

A NOVEL, NH₂-TERMINAL SEQUENCE-CHARACTERIZED
HUMAN MONOKINE POSSESSING NEUTROPHIL
CHEMOTACTIC, SKIN-REACTIVE, AND
GRANULOCYTOSIS-PROMOTING ACTIVITY

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Infiltration of tissues with neutrophil granulocytes is an important element of the acute local inflammatory response. It is brought about by adherence of neutrophils to the endothelial linings of small blood vessels, followed by movement through the vessel wall and into the inflamed tissue. These phenomena are known to involve the action of membrane-bound receptors, as well as soluble chemotactic factors. Some of these factors are chemotactic per se, while others act by changing the expression of membrane-associated molecules involved in adhesion.

Chemotactic agents that play a role in the inflammatory reaction may be exogenous (e.g., of bacterial origin) or endogenous (reviewed in reference 1). The classical example of an endogenous factor is the plasma-derived, activated complement factor C5a. Other endogenous chemotactic factors are those released locally by any of the cells that take part in inflammation: endothelial cells, platelets, monocytes, lymphocytes, or granulocytes themselves. Examples of such cell-released chemotactic agents are leukotriene B₄ (LTB₄)¹ and platelet-activating factor (PAF). Certain cytokines, e.g., IL-1, have also been described to possess chemotactic activity (2).

We here describe the biological and biochemical characterization of a human macrophage-derived factor that, in vitro, possesses chemotactic activity for granulocytes and, in vivo, induces granulocytosis upon systemic injection and skin reaction upon local injection in experimental animals. The kinetics of appearance of the in vivo effects suggest that this factor is a mediator for similar effects

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¹ *Abbreviations used in this paper:* βTG, β-thromboglobulin; EMEM, Eagles minimum essential medium; FPLC, fast protein liquid chromatography; HGF, hybridoma growth factor; HPLC, high-pressure liquid chromatography; HSF, hepatocyte-stimulating factor; LTB₄, leukotriene B₄; PAF, platelet-activating factor; PF4, platelet factor 4.

induced by IL-1. The factor was purified to homogeneity and its NH₂-terminal sequence was determined. The sequence was found to correspond to that deduced from a cDNA sequence "3-10C" coding for a protein that has itself not been isolated, to which no biological activity could as yet be assigned, but that has sequence homology with the platelet factors β -thromboglobulin (β TG) and platelet factor 4 (PF4).

Materials and Methods

Production of Chemotactic Factor and β TG. Human peripheral blood leukocytes were isolated and fractionated from pooled buffy coats provided by the Blood Transfusion Centers of Leuven and Antwerp (Belgian Red Cross). Red blood cells were removed by sedimentation (30 min) in hydroxyethyl starch (Plasmasteril[®]; Fresenius AG, Bad Homburg, Federal Republic of Germany). Platelets were separated by centrifugation at low speed (300 g, 10 min). Mononuclear cells were isolated by gradient centrifugation (400 g, 30 min) on Ficoll-sodium metrizoate (Lymphoprep; Nyegaard, Oslo, Norway). Cells were suspended at 5×10^6 cells/ml in RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 2% FCS using spinner flasks. The adhering cell population was obtained by incubating the mononuclear cell fraction in stationary cultures for 2 h at 37°C. Non-adherent cells were then removed by repeated washes and the cultures were replenished with Eagle's minimum essential medium (EMEM) supplemented with 2% FCS. The adherent cell fraction consisted of 80% monocytes as tested microscopically using phycoerythrin-conjugated human Leu-M3 antibody (Becton Dickinson & Co., Mountain View, CA).

Suspended platelets, free of leukocytes, were stimulated at 37°C with thrombin (Topostasine, Hoffmann-La Roche, Basel, Switzerland) at 1 U/ml for 2 h or were left untreated for 24 h. Total mononuclear cell suspension and adhering cell fractions were stimulated for 48 h at 37°C with Con A (Calbiochem-Behring Corp., San Diego, CA) or LPS from *Escherichia coli* (0111:B4; Difco Laboratories, Detroit, MI).

