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Cold Urticaria

RECOGNITION AND CHARACTERIZATION OF A NEUTROPHIL CHEMOTACTIC FACTOR WHICH APPEARS IN SERUM DURING EXPERIMENTAL COLD CHALLENGE

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ABSTRACT Sera were obtained from the venous effluents of cold-challenged arms of patients with idiopathic cold urticaria without plasma or serum cryoproteins; these sera exhibited increased neutrophil chemotactic activity without alterations of the complement system. A two- to fourfold augmentation of the base-line neutrophil chemotactic activity of serum from the immersed extremity began within 1 min, peaked at 2 min, and returned to base-line levels within 15 min, whereas there was no change in the serum chemotactic activity in the control arm. The augmented chemotactic activity in the serum specimens from the challenged arm of each patient appeared in a high molecular-weight region, as assessed by the difference in activity recovered after Sephadex G-200 gel filtration of the paired lesional and control specimens. Sequential purification of this high molecular-weight activity by anion- and cation-exchange chromatography revealed a single peak of activity at both steps. The partially purified material continued to exhibit a high molecular weight, being excluded on Sepharose 4B, and had a neutral isoelectric point. The partially purified material showed a preferential chemotactic activity for neutrophilic polymorphonuclear leukocytes, required a gradient for expression of this function, and exhibited a capacity to deactivate this cell type. This

active principle, termed high molecular-weight neutrophil chemotactic factor, exhibited a time-course of release that could be superimposed upon that of histamine and the low molecular-weight eosinophil chemotactic factor and may represent another mast cell-derived mediator.

INTRODUCTION

Idiopathically acquired, cold-induced urticaria is a disorder in which patients, when exposed to cold, experience an urticarial eruption that may evolve into angioedema and is rarely associated with syncope or wheezing. A substantial number of these patients possess a serum factor capable of transferring cold urticaria to a skin site of a normal recipient as assessed by local cold challenge 24 h later (1). Isolation and immunochemical characterization of the principle involved in passive transfer have shown that it is IgE (2). Participation of the mast cell in the clinical entity is suggested by the involvement of IgE and by the recognition of histamine in whole blood, plasma, or serum (3-7) after the challenge of patients with local cold. Further evidence for involvement of the mast cell is derived from finding a low molecular-weight eosinophil chemotactic factor, comparable in size to the eosinophil chemotactic factor of anaphylaxis, in the venous effluent from an arm of the affected patient after cold immersion (7). During the characterization of the eosinophil chemotactic activity in the serum, an additional chemotactic principle with specificity for neutrophils is noted. This neutrophil chemotactic factor has a high molecular weight and a neutral isoelectric point.

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METHODS

Phenol red (Sigma Chemical Co., St. Louis, Mo.), Hanks' balanced salt solution (HBSS)¹ and Medium 199 (Microbiological Associates, Bethesda, Md.), Sephadex G-200, Sepharose 4B, Ficoll, quaternary amino ethyl (QAE)-Sephadex, blue dextran, and sulfopropyl (SP)-Sephadex (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), ovalbumin (Miles Laboratories, Kankakee, Ill.), Hypaque (diatrizoate, Winthrop Laboratories Inc., Miles Research Products, New York), and Ampholine carrier ampholytes (LKB Instruments, Inc., Rockville, Md.) were obtained from the manufacturers.

Seven patients with idiopathically acquired, cold-induced urticaria participated in a protocol, after giving informed consent, in which one arm was immersed in ice water up to the antecubital fossa for 3 min, while the control arm remained at room temperature. Indwelling intravenous needles were placed in both arms at the level of the antecubital fossae, and blood was obtained from both just before and serially at 0.5, 2, 5, 10, 15, 30, and 60 min after immersion of one arm. Blood was collected into plastic tubes and allowed to clot for 60 min at 4°C; serum was separated by centrifugation at 800 g for 15 min in the cold, divided into aliquots and stored at -70°C for subsequent analyses. Challenge of two normal laboratory volunteers was performed in an identical fashion.

