes in $[Ca^{2+}]_i$. Monocytes incubated in a Ca^{2+}-free buffer in the presence of 3 mM EGTA were challenged with 1 μM A23187 to cause the discharge of intracellular Ca^{2+} stores and to form Ca^{2+}-permeable pores in the membrane (31). $[Ca^{2+}]_i$ was regulated by the addition of different concentrations of Ca^{2+} to the EGTA buffer (32). Fig. 5 shows that stimulation of permeabilized monocytes in EGTA buffer or EGTA/Ca^{2+} buffer (784 nM free $[Ca^{2+}]$) with rMCP-1 or fMLP was not associated with any change of FURA-2 fluorescence. Using this experimental model it was found that rMCP-1 did not induce any [^{3}H]arachidonic acid release in the absence of Ca^{2+} added or with 70 nM free $[Ca^{2+}]$ (Fig. 6). Accumulation of [^{3}H]arachidonic acid started at 350 nM and reached a maximum at 784 nM free $[Ca^{2+}]$ (Fig. 6). Monocyte activation with 10^{-7} M fMLP gave comparable results, with the only exception that [^{3}H]arachidonic acid release was observed starting at 70 nM free $[Ca^{2+}]$ (Fig. 6). At the concentration of 784 nM free $[Ca^{2+}]$ the increase of [^{3}H]arachidonic acid release was 63 ± 16% ($p < 0.05$) and 181 ± 29% ($p < 0.05$) for rMCP-1 and fMLP, respectively (n = 3). Also, in permeabilized monocytes the effect of both rMCP-1 and fMLP was inhibited by pretreatment of the cells with 1 μM PT (88 ± 3% and 80 ± 5% for rMCP-1 and fMLP, respectively; n = 3) and by 5 μM manoalide (95% inhibition).

Importance of [^{3}H]Arachidonic Acid Release for Monocyte Chemotaxis—The role of arachidonic acid release in monocyte chemotaxis was investigated by the use of phospholipase A_2 inhibitors. Fig. 7*A* shows that all three inhibitors tested (BPB, manoalide, and mepacrine) decreased the number of monocytes migrated across a polycarbonate filter in response to an optimal concentration of rMCP-1 (50 ng/ml), although with a different potency (IC_{50} = 0.5 ± 0.4, 1.2 ± 0.5, 100 ± 11 μM for BPB, manoalide, and mepacrine, respectively; n = 3). Mepacrine and manoalide also inhibited monocyte polarization in response to rMCP-1 in a 10-min assay (IC_{50} = 120 ± 20 μM, and 1 ± 0.5 μM, respectively; n = 3), suggesting that phospholipase A_2 activation plays an important role at the early steps of the locomotor response. The action of mepacrine was not simply a membrane perturbation effect because 100 μM mepacrine did not reduce the increase of $[Ca^{2+}]_i$ in response to 50 ng/ml rMCP-1[3] and did not alter protein kinase C translocation in response to 100 ng/ml PMA in human monocytes.[4]

Induction of Arachidonic Acid Release by Other Members of the Cys-Cys Chemokine Subfamily in Human Monocytes—To evaluate if release of arachidonate is a common step in monocyte activation by Cys-Cys chemokines, we investigated the effect of LD78/MIP1α and RANTES on human monocytes. These two proteins show a high level of homology with human MCP-1 (5, 6) and share aspects of the signaling mechanisms in human monocytes (14). As reported in Fig. 8, both LD78/MIP1α and RANTES, tested at optimal concentrations for the chemotactic response, induced an increase of [^{3}H]arachidonic acid levels. The magnitude of the effect was comparable to the one observed with either rMCP-1 or human natural purified MCP-1. In the same experimental conditions IL-8, a member of the Cys-*X*-Cys branch of the chemokine superfamily, was not active (Fig. 8). Also, lipopolysaccharide at concentrations up to 100 ng/ml did not induce release of [^{3}H]arachidonic acid (Fig. 8). LD78/MIP1α and RANTES activate human monocytes and basophils through a PT-sensitive step (12, 33). The results presented in Fig. 3 also show that the [^{3}H]arachidonic acid release induced by these proteins is mediated by the activation of a PT-sensitive GTP-binding protein. In the presence of mepacrine (data not shown) and BPB (Fig. 7*B*), monocyte migration in response to RANTES and LD78/MIP1α was strongly decreased (IC_{50} = 0.4 and 0.3 μM BPB, respectively; n = 2).