Purification of Chemotactic Factor and β TG. Crude supernatants from stimulated mononuclear cells, adhering cells, or platelets were concentrated and partially purified by adsorption to silicic acid (Mallinckrodt Inc., Paris, KY) and elution with 50% ethylene glycol containing PBS at pH 7.4 as described previously (3). The second purification step consisted of adsorption of impurities to DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) in 20 mM Tris-HCl buffer, pH 7.5. The DEAE-Sephacel-unsorbed fraction was concentrated by dialysis against 20 mM Tris-HCl buffer, pH 7.5, containing 15% polyethylene glycol 20,000 (Serva, Heidelberg, Federal Republic of Germany) and further fractionated by gel filtration on Ultrogel AcA 54 (LKB Produkter, Bromma, Sweden) using a 2.6 cm (inner diameter) \times 100 cm column equilibrated with 18% ethylene glycol in 1.55 M NaCl, 8 mM phosphate, pH 7.2.

The 15–30-kD fractions from gel filtration were then concentrated and purified by fast protein liquid chromatography (FPLC). The material was applied to a Mono S cation exchange column (Pharmacia Fine Chemicals, Uppsala, Sweden) in 50 mM formate buffer, pH 4.0. Biological activity was eluted at a flow rate of 1 ml/min with a linear NaCl gradient (0–0.65 M), followed by a step-wise gradient to 1 M NaCl.

SDS-PAGE was done as described by Laemmli (4). Samples were loaded onto linear gradient (8–30% wt/vol) polyacrylamide gels containing 0.1% (wt/vol) SDS with a 5% stacking gel containing 0.25% SDS. The gels (17 \times 13 \times 0.1 cm) were stained with Coomassie blue. The molecular weight markers (Bio-Rad Laboratories, Richmond, CA) used were phosphorylase b (M_r 92,500), BSA (M_r 66,200), OVA (M_r 45,000), carbonic anhydrase (M_r 31,000), soybean trypsin inhibitor (M_r 21,500), lysozyme (M_r 14,400) and the low molecular mass markers (Pierce Chemical Co., Rockford, IL), cytochrome *c* (M_r 12,500), and aprotinin (M_r 6,500).

Induction of Skin Reactivity and Granulocytosis In Vivo. Skin reactivity was measured in triplicate in New Zealand White rabbits. Rabbits were shaved at the abdomen and

injected subcutaneously with 150 μ l of FPLC column fractions (12 samples per rabbit), concentrated, and dialyzed against PBS + 15% polyethylene glycol. Skin reactivity was measured with 3-h time intervals by measuring the diameter of the swelling and the intensity of coloring. The reaction was scored arbitrarily but twice independently at each time interval. Scores for the different rabbits were averaged and expressed as a percentage of maximal skin reactivity (1.5-cm diameter of redness) as obtained with a concentrated standard preparation of partially purified (FPLC input) material.

Induction of granulocytosis was measured in rabbits (\sim 3 kg) by intravenous injection (3 ml) of different doses of the test material, verified for purity by SDS-PAGE, and dialyzed against pyrogen-free saline. Blood samples (1 ml) were collected in heparinized tubes at several time intervals after treatment by bleeding at a peripheral ear vein. Total leukocytes were counted in duplicate using a hemacytometer. The percentage of granulocytes was determined in duplicate by 100-cell differential cell counts of a smear stained with Wright-Giemsa solution. Control rabbits injected with pyrogen-free physiological saline showed during the 30-h test period a $<25\%$ change in total or differential cell counts as compared with the average cell number ($n = 3$) before injection. Average changes in cell counts of $>50\%$ of the cell number before treatment must thereby be considered as significant.

In Vitro Assay for Granulocyte Chemotaxis. Populations of polymorphonuclear neutrophils and mononuclear cells were separated by gradient centrifugation (30 min, 400 g) of heparinized human peripheral blood from a single donor on Ficoll-sodium metrizoate. The pellet, containing granulocytes and erythrocytes, was suspended in hydroxyethyl starch for 30 min to remove erythrocytes by sedimentation. Residual erythrocytes were eliminated by lysis in bidistilled water (30 s). Purified neutrophils were finally obtained by centrifugation (30 min, 20,000 g) in a Percoll gradient ($d = 1.054$). Granulocytes were washed, counted, and resuspended at 2×10^7 cells/ml in HBSS (Gibco), supplemented with pyrogen-free human plasma protein (1 mg/ml albumin, Cohn fraction V).

