Vol. 152, No. 1, 1988
April 15, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 277-284

IDENTIFICATION OF DIFFERENT CHARGED SPECIES OF A HUMAN MONOCYTE DERIVED NEUTROPHIL ACTIVATING PEPTIDE (MONAP)

J.-M. Schröder, U. Mrowietz, and E. Christophers

Department of Dermatology

University of Kiel

2300 Kiel, FRG

Received February 19, 1988

Summary Lipopolysaccharide-stimulated human monocytes secrete a 10 kD peptide (MONAP) of high neutrophil, not however monocyte or eosinophil stimulating activity. By reversed phase HPLC MONAP could be distinguished from Interleukin 1. Analytic isoelectrofocusing of pure MONAP (single line upon sodiumdodecylsulfate polyacrylamide gel electrophoresis, single peak after RP-18-HPLC), obtained by size exclusion HPLC followed by two different reversed phase HPLC steps revealed charge heterogeneity giving major components with isoelectric points at 4.7, 4.9, 6.4 and 6.9, all of which exhibited chemotactic activity.

Human IL-1- as well as ETAF-preparations have been shown to elicit chemotactic as well as secretory responses of human neutrophils in vitro (1-4). Recently, however, it has been reported that recombinant human IL-1 lacks chemotactic activity for human neutrophils in vitro (5,6), raising the question whether monocyte derived IL-1 and ETAF-preparations may contain a yet unrecognized chemotaxin.

Abbreviations: IL-1, Interleukin 1, ETAF; epidermal cell derived thymocyte activating factor, PMNL; polymorphonuclear leukocyte, HPLC; high performance liquid chromatography, MONAP; monocyte derived neutrophil activating peptide, BSA; bovine serum albumine, PBS; Dulbecco's phosphate buffered saline, LPS; lipopolysaccharide, TFA; trifluoroacetic acid, HPLC; high performance liquid chromatography, SDS-PAGE; sodium dodecylsulfate-polyacrylamide gel electrophoresis, pI; isoelectric point, IEF; isoelectrofocusing, kD; kilo Dalton.

0006-291X/88 $1.50

Moreover, Yoshimura et al. (7) by size exclusion-HPLC were able to separate PMNL- stimulating activity from murine thymocyte co-stimulating (IL-1) activity present in monocyte supernatants indicating a novel chemotactic cytokine. We have purified this neutrophil activating peptide (MONAP) to apparent homogeneity and we detected a 10 kD polypeptide which is a potent neutrophil activator and lacks IL-1 activity (8).

In the present report we are able to show that this newly discovered chemotaxin consists of different charged species.

MATERIALS AND METHODS

Monocyte purification: Human monocytes were purified as previously described (9) by receptor mediated reversible attachment to human fibronectin coated plastic surface according to Freundlich and Avdalovic (10).
Neutrophil isolation: Polymorphonuclear leukocytes were isolated from the pellet after Ficoll gradient centrifugation as described under "monocyte purification" using a modification of a previously described method (8,11).
MONAP assays: PMNL stimulating activity was investigated by three different methods: PMNL-chemotactic activity in samples was assayed using a modification of the "endogenous component chemotactic assay" method (12) using ß-glucuronidase as marker enzyme (8,13). Release of azurophilic granule enzymes was performed as previously described using ß-glucuronidase or myeloperoxidase as marker enzymes (8,15).
MONAP-production: Purified human blood monocytes were allowed to adhere to tissue culture flasks (Falcon) in medium 199 containing Earles salts and 20 mM HEPES (1-2 x 10^6 cells/ml).
Thereafter LPS (Salmonella minnesota RE 595, Calbiochem, Marburg, FRG) was added and cultures maintained at 37° C in a humidified atmosphere containing 5 % CO_2.
Media were collected after a 24 - 40 h incubation and stored below -70° C until further processing.
MONAP-purification: Acidified (pH 3-4) culture media were concentrated on an Amicon YM-5 membrane and applied directly to a TSK-2000-SW-HPLC-column (LKB, Bromma, Sweden), equilibrated with 0.1 % (v/v) TFA. Chromatography was performed with 0.1 % TFA using a flow rate of 1 ml/min, and fractions of 0.5 ml were collected. Samples from each fraction were taken for biological assays (chemotaxis, enzyme release and occasionally IL-1-assay).
Biological active fractions were pooled, lyophilized and after solubilization of the residue in 0.1 % TFA applied to a 300 Å reversed phase (RP-8)-HPLC-column (Zorbax PEP-RP1, Dupont). Peptides were eluted by a gradient of acetonitrile in 0.1 % TFA and fractions were taken manually. Samples from each fraction were tested in bio-assays.
Fractions with PMNL-stimulating activity of the wide pore RP-8-HPLC-separation were further purified by RP-18-reversed phase HPLC (Nucleosil 5 µ, Merck, Darmstadt, FRG) using an acetonitrile/0.1 % TFA gradient and monitoring the effluent absorption