Synergism between PAF and rMCP-1—It was shown recently

[3] C. Bizzarri and S. Sozzani, unpublished results.
[4] M. Rieppi and S. Sozzani, unpublished results.

TABLE II

Role of extracellular Ca^{2+} in rMCP-1-induced [^{3}H]arachidonic acid release in human monocytes

Monocytes were separated and labeled as described under "Experimental Procedures." Monocytes (10^7/ml) were stimulated with 50 ng/ml rMCP-1 in Hanks' in the presence of 0.2% bovine serum albumin for 3 min. For some experiments cells were incubated with 25 μM BAPTA/AM or Me_2SO at 37 °C for 30 min (30). Samples were treated as described in the Table I legend. Results are the average values ± S.E. of three separate experiments.

Agonist	Treatment	^{3}H in the FA band[a]	% Activity[b]
		%	%
Medium		0.541 ± 0.06	
rMCP-1		0.989 ± 0.13	183
Medium	5 mM EGTA	0.530 ± 0.06	
rMCP-1	5 mM EGTA	0.640 ± 0.08[c]	120
Medium	5 mM Ni^{2+}	0.538 ± 0.09	
rMCP-1	5 mM Ni^{2+}	0.541 ± 0.08[c]	101
Medium	25 μM BAPTA/AM	0.509 ± 0.10	
rMCP-1	25 μM BAPTA/AM	0.558 ± 0.05[c]	109

[a] Results are expressed as the percent of ^{3}H present in the fatty acid (FA) band on the total radioactivity recovered from the TLC lane.
[b] Percent activity was calculated on respective control (medium) value (100%).
[c] $p < 0.01$ by Student's t test against respective untreated group (medium).