Whole serum complement activity determinations (CH_{50}) (8), effective molecule titrations for C1 (9), C4 (10), C2 (11), C3 (12), and C9 (13), and immunochemical measurements (14) of Clq (15), C4 (16), C3 (15), C5 (17), B (18), properdin (19), and the Cl inhibitor (20) were conducted as described. For assessment of cryoproteins, blood was drawn into plastic syringes at 37°C, distributed into glass tubes with and without disodium EDTA, clotted for 1 h at 37°C, and sedimented at the same temperature. The cryocrits of the serum and the plasma were determined in calibrated tubes that were maintained at 4°C for 24 h and then centrifuged at 250 g for 10 min at 4°C (21).

Chemotaxis. In order to obtain leukocytes for chemotaxis, 6-ml portions of venous blood from normal donors and from a patient with hypereosinophilia of 35% were incubated with 1 ml dextran and 0.75 ml citrate anticoagulant, pH 5.2, for 45 min at 37°C to allow sedimentation of the erythrocytes. The leukocyte-rich supernatant fraction was aspirated and suspended in 0.84% NH_4Cl to lyse contaminating erythrocytes. The leukocytes then were sedimented at 400 g for 10 min at room temperature, washed twice in HBSS containing 0.4% ovalbumin and 0.005 M Tris, pH 7.4, and resuspended at a concentration of $2.4-2.8 \times 10^6/ml$ in Medium 199 made 0.4% with ovalbumin and 0.005 M Tris, pH 7.4. In some experiments the cells were separated into polymorphonuclear leukocyte- and mononuclear leukocyte-rich fractions by centrifugation upon Hypaque-Ficoll cushions (22).

Chemotaxis was assessed by a modification (23) of the Boyden chamber assay (24) with $2.4-2.8 \times 10^6$ cells separated from the chemotactic stimulus or buffer by micropore filters (Millipore Corp., Bedford, Mass.) fixed to polystyrene disposable chemotactic chambers (Adaps Corp., Dedham, Mass.). 3- μm pore size micropore filters were employed for chemotactic studies of neutrophilic and eosinophilic polymorphonuclear leukocytes, and 5- μm pore size filters were used for

mononuclear leukocytes. The lower compartment was filled with 1 ml of buffer alone or of material being assessed for chemotactic activity. Chemotaxis was carried out for 2.5-3 h at 37°C in moist chambers; the cell response was determined by counting the cells at a fixed distance into the stained filter. This distance was selected to achieve background counts in the absence of a chemotactic stimulus of zero to six cells for mixed leukocytes or for neutrophilic and eosinophilic leukocytes and zero to four cells for mononuclear leukocytes. Cells then were counted without knowledge of the protocol in 5 high power fields (hpf) from each of duplicate filters, and these values were expressed as the mean net cells per high power field per milliliters of chemotactic factor by correction for spontaneous migration in additional controls. The mean values of the duplicate filters differed by <10%.

Deactivation refers to the failure of leukocytes to migrate along a concentration gradient of a chemotactic stimulus as a result of a previous interaction with an active chemotactic factor (25). The deactivation reaction has the same specificity as chemotactic activation but differs in that the concentrations of factors interacting with target cells are defined because a gradient is not involved. The degree of deactivation was determined by comparing the migration of cells pretreated with a chemotactic factor with the migration of untreated cells in response to identical chemotactic gradients. The net response of treated and untreated cells was quantitated as described for routine chemotactic experiments, and the percent deactivation was calculated by $(1 - [\text{net response of deactivated cells}]/[\text{net response of buffer treated cells}]) \times 100$.

Isoelectric focusing. Isoelectric focusing was performed both in a sucrose density gradient column and in a thin layer of Sephadex G-75 gel. A 15-ml linear 10-40% sucrose gradient containing 2% vol/vol ampholytes, pH 3-10, was prepared in a glass column (Metaloglass, Boston, Mass.) at room temperature with a gradient maker (Buchler Instruments Div., Seale Analytic Inc., Ft. Lee, N. J.). 1 ml of sample was mixed with the 40% sucrose in the well of the gradient maker after 1 ml of the gradient had been generated and was subsequently dispersed throughout the entire gradient. Isoelectric focusing was performed at a constant 800 V at 10°C until the current dropped to 2 mA. The gradient was decanted in 0.5-ml portions, and the pH of each portion was measured. The fractions were then dialyzed overnight at 4°C against 1 liter of HBSS and assessed for their chemotactic activity.