Chemotaxis was measured under agarose as described by Nelson et al. (5). In each agarose dish, six series of three wells (2.8-mm diameter) were cut. The center well of each series received 10 μ l of the purified granulocyte population (2×10^5 cells/well). The inner and outer wells were filled with 10 μ l of test sample and control nonchemotactic medium (HBSS + albumin), respectively. FMLP (Sigma Chemical Co., St. Louis, MO) at 10^{-7} M was used as a positive control chemotactic factor in each dish. After incubation (37°C, 5% CO₂) for 2–3 h maximal chemotaxis could be observed in the FMLP⁺ control. At that time cells were fixed with methanol and formaldehyde (37%). Test samples were scored microscopically for chemotactic activity by measuring the migration distance of the cells. Quantification for effective migration was calculated from the actual migration distance (toward the chemotactic factor) by subtraction of the spontaneous migration distance (toward the negative control medium). For each sample at least three dilutions were tested in duplicate. The titration endpoint corresponding to 1 U/ml was calculated from the dilution resulting in half-maximal effective migration distance as compared with that obtained with FMLP (10^{-7} M). Titers for chemotactic activity were expressed in log₁₀ U/ml.

Determination of β -TG. The concentrations of β TG in fractions obtained by FPLC were determined with a commercially available radioimmunoassay kit (Amersham International, Buckinghamshire, UK). Since the standard solutions present in this kit were ranging from 10 to 225 ng/ml, column fractions containing β TG at the microgram level were appropriately prediluted (in bidistilled water) to obtain a final concentration between 20 and 150 ng/ml. Interference in the assay by other compounds present in the column fractions was not likely, in view of the high purification rate achieved at the FPLC stage.

IL-1 Assay. IL-1 activity was measured by its antiviral effect against vesicular stomatitis virus on human MG-63 osteosarcoma cells as described (6), 1 U/ml of IL-1 corresponding to half-maximal protection of the test cells against the virus. The titers for IL-1 (log₁₀ U/ml) as obtained by this method correspond to those obtained in the murine

thymocyte costimulatory assay. The preparation and biological determination of other natural cytokines is described in (6).

NH₂-terminal Amino Acid Sequence Analysis. NH₂-terminal amino acid sequence analysis was carried out on a gas-phase sequencer (Model 470A; Applied Biosystems, Inc., Foster City, CA) with off-line identification of the PTH-amino acids by HPLC on a 4.6 × 250 mm IBM cyanopropyl column (7). Methanolic HCl was used as reagent to convert the thiazolinones so that the PTH derivatives of Asp, Glu, and carboxymethylcysteine were automatically methylated. The latter compound was identified following the protocol described in (8).

Results

Isolation of a Skin-reactive Factor Different from IL-1. IL-1 β produced by mitogen-stimulated leukocytes was purified by consecutive adsorption to silicic acid, DEAE-Sephacel, and gel filtration as described (3, 9). The 15–30-kD fractions from gel filtration, containing all IL-1 (22 kD) activity, were then pooled and separated on a cation-exchange FPLC column at pH 4.0. Fractions from FPLC dialyzed against PBS were tested for *in vitro* and *in vivo* biological effects of IL-1. Antiviral activity on fibroblasts (6) was taken as an *in vitro* test for IL-1 while

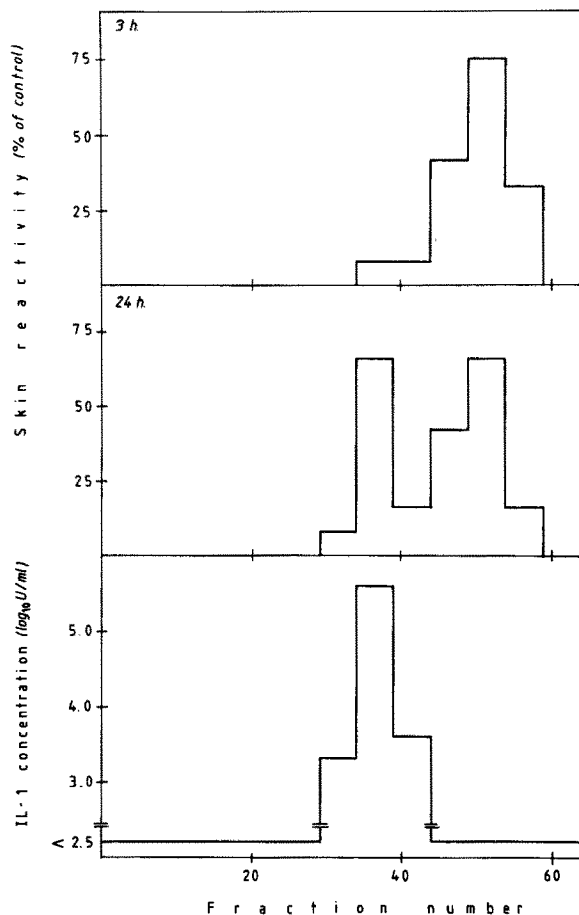


FIGURE 1. Separation of skin-reactive factor different from IL-1 by cation exchange chromatography. Fractions (150 μ l) of mitogen-stimulated leukocyte supernatants purified by FPLC (Mono S, pH 4.0) were injected subcutaneously and skin reactivity was registered after 3 h and 24 h. IL-1 was detected in the same fractions by its *in vitro* antiviral effect on fibroblast cells.