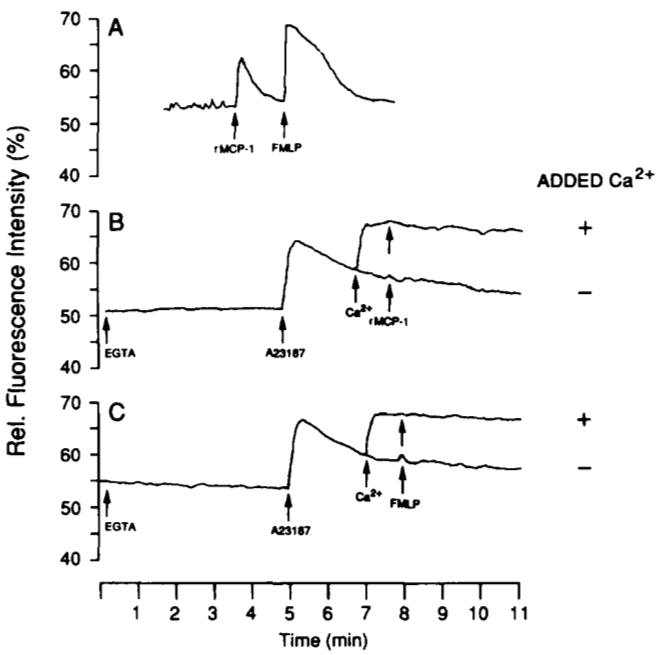

FIG. 5. **Ca^{2+} transients in normal and A23187-permeabilized FURA-2-loaded monocytes.** Human monocytes were loaded with 1 μM FURA-2/AM at 37 °C for 15 min as detailed under "Experimental Procedures." *Panel A*, monocytes (5 × 10^6/ml) were resuspended in Hanks' (1.2 mM Ca^{2+}) and stimulated with 50 ng/ml rMCP-1 or 10^{-7} M fMLP. *Panels B* and *C*, monocytes (5 × 10^6/ml) were resuspended in Ca^{2+}-free Hanks' buffer in the presence of 3 mM EGTA. After a 5-min prewarming, cells were challenged with 1 μM A23187 to discharge intracellular Ca^{2+} stores (31). After 2 min buffer or $CaCl_2$, to give a final free [Ca^{2+}] of 784 nM, was added (32). After 1 additional min cells were stimulated with 50 ng/ml rMCP-1 (*panel B*) or 10^{-7} M fMLP (*panel C*). These traces are representative of at least three independent experiments.

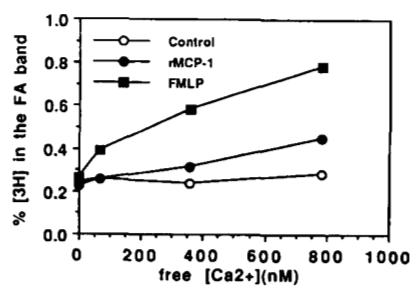

FIG. 6. **Release of [^{3}H]arachidonic acid in A23187-permeabilized human monocytes.** Monocytes were obtained as detailed under "Experimental Procedures" and labeled with [^{3}H]arachidonic acid for 16–18 h. For permeabilization, monocytes (10^7/ml) were incubated in Ca^{2+}-free Hanks' with 0.2% bovine serum albumin in the presence of 3 mM EGTA at 37 °C for 5 min. Cells were then exposed to 1 μM A23187 for 2 min and subsequently to different concentrations of $CaCl_2$ to give the desired final free [Ca^{2+}] (31, 32). After 1 min monocytes were stimulated with 50 ng/ml rMCP-1 or 10^{-7} M fMLP for 3 min. The reaction was stopped by the addition of 3 ml of chloroform/methanol/formic acid (1:2:0.2; v/v). The organic phase was extracted and the [^{3}H] arachidonic acid separated by TLC chromatography as described under "Experimental Procedures." Results are the mean values of two independent experiments. The variation between the two experiments was less than 15%.

that PAF pretreatment enhances NADPH oxidase activity in neutrophils stimulated with IL-8 (34). Fig. 9 shows that PAF-pretreated human monocytes, upon stimulation with rMCP-1, release greater amounts of [^{3}H]arachidonic acid from prelabeled cells. With 100 nM PAF for 5 min, the increase in [^{3}H]arachidonic acid measured after 50 ng/ml rMCP-1 was about 3-fold higher. Further, in the presence of PAF, the concentration of 5 ng/ml rMCP-1, inactive by itself, was as active as 50 ng/ml. The interaction between PAF and rMCP-1 was statistically significant when analyzed by two-way analysis of variance ($F = 13.67$, $p < 0.0006$ and $F = 12.42$, $p < 0.0008$ for 5 and 50 ng/ml rMCP-1, respectively). The order of the addition of rMCP-1 with respect to PAF was not crucial for the effect. Comparable results were obtained if PAF was added subsequently to rMCP-1 or if rMCP-1 and PAF were added simultaneously to labeled monocytes (data not shown). To evaluate if the observed synergism could also result in potentiation of monocyte chemotaxis, monocytes were exposed to different concentrations of rMCP-1 in the presence of PAF. As reported in Fig. 10, in the presence of the two agonists the number of monocytes migrated was higher than in the presence of only rMCP-1 or PAF. The effect was present when suboptimal (1–10 ng/ml) concentrations of rMCP-1 were used, and the interaction between PAF and rMCP-1 was statistically significant by two-way analysis of variance ($F = 311$, $p < 0.0001$).

DISCUSSION

It has been known for a number of years that leukocytes infiltrate tissues during inflammatory processes and tumor growth. Nevertheless, the mechanisms underlying this process have only recently started to be disclosed. The identification of the chemokine superfamily, a new family of chemotactic proteins, has greatly contributed to this process giving clues to the rules regulating the selective recruitment of the different leukocyte populations.

