Urate Crystal-Induced Chemotactic Factor

ISOLATION AND PARTIAL CHARACTERIZATION

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ABSTRACT A factor with chemotactic properties for neutrophils and mononuclear cells was extracted from the lysosomal fraction of both human and rabbit neutrophils that had been allowed to phagocytose monosodium urate crystals. The chemotactic factor was found to be a glycoprotein with a mol wt of 8,400 daltons. The factor is heat labile and has chemotactic activity for human as well as rabbit cells. Preincubation of the cells with the urate induced chemotactic factor or with complement activated plasma prevents the cell from migrating chemotactically when challenged with either factor in the chemotactic chamber. The chemotactic factor induces release of lysosomal enzymes from cytochalasin B treated human neutrophils.

INTRODUCTION

A substance with chemotactic properties for polymorphonuclear neutrophils appears in their lysosomal fraction after phagocytosis of microcrystalline monosodium urate crystals. Previous studies indicate that the appearance of factor activity is abolished by preincubation of the cells with inhibitors of protein synthesis (1, 2). The purpose of the present study was to isolate and to characterize the factor.

METHODS

Isolation and molecular weight determination of urate induced chemotactic factor. The factor was extracted from human and from rabbit neutrophils. Human neutrophils were obtained from the peripheral blood by a procedure that utilized Ficoll-Hypaque centrifugation followed by dextran sedimentation (3). The final cell preparation was 90% neutrophils, the contaminating cells being mainly erythrocytes. The rabbit cells were obtained from their peritoneum as described by Cohn and Hirsh (4) and were 95% neutrophils. The chemotactic factor was prepared as follows: approximately 60×10^6 cells were incubated at 37°C for 45 min in 5 ml of Hanks solution with 6 mg of synthetic monosodium urate crystals (1) made pyrogen free by heating for 1 h at 200°C. The cells were then disrupted, and the lysosomal fraction was obtained by differential centrifugation as previously described (2). The lysosomal fraction of the cell was disrupted by ultrasonication for 45 s (MSE Mullar Ultrasonic Disintegrator, model 60 W, London, England) on ice and centrifuged at 20,000 *g* for 20 min in a Beckman model L Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.), and the supernate (lysosomal extract) was saved. The specific activity for β glucuronidase was found to have increased by 6.9-fold in the lysosomal extract and the specific activity of β galactosidase by 8-fold as compared to the activity in the whole cell lysate which was disrupted by sonication in 0.1% Triton $\times$ 100 (Sigma Chemical Co., St. Louis, Mo.). The total yield for both enzymes was greater than 85%. The specific activity of the chemotactic factor was increased by sixfold in the lysosomal fraction as measured by the Boyden Chamber technique. The lysosomal extract was passed over a 63×1.5 cm G-50 Sephadex column (Sigma Chemical Co.), in isotonic saline solution, and the fractions were tested for chemotactic activity. The fractions containing activity were pooled and repassed over the same column. The preparation of the nonchemotactic control followed the same procedure but the cells were killed by freezing before incubation with the crystals. The substances used as molecular weight standards were as follows: bovine parathyroid hormone labeled with ^{125}I, mol wt 9,000 (courtesy of Dr. E. Slatopolsky), porcine insulin, mol wt 6,000 (E. R. Squibb and Sons, New York) and porcine ACTH, mol wt 3,500 (Parke-Davis and Co., Detroit, Mich.). The column fractions were measured for ^{125}I PTH with the aid of a gamma counter (Baird Atomic, Inc., Bedford, Mass., model 707). The column fractions containing the bulk of the insulin and ACTH were determined by optical absorbance. The chemotactically active fraction (3 ml) from several experiments were pooled, dialyzed against deionized water, lyophilized, and run (50 μg) in preparative polyacrylamide gel electrophoresis in glycine buffer (0.38 M glycine and 0.005 M Tris) pH 8.3 at 2 mA per tube (5), for 1 h. The gels measuring 5×75

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mm were stained with Coomassie Blue (6) for protein and for carbohydrate by the method of Zacharius et al. (7). The lowest amount of protein and carbohydrate that we can visually detect using the above methods is 1 μg for protein and 2 μg for carbohydrate. Unstained gels were sliced in equal fractions, and their contents were eluted with distilled water. The eluates were then tested for chemotactic activity. The chemotactically active fraction gave three lines in polyacrylamide gel electrophoresis after staining, but only one was shown to have chemotactic properties (details are given in the Results section). All subsequent studies were performed by utilizing the eluate of gel slices containing the single chemotactic line.