at 215 nm. Fractions were collected manually and aliquots tested after lyophilisation for inherent biological activity.
SDS-PAGE: Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) for peptides was performed as described by Swank and Munkres (17) under non-reducing conditions (8).
Proteins were visualized by silver staining (Amersham, Braunschweig, FRG).
Isoelectrofocusing: Isoelectrofocusing was performed using commercial available IEF-precotes (range: pH 3 - 10, Serva, Heidelberg, FRG). IP's were determined by calibration with samples of known pI (Pharmacia, Uppsala). Proteins were visualized by a combination of Coomassie Blue staining followed by silver staining or silver staining (Amersham, Braunschweig, FRG) alone.
In some experiments IEF-analyses were performed in duplicate: One lane was stained with Coomassie Blue and silver, and subsequently in the second lane the whole gel-area was sliced and extracted with 30 µl acetonitrile containing 0.1 % TFA over night at 4° C. Extracts were thereafter lyophilized in the presence of 0.1 % BSA and residues solubilized in PBS/0.1 % BSA before testing in the chemotaxis assay at 1:50 and 1:500 dilution.

RESULTS AND DISCUSSION

In the past a number of observations dealing with peptide-like neutrophil-chemoattractants secreted by human monocytes or macrophages have been reported (1,7, 18-22). The molweight of these isolates has been found to vary from 9.5 to 12 kD as determined by gel-filtration (4, 7,8,18,22).

The monocyte derived neutrophil activating peptide (MONAP) we recently purified to apparent homogeneity binds to an anion-exchanger (8) indicating the presence of an predominantly acidic form of this chemotaxin. In that study we used ion-exchange HPLC for purification of MONAP, which principally makes it possible to enrich a single charged species as it has been found for IL-1 (23,24). In the present study we asked for a possible charge heterogeneity, therefore purification of MONAP was attempted by an alternative chromatographic procedure avoiding ion-exchange-steps during isolation of this chemotaxin.

Subsequently performed size-exclusion-HPLC followed by wide pore RP-8-HPLC and narrow pore RP-18-HPLC resulted in virtually pure MONAP which eluted in a single, at 215 nm absorbing sharp peak

