Clin. exp. Immunol. (1986) **64**, 214–222.

Novel neutrophil chemotactic factor derived from human peripheral blood mononuclear leucocytes

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(Accepted for publication 22 November 1985)

SUMMARY

Human mononuclear leucocytes isolated from the peripheral blood by centrifugation on Ficoll-Hypaque cushions and adherent on plastic petri dishes, produced a chemotactic factor that attracted human neutrophilic granulocytes to the same extent as did optimal concentrations of the complement split product C5a and the leukotriene B_4. The active component eluted from a Sephadex G-50 gel filtration column as a single peak with an apparent molecular weight of 10,000. The chemotactic activity was resistant to reductive cleavage of disulfide bonds and heating at 100° C for 30 min but was lost when reduction and heating were combined. Digestion with a proteolytic enzyme eliminated the attractive potential. The data suggest that this is a novel chemotactic peptide. It is conceivable that it has been seen previously and was mistaken for a lymphokine or interleukin 1.

Keywords chemotactic factor human peripheral blood mononuclear leucocytes chemotaxis neutrophilic granulocytes interleukin

INTRODUCTION

Chemotactic factors appear to play an important role in the accumulation of leucocytes at sites of inflammatory reactions. By various *in vitro* chemotaxis assays numerous substances have been identified as chemotaxins (Schiffmann & Gallin, 1979; Snyderman & Goetzl, 1981). However, they differ widely in their attractive potential as determined by the concentration required to induce migration, the number of cells mobilized and the distance migrated by the leading cells. The most potent chemotactic factors known to occur *in vivo* that attract polymorphonuclear neutrophilic granulocytes (PMN) are the split product of the fifth complement component, C5a (Hugli, 1981), and the leukotriene B_4 (Borgeat & Samuelson, 1979; Ford-Hutchinson *et al.*, 1980). Of similar potency is the synthetic tripeptide formyl-methionyl-leucyl-phenylalanine (f-MLP) (Schiffmann, Corcoran & Wahl, 1975).

In the present communication a chemotaxin is described which attracted PMN to a similar extent as did optimal concentrations of the three above chemotactic factors. It was released from adherent human mononuclear leucocytes (MNL) in the absence of additional stimulation. It had an apparent molecular weight of 10,000, resisted heating at 100° C for 30 min, but was sensitive to proteolytic degradation. This chemotactic factors has some resemblance to a product shown previously to be released from human alveolar macrophages (Merril *et al.*, 1980), to an alleged lymphokine (Altman *et al.*, 1973; Snyderman, Meadows & Amos, 1977) and to the chemotactic activity attributed to interleukin 1 (IL-1) (Sauder *et al.*, 1984).

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MATERIALS AND METHODS

Reagents. The molecular weight marker peptides bacitracin, chymotrypsinogen, cytochrome C, insulin B chain, horse myoglobin, and trypsin inhibitor from bovine lung, the proteolytic enzyme pronase P from streptomyces griseus and iodoacetamide were purchased from Serva Feinbiochemica, (Heidelberg, FRG). Lyophilized bovine serum albumin (BSA) was obtained from Behringwerke (Marburg, FRG); the chemotactic tripeptide f-MLP, fumed silica (particle size 0·007 μm) zymosan A from saccharomyces cerevisiae and cycloheximide from Sigma GmbH (München, FRG); the ionophore A 23187 and dithiothreitol from Calbiochem GmbH (Frankfurt, FRG); phytohaemagglutinin (PHA) from Wellcome Diagnostica (Burgwedel, FRG); media and fetal calf serum (FCS) from Biochrom-Seromed (Berlin, FRG); Lipopolysaccharide (LPS) from *Salmonella abortus equi* was a kind gift of Dr C. Galanos (Galanos & Lüderitz, 1975).

