BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 32–35 (1997)
ARTICLE NO. RC977601

The Monocyte Chemotactic Protein-4 Induces Oxygen Radical Production, Actin Reorganization, and CD11b Up-regulation via a Pertussis Toxin-Sensitive G-Protein in Human Eosinophils[1]

Kirsten Tenscher, Beatrix Metzner, Clemens Hofmann, Erwin Schöpf, and Johannes Norgauer
Department of Dermatology, Divisions of General and Experimental Dermatology, University of Freiburg, Hauptstrasse 7, Freiburg i. Br. D-79104, Germany

Received September 29, 1997

The novel human CC-chemokine monocyte chemotactic protein-4 (MCP-4) is a chemotaxin for eosinophils. Here, the biological activities and the activation profile of MCP-4 was further characterized in eosinophils and compared to other activators such as platelet activating factor (PAF), Eotaxin and RANTES. As demonstrated by lucigenin-dependent chemiluminescence and superoxide dismutase-inhibitable cytochrome C reduction MCP-4 stimulated the production of reactive oxygen metabolites. Furthermore, MCP-4 induced up-regulation of the integrin CD11b. Flow cytometric studies revealed rapid and transient actin polymerization upon stimulation with MCP-4. At optimal concentrations the changes induced by MCP-4 were weaker than the effects after stimulation with PAF and comparable to those obtained by RANTES and Eotaxin. Cell responses elicited by MCP-4 were inhibited by pertussis toxin indicating involvement of G_i-proteins in this signal pathway. These findings point to a role of MCP-4 in the pathogenesis of eosinophilic inflammation as chemotaxin as well as activator of pro-inflammatory effector functions.

Human eosinophils are considered as major effector cells in several inflammatory condi tions such as parasitic infections, atopic diseases, bullous dermatoses or vasculitis [1-6]. Accumulation of eosinophils at inflammatory sites is presumably caused by various chemotactic agents. The phosphatidylcholin-derivate platelet activating factor (PAF) and the CC-chemokine RANTES as well as Eotaxin are well characterized eosinophil chemo taxins [7-9]. In addition to migration PAF, RANTES and Eotaxin stimulate eosinophil effector functions, such as the production of reactive oxygen metabolites and up-regulation of CD11b [10]. Stimulation of leukocytes by chemotaxins requires binding to membrane-spanning ligand-specific receptors [11]. These receptors interact at the intracellular site of the plasma membrane with pertussis toxin- or cholera toxin-sensitive heterotrimeric guanine nucleotide-binding proteins (G-proteins) [12-14]. Activated G-proteins regulate actin polymerization, which in concert control cell response such as the migration, the production of reactive oxygen species and upregulation of integrins [10,15].

Recently, monocyte chemotactic protein-4, a novel peptide of the CC-chemokine family, has been cloned, sequenced and expressed as recombinant protein [16, 17]. At the amino acid level this peptide is related to MCP-1, -2, -3 and Eotaxin with 65, 57, 65 and 66 %, respectively [17]. It posesses chemotactic activity for monocytes and eosinophils and stimulates the release of histamin in basophils [16-18]. Reconstitution experiments with heterologous expressed CC-chemokines receptors suggested that MCP-4 binds with high affinity to the CC-chemokine receptor type 2 and 3 (CCR-2& 3) triggering intracellular Ca^{2+}-transients [17, 19]. In eosinophils the biological activity of MCP-4 is mediated by CCR-3. This receptor binds also Eotaxin and RANTES [19]. In the present study, the signal pathways and cell effector functions elicited by MCP-4 in human eosinophils were further characterized and compared to the effect induced by other eosinophil activators.

MATERIALS AND METHODS

Materials. L-α-phophatidylcholine-β-acetyl-γ-O-alkyl (PAF), phorbol 12-myristate 13-acetate (PMA), lysophosphatidylcholine, fi-

[1] This work was supported by the Deutsche Forschungsgemeinschaft (No.266/2-1).

Abbreviations used: Platelet activating factor, PAF; monocyte chemotactic protein-4, MCP-4, heterotrimeric guanine nucleotide-binding proteins, G-proteins; phorbol 12-myristate 13-acetate, PMA.