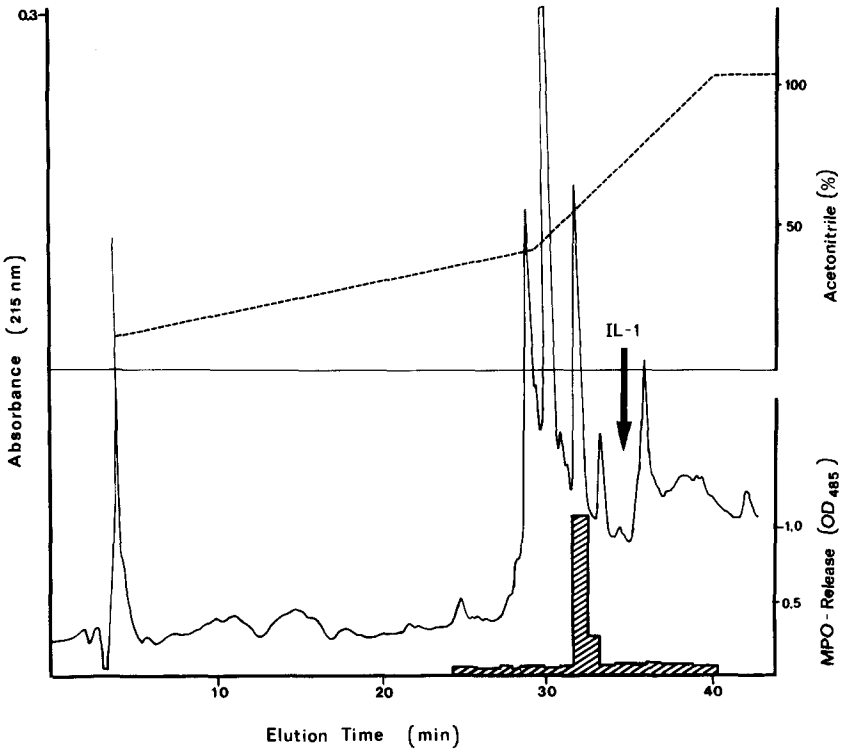

Figure 1. Reversed phase (RP-18)- narrow pore HPLC of TSK-2000-HPLC and RP-8 wide pore HPLC purified MONAP. The effluent was monitored at 215 nm (—). An 5 µl-aliquot of fractions number 25 to 41 was tested for myeloperoxidase-release by human PMN (shaded area). Note association of peak biological activity with a single, sharp peak absorbing at 215 nm.
The arrow indicates the elution position of peak interleukin-1-activity.

(Figure 1) and gave a single line on SDS-PAGE at 10 kD under non reducing conditions (not shown). This material has been applied to ultrathinlayer isoelectrofocusing precotes. The lower part of Figure 2 shows a typical result of an apparent homogeneous MONAP-preparation (in m.w. by SDS-PAGE as well as RP-18-HPLC). As demonstrated by several silver stained lines, MONAP seems to exist in different isoelectric species. Analytical IEF typically reveals intense bands corresponding to pI's at 4.7, 4.9, 6.4 and 6.9. In addition, several weak lines could be detected, especially in the area pI = 7.0-7.5. Some MONAP preparations, however, contained additionally major lines with pI = 6.7 and 5.4 (not shown). In order to verify that the bands obtained were not the result of