Although many of the members of the chemokine family were shown to be chemotactic *in vitro* and *in vivo*, the molecular

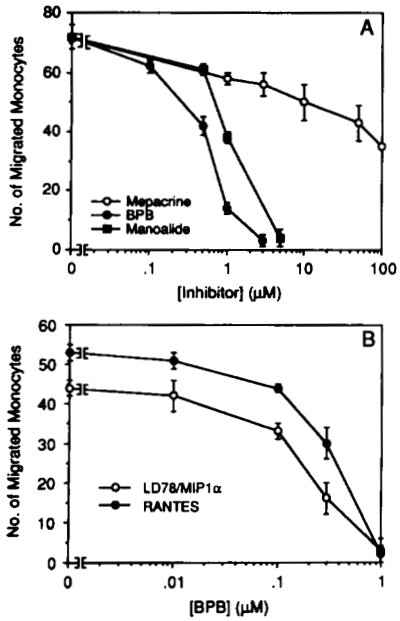

FIG. 7. **Effect of phospholipase A_2 inhibitors on monocyte chemotaxis induced by rMCP-1, RANTES, and LD78/MIP1α.** Human monocytes (1.5×10^6/ml in PBMC) were tested for their ability to migrate across a polycarbonate filter in response to an optimal concentration of the chemotactic factors (50 ng/ml rMCP-1, 100 ng/ml RANTES, 50 ng/ml LD78/MIP1α) in the presence of different concentrations of the inhibitors. Mepacrine was dissolved in buffer, BPB and manoalide in Me_2SO. The final concentration of Me_2SO was < 0.01% and did not affect monocyte migration. Inhibitors did not affect monocyte viability as assessed by trypan blue dye exclusion. One representative of three (*panel A*) and two (*panel B*) different experiments is shown. Results are expressed as number of migrated monocytes at the net of basal migration (33 ± 1 and 37 ± 2, *panels A* and *B*, respectively). Monocyte migration in response to an optimal fMLP concentration (10^{-8} M) was 92 ± 3 and 78 ± 4 for *panels A* and *B*, respectively.

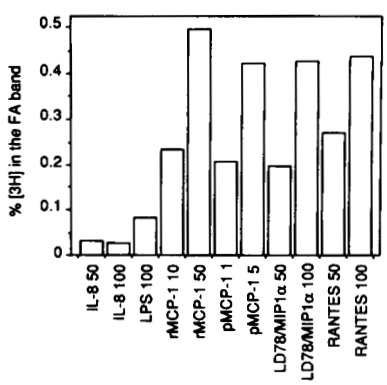

FIG. 8. **Effect of Cys-Cys chemokines on [^{3}H]arachidonic acid release in human monocytes.** Human monocytes were separated and labeled as detailed under "Experimental Procedures" and then activated with different concentrations (ng/ml) of the cytokines for 5 min at 37 °C. Accumulation of [^{3}H]arachidonic acid in the extracted organic phase was evaluated by TLC on Silica Gel G plates. The results are the average of two to four experiments and are expressed as the percentage of ^{3}H in the fatty acid (*FA*) band at the net of control value (0.551%). pMCP-1, natural human purified MCP-1. The variation between the experiments was less than 15%.

basis for this action is still largely unknown, and only recently was a Cys-Cys chemokine receptor identified and cloned (35, 36). In previous investigations it was found that activation of monocytes by rMCP-1, a member of the Cys-Cys branch of the chemokine superfamily, is associated with a rapid and PT-sensitive influx of Ca^{2+} across plasma membrane channels (12–14). In this paper, we report that rMCP-1-induced Ca^{2+} influx