The chemotactic split peptide C5a was partially purified by passage over Sephadex G-200 of normal human serum that had been treated with baker's yeast, 50 mg/ml, for 1 h at 37° C. No provisions were taken to avoid cleavage of the *N*-terminal arginine, so that the preparation presumably consisted of C5a des-Arg mainly (Bokisch & Müller-Eberhard, 1970; Gerard & Hugli, 1981).

Leukotriene B_4 (LTB_4) was produced by stimulating peripheral blood neutrophils at a concentration of 10^7 cells/ml with ionophore A 23187, 2 μg/ml, for 5 min. The chemotactic factor in the supernatant was partially purified by extraction into ether (Grabbe *et al.*, 1984). After evaporation of the solvent the remaining chemotaxin was dissolved in the original volume of Hanks' balanced salt solution (HBSS).

Zymosan was washed repeatedly in hot saline solution to remove any soluble material and opsonized by incubating 10 mg zymosan with 1 ml fresh human serum for 30 min at 37° C followed by washing with HBSS.

White blood cells. Buffy coats from health donors were provided by the local blood bank. The cells were suspended in HBSS, layered over Ficoll-Hypaque (Deutsche Pharmacia, Freiburg, FRG) and centifuged at 1000 ***g*** for 25 min at room temperature. The mononuclear cell fraction at the interface was removed, washed three times with HBSS, resuspended in HEPES-buffered Eagle's minimal essential medium (MEM) containing 1 mg/ml BSA and seeded in plastic petri dishes (Nunc, Roskilde, Denmark). One dish of 90 mm diameter received 10 ml of cell suspension containing 2×10^7 cells. After 24 h at 37° C in 5% CO_2 the supernatant was removed and either analysed for chemotactic activity immediately or stored at −20° C.

For purification of the adherent cell fraction 10 ml MNL suspension containing 2×10^6 cells/ml in Eagle's MEM with 20% heat-inactivated FCS were incubated in 90 mm diameter plastic petri dishes for 60 min at 37° C. The nonadherent fraction was removed and the plates were extensively washed with medium. Following incubation at 4° C for 30 min the adherent cells were removed by flushing medium through a needle and were subjected to the same procedure once more. Greater than 95% of the cells were viable as tested by trypan blue exclusion (Hanks & Wallace, 1958). Monocytes were identified by esterase staining as described by Tucker, Pierre and Jordan (1977) using an assay kit obtained from Technicon GmbH (Bad Vilbel, FRG).

Human neutrophilic granulocytes were prepared according to Chodirker, Bock and Vaughan (1968). Heparinized venous blood was diluted 1:3 with 0·15 M NaCl and centrifuged. The red cells were lysed repeatedly by the addition of 0·03 M NaCl followed by the addition of an equal amount of 0·27 M NaCl. The remaining white cells contained between 80 and 90% PMN. Prior to the experiment the cells were kept at 0° C for a maximum of 2 h. Viability as assessed by the percentage of trypan blue excluding cells was always more than 95%.

Migration. Cell migration was measured with the double chamber filter method of Boyden (1962) using blind well chambers (Neuroprobe, Bethesda, MD, USA) with a volume of 200 μl attractant or control medium in the bottom and 800 μl cell suspension in the top compartment. Cellulose nitrate filters of 3 μm pore size and 150 μm thickness were purchased from Sartorius GmbH (Göttingen, FRG). One million cells suspended in HBSS containing 5 mg/ml BSA (HBSS-BSA) were filled into the top compartment and incubated at 37° C for 1 h. The filters were fixed in

propanol, stained with Harris' haematoxylin, dried by successive rinses in propanol, made translucent with xylene and mounted on microscopic slides with Canada balsam. They were evaluated with the leading front method (Zigmond & Hirsch, 1973). Values presented are the means ± s.d. of triplicate filters, each evaluated at four different sites.