skin reactivity after subcutaneous injection in rabbits (9) was used for in vivo detection. Fig. 1 shows that on FPLC all antiviral activity eluted in a single peak at 0.37 M NaCl. Concordantly, a late (24 h) skin reactivity consisting of redness and swelling was observed with this antivirally active fraction. SDS-PAGE of such peak fraction revealed a single protein of 17,000 kD identifiable as IL-1 β by NH₂-terminal sequence analysis (9).

In addition to the late skin reactivity, as observed with IL-1 β , an early (3 h) but persisting reaction in rabbits was seen with a fraction eluting at 1 M NaCl, but containing no antiviral (IL-1) activity (Fig. 1). Such reaction consisted of a swelling and red coloring of \sim 1 cm in diameter. The nature of this biologically active component and its relation to IL-1 were further investigated.

Chemotactic Activity In Vitro with Fractions Inducing Skin Reactivity. Pathological examination of skin biopsies from rabbits in which skin reactions were induced revealed strong neutrophil granulocyte infiltration. This led us to test whether skin reactivity containing fractions from FPLC were chemotactic for granulocytes in vitro (Table I). Several cytokine preparations were used as controls, including pure natural IL-1 β (from FPLC), IFN- β , and IL-6 (10), as well as the inducer substances Con A and LPS. As shown in Table I, none of

TABLE I
Chemotactic Activity of FPLC Fractions Inducing Early Skin Reactivity

Batch number or substance	Fraction number or dose	Early skin reaction* (percent of control)	Chemotactic activity [‡] (end point dilution)
42029	Input	100	ND
	49-50	<10	ND
	51-52	<10	ND
	53-54	72	ND
	55-56	<10	ND
	57-58	<10	ND
43053	51	<10	<1/3
	52	<10	<1/3
	53	77	1/250
	54	11	1/25
	55	<10	<1/3
41780	51	ND	<1/3
	52	ND	<1/3
	53	ND	1/400
	54	ND	1/100
	55	ND	1/6
IL-1 β	2,000 U/ml	<10	<1/3
IFN- β	10,000 U/ml	ND	<1/3
IL-6	10,000 U/ml	ND	<1/3
LPS	1,000 ng/ml	ND	<1/3
Con A	1,000 ng/ml	ND	<1/3

* Skin reactivity at 4 h after subcutaneous injection in rabbits of 150 μ l/fraction (values are averages of three independent scores); a crude cytokine preparation was used as positive control (FPLC input material).

[‡] Expressed as endpoint dilution resulting in half-maximal migration distance as compared with FMLP.

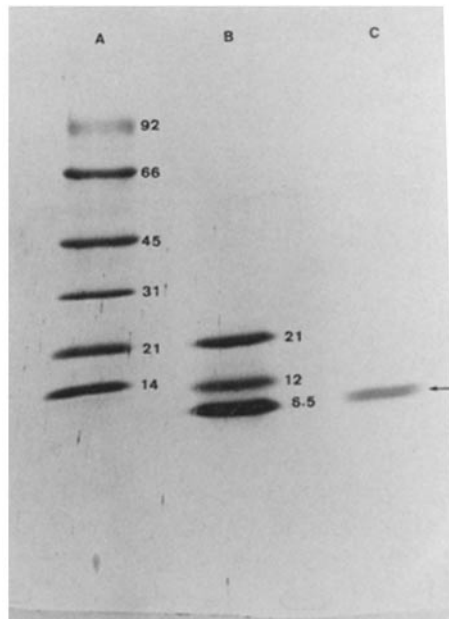


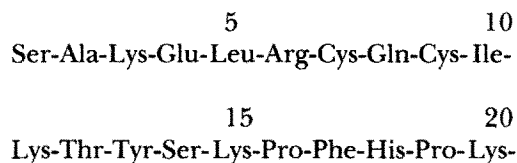
FIGURE 2. SDS-PAGE of purified chemotactic factor. A linear gradient gel was run with 10 μ l (C) of FPLC fraction no. 53 (20 μ g/ml) under reducing conditions and stained with Coomassie blue. M_r markers ($\times 10^{-3}$) are shown (A and B).