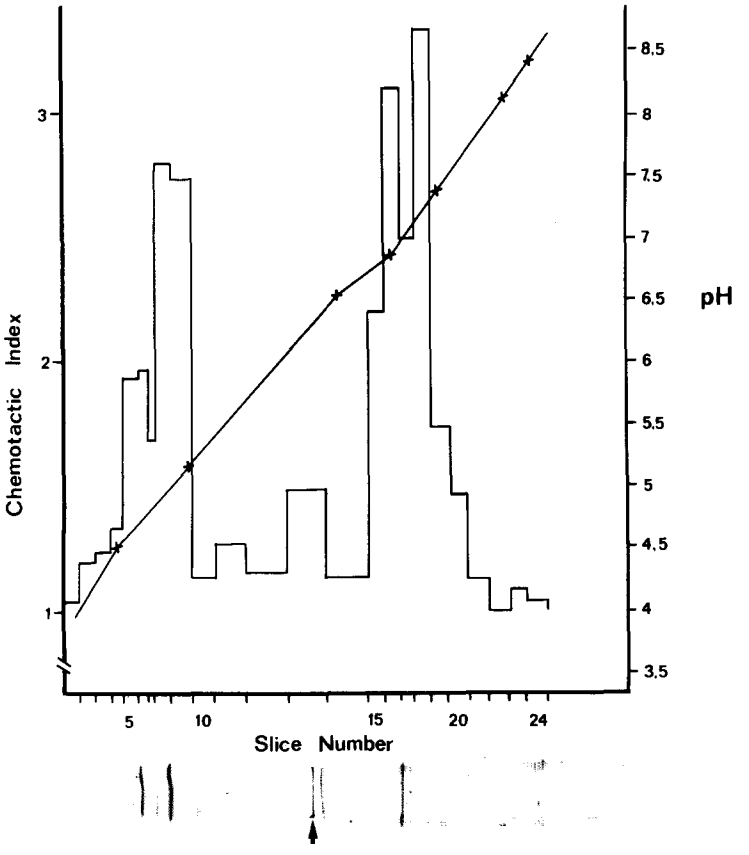

Figure 2. Analytical IEF of purified MONAP. 5 ug of HPLC-purified MONAP was applied to two adjacent lanes of an IEF gel (pH 3.5-10) by means of an applicator template. Proteins of known pI were applied to separate lanes in order to calibrate the pH gradient of the gel. After focusing, lanes bearing standard proteins and one of the lanes bearing MONAP were fixed, prestained with Coomassie Brilliant Blue followed by staining with silver (see materials and methods) (lower part of this figure).
The remaining gel slice bearing MONAP was sliced, extracted and the extract assayed for chemotactic activity at two different concentrations (1:50 and 1:500 of the initial extracts). Chemotactic indices of the 1:500 dilutions are shown in the upper part of this figure.
The arrow indicates a staining artifact resulting from scoring of the gel surface by the applicator template.

contaminating impurities we performed a number of duplicate IEFs, whereby one lane was stained with silver and the second lane sliced into 24 sections, which were extracted and tested for inherent chemotactic activity. In the upper part of figure 2 the pattern of chemotactic activity, extractable from the IEF-gel, is demonstrated in a typical experiment.

These data show that chemotactic activity apparently is present in all sections corresponding to intense silver stained bands. However, also moderate stained bands, especially in the neutral pH-area, represent chemotactic components.

Our results are in agreement with a recent report, where upon tube gel isoelectrofocusing of crude leukocytic pyrogen chemotactic activity eluted as a broad peak between pH 4 and 5.5 as well as a second one near pH 6.8 (1). In addition, an alveolar macrophage derived chemotactic peptide eluted upon tube gel isoelectrofocusing between pH 4.5 and 5.5 (18). It is likely that the material used in both reports contained MONAP as a contaminating chemotaxin. In a more recent study, Yoshimura et al. partially characterized a mononuclear cell derived chemotaxin, which appears to be identical with the chemotaxin we recently purified to apparent homogeneity. In their report (7) an IP, determined upon chromatofocusing, was found near 8-8.5, which is different from the IP reported by others as well as data we obtained with highly purified material by analytical IEF (Figure 2).

The reasons for these discrepancies are presently unclear but may be related to technical causes. In a study on the molecular nature of IL-1 Auron et al. recently demonstrated that in general pI's determined by flat-bed isoelectrofocusing technique tend to be more acidic than those derived from chromatofocusing (25). The key question in consideration of these isoelectric focusing results is whether the heterogeneity of MONAP we observe arises from multiple-gene products, multiple-acidic carbohydrate moieties or from deamidation reactions.

Deamidation of asparagin and glutamin residues is commonly observed in proteins (27) and leads to characteristic series of increasingly acidic species on IEF gels possibly with retention of bio-

logic activity. This has been discussed by several authors for IL-1 (23,24,26). In this case the most basic (non-deamidated) form can be considered to be the original biosynthesis product. It is possible, that deamidation reactions in part may be responsible for the charge heterogeneity of MONAP, because data obtained from amino acid sequence analysis indicate the presence of glutamine and asparagine residues (27).

In addition to this, acidic carbohydrate-moities may similarily result in acidic components. However, it is not clear yet whether MONAP is glycosylated. Studies to investigate this, are now in progress.

On the other hand it appears to be unlikely, that MONAP-preparations contain multiple gene products - as has been seen for IL-1 (28) - because amino acid sequence analysis of a single lot of MONAP, which demonstrated charge heterogeneity upon IEF-analysis, reveals a constant sequence of a single peptide backbone (H. Gregory, unpublished results).

ACKNOWLEDGEMENTS

This investigation was supported by DFG grant Schr 305/1-1. We are gratefully indebted to Miss C. Gerbrecht, Miss A. Stützl-Studt and Miss E. Ohm for their excellent technical assistance and Mrs. I. Böttjer for typing the manuscript.