RESULTS

The migration of PMN with several dilutions of the 24 h supernatant of human MNL added to the lower compartment of the Boyden chamber is demonstrated in Table 1. The table also lists the response of the cells to other chemotactic factors, which were used at the lowest concentrations which still induced maximal migration. As can be seen from the table, the distance migrated by the leading cells in response to the MNL supernatant was comparable to the stimulation by C5a, LTB_4 and f-MLP. Maximum migration was observed when the supernatant was added to the lower compartment of the chamber, thus presumably establishing a concentration gradient (Damerau, Wüstefeld & Vogt, 1983). There was a marked chemokinetic activation (Keller *et al.*, 1977), when the attractive material was added to the upper compartment of the chamber.

Ficoll-Hypaque purified mononuclear cells were further fractionated by adherence on plastic petri dishes in the presence of FCS. Unfractionated, adherent and non-adherent MNL were brought to equal cell concentration and incubated for 24 h in Eagle's MEM with 1 mg/ml BSA. As presented in Table 2, amounts of chemotaxin corresponding to the activity of the unfractionated mononuclear cell supernatant were released from the adherent fraction, and only small amounts were obtained from the non-adherent cells. Since the adherent fraction was enriched in esterase-staining cells, monocytes appear to be a likely source of the chemotaxin. The small amount of activity detected in the non-adherent mononuclear cell supernatant could be released from monocytes that had not become attached to the petri dish during the adherence step. Although there was presumably a higher density of chemotaxin producing cells in the adherent cell fraction, there was no increased factor production as compared to the unfractionated cells.

The chemotaxin could be detected in the MNL cultures after 9 h of incubation and increased in concentration up to 48 h (Fig. 1). There was no significant production beyond this time. It should be noted that the distance of migration illustrated or the ordinate of Fig. 1 is not an accurate assessment of the amount of chemotaxin. There may well be factor production within the first 6 h

Table 1. Migration of human PMN towards MNL supernatant and established chemotactic factors in a Boyden chamber assay

Attractant in lower chamber	Migration (μm) with PMN suspended in HBSS-BSA	Migration with PMN suspended in MNL supernatant 1:10†
HBSS	38 ± 6*	81 ± 7
MNL supernatant 1:10†	135 ± 4	87 ± 6
MNL supernatant 1:50	134 ± 2	73 ± 5
MNL supernatant 1:250	85 ± 4	nt
C5a‡	142 ± 2	nt
LTB_4‡	134 ± 2	nt
f-MLP‡	122 ± 5	106 ± 3

* Values are mean ± s.d.

† Supernatant was diluted with HBSS.

‡ Substances were used at lowest concentrations which still induced maximal migration.

nt Not tested.

Table 2. Release of chemotactic activity following fractionation of MNL by adherence

Source of supernatant	Esterase-staining fraction (%)	Dilution of supernatant	Migration (μm)*
Unfractionated MNL	15	1:10	124±4
		1:50	76±4
Adherent MNL	87	1:10	131±6
		1:50	71±2
Non-adherent MNL	1	1:10	66±4
		1:50	57±4

* Background migration with HBSS in lower compartment was 38±4 μm.

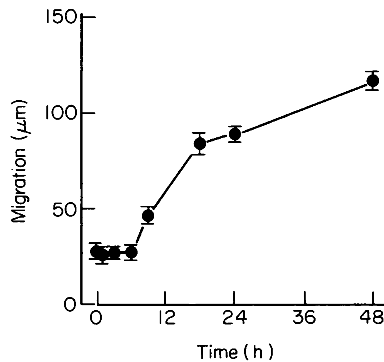

Fig. 1 Time course of the production of MNL-derived chemotaxin. Values are means of triplicate filters ± s.d.

which is under the level of detection by the chemotaxis assay, and the convex shape of the curve does not rule out a constant release rate of the factor.