A 20 × 20-cm thin-layer plate was filled with a suspension of 200 ml Sephadex G-75 in distilled water containing 1.25% ampholytes, pH 3-10, and dried overnight to obtain the consistency of a gel. After refocusing at 400 V for 6 h in a Brinkmann/Desaga double-chamber apparatus (Brinkmann Instruments, Inc., Westbury, N. Y.), 1 ml of sample was applied to the center of the gel; and the gel was subjected to 500 V for 6 h at 10°C. The gel was then sectioned into 20 equal fractions; the pH of each fraction was measured; and the fractions were diluted in 10 ml of HBSS and centrifuged at 1,000 g for 10 min at room temperature. The supernatant fractions were decanted, and the pellets were resuspended in the same buffer and sedimented twice more. The three supernatant fractions from each of the 20 gel slices were combined, concentrated, dialyzed against 400 vol of HBSS for 48 h at 4°C, and assessed for chemotactic activity.

RESULTS

Appearance of neutrophil chemotactic activity in serum of patients with cold urticaria upon cold

¹Abbreviations used in this paper: HBSS, Hanks' balanced salt solution; hpf, high power field(s); QAE-Sephadex, quaternary amino ethyl-Sephadex; SP-Sephadex, sulfopropyl-Sephadex.

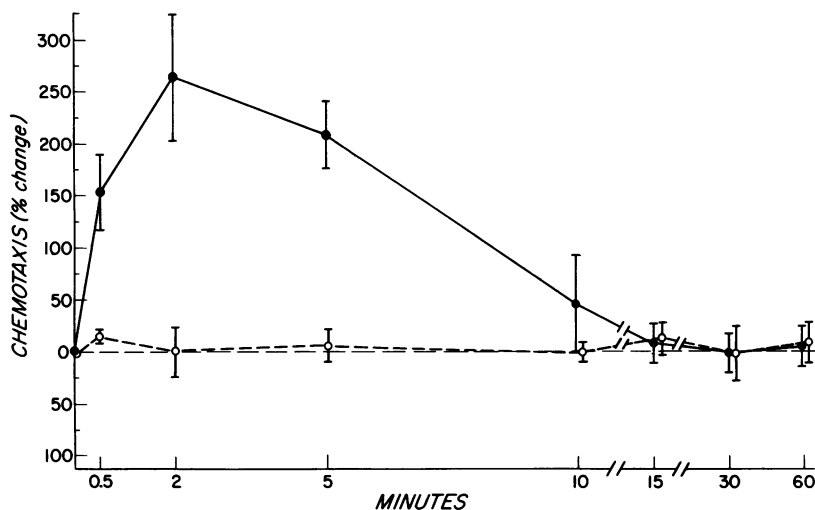


FIGURE 1 Time-course of appearance of neutrophil chemotactic activity obtained from venous effluent sera from seven patients after experimental cold challenge. Each point represents the mean (± 1 SD) net percent change in the neutrophil chemotactic activity in lesional (●) and control arms (○), as compared to time zero.

challenge. The seven patients with cold-induced urticaria noted pain that lasted 60–90 s followed by warmth and pruritus in the immersed extremity. Erythema was noted at the time that the immersed extremity was withdrawn from the ice water, and urticaria and angioedema developed over the next 4–6 min. Urticarial lesions were apparent on the arm at and just below the level of immersion, while the remainder of the extremity was diffusely enlarged with nonpitting edema. Urticarial streaks with erythema overlying veins developed above the point of immersion. No lesions appeared on the control arm. Normal subjects experienced only pain followed by numbness during immersion and did not develop urticaria or angioedema.