these control substances scored positive in this assay for chemotaxis. In contrast, FPLC fractions from mitogen-stimulated leukocyte supernatants showing the early skin reaction did stimulate *in vitro* chemotaxis (Table I) indicating that they contained a factor different from the controls. It can further be seen that the peaks of chemotactic activity and skin reactivity coincided with FPLC fraction No. 53. SDS-PAGE of such peak fraction revealed a single protein band of M_r 6,500, indicating that both the *in vitro* and *in vivo* activities reflected the effect of the same molecule (Fig. 2). Confirmation for this was obtained by an experiment in which an FPLC fraction No. 53 was run on a gel under nonreducing conditions, followed by slicing of the gel and detection of biological activity in the eluates from each slice. This experiment demonstrated that the biological activity fully coincided with the 6.5-kD protein band.

NH₂-Terminal Amino Acid Sequence of the Chemotactic Factor. Since the chemotactic factor was found to copurify with IL-1, the different purification steps were verified in respect of the yield of activity recovered. Although the chemotactic assay might detect several related factors, it was noticed that $\sim 50\%$ ($n = 8$) of the biological activity could be recovered after the first two purification steps (adsorption to silicic acid and DEAE-Sephacel). It was verified that most of the chemotactic activity indeed coeluted with IL-1 upon subsequent gel filtration at a higher M_r ($15\text{--}30 \times 10^3$) than 6.5×10^3 , suggesting that this molecule could occur as a multimer. Finally, no loss of chemotactic activity was observed ($n = 5$) during cation exchange FPLC.

Several preparations from FPLC (about 40 μ g each), tested for purity by SDS-PAGE and for chemotactic activity, were sequenced in a gas-phase sequenator. One of these preparations was carboxymethylated before sequence analysis in order to detect cysteine residues. For the different runs a single and identical

NH₂-terminal protein sequence was obtained which reads:



The sequence showed strong homology with that of the human platelet factors β TG (11) and PF-4 (12). Moreover, complete identity was observed with a cDNA-derived sequence (3-10C) that in its entirety codes for a protein (M_r 11,000) of 99 amino acid residues (13). Since this cDNA has so far not been expressed and tested for biological activity, it is not known which activity, if any, the corresponding protein may have. The location of the NH₂-terminus of the naturally produced chemotactic factor isolated in our laboratory is different from that of the amino acid sequence inferred from the 3-10C cDNA on the basis of hydrophobicity analysis. In addition, the molecular mass of our chemotactic factor is significantly lower than that postulated for the 3-10C putative protein. A weak homology was also seen with another human cDNA-derived sequence (14) coding for a protein called γ IP-10.

Cell Source for Human Chemotactic Factor. Since the platelet factor β TG, related to the isolated chemotactic factor, was also detected in partially purified preparations from mitogen-stimulated leukocytes, it was investigated which cell types were the producers of these molecules in our system. Different cell fractions were isolated, cultured, and stimulated. The supernatants were purified following the same procedure as that used for supernatants of unfractionated leukocytes. It can be seen from Fig. 3 (*left*) that, with FPLC as a final purification