0006-291X/97 $25.00

coll paque and lucigenin were obtained from Sigma (Deisenhofen, Germany); recombinant human MCP-4, RANTES and Eotaxin from Peprotech (London, England); N-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-phallacidin (NBD-phallacidin) from Becton Dickinson (Heidelberg, Germany); the monoclonal anti-CD16 antibody (BW 209/2) was obtained from Dr. R. Kurrle, Behring-Werke (Marburg, Germany); immunomagnetic beads (Dynabeads M-450) were purchased from Dianova (Hamburg, Germany); phycoerythrin-conjugated CD11b (LEU 15) was from Becton Dickinson (Heidelberg, Germany); pertussis toxin was a kind gift from Prof. Dr. K. Aktories (University of Freiburg, Germany).

Isolation of eosinophils. Human eosinophil granulocytes were purified from heparin-anticoagulated (10 units/ml) blood of healthy non-atopic volunteers by ficoll separation and negative selection with anti-CD16 antibody-coated Dynabeads [15]. Pappenheim stain judged the purity of isolated eosinophils $\geq$ 96%.

Actin polymerization. The filamentous actin content was quantified analyzed by flow cytometry with NBD-phallacidin staining [20]. Briefly, cells were stimulated and fixed after the indicated time intervals in 7.4 % formaldehyde buffer. The f-actin was stained using a cocktail containing 7.4 % formaldehyde, 0.33 μM NBD-phallacidin and 1 mg/ml lysophosphatidylcholine. The flourescence intensity was measured.

Lucigenin-dependent chemiluminescence. Eosinophils were resuspended to a density of 5×10^4 cells/ml containing 200 μM lucigenin. Measurements were performed in triplicate at 37 °C. The reaction over a 60 min time period after addition of stimuli to the cells were followed and expressed as intensity integral counts [10].

Superoxide anion production. The production of superoxide anions was measured as superoxide dismutase-inhibitable reduction of cytochrome C at 550nm (Ultrospec III, Pharmacia, Freiburg, Germany) [21].

CD11b-Expression. The integrin CD11b was analyzed by flow cytometry with phycoery thrin-conjugated anti-CD11b antibodies. Eosinophils were activated with the indicated stimuli for 30 min at 37 °C. The reaction was stopped by diluting the sample with 100-fold ice-cold buffer. Samples were incubated on ice for 40 min with phycoerythrin-conjugated anti-CD11b antibodies [15].

RESULTS

Actin Response and CD11b Upregulation by MCP-4

The influence of MCP-4 on the f-actin network in human eosinophils was analyzed. This novel CC-chemokine caused a rapid and transient polymerization of actin molecules (Fig. 1). Within 30 s was a transient increase of the f-actin content by about 50 %. Half-maximal and maximal effects were observed at 10 ng/ml and 100 ng/ml, respectively. The time for subsequent recovery to initial values depended on the added concentration of MCP-4.

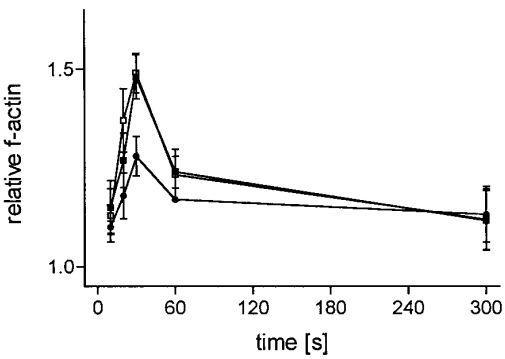

FIG. 1. Effect of MCP-4 on the actin polymerization in eosinophils. Cells were stimulated with 10 ng/ml (●), 100 ng/ml (■) and 1000 ng/ml (□) of MCP-4. The relative f-actin content was determined at the indicated time points by flow cytometry. Data are means +/− SEM (n=5).

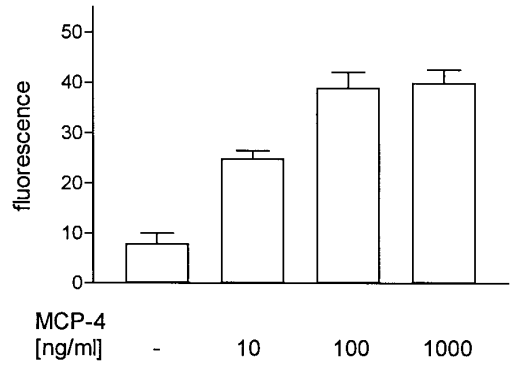

FIG. 2. Dose-dependency of the MCP-4-induced CD11b expression in eosinophils. Eosinophils were activated without or with 10 ng/ml, 100 ng/ml and 1000 ng/ml MCP-4 for 30 min at 37°C. Expression of CD11b was analyzed by flow cytometry with phycoerythrin-conjugated anti-CD11b antibodies. Data are means +/− SEM (n=5).