Paired serum specimens obtained from the cold-challenged and control arms of each of the seven patients showed comparable base-line levels of neutrophil chemotactic activity at time zero. Increased chemotactic activity appeared in the venous effluent of the cold-challenged extremity within 30 s, reached a peak at 2 min, and returned to base-line levels within approximately 15 min (Fig. 1). The base-line chemotactic activity at time zero assayed with different target cell pools for each of the seven patients ranged from 52 to 170 cells/hpf per ml, and the net chemotactic activity in the 2-min samples ranged from 125 to 700 cells/hpf per ml. Normal individuals subjected to cold immersion did not develop augmented chemotactic activity in the venous effluent of the challenged extremity at any time.

Paired 1.5-ml control and lesional serum samples from four of the seven patients (A,C,D,Z) were studied further by separate application to a 1.5×90 -cm column of Sephadex G-200 equilibrated in HBSS and run at

4°C with a flow rate of 10 ml/h. 2-ml fractions were collected, and 100- μ l aliquots of alternate fractions were assessed for chemotactic activity. Sera from the control arms showed three areas of chemotactic activity at 35, 45, and 75% bed volume, as illustrated for patient C (Fig. 2). The sera obtained from the challenged arms of four patients (A,C,D,Z) differed in that the peak of chemotactic activity that filtered at the void volume was greatly augmented, as represented by patient C (Fig. 2), and ranged from 75 to 160 cells/hpf per ml for peak tubes, as compared to base-line values from 26 to 42 cells/hpf per ml. Patient Z also

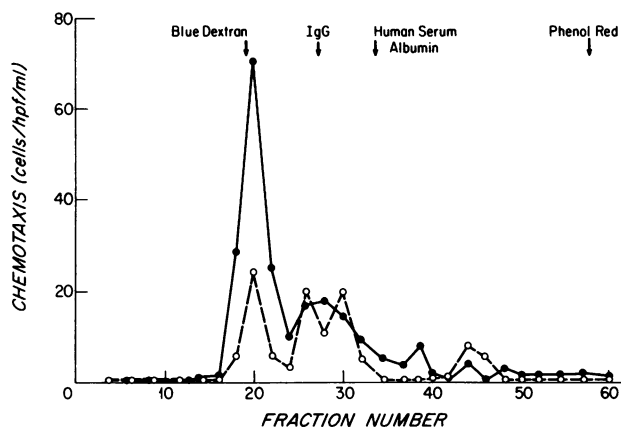


FIGURE 2 Neutrophil chemotactic activity after Sephadex G-200 chromatography of serum from cold-challenged (●) and control (○) arms of patient C. Neutrophil chemotactic activity is depicted as net leukocytes per high power field per milliliter in each column fraction.

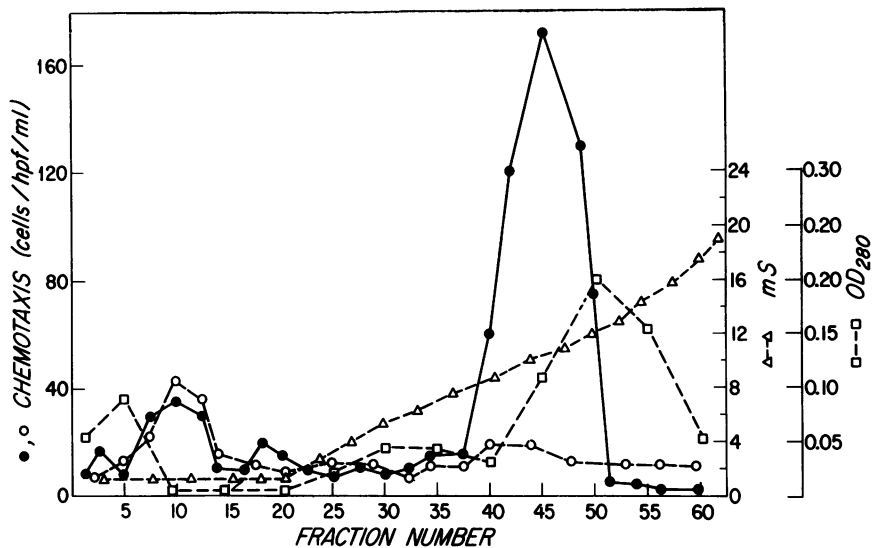


FIGURE 3 QAE-Sephadex chromatography of high molecular-weight neutrophil chemotactic activity obtained from exclusion volume of Sephadex G-200 gel filtration of serum from cold-challenged (●) and control (○) arms of patient C. Protein estimated by optical density at 280 nm (□) and salt gradient in mS (△) are presented for the lesional arm only but were not significantly different in the control specimens.