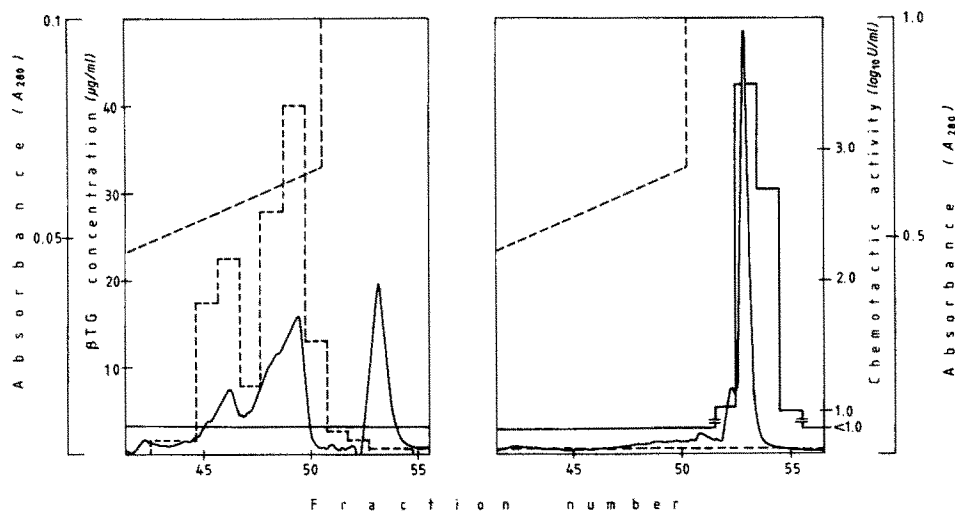


FIGURE 3. Determination of chemotactic activity and β TG in FPLC fractions of supernatants from stimulated platelets (*left*) and monocytes (*right*). Supernatants were purified and tested for activity as described in Materials and Methods. Histograms are indicating chemotactic activity (—) and β TG (---) respectively. The FPLC column was subjected to a linear NaCl gradient (---) and the absorbance was monitored at 280 nm (—).

step, β TG produced from platelets eluted at a position different from that of the chemotactic factor from unfractionated cells. In addition, platelets did not produce this chemotactic activity. It was further found that the chemotactic factor was not released by the granulocyte fraction, but was produced by mononuclear cells. It was then shown (Fig. 3, right) that Con A- or LPS-stimulated adherent cells were potent producers of the chemotactic factor, while no β TG activity was released by those cells. This is in agreement with the finding of others that the mRNA that corresponds to the protein occurs in the histiocytic lymphoma cell line U937 (13). It thus appears that the chemotactic protein and β TG, although structurally related, are produced by different cell types.

Human Chemotactic Factor Induces Granulocytosis In Vivo. Although IL-1 β failed to act chemotactically in vitro, it is known to exert a profound in vivo influence on leukocyte counts. Our studies in this regard (9, 15) revealed that the effect of IL-1 β was biphasic, in that an immediate granulopenia was followed by a later granulocytosis. As demonstrated by Fig. 1, both the chemotactic factor and IL-1 β induce skin reactivity, although with different kinetics. To further investigate the role of this chemotactic factor for granulocyte physiology and its possible role in the hematological effects of IL-1, pure factor was injected intravenously into rabbits (Fig. 4). It was observed that the chemotactic factor rapidly induced a profound granulocytosis (up to fourfold increase in circulating granulocyte numbers). This granulocytosis peaked at 1/2 to 2 h after injection and normal cell counts could be restored within 8 h. This effect was found to be dose dependent, 100 U/kg of in vitro chemotactic activity being the minimum effective in vivo dose. The fast granulocytosis as induced by the chemotactic fac-

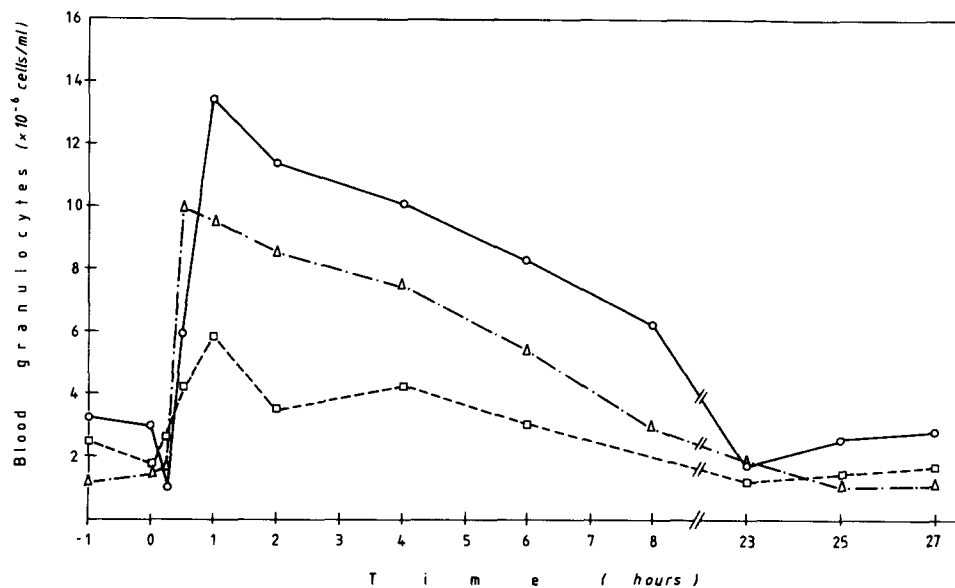


FIGURE 4. Effect of human chemotactic factor on circulating granulocyte counts. Rabbits were injected intravenously with 50 μ l (—□—), 100 μ l (—△—), and 200 μ l (—○—), respectively of FPLC-purified chemotactic factor (50 μ g/ml). Peripheral blood cell counts and control rabbits were as described in Materials and Methods.