Carbohydrate and protein estimations. The carbohydrate content of the samples was estimated by the phenol-sulfuric acid technique (8) and expressed as percent of dry weight. The samples were tested for protein using Coomassie Blue (6) because the Lowry technique (9) proved ineffective since the factor contains only one tyrosine residue and no tryptophan (unpublished data).

Evaluation of chemotactic activity. Chemotactic activity was evaluated by a modification of the Boyden Chamber technique (2, 10–12). Human and rabbit neutrophils were obtained as described above. Rabbit mononuclear cells were obtained from the peritoneum of rabbits after intraperitoneal injection of sterile mineral oil (13, 14). Approximately 91% of the cells were considered to be mononuclear cells by light microscopic examination. The contaminant cells were mainly erythrocytes. The cells were incubated with sodium chromate ^{51}Cr (Mallinckrodt Inc., St. Louis, Mo.) at a concentration of 5 μCi/million cells. The mixture of ^{51}Cr and cells was incubated in plastic tubes at 37°C for 50 min with constant agitation. The cells were then washed three times and resuspended in Hanks solution at a concentration of 5 million cells/ml. Approximately 3,600 cpm/million cells were obtained by using this technique.

The chemotactic chambers consisted of two 1-ml Lucite compartments (courtesy of Sherwood Medical Industries, Inc., St. Louis, Mo.) separated by two micropore filters of 5 μm pore size (Millipore Corp., Bedford, Mass. lot 367-145). The neutrophils were placed in the top chamber while the chemotactic material or control was placed in the bottom (test) chamber. After 3 h of incubation in an environmental room (Hotpack Corp., Philadelphia, Pa.) in room air at 37°C, the fluid from the chambers was aspirated, and the upper (UF)[1] and lower (LF) filters were separated with fine forceps and counted separately in a gamma counter for 2 min. Results were expressed as the net percent radioactivity: $LF \times 100/UF + LF$ less control (2, 11): the chemotactic index.

The chemotactic assay for mononuclear cells was performed as described for neutrophils except that the incubation period was 4 h. After 4 h the chemotactic index reaches a plateau for mononuclear cells, as measured by the described method.

Chemotactic activity was expressed as the mean and standard deviation of three experiments each consisting of triplicate chambers.

Assay of enzyme activity. β galactosidase levels were measured by a fluorimetric method previously described (2, 15) by using 4-methylumbelliferyl-β-D-galactopyranoside monohydrate (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England) as substrate. β glucuronidase was assayed by using phenolpthalein glucuronide as described by Stahl and Trouster (16). Lactic dehydrogenase (LDH) was measured by using a coenzyme (reduced NAD)—linked assay (Sigma kit 340-LD, Sigma Chemical Co.).