demonstrated augmented chemotactic activity in the region of 45% bed volume; but inasmuch as this difference was not noted in the other three patients, only the augmented chemotactic activity in the void volume was further characterized.

The complement system was assessed in terms of C1, C4, C2, C3, and C9 functional activity and Clq, C4, C3, C5, B, properdin, and C1 inhibitor protein concentrations. No differences were found between the normal and challenged extremities nor were there alterations from the base-line values.

Partial purification and physical characterization of the high molecular-weight neutrophil chemotactic factor. The high molecular-weight peak of chemotactic activity found after Sephadex G-200 chromatography of serum from the lesional arm was further isolated and characterized in patients A, C, and Z. The fractions in the void volume of Sephadex G-200 chromatography of the 2-min specimens from both the lesional and control arms were separately pooled and dialyzed at 4°C overnight against one change of 200 vol of 0.0035 M phosphate buffer, pH 7.8. The dialyzed material was concentrated by ultrafiltration to 10 ml (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and applied to a 0.9 × 30-cm column of QAE-Sephadex equilibrated in the dialysis buffer. The column was washed with 10 bed volumes of the same buffer and eluted at 4°C with a linear salt gradient of 400 ml to 0.5 M NaCl. 4-ml fractions were obtained, and 250-μl aliquots of alternate fractions were assessed for chemotactic activity. An increased peak of chemotactic activity in sera from the lesional, as compared

to the control arm, eluted between 6 and 12 mS in all three patients, as illustrated for patient C (Fig. 3). Because of minimal activity, fractions from the control extremities were not further purified.

Fractions eluting between 6 and 12 mS from the lesional arms of all three patients were separately pooled, concentrated to 4 ml, and dialyzed against 200 vol of 0.01 M acetate buffer, pH 6.0, overnight at 4°C with one change of dialysis buffer. The dialyzed material was applied to an SP-Sephadex column of 20-ml bed volume, which was equilibrated and then washed with 5 bed volumes of the same buffer. Elution was carried out at 4°C with a 300-ml linear salt gradient to 0.4 M NaCl; 4-ml fractions were collected and 250-μl aliquots were assessed for chemotactic activity. A single peak of chemotactic activity was found in the effluent in all patients, as illustrated for patient C (Fig. 4).

The effluent fractions containing chemotactic activity designated as partially purified high molecular-weight neutrophil chemotactic factor were pooled, concentrated to 6.0 ml, and dialyzed overnight at 4°C against 1 liter of HBSS. 1 ml of material from patient C was dialyzed overnight at 4°C against distilled water and subjected to isoelectric focusing in sucrose, and 1 ml of material from patient Z was similarly dialyzed against distilled water and was focused in thin-layer Sephadex G-75 plates. Neutrophil chemotactic activity appeared in a single peak that focused between pH 6.7 and 7.4 in sucrose (Fig. 5a) and between pH 6.8 and 7.4 in Sephadex G-75 plates (Fig. 5b). Thus, in both patients when wide-range ampholytes were used, the iso-

electric point of the high molecular-weight neutrophil chemotactic factor was in a neutral range. Further, in no instance was sufficient heterogeneity observed to suggest that the partially purified material contained more than one major active principle.