tor suggests that the delayed granulocytosis promoting effect of IL-1 β could be mediated by this factor. This would also explain the delayed (24 h) skin reactivity with IL-1 β as compared with the early effect (3 h) with the chemotactic factor, as well as the lack of chemotactic activity of IL-1 β in vitro (Table I). With respect to the effect of the chemotactic factor on the number of circulating mononuclear cells, no significant changes were observed even at the highest dose (400 U/kg) tested. This is also in contrast with IL-1 β , which showed lymphopenia upon intravenous injection in rabbits (9, 15).

Since LPS is known to possess an effect on circulating granulocytes similar to that of IL-1, contamination of the pure chemotactic factor with LPS had to be excluded. First it was observed that surrounding fractions from FPLC containing no IL-1 β nor chemotactic factor had no significant effect (<25% increase or decrease) on granulocyte counts, excluding LPS contamination coming from the column. Second, peak fractions of chemotactic factor from FPLC contained only low amounts of endotoxin (<150 pg/ml as tested in a *Limulus* amoebocyte lysate assay), corresponding to a maximal injected dose of <30 pg per rabbit. Such low doses of LPS (*E. coli*) could not explain the effect on granulocyte counts in rabbits, since doses of LPS as high as 30 ng per rabbit did not have such an effect (data not shown). Third, LPS-induced hematological effects (300 ng/rabbit) have a different pattern in that an early (2 h) granulopenia is followed by granulocytosis (10 h). Finally, peak fractions of chemotactic factor did not provoke fever upon injection in rabbits, whereas LPS (100 ng/rabbit) was indeed pyrogenic (data not shown).

Discussion

Macrophage-derived factors such as IL-1 and TNF have been found to play an important role in host reactions against infection, cancer, allergens, and autoantigens. Numerous biological in vivo effects, including induction of fever, acute-phase reactants, and neutrophilia, have been attributed to these factors (16). However, in vitro studies have demonstrated that several of these activities are in fact indirect, i.e., mediated by other factors induced by IL-1 or TNF. Thus it is well known that IL-1-induced thymocyte activation is mediated by induction of IL-2. Similarly, as evident from our work, IL-1 has IFN-like (6, 9) and colony-stimulating factor (9, 17) activities which can be explained by induction of the corresponding cytokines. The finding that IL-1 is a potent inducer (10) of IL-6, a growth factor for B cells (IL-6 or HGF) and a stimulating factor for hepatocytes (IL-6 or HSF) (18), suggests that the B cell-stimulating effect of IL-1 and its ability to induce acute-phase proteins are mediated by this molecule. Induction of neutrophilia by IL-1 is not paralleled by in vitro effects on granulocytes (19, 20), again suggesting intervention of a mediator.

One of the in vivo reactions possibly to be attributed to macrophage-derived factors is the elicitation of skin inflammation, an activity first described as skin-reactive factors, present in supernatants of mitogen-stimulated leukocytes (21). While studying the possible role of IL-1 (9) in the causation of such skin reactions, we discovered and purified a 6.5-kD protein distinct from IL-1 that could induce skin reaction at an earlier time than IL-1. Such acute inflammatory lesions have also been shown to be induced by a number of exogenous agents,

including Con A and LPS (22). However, in contrast to those agents as well as the cytokines IL-1, IFN- β , and IL-6, this 6.5-kD factor possessed in vitro chemotactic activity for granulocytes. These in vitro findings confirm the lack of chemotactic activity of IL-1 as described by others (19, 20). Furthermore, we found that our newly isolated chemotactic factor could induce an immediate and dose-dependent granulocytosis in vivo, while, as evident from our previous work (9, 15), IL-1 β induced a more delayed neutrophilia. These differences between IL-1 β and the chemotactic factor with respect to kinetics of both skin reactivity and neutrophilia, together with the absence of an in vitro chemotactic effect of IL-1 suggest that these in vivo effects of IL-1 are mediated by this or related chemotactic factor(s). Direct evidence for such inducer hypothesis was provided by demonstration of chemotactic activity in monocyte cultures treated with IL-1. However, unstimulated adherent cell populations also produced a significant amount of chemotactic activity. Whether this activity is mediated by a spontaneous release of IL-1 from the monocytes is presently being investigated. Similar observations were done on monocytes with respect to the production of IL-6 after treatment with IL-1.