Ability of the chemotactic factor to induce lysosomal enzyme release. Approximately 1×10^7 human peripheral blood neutrophils, in quadruplicate, were layered in a total volume of 2 ml on a millipore filter, 25 mm diameter and 5 μ pore size, on the bottom of a 15-ml plastic test tube in the presence or absence of different concentrations of the chemotactic factor and incubated for 1 h at 37°C. The cells were in complete Hanks solution pH 7.2 with 1 mg/ml glucose (17). At the end of the incubation period, the media was removed and centrifuged in the cold for 10 min at 1,300 *g*, and the supernate assayed was for LDH and β glucuronidase as described. The total activity of the enzymes was measured in separate tubes containing 1×10^7 neutrophils in 2 ml of the Hanks solution after cell lysis with 0.1% Triton × 100 (17). The results are expressed as the percent±SE of the total enzyme activity. In a separate set of experiments, the human neutrophils were incubated in media containing cytochalasin B (Calbiochem, San Diego, Calif.) 5 μg/ml, dissolved in 0.885% dimethyl sulfoxide in saline (Nutritional Biochemical Co., Cleveland, Ohio) for 10 min at 37°C. The chemotactic factor, 50 or 100 μg/ml, was subsequently added, and the experiment was performed as described above. Additional enzyme assays were performed on cell homogenates in the presence of 300 μg/ml of chemotactic factor. The presence of chemotactic factor at this concentration did not affect the activity of either β-glucuronidase or lactic dehydrogenase as measured by the described methods.

Suppression of the chemotactic response. Human neutrophils were incubated at 37°C for 20 min with the purified human urate induced chemotactic factor or complement activated plasma at various concentrations. After washing three times with Hanks solution the cells were labeled with ^{51}Cr and challenged in the chemotactic chamber with urate induced chemotactic factor or complement activated plasma. Complement associated chemotactic factors were generated by the addition of goat anti-human IgG (Hyland Laboratories, Los Angeles, Calif.) to fresh human plasma followed by incubation at 37°C for 1 h with subsequent removal of the precipitate by centrifugation (18).

RESULTS

Isolation and molecular weight determination of the chemotactic factor. A single peak of chemotactic activity was obtained when the lysosomal extract from human neutrophils incubated with Na urate crystals was passed twice through a Sephadex G-50 column. It appeared consistently at 0.11 of the effluent volume. Bovine parathyroid hormone, porcine insulin, and porcine ACTH were passed through the column to obtain reference points for an estimation of the molecular weight of the factor. By this method, the factor was estimated to have a mol wt of 8,400 daltons, (Results not shown). The rabbit lysosomal extract also gave a single peak of chemotactic activity at the same effluent point as the one obtained from human lysosomal extract. The lysosomal extract of neutrophils killed by freezing, before incubation with crystals, was used as control. No chemotactic ac-

[1] *Abbreviations used in this paper:* LDH, lactic dehydrogenase; LF, lower filters; UF, upper filters.

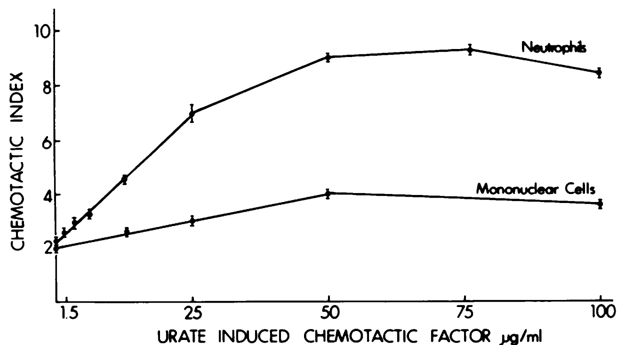

FIGURE 1 Chemotactic activity of urate induced chemotactic factor for neutrophils and mononuclear cells.

tivity was observed when the chromatographic fractions of the control were tested in the Boyden Chamber.

The chemotactically active fraction gave a strong single line in polyacrylamide gel electrophoresis after carbohydrate or protein staining. The line was present at 2.7 cm from the cathode. Two slow migrating lines that stained weakly for protein but not for carbohydrate, were also observed at 1.05 and 1.07 cm from the cathode. Unstained gels were divided into several slices, and their contents were eluted and tested for chemotactic activity. The activity was confined to the gel slice corresponding to the carbohydrate and protein line seen in the stained gels. All subsequent studies were performed by utilizing the eluate of gel slices containing the single chemotactic line.

The purified human chemotactic factor was found to have a carbohydrate content of 3.2% of its dry weight. The yield of the chemotactic factor was about 15–20 µg dry weight/100 million cells in five consecutive experiments.