The effect of MCP-4 on the expression of the integrin CD11b at the cell surface was measured by flow cytometry. Again, MCP-4 induced a concentration dependent response with half-maximal and maximal responses at 10 ng/ml and 100 ng/ml, respectively (Fig. 2).

Superoxide Anion Production and MCP-4

Next, the production of reactive oxygen species in eosinophils by MCP-4 was studied with lucigenin-dependent chemiluminescence. These experiments revealed activation of the respiratory burst in a concentration-dependent manner; half-maximal and maximal effects were observed at 10 ng/ml and 100 ng/ml, respectively (Fig. 3). At optimal concentrations continuous measurements indicated a rapid induction of the response with maximum values after 5 min.

Superoxide anion production was analyzed by cytochrome C reduction (Fig. 4). Dose dependent responses were also observed in these experiments. Optimal doses of MCP-4 generated about 15 nmoles superoxide anion / 10^6 eosinophils within 30 min. Similar amounts of superoxide anion were also found after longer stimulation periods (up to 60 min, data not shown).

Comparison of the Activation Profiles of Different Eosinophil Stimuli

The activation profile of MCP-4 was compared to the responses provoked by other well defined eosinophil activators such as PAF, RANTES and Eotaxin. Similar to MCP-4, the other eosinophil activators induced actin

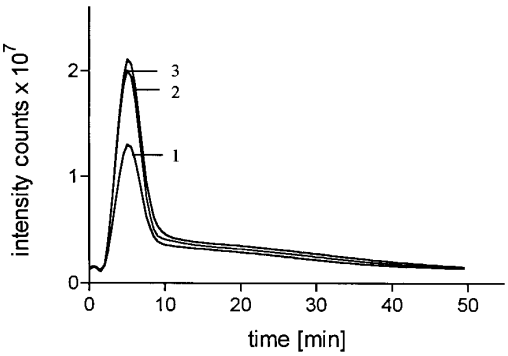

FIG. 3. Dose-dependency of MCP-4 on the chemiluminescence response in eosinophils. The time courses of the lucigenin-dependent chemiluminescence response in eosinophils upon stimulation with 10 ng/ml (line 1), 100 ng/ml (line 2) and 1000 ng/ml MCP-4 (line 3) are shown. Representative data of one experiment are shown. The experiment was repeated five times with identical results.

TABLE I

Influence of Different Eosinophil Activators on Actin Response, Lucigenin-Dependent Chemiluminescence, and CD11b Expression

Stimulus	f-actin	Chemiluminescence	CD11b
Control	1.00 (±0.00)	15 (±4)	11.25 (±1.76)
MCP-4	1.49 (±0.07)	362 (±38)	40.25 (±2.35)
RANTES	1.48 (±0.05)	383 (±51)	36.72 (±0.84)
Eotaxin	1.48 (±0.06)	380 (±58)	41.7 (±0.98)
PAF	1.53 (±0.05)	514 (±50)	41.87 (±2.00)

Eosinophils were stimulated without or with 1000 ng/ml MCP-4, 1000 ng/ml RANTES, 1000 ng/ml Eotaxin and 100 nM PAF. The relative f-actin content was taken after 10 s. Chemiluminescence response is given as integral (counts $\times 10^6$) after 60 min. The mean channel number of CD11b expression was analyzed after 30 min. Data are means ± SEM (n = 3).

reorganization, triggered the respiratory burst and stimulated expression of CD11b in a concentration dependent manner (data not shown). At optimal concentrations all tested activators induced comparable CD11b and actin responses (Table I). By chemiluminescence the effect of MCP-4 was comparable to the response of the other two tested CC-chemokines, RANTES and Eotaxin. Platelet activating factor induced a stronger respiratory burst as MCP-4.