No stimulus was required to release the chemotactic material and no significant differences in the amount of chemotaxin produced were detected with the following variations of the incubation conditions: addition of bacterial lipopolysaccharide (LPS), 0·5 μg/ml, silica, 50 μg/ml, or PHA 5 μg/ml; preincubation of the MNL with phorbol myristate acetate (PMA), 10 ng/ml followed by washing; omission of BSA with petri dishes untreated or preincubated with BSA and washed; variation of the BSA concentration from 0·2 to 5·0 mg/ml; addition of opsonized zymosan, 2 mg per 5×10^6 MNL. Thus, if the cells did not produce the factor spontaneously the only apparent stimulation was the adherence on the plastic petri dishes.

The supernatant of MNL cultured in the presence of 20 μg/ml cycloheximide contained only small amounts of chemotactic material when compared with the supernatants of control cultures without cycloheximide (Table 3). The later addition of cycloheximide to these control supernatants did not affect its chemotactic activity. The results suggest that protein synthesis was required for elaboration of the chemotaxin.

From a column of Sephadex G-50 the chemotactic activity eluted as a single peak shortly following the elution of cytochrome C (Fig. 2). In contrast, the chemotactic activity generated in normal human serum by complement activation, and isolated C5a eluted ahead of the cytochrome C marker. This suggested that the MNL-derived chemotactic activity could be attributed to a distinct component for which the term mononuclear cell-derived chemotaxin (MOC) will be used. A plot of the Kav values, which is an assessment of the gel fraction available to diffusion by a given molecule (Andrews, 1965; 1970), of various marker peptides and MOC against the log mol. wt (Fig. 3) revealed an apparent molecular weight of 10,000.

Table 3. Effect of cycloheximide on the production of MNL-derived chemotaxin

Chemoattractant	Migration (μm)
HBSS	42±3
f-MLP 10^{-8} M	118±5
MNL supernatant	
1:25	142±4
1:50	128±7
Cycloheximide, 20 μg/ml, present during culture	
1:25	65±8
1:50	47±5
Cycloheximide added after culture	
1:25	138±4
1:50	124±7

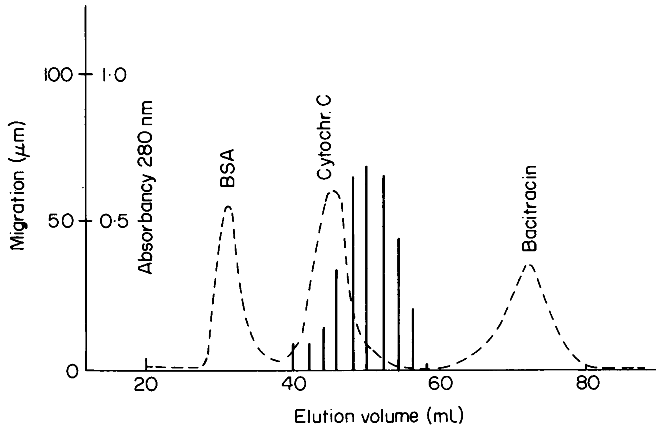

Fig. 2 Elution profile on the chemotactic activity of the MNL supernatant (vertical bars) from a Sephadex G-50 column compared with the elution of three marker peptides (dashed line). Only migration values exceeding the background are shown. Variability was below 10%.

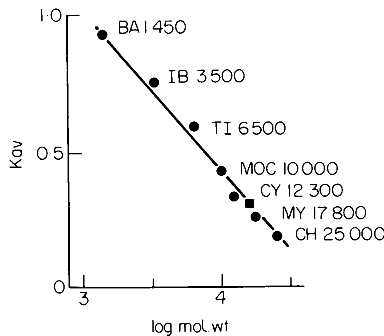

Fig. 3 Molecular weight determination of MOC by gel filtration on Sephadex G-50. Kav values were plotted against the log MW. Marker peptides: (BA) bacitracin; (IB) insulin B chain; (TI) trypsin inhibitor; (CY) cytochrome C; (MY) myoglobin; (CH) chymotrypsinogen. Figures represent respective mol. wt's. (■) marks position of human C5a with an apparent mol. wt of 15,800.