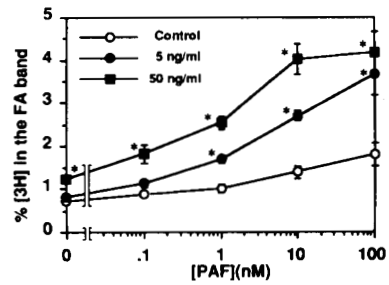

FIG. 9. **Synergism between PAF and rMCP^{-1} for [^{3}H]arachidonic acid release in human monocytes.** Human monocytes (10^7/ml), prepared as detailed under "Experimental Procedures," were incubated with different concentrations of PAF for 5 min before stimulation with rMCP-1 for an additional 3 min. The reaction was blocked and the extracted organic phase loaded on Silica Gel G TLC plates. Results are expressed as percent of radioactivity in the fatty acid band (*FA*) on the total radioactivity recovered from the lane. Each point is the average value of three independent experiments ± S.E. * $p < 0.05$ by Tukey's test with respect to the respective experimental group pretreated with PAF. Groups stimulated with 5 and 50 ng/ml rMCP-1 in the presence of 1, 10, and 100 nM PAF were statistically different ($p < 0.05$) from the respective group not treated with PAF. The interaction between PAF and rMCP-1 was also statistically significant by two-way analysis of variance (see details under "Results").

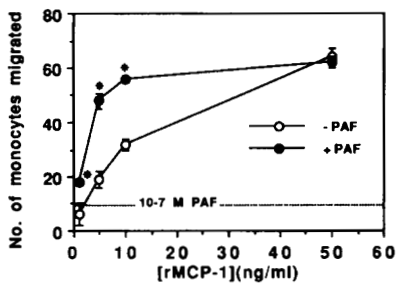

FIG. 10. **Synergism between PAF and rMCP-1 for monocyte chemotaxis.** Chemotaxis assay was performed as described in the Fig. 7 legend. PAF (10^{-7} M) and rMCP-1 were seeded in the lower compartment of the chemotactic chamber. One experiment, performed in triplicate, representative of three similar experiments is shown. Results are expressed as number of monocytes migrated at the net of basal migration value (31 ± 2). Monocyte migration in response to an optimal fMLP concentration (10^{-8} M) was 85 ± 2. * $p < 0.01$ with respect to the corresponding rMCP-1 concentration without PAF. The interaction between PAF and rMCP-1 was also statistically significant by two-way analysis of variance (see details under "Results").

supports the accumulation of [^{3}H]arachidonic acid in human monocytes and that this effect may be relevant for induction of the chemotactic response by this and related cytokines.

The effect of rMCP-1 was rapid (<15 s) and dose-dependent (Figs. 1 and 2*A*). Maximal activation was observed at 50–100 ng/ml (Fig. 1), concentrations active for the chemotactic response (Fig. 10 and Ref. 12). Arachidonate accumulation was observed in human monocytes and THP-1 cells but not in undifferentiated HL-60 (Fig. 2) which lack specific binding sites for rMCP-1. rMCP-1 action was blocked by PT pretreatment of monocytes (Fig. 3) and by a specific antiserum (Fig. 4). No accumulation of [^{3}H]arachidonic acid was observed in monocytes challenged with up to 100 ng/ml IL-8 (Fig. 8), a member of the Cys-*X*-Cys branch of the chemokine superfamily which shows 21% homology with human MCP-1 (5, 6). On the other hand, RANTES and LD78/MIP1α, two other members of the Cys-Cys chemokine superfamily, induced a dose-dependent accumulation of [^{3}H]arachidonic acid comparable to that induced by rMCP-1 (Figs. 3 and 8). These results, taken together, indicate that the rMCP-1-induced release of [^{3}H]arachidonic acid is mediated by the interaction of the protein with a specific receptor(s) and not by nonspecific membrane perturbation. Further, this effect is not the result endotoxin contamination be-

cause (i) it was blocked by heat treatment of the protein (Fig. 4); (ii) lipopolysaccharide alone was a weak inducer of [^{3}H]arachidonic acid release (Fig. 8); and (iii) lipopolysaccharide (100 ng/ml for 30 min) did not prime monocytes for [^{3}H]arachidonic acid release by rMCP-1 (data not shown).