REFERENCES

1. Sauder, D.N., Mounessa, N.L., Katz, S.I., Dinarello, C.A., and Gallin, J. I. (1984) J. Immunol. 132, 828-832.
2. Luger, T.A., Charon, J.A., Colot, M., Micksche, M., and Oppenheim, J.J. (1983) J. Immunol. 132, 816-820.
3. Smith, R.J., Bowman, B.J., and Speziale, S.C. (1986) Int. J. Immunopharmacol. 8, 33-40.
4. Klempner, M.S., Dinarello, C.A. and Gallin, J.I. (1978) J. Clin. Invest. 61, 1330-1336.
5. Issekutz, A.C., Megyeri, P., and Issekutz, T.B. (1987) Lab. Invest. 56, 49-59.
6. Georgilis, K., Schaefer, C., Dinarello, C.A., and Klempner, M.S. (1987) J. Immunol. 138, 3403-3407.

7. Yoshimura, T., Matsushima, K., Oppenheim, J.J., Leonard, E.J. (1987) J. Immunol. 139, 788-793.
8. Schröder, J.M., Mrowietz, U., Morita, E., and Christophers, E. (1987) J. Immunol. 139, 3474-3484.
9. Mrowietz, U. and Ternowitz, T. (1987) Acta path. microbiol. immunol. Scand. C. 94, 1-7.
10. Freundlich, B., and Avdalovic, N. (1983) J. Immunol. Methods 52, 31-37.
11. Henson, P.M. (1971) J. Immunol. 107, 1535-1557.
12. Creamer, H.R., Gabler, W.L. and Bullock, W.W. (1983) Inflammation 7, 321-329.
13. Schröder, J.-M., and Christophers, E. (1985) J. Invest. Dermatol. 85, 194-198.
14. Fernandez and Hugli, T.E. (1976) J. Immunol. 117, 1688-1694.
15. Preissner, W.C., Schröder, J.-M., and Christophers, E. (1983) Brit. J. Dermatol. 109, 1-8.
16. Bergmeyer, H.U., editor (1963) in: Methods in enzymatic analysis, Academic Press Inc. New York, 737-754.
17. Swank, R.T., and Munkres, K.D. (1971) Anal. Biochemistry 39, 462-479.
18. Merril, W.W., Naegel, G.A., Matthay, R.A., and Reynolds, H.Y. (1980) J. Clin. Invest. 65, 268-276.
19. Altman, L.C., Snyderman, R.S., Oppenheim, J.J., and Mergenhagen, S.E. (1973) J. Immunol. 110, 801-810.
20. Snyderman, R., Meadows, L., and Amos, D.B. (1977) Cell. Immunol. 30, 225-235.
21. Tono-Oka, T., Nakayama, M., and Matsumoto, S. (1980) Immunology 39, 607-613.
22. Kownatzki, E., Kapp, A., and Uhrich, S. (1986) Clin. exp. Immunol. 64, 214-222.
23. Kronheim, S.R., March, C.J., Erb, S.K., Conlon, P.J., Mochizuki, D.Y., and Hopp, T.P. (1985) J. Exp. Med. 161, 490-502.
24. Cameron, P.M., Limjuco, G.A., Chin, J., Silberstein, L., Schmidt, J.A. (1986) J. exp. Med. 164, 237-250.
25. Auron, P.E., Warner, S.J.C., Webb, A.C., Cannon, J.G., Bernheim, H.A., McAdam, K.J.P.W., Rosenwasser L.J., LoPreste, G., Mucci, S.F., Dinarello, C.A. (1987) J. Immunol. 138, 1447-1456.
26. Hopp, T.P., Dower, S.K., March, C.J. (1986) Immunol. Res. 5, 271-280.
27. Gregory, H., Young, J., Schröder, J.-M., Mrowietz, U., and Chrisophers, E. (1988) Biochem. Biophys. Res. Comm., in press.
28. Robinson, A.B., and Rudd, C.J. (1974) In Current Topics of Cellular Regulation. Vol. 8, B.J. Horecker and E.R. Stadtman, editors. Academic Press, New York, 247-261.