Table 4. Effect of heat treatment and reductive cleavage of disulfide bonds on the chemotactic activity of C5a and MOC

	Chemotaxin		
Treatment	HBSS	MOC	C5a
None	49±4*	120±3	138±2
100° C 30 min	nt	114±3	133±3
Reduction	55±4	113±3	134±4
100° C 30 min + reduction	52±4	59±4	136±3

* Migration of leading front in μm, means ± s.d.
nt Not tested

Table 5. Pronase digestion of the mononuclear cell-derived chemotaxin*

Chemoattractant	Control	Pronase-digested
HBSS	61±3	65±4
f-MLP	113±3	69±5
C5a	130±4	70±5
MOC	119±3	66±3
LTB_4	136±4	134±5

* Migration in μm (means ± s.d.) following 2 h 37° C incubation without and with pronase.

Sephadex G-50-purified MOC and C5a were subjected to heating for 30 min at 100°C and reductive disulfide bond cleavage using dithiothreitol, 10^{-3} M, for reduction and iodoacetamide at 10% molar excess for alkylation. As shown in Table 4 neither of these treatments alone affected either of the two chemotaxins, however, when reduction and alkylation was followed by the heat treatment, the activity of MOC was lost while that of C5a was retained.

In order to test whether MOC activity was sensitive to proteolytic cleavage several peptide and non-peptide chemotactic factors at 20 times the chemotactic concentrations were incubated for 2 h at 37°C with pronase, 0·2 mg/ml, a concentration determined beforehand not to influence chemotactic migration after a 1:20 dilution. Controls were incubated for the same time in the absence of pronase. After the 2 h incubation the materials were diluted 1:20 and assayed for chemotactic activity. As shown in Table 5, there was a complete loss of the chemotactic activity of C5a, f-MLP and MOC, whereas the migration towards LTB_4 was unaffected. This result suggested that the MOC was indeed a polypeptide.

DISCUSSION

There are considerable differences in the potency of substances reported to induce directed migration of PMN. The chemotactic factors C5a, LTB_4 and f-MLP at low concentrations stimulate many cells to migrate long distances. The distance migrated by PMN in response to MOC, which correlates well with the number of cells mobilized from the filter surface, was comparable to their stimulation by the three above chemotactic factors. Since the MOC preparation has not been tested

for its purity, we have no information yet about the concentration which is required for PMN stimulation.

The mol. wt of 10,000, the destruction by a proteolytic enzyme and the requirement of active protein synthesis argued in favour of MOC being a protein. It shared a remarkable heat resistance with C5a, however, it clearly differed from C5a in both its size and its heat sensitivity following reductive cleavage of disulfide bonds.

MOC was released from adherent human peripheral blood mononuclear cells, presumably monocytes, cultured *in vitro* in plastic petri dishes in the absence of serum. Release of the factor could not be stimulated by LPS, Silica, PHA or phagocytosis of opsonized zymosan. We assume that adherence to the petri dish was the stimulus for the release of the chemotaxin, but it cannot be excluded that the cells produced the factor spontaneously in the absence of any stimulation.

Monocytes and macrophages have been shown previously to release chemotactic factors that attract PMN. Low molecular weight (400–600) chemotactic factors of lipid nature derived from arachidonic acid were detected in the supernatant of macrophages from various species and different locations (Merril *et al.*, 1980; Hunninghake *et al.*, 1980; Valone *et al.*, 1980; Czarnetzki, 1980; Hunninghake, Gadek & Lawley, 1981). The best known and probably most active of these is LTB_4 (Borgeat & Samuelson, 1979; Ford-Hutchinson *et al.*, 1980). On the basis of its size and the protein nature MOC was distinct from LTB_4 and the only property shared by both of these mediators was their stability at 100° C. Fractionation of the MOC containing mononuclear cell supernatants did not reveal a low mol. wt chemotactic activity that could represent LTB_4.