Pertussis Toxin Inhibition of MCP-4-Induced Cell Response

Pertussis toxin blocks cell activation induced by G_i-protein-coupled receptors [13]. Pretre atment of eosinophils with pertussis toxin completely inhibited the MCP-4-induced chemiluminescence response (Fig. 5) and the transient increase of actin polymerization (data not shown). To prove the metabolic activity of eosinophils after pertussis toxin treatment, the chemiluminescence response with PMA was followed. Toxin treatment did not influence the phorbol ester-triggered response (data not shown).

DISCUSSION

Well defined chemotaxins for eosinophils are PAF, Eotaxin and RANTES [7-9]. Except Eotaxin these chemotaxins act on various types of leukocytes [15]. Recently, a novel CC-chemokine, MCP-4, has been identified [16, 17]. This peptide has chemotactic activity for eosinophils as well as monocytes. In addition, it stimulates release of histamin in basophils [16-19]. Tumor necrosis factor α, interleukin-1α and interferon γ stimulate mRNA expression of MCP-4 in bronchial epithelial and endothelial cells [17, 18]. Enhanced MCP-4 mRNA transcripts have been found in allergic

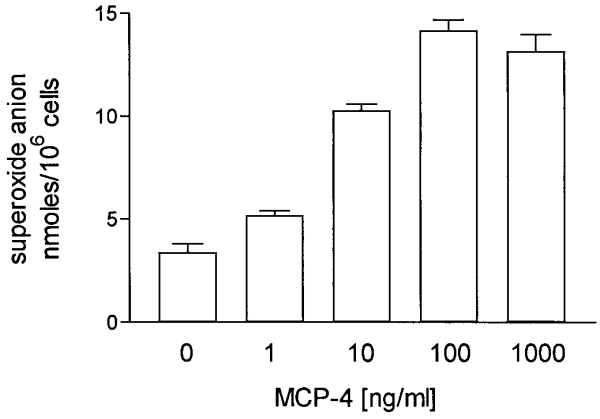

FIG. 4. Influence of MCP-4 on superoxide-anion production. Eosinophils were stimulated with the indicated concentrations of MCP-4 for 30 min at 37°C. Superoxide-dismutase-inhibitable cytochrome C reduction was analysed. Data are means +/− SEM (n=5).

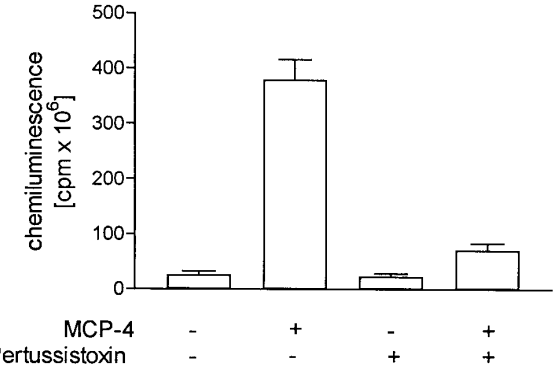

FIG. 5. Pertussis toxin inhibits MCP-4 induced lucigenin-dependent chemiluminescence in eosinophils. Cells were preincubated without or with 1 μg/ml pertussis toxin for 90 min at 37 °C and further incubated without or with 100 ng/ml MCP-4 for 60 min at 37 °C. Data are means +/− SEM (n=5).

sinusitis [17]. Expression of MCP-4 at other sites as well as diseases or cells has not yet been analyzed. To improve our understanding of the biological activities of MCP-4, we analyzed various intracellular signal mechanisms and cell effector functions in eosinophils. As could be expected for a chemotactic agent we have shown here that MCP-4 induced a transient reorganization of the actin network. The precise regulation mechanisms for the actin response is not fully understood, however, it is believed to involve interaction of phospholipids with actin binding proteins [22]. Actin reorganisation have been implicated in many biological regulative mechanisms including the activation of the NADPH oxidase and translocation of CD11b from intracellular stores to the plasma membran [10,11]. Production of reactive oxygen metabolites and upregulation of the CD11b integrin by MCP-4 was demonstrated here. Cell-studies evaluating the proinflammatory activity of MCP-4 revealed that this peptide has a lower activity than PAF but is comparable to Eotaxin and RANTES. The similar activation profiles of the three tested CC-chemokines might be explained by interacting with the same receptor [19].