The size of the high molecular-weight neutrophil chemotactic factor obtained at the isoelectric focusing step of serum from patient Z and the effluent from SP-Sephadex chromatography from patient C was determined on Sepharose 4B. 1 ml of material obtained after a sixfold concentration of the peak fraction from isoelectric focusing, and 1 ml of concentrated effluent from SP-Sephadex were each applied to a 1.5×90 -cm column of Sepharose 4B equilibrated in HBSS and calibrated with dextran blue and phenol red. The columns were run at flow rates of 8 ml/h, 2.5-ml fractions were collected, and 200- μ l aliquots of consecutive fractions were assessed for neutrophil chemotactic activity. The chemotactic activity was excluded, indicating a molecular weight in excess of 750,000, as illustrated for patient C (Fig. 6).

Functional characterization of partially purified high molecular-weight neutrophil chemotactic activity. Although the chemotactic activity that appeared in serum as a result of cold challenge was assessed throughout with the mixed leukocytes of normal donors, the predominant responding cell type was the neutrophil. To define the range of chemotactic activity of the factor purified through SP-Sephadex from patient C and isoelectric focusing in patient Z, the response of neutrophilic, eosinophilic, and mononuclear leukocytes was compared. A dose-response relationship was obtained between the concentration of chemotactic factor and the number of responding neutrophils with a plateau as illustrated with material obtained from patient C (Fig. 7). The response of mononuclear leukocytes was observed only at the highest dose. There was no neutrophil migra-

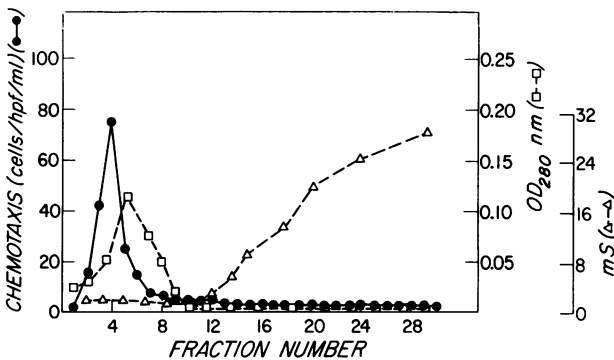


FIGURE 4 SP-Sephadex chromatography of neutrophil chemotactic activity eluting between 6 and 12 mS (●) from QAE-Sephadex in patient C. The protein estimated by optical density at 280 nm is depicted by (□) and salt gradient by (Δ).

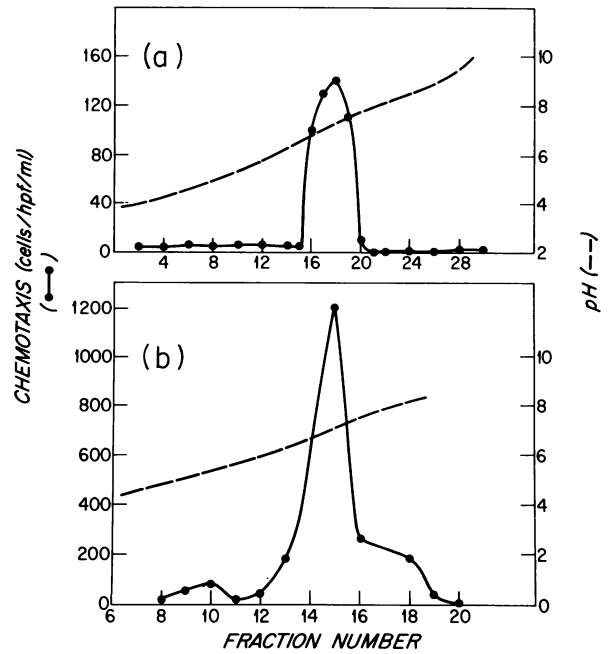


FIGURE 5 (a) Isoelectric focusing in sucrose of partially purified high molecular-weight neutrophil chemotactic activity obtained from patient C. (b) Isoelectric focusing in thin-layer Sephadex G-75 gel of high molecular-weight neutrophil chemotactic activity obtained from patient Z.

tion when the gradients were eliminated (Fig. 7). Thus, the purified high molecular-weight principle is a chemotactic factor with a preferential specificity for neutrophilic over mononuclear leukocytes.