Attraction of neutrophils and mononuclear cells. The chemotactic factor obtained from human cells was observed to attract human neutrophils in a dose-dependent fashion from 1.5 to 50 µg/ml. At higher concentrations the response reached a plateau (Fig. 1). The chemotactic factor was also seen to attract mononuclear cells but to a much lesser extent than neutrophils (Fig. 1).

The chemotactic factor obtained from human neutrophils attracted rabbit and human cells to a similar degree. Likewise, the factor obtained from rabbit cells was equally effective in attracting human or rabbit cells (Table I).

Suppression of the chemotactic response. Human neutrophils were incubated with the urate induced chemotactic factor or complement activated plasma, and the ability of the cells to respond chemotactically to either factor was evaluated. The chemotactic response of neutrophils preincubated with human urate induced chemotactic factor and then challenged in the chemotactic chamber with the same chemotactic factor was inhibited.

TABLE I
Chemotactic Response of Neutrophils and Mononuclear Cells to Urate Induced Chemotactic Factors from Human or Rabbit Neutrophils

Chemotactic factor*	Chemotactic index‡ Neutrophils Human	Neutrophils Rabbit	Mononuclear cells Rabbit
Human UCF	8.3±0.2	8.5±0.5	3.0±0.4
Rabbit UCF	7.7±1.2	8.7±0.6	3.9±0.6

Abbreviations: UCF, urate-induced chemotactic factor.
* 50 µg per ml (dry weight).
‡ Number represents mean±standard deviation of three experiments each performed in triplicate.

The degree of inhibition was related to the concentration of chemotactic factor used in the preincubation. Inhibition was again seen when complement activated plasma was used as the chemotactic challenge (Table II). When the cells were preincubated with activated plasma and in the chemotactic chamber, challenged with complement activated plasma or urate induced chemotactic factor, an inhibition in the ability of the cells to migrate was again observed (Table II).

Ability of the chemotactic factor to induce lysosomal enzyme release. When human neutrophils were incubated with the chemotactic factor at various concentrations from 50 to 500 µg/ml no increase of β glucuronidase release above control levels was observed. Likewise, the activity of the cytoplasmic enzyme LDH was not significantly different in the control media than in the media of samples incubated with the chemotactic factor (Table III). When the lysosomal enzyme release was measured in the presence of cytochalasin B and the chemotactic factor (100 µg/ml), a significant release of β glucuronidase but not LDH above control levels was observed.

TABLE II
Suppression of Neutrophil Chemotactic Response by Preincubation with Chemotactic Factors

Neutrophil preincubated with	Chemotactic factor challenge	Inhibition of chemotaxis*
		%
Urate CF 50.0, µg/ml	Urate CF 50.0, µg/ml	88.3±7.2
Urate CF 25.0 µg/ml	Urate CF 50.0, µg/ml	67.5±8.1
Urate CF 12.5, µg/ml	Urate CF 50.0, µg/ml	46.0±6.0
Urate CF 50.0, µg/ml	Activated plasma, 50%	70.0±3.2
Activated plasma, 50%	Activated plasma, 50%	87.8±7.0
Activated plasma, 25%	Activated plasma, 50%	47.4±5.5
Activated plasma, 50%	Urate CF 50.0, µg/ml	98.5±2.1

Concentration of Chemotactic Factor (CF) determined by dry weight.
* Results represents mean and SD of three experiments each performed in triplicate.

TABLE III

Release of β Glucuronidase and LDH by Urate Induced Chemotactic Factor

Chemotactic factor*	β Glucuronidase		LDH total
μg/ml	*μmol‡ ±SE*	%	%
0	0.029±0.005	3.4	3.2
50	0.040±0.008	4.6	3.8
75	0.028±0.007	3.3	3.1
100	0.041±0.010	4.8	3.6
150	0.042±0.005	4.8	3.6
500	0.030±0.001	3.6	3.2
0 + cytochalasin B	0.036±0.005	4.5	3.6
50 + cytochalasin B	0.030±0.010	3.8	3.5
100 + cytochalasin B	0.075±0.008	9.8	3.6

Cytochalasin B used at a concentration of 5 μg/ml.
Pair analysis between samples containing cytochalasin B and 100 μg of chemotactic factor vs. samples with cytochalasin B but not chemotactic factor was statistically significant (<0.025).
* Dry weight.
‡ Phenolphthalein 24 h/10^7 neutrophils.