Reconstitution experiments with heterologous expressed CC-chemokine receptors indicate interaction of MCP-4 with CCR-2 or CCR-3 [17, 19]. In eosinophils the activity seems to be mediated by CCR-3 [19]. Cell biology studies revealed that CC-chemokine receptors couple to pertussis toxin-sensitive G_i-proteins [13]. Here, we have shown that all cell responses induced by MCP-4 were inhibited by pertussis toxin, indicating involvement of G_i-proteins in the intracellular signal pathway.

Our results indicate that MCP-4 in eosinophils induce actin polymerization via pertussis toxin sensitive G-proteins. In addition, it is a strong activator of the respiratory burst as well as upregulation of CD11b. Therefore, one can assume that MCP-4 similarly to other chemotaxins not only recruit eosinophils to the site of inflammation, but also possesses proinflammatory activity.

REFERENCES

1. Bruijnzeel, P. (1994) *Ann. Acad. Sci.* **725,** 259–267.
2. Kay, A. B. (1992) *Am. Rev. Respir. Dis.* **145,** S22–S26.
3. Walker, C., Kaegi, M. K., Braun, P., and Blaser, K. (1991) *J. Allergy Clin. Immunol.* **88,** 935–942.
4. Gounni, A. S., Lamkhioued, B., Delaporte, E., Dubost, A., Kinet, J. P., Capron, A., and Capron, M. (1994) *J. Allergy Clin. Immunol.* **94,** 1214–1216.
5. Weller, P. F. (1992) *Curr. Opin. Immunol.* **4,** 782–787.
6. Gleich, G. J., Adolphson, C. R., and Leiferman, K. M. (1993) *Annu. Rev. Med.* **44,** 85–101.
7. Weber, M., and Dahinden, C. A. (1995) *Int. Arch. Allergy Immunol.* **107,** 148–150.
8. Valone, F. H. (1980) *Clin. Immunol. Immunopathol.* **15,** 5265–5271.
9. Wardlaw, A. J., Moqbel, R., Cromwell, O., and Kay, A. B. (1986) *J. Clin. Invest.* **78,** 1701–1706.
10. Kapp, A., Zeck-Kapp, G., Czech, W., and Schöpf, E. (1994) *J. Invest. Dermatol.* **102,** 906–914.
11. Baggiolini, M., and Dahinden, C. A. (1994) *Immunol. Today* **15,** 127–133.
12. Baggiolini, M., Boulay, F., Badwey, J. A., and Curnutte, J. T. (1993) *FASEB J.* **7,** 1004–1010.
13. Gierschik, P., Sidiropoulos, D., and Jakobs, K. H. (1989) *J. Biol. Chem.* **264,** 21470–21473.
14. Sozzani, S., Luini, W., Molino, M., Jilnek, P., Bottazzi, B., Cerletti. C., Matsushima K., and Mantovani, A. (1991) *J. Immunol.* **147,** 2215–2221.
15. Tenscher, K., Metzner, B., Schöpf, E., Norgauer, J., and Czech, W. (1996) *Blood* **88,** 3195–3199.
16. Uguccioni, M., Loetscher, P., Forssmann, U., Dewald, B., Li, H., Lima, S. H., Li, H., Kreider, B., Barotta, G., Thelen, M., and Baggiolini, M. (1996) *J. Exp. Med.* **183,** 2379–2384.
17. Garcia-Zepeda, E., Combadiere, C., Rothenberg, M., Sarafi, M., Lavigne, F., Hamid, Q., Murphy, P., and Luster, A. (1996) *J. Immunol.* **157,** 2613–2626.
18. Stellato, C., Collins, P., Ponath, P., Suler, D., Newman, W., La Rosa, G., Li, H., White, J., Schwiebert, L., Bickel, C., Liu, M., Bochner, B., Williams, T., and Schleimer R. (1997) *J. Clin. Invest.* **99,** 926–936.
19. Heath, H., Qin, S., Rao, P., Lijun, W., La Rosa, G., Kassam, N., Ponath, P., and Mackay, C. (1997) *J. Clin. Invest.* **99,** 178–184.
20. Norgauer, J., Just, I., Aktories, K., and Sklar, L. S. (1989) *J. Cell. Biol.* **109,** 1133–1140.
21. Norgauer, J., Kownatzk,i E., Seifert, R., and Aktories, K. (1988) *J. Clin. Invest.* **82,** 1376–1382.
22. Stossel, T. P. (1989) *J. Biol. Chem.* **264,** 18261–18264.