The material obtained from patient Z and further purified by isoelectric focusing was used to compare the response of eosinophilic and neutrophilic leukocytes from a single donor whose peripheral blood contained 35% eosinophils and 50% neutrophils.

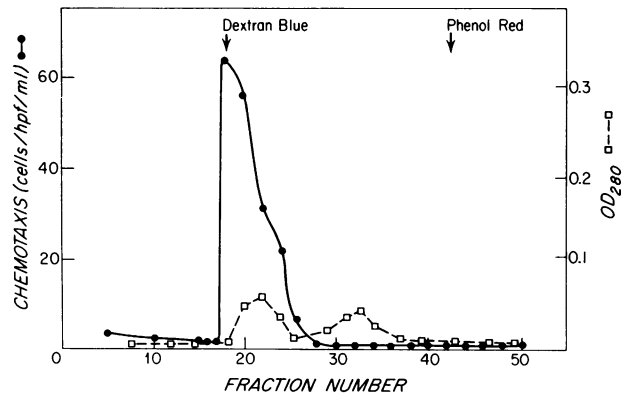


FIGURE 6 Sepharose 4B chromatography of purified neutrophil chemotactic activity (●) in the effluent from SP-Sephadex chromatography in patient C. Protein is estimated as optical density at 280 nm (□).

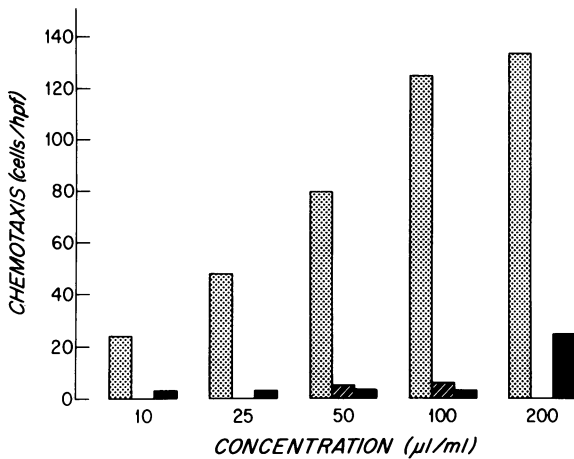


FIGURE 7 Chemotactic responses of neutrophils and mononuclear leukocytes to the purified chemotactic factor from patient C. Neutrophil (stippled bars) chemotaxis was assessed with a cell pool purified to contain more than 95% neutrophils, while the mononuclear leukocyte response (closed bars) was assessed with a cell pool purified to contain 95% mononuclear leukocytes. The effect of eliminating the gradient for neutrophils by placement of equal amounts of purified chemotactic factor into both compartments of the chemotactic chamber is depicted by hatched bars.

Each cell type responded in a dose-response fashion. The maximum net neutrophil response exceeded that of the eosinophils by 2½-fold and occurred at one-quarter the concentration of chemotactic factors required for the maximum eosinophil response.

The time-dependent ability of the factor from patient C (purified through SP-Sephadex chromatography) to deactivate neutrophils was examined at two doses. Isolated neutrophils were incubated with buffer alone or with a subchemotactic (5 µl/ml) or minimally chemotactic (25 µl/ml) amount of purified material from patient C at room temperature for intervals up to 30 min. The cells were sedimented at 500 g at room temperature, washed twice in HBSS, and resuspended at 2.6×10^6 cells/ml for assessment of their chemotactic response to 100 µl of the same material at 37°C. The purified high molecular-weight chemotactic activity deactivated the neutrophils in a dose- and time-dependent manner (Fig. 8).

DISCUSSION

A factor chemotactic for neutrophilic polymorphonuclear leukocytes increased in amount in the venous effluent of the cold-immersed extremity as compared with the contralateral control arm in each of seven patients with idiopathically acquired cold-induced urticaria. The neutrophil chemotactic activity in the venous effluent of the challenged extremity was uniformly noted at 30 s, reached a peak at 2 min, and was dissipated after 15 min (Fig. 1). At time zero there was

no difference in base-line neutrophil chemotactic activity between the challenged and control arms, and the chemotactic activity remained at base-line levels in the unchallenged extremity.