The release of [^{3}H]arachidonic acid was blocked in the presence of mepacrine (37), manoalide (38), and dexamethasone (39), suggesting that the activation of a phospholipase A_2 was responsible for arachidonate release. At present, experiments are in progress to characterize the type(s) of phospholipase A_2 involved in rMCP-1 action.

Extracellular Ca^{2+} was strictly required for rMCP-1-induced [^{3}H]arachidonic acid release. In conditions in which Ca^{2+} influx was blocked, such as by 5 mM EGTA or 5 mM Ni^{2+} in the incubation buffer or in BAPTA-loaded monocytes, rMCP-1 failed to induce arachidonate release (Table II). These results are in agreement with previous observations showing that in macrophages (40, 41), C62B glioma cells (42), Swiss 3T3 fibroblasts (43), Mono Mac 6 cells (44), and human monocytes (45) the influx of extracellular Ca^{2+}, rather than release of Ca^{2+} from intracellular stores, is required for arachidonate release. By the use of ionophore-permeabilized monocytes and controlled Ca^{2+} concentrations (31) it was possible to dissociate receptor stimulation from Ca^{2+} transients (Fig. 5) and to show that rMCP-1 does not act merely as a Ca^{2+} ionophore (Fig. 6). Thus, Ca^{2+} is required but not sufficient for activation of phospholipase A_2 by rMCP-1, and a PT-sensitive step, other than the activation of Ca^{2+} influx triggered by receptor-ligand interaction, is necessary for this response.

Arachidonate release appears to be an important step in the induction of chemotaxis by members of the Cys-Cys chemokine superfamily. Mepacrine, manoalide, and BPB (46), three inhibitors of phospholipase A_2, inhibited monocyte chemotaxis in response to rMCP-1 with different potency (BPB ≥ manoalide >> mepacrine) (Fig. 7*A*). BPB and manoalide differ from mepacrine in their apparent mechanism of action. BPB and manoalide have been shown to modify phospholipase A_2 covalently, whereas mepacrine is believed to limit substrate availability (47, 48). BPB and mepacrine were also effective in reducing monocyte migration to RANTES and LD78/MIP1α (Fig. 7*B* and data not shown).

Monocytes exposed to rMCP-1 in the presence of PAF showed a 3-fold increased [^{3}H]arachidonic acid release (Fig. 9) and a potentiation of the chemotactic response (Fig. 10). This finding is potentially of great interest and may mimic an *in vivo* physiological situation. In fact, at sites of inflammation, the same cell type, such as endothelial cells, could be induced by the same agonists (*i.e.* tumor necrosis factor and IL-1) to produce and present both PAF and MCP-1 with a similar kinetics (4, 49–51), giving rise to an amplification loop.

A direct role for arachidonic acid in monocyte and macrophage adherence and in the expression of adhesion molecules was recently suggested (18–20). In addition, recent data have shown that membrane lipid composition may play a crucial role in determining the characteristics of integrin binding (52). These observations support the emerging role of arachidonic acid, by itself, as second messenger, active for instance, as cofactor for protein kinase C activation (17, 53, 54) and as regulator of ion channel conductances (55, 56).

In conclusion, we report that three proteins, members of the Cys-Cys branch of the chemokine superfamily, activate the release of arachidonic acid in human monocytes and that this effect might be an important step in the induction of monocyte migration. Since these proteins are believed to be important mediators in immunity and inflammation, a better understanding of their signaling pathways will improve the level of knowledge of these processes and provide a basis for novel therapeutic strategies.

Acknowledgments—We thank Dr. C. Cerletti for the discussion and criticism of the manuscript.