In the supernatant of human alveolar macrophages cultured *in vitro* Kazmierowski, Gallin & Reynolds (1977) found a PMN chemotactic factor that was destroyed by heating at 56° C and had a mol. wt below 5,000. In our culture supernatants of peripheral blood monocytes we were unable to discover a PMN chemotactic factor of this size. It is conceivable that this component was produced by alveolar macrophages only.

A PMN attracting substance with a mol. wt of 9,500 that was sensitive to trypsin digestion and immunologically distinct from C5a was detected in the culture supernatants of human alveolar macrophages (Merril *et al.*, 1980). The authors suggest that simple adhesion was sufficient for its production, however, the rate of release was maximally stimulated by aggregated human IgG or zymosan particles. This factor from human alveolar macrophages is quite similar to our chemotaxin derived from human peripheral blood monocytes. However, we have not been able to observe enhanced production of our material with LPS or zymosan phagocytosis.

Interleukin 1 (IL-1) or formerly called lymphocyte-activating factor (LAF) is a product of mononuclear phagocytes, which stimulates thymus-derived lymphocytes (Meltzer & Oppenheim, 1977; Mizel & Mizel, 1981). It appears to be similar if not identical with human leucocytic pyrogen (Rosenwasser, Dinarello & Rosenthal, 1979) and the epidermal cell-derived thymocyte activating factor (ETAF) (Luger *et al.*, 1983). All of these factors were reported to induce directed migration of PMN (Luger *et al.*, 1983; Sauder *et al.*, 1984). Therefore, the question was rasied whether MOC was IL-1. For several resons this is unlikely. The mol. wt of human IL-1 is reported to be 15,000 (Mizel & Mizel, 1981). From Sephadex G-50 MOC clearly eluted after the cytochrome C marker indicating that its mol. wt was below 12,300. While the activity of IL-1 was reportedly destroyed by heating at 60° C (Luger *et al.*, 1983), MOC was resistant to 100° C. Whereas the release of IL-1 required a stimulus like LPS, MOC production from MNL proceeded in the absence of additional stimulation and could not be enhanced by LPS.

While there is no indication to suspect that MOC was identical with IL-1, ETAF or human leucocytic pyrogen, we wondered whether some of the chemotactic activity attributed to these factors could be due to contaminating MOC rather than the thymocyte stimulating substances themselves. This was supported by preliminary findings indicating that thymocyte stimulating and chemotactic activities of an ETAF preparation after passage over a TSK 125 HPLC column had different elution patterns (Luger & Kownatzki: unpublished observation).

In numerous attempts we have never been able to detect PMN chemotactic activity in the supernatant of antigen or mitogen stimulated lymphocyte cultures, as has been reported by others (Altman, 1978; Rocklin, Bendtzen & Greineder, 1980). However, a monocyte chemotactic factor found in the supernatant of mitogen or antigen stimulated MNL or of mixed lymphocyte cultures

was similar to MOC (Altman *et al.*, 1973; Snyderman et al. (1977)). It was heat stable at 56° C, and from a Sephadex G-100 column it eluted directly following the cytochrome C marker. These authors also noticed high levels of activity in unstimulated control cultures (Snyderman *et al.*, 1977). There is no information whether this alleged lymphokine attracted PMN, and it has not yet been tested whether MOC was chemotactic for monocytes.

The strong attraction of PMN by MOC puts this factor in one line with C5a and LTB_4. If one accepts that chemotaxis is not only an *in vitro* phenomenon but is relevant for cell accumulation *in vivo*, MOC would be expected to play an important role in the infiltration of PMN. What makes it different from the two other potent attractants is the rate of release. While LTB_4 and C5a are generated within minutes following cell stimulation and complement activation respectively, MOC generation could be observed only after hours of incubation. This raises the possibility that MOC is responsible for PMN infiltration in sustained or chronic inflammatory processes.

Supported by the Deutsche Forschungsgemeinschaft.

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