Heat sensitivity. The chemotactic activity of the factor was abolished by heating it at 80°C for 30 min.

DISCUSSION

The interaction of neutrophils with urate crystals leads after phagocytosis of the crystals to the appearance of a chemotactic factor in the lysosomal fraction of the cell (1, 2). Previous studies have suggested that the appearance of factor activity is due to the induction of new protein synthesis (1). The current experiments show that the human urate induced chemotactic factor is a glycoprotein with a mol wt of approximately 8,400 daltons. It has the ability to attract human as well as rabbit neutrophils and, to a lesser degree, rabbit mononuclear cells; likewise, rabbit urate induced chemotactic factor can attract human and rabbit neutrophils and rabbit mononuclear cells to a lesser degree. The chemotactic factor dose-response curve for neutrophils and mononuclear cells was observed to reach a broad plateau with increasing concentration of the chemotactic factor. Similar dose response curves for other chemotactic factors have been described (19–21). Comparison of the chemotactic activity for neutrophils between urate induced chemotactic factor and C5a, as reported by Gallin et al. (22), suggest that the urate induced chemotactic factor is less active on a molar basis. The comparison was performed by calculating the concentration of the chemotactic factors required to give 50% of maximal chemotactic activity. Direct comparative studies between urate induced chemotactic factor and C5a, however, have not been performed.

A statistically significant release from neutrophils of β glucuronidase but not LDH by the chemotactic factor was observed when the cells were pretreated with cytochalasin B and placed upon a Millipore filter. (Millipore Corp.) This effect was not observed in the absence of cytochalasin B by using a maximal concentration of the chemotactic factor 100-fold above the one needed to demonstrate chemotactic activity. It is possible, however, that the concentrations of the factor used were not sufficient to induce lysosomal enzyme release in the absence of cytochalasin B (17). The concentration of the urate induced chemotactic factor that produced a onefold increase of β glucuronidase release above control levels from cytochalasin B treated cells was approximately 10 times greater than the corresponding concentration required to elicit an equivalent degree of chemotaxis. This is about twice the corresponding relationship between the ability of C5a to elicit β glucuronidase release from cytochalasin B treated cells (23) and chemotaxis from human granulocytes (22).

The preincubation of human neutrophils with complement activated plasma or human urate induced chemotactic factor was shown to prevent the human neutrophils from migrating towards a chemotactic gradient when, after washing, the cells were challenged with either of the factors in the Boyden Chamber. The chemotactic activity of complement activated plasma has been shown to be due mainly to C5a and $\overline{C567}$ (17). The inhibition of the PMNs chemotactic response by preincubation of the cells with the same chemotactic factor has been called deactivation by Ward and Becker (18). Cross-deactivation between the chemotactic factors C5a and kallikrein (24) and between different complement derived chemotactic factors (25) has been described. Cross-deactivation of PMNs is consistent with a concept that chemotactic factors share a general recognition site(s) on the surface of the cell which can be saturated with chemotactic factor, thus preventing the recognition of a chemotactic gradient. Alternatively, different recognition sites may exist which, when bound to chemotactic factors, lead to the activation of a common pathway; once fully activated, the cell would be prohibited from responding with directional migration to another chemotactic factor which it is, however, capable of recognizing.

In conclusion, the described cell derived glycoprotein possesses several of the characteristics of other reported chemotactic factors for neutrophils such as chemoattraction of neutrophils and mononuclear cells, cross-deactivation of neutrophils, and the ability to induce lysosomal enzyme release from cytochalasin B treated cells.

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