REFERENCES

1. Mantovani, A., and Dejana, E. (1989) *Immunol. Today* **10,** 370–375
2. Pober, J., and Cotran, R. S. (1990) *Physiol. Rev.* **70,** 427–451
3. Mantovani, A., Bussolino, F., and Dejana, E. (1992) *FASEB J.* **6,** 2591–2599
4. Bussolino, F., Breviario, F., Tetta, C., Aglietta, M., Mantovani, A., and Dejana, E. (1986) *J. Clin. Invest.* **77,** 2027–2033
5. Oppenheim, J. J., Zachariae, C. O. C., Mukaida, N., and Matsushima, K. (1991) *Annu. Rev. Immunol.* **9,** 617–648
6. Schall, T. J. (1991) *Cytokine* **3,** 165–183
7. Wolpe, S. D., and Cerami, A. (1989) *FASEB J.* **3,** 2565–2573
8. Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. (1992) *Immunol. Today* **13,** 265–270
9. Baggiolini, M. A., Walz, A., and Kunkel, S. L. (1989) *J. Clin. Invest.* **84,** 1045–1049
10. Zachariae, C. O. C., Anderson, A. O., Thompson, H. L., Appella, E., Mantovani, A., Oppenheim, J. J., and Matsushima, K. (1990) *J. Exp. Med.* **171,** 2177–2182
11. Yoshimura, T., and Leonard, E. J. (1990) *J. Immunol.* **145,** 292–297
12. Sozzani, S., Luini, W., Molino, M., Jílek, P., Bottazzi, B., Cerletti, C., Matsushima, K., and Mantovani, A. (1991) *J. Immunol.* **147,** 2215–2121
13. Rollins, B. J., Walz, A., and Baggiolini, M. (1991) *Blood* **78,** 1112–1116
14. Sozzani, S., Molino, M., Locati, M., Luini, W., Cerletti, C., Vecchi, A., and Mantovani, A. (1993) *J. Immunol.* **150,** 1544–1553
15. Goldyne, M. E., Burrish, G. F., Poubelle, P., and Borgeat, P. (1984) *J. Biol. Chem.* **259,** 8815–8819
16. Hirata, F., Corcoran, B. A., Venkatasubramanian, K., Schiffmann, E., and Axelrod, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 2640–2643
17. McPhail, L. C., and Harvath, L. (1993) in *The Natural Immune System: The Neutrophil* (Wheeler, J. G., and Abramson, J., eds) pp. 63–107, Oxford University Press, Oxford
18. Lefkowith, J. B., Rogers, M., Lennartz, M. R., and Brown, E. J. (1991) *J. Biol. Chem.* **266,** 1071–1076
19. Lefkowith, J. B., Lennartz, M. R., Rogers, M., Morrison, A. R., and Brown, E. J. (1992) *J. Immunol.* **149,** 1729–1735
20. Bates, E. J., Ferrante, A., Harvey, D. P., and Poulos, A. (1993) *J. Leukocyte Biol.* **53,** 420–426
21. Van Damme, J., Decock, B., Lenaerts, J.-P., Conings, R., Bertini, R., Mantovani, A., and Biliau, A. (1989) *Eur. J. Immunol.* **19,** 2367–2373
22. Wang, J. M., Cianciolo, G. J., Snyderman, R., and Mantovani, A. (1986) *J. Immunol.* **137,** 2726–2732
23. Pawlowski, N. A., Kaplan, G., Hamill, A. L., Cohn, Z. A., and Scott, W. A. (1983) *J. Exp. Med.* **158,** 393–412
24. Colotta, F., Peri, G., Villa, A., and Mantovani, A. (1984) *J. Immunol.* **132,** 936–944
25. Bauldry, S. A., Wykle, R. L., and Bass, D. A. (1988) *J. Biol. Chem.* **263,** 16787–16795
26. Grynkiewicz, G., Poenie M., and Tsien, R. V. (1985) *J. Biol. Chem.* **260,** 3440–3450
27. Falk, W., Goodwin, R. H., Jr., and Leonard, E. J. (1980) *J. Immunol. Methods* **33,** 239–247
28. Wang, J. M., McVicar, D. W., Oppenheim, J. J., and Kelvin, D. J. (1993) *J. Exp. Med.* **177,** 699–705