The possibility that the observed biological effects were due to contaminating endotoxin was excluded by several observations: (a) inability of LPS to induce in vitro chemotaxis of granulocytes; (b) absence of significant amounts of LPS (as tested by the Limulus amoebocyte lysate assay) sufficient to induce granulocytosis; and (c) lack of pyrogenicity of purified factor in rabbits.

The chromatographical procedure used to isolate this chemotactic factor was that originally developed for purification of IL-1. Nevertheless, it was observed that as much as 50% of the chemotactic activity present in the crude material was recovered after the final purification step. However, we have recently been able to purify this molecule to homogeneity by a more efficient sequence of purification steps, including adsorption to silicic acid, heparin-Sepharose chromatography, and FPLC (manuscript in preparation).

Evidence for a single protein being responsible for the observed activities was obtained by SDS-PAGE analysis of the FPLC-purified factor, showing a protein band of M_r 6,500 coeluting with the chemotactic activity. NH₂-terminal amino acid sequence analysis identified the protein as being different from all chemotactic factors known so far. However, this stretch of sequence was identical with that derived from the nucleotide sequence of a cDNA clone (3-10C) isolated from mitogen-stimulated human leukocytes (13). In addition, some 50% homology was also observed with the low M_r platelet factors β TG and PF-4 (11, 12) to which some chemotactic activity has been ascribed (23). We could verify that monocytes but not platelets were the producer cells of this novel chemotactic protein. Furthermore, platelet-derived β TG was found to be separable from the chemotactic factor by cation exchange chromatography. Purified β TG failed to exert significant chemotactic activity on granulocytes at concentrations up to 10 μ g/ml. In contrast, pure chemotactic factor showed a much higher specific activity ($\sim 10^5$ U/mg). It can therefore be concluded that the observed in vitro and in vivo effects are reflecting the physiological role of this novel molecule. The 6.5-kD factor also showed homology with an IFN- γ -inducible cytokine (γ IP-10). Although the γ IP-10 gene codes for a predicted protein of 10 kD (14), it has

recently been shown that, due to posttranslational processing, the mature protein also has an M_r of $6-7 \times 10^3$ (24). A similar posttranslational modification could explain the difference in M_r between our chemotactic factor (6.5×10^3) and that of the protein predicted from the identical 3-10c sequence (11×10^3). In addition, biological similarity between our factor and the γ IP-10 protein exists in that both are involved in skin reactivity (25).

It will be of interest to determine whether the chemotactic factor also exerts some of the other biological properties ascribed to the platelet factors β TG and PF-4, such as affinity for heparin (26), immunoregulatory activity (27), histamine release from basophils (28), stimulation of leukocyte elastase (29), inhibition of PGI₂ production in endothelial cells (30), chemotaxis for fibroblasts (31). It will also be important to further clarify the relation between this new neutrophil-chemotactic factor and other agents that have been described to possess similar activities but that are still not fully characterized (19, 32-35).

Summary

A factor able to induce an early local inflammation in rabbit skin was detected in the supernatant of mitogen-stimulated human blood leukocytes. The factor was different from IL-1 which, although present in the supernatants, was chemically separable from the factor and induced a late rather than an early skin response.

Other biological effects of the principal factor were its *in vitro* chemotactic effects on granulocytes and its ability to induce rapid granulocytosis upon intravenous injection in rabbits. When tested under the same conditions, IL-1 β did not act chemotactically and induced granulocytosis at a later time.

The factor was purified to homogeneity and identified by electrophoretic mobility as a protein of M_r 6,500. Amino acid sequence analysis revealed the presence of an uncontaminated NH₂-terminal sequence identical to a segment of the sequence previously predicted (13) from the cDNA clone (3-10C) copied from an mRNA isolated from human leukocytes and coding for a protein of unknown function. The NH₂-terminal sequence of the factor also showed extensive homology to that of the platelet factors β -thromboglobulin (β TG) and platelet factor 4 (PF-4). Studies done to identify the cell source of the factor revealed that it was produced by adherent mononuclear cells but not by platelets, while the opposite was true for β TG.

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