29. Rehfeldt, W., Hass, R., and Goppelt-Struebe, M. (1991) *Biochem. J.* **276,** 631–636
30. Kuijpers, T. W., Hoogerwerf, M., and Roos D. (1992) *J. Immunol.* **148,** 72–77
31. Halenda, S. P., and Rehm, A. G. (1987) *Biochem. J.* **248,** 471–475
32. Fabiato, A., and Fabiato, F. (1979) *J. Physiol.* **75,** 463–505
33. Kuna, P., Reddigari, S. R., Shall, T. J., Rucinski, D., Viksman, M. Y., and Kaplan, A. P. (1992) *J. Immunol.* **149,** 636–642
34. Peveri, P., Walz, A., Dewald, B., and Baggiolini, M. (1988) *J. Exp. Med.* **167,** 1547–1559
35. Neote, K., DiGregorio, D., Mak, J. Y., Horuk, R., and Shall, T. J. (1993) *Cell* **72,** 415–425
36. Gao, J. L., Kuhns, D. B., Tiffany, H. L., McDermott, D., Li, X., Francke, U., and Murphy, P. M. (1993) *J. Exp. Med.* **177,** 1421–1427
37. Yorio, T., and Bentley, P. J. (1978) *Nature* **271,** 79–81
38. Lombardo, D., and Dennis, E. (1985) *J. Biol. Chem.* **260,** 7234–7240
39. Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D., and Axelrod, J. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2533–2536
40. Aderem, A. A., Scott, W. A., and Cohn, Z. A. (1986) *J. Exp. Med.* **163,** 139–154
41. Balsinde, J., Fernandez, B., and Diez, E. (1990) *J. Immunol.* **144,** 4298–4304
42. Brooks, R. C., McCarthy, K. D., Lapetina, E. G., and Morell, P. (1989) *J. Biol. Chem.* **264,** 20147–20153
43. Takuwa, N., Kumada, M., Yamashita, K., and Takuwa, Y. (1991) *J. Biol. Chem.* **266,** 14237–14243
44. Aepfelbacher, M., Ziegler-Heitbrock, H. W. L., Lux, I., and Weber, P. C. (1992) *J. Immunol.* **148,** 2186–2193
45. Hoffman, T., Brando, C., Lizzio, E. F., Lee, Y. L., Hansen, M., Tripathi, A. K., Taplits, M., Puri, J., Bonvini, E., Abrahamsen, T. G., Carter, C. S., Reid, G. K., and Evans, J. (1991) *J. Immunol.* **146,** 692–700
46. Volwerk, J. J., Pieterson, W. A., and deHaas, G. H. (1974) *Biochemistry* **13,** 1446–1454
47. Glaser, K., and Jacobs, R. (1986) *Biochem. Pharmacol.* **35,** 449–453
48. Dise, C. A., Burch, J. W., and Goodman, D. B. (1982) *J. Biol. Chem.* **257,** 4701–4704

49. Valone, F. H., and Epstein, L. B. (1988) *J. Immunol.* **141,** 3945–3950
50. Sica, A., Wang, J. M., Colotta, F., Dejana, E., Mantovani, A., Oppenheim, J. J., Larsen, C. G., Zachariae, C. O. C., and Matsushima, K. (1990) *J. Immunol.* **144,** 3034–3038
51. Kuijpers, T. W., Hakkert, B. C., Hoogerwerf, M., Leeuwenberg, J. F. M., and Roos, D. (1991) *J. Immunol.* **147,** 1369–1376
52. Conforti, G., Zanetti, A., Pasquali-Ronchetti, I., Quaglino, D., Neyroz, P., and Dejana, E. (1990) *J. Biol. Chem.* **265,** 4011–4019
53. McPhail, L. C., Clayton, C. C., and Snyderman, R. (1984) *Science* **224,** 622–625
54. Asaoka, Y., Nakamura, S., Yoshida, K., and Nishizuka, Y. (1992) *Trends Biochem. Sci.* **17,** 414–417
55. Kim, D., and Clapham, D. E. (1989) *Science* **244,** 1174–1176
56. Ordway, R. W., Walsh, J. V., and Singer, J. J. (1989) *Science* **244,** 1176–1179