Sephadex G-200 gel filtration of the 2-min serum specimens from the lesional and control arms of four patients revealed the major difference between the paired specimens to be consistently the neutrophil chemotactic activity that filtered in the void volume (Fig. 2). This activity was further purified by QAE-Sephadex chromatography (Fig. 3); in each patient the activity from the lesional arm eluted between 6 and 12 mS, whereas activity from the control arm was too minimal to define. The high molecular-weight neutrophil chemotactic activity was further purified on SP-Sephadex chromatography, where it appeared in the effluent (Fig. 4), and by isoelectric focusing (Fig. 5a, b). Although the starting sera, both lesional and control, contained several peaks of chemotactic activity as assessed by Sephadex G-200 gel filtration as noted previously (26), the neutrophil chemotactic activity, which appeared in serum from the lesional arm and was selected because of its exclusion by gel filtration on Sephadex G-200, presented as a single peak on anion- and cation-exchange chromatography and on isoelectric focusing.

The purified high molecular-weight neutrophil chemotactic factor isolated from the lesional arms of two patients exhibited a neutral isoelectric point when focused in two different support media (Fig. 5). The neutral isoelectric point was not a loading artifact, since in both techniques the material moved at least 5 cm from the loading point. Material purified through the SP-Sephadex step or the isoelectric focusing stage was excluded on Sepharose 4B, indicating a molecular weight in excess of 750,000 (Fig. 6).

The responding cell from the mixed leukocyte source used during isolation of the high molecular-weight

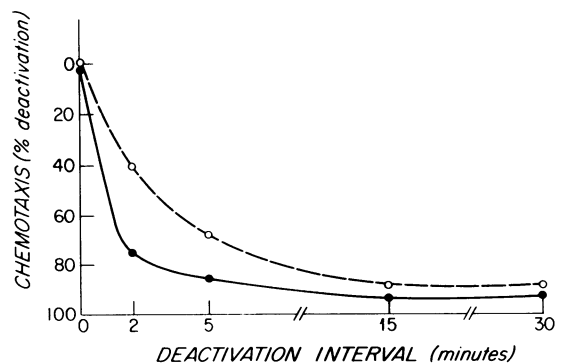


FIGURE 8 Time-dependent deactivation of neutrophils by 5 (○) or 25 (●) µl of purified chemotactic factor from patient C as assessed by their subsequent chemotactic response to 100 µl of the same stimulus.

factor was invariably the neutrophil. This point was more clearly demonstrated by the ability of the purified high molecular-weight activity to attract purified neutrophils in a dose-response fashion at concentrations that had little or no effect on mononuclear leukocytes (Fig. 7). The purified high molecular-weight neutrophil chemotactic factor also demonstrated a preference for neutrophils over eosinophils in a dose-response experiment. The neutrophil response was abrogated if the concentration gradient was abolished by placing identical concentrations of the factor on the cell and stimulus sides (Fig. 7), thus indicating that the activating principle was a chemotactic factor. Chemotactic factors characteristically deactivate their specific target cell in terms of a subsequent chemotactic response to the same or a different chemotactic stimulus (25). Further, studies with the eosinophil chemotactic factor of anaphylaxis have revealed that the concentrations required for deactivation are considerably less than those needed to establish an effective gradient for chemotactic migration (27, 28). As illustrated in Fig. 8, the high molecular-weight neutrophil chemotactic factor deactivated neutrophils in a time- and dose-related fashion. The concentrations effective in deactivation were subthreshold to minimal when compared to the chemotactic stimulus utilized to demonstrate that the cells were deactivated.

There is no evidence that the high molecular-weight neutrophil chemotactic factor is derived from the activation of the complement system as complement protein concentrations and their specific functional activities were not altered from base-line levels in the lesional or the control sera. The time-course of the appearance of serum chemotactic activity for neutrophils was indistinguishable from that of histamine and of material similar to the eosinophil chemotactic factor of anaphylaxis reported in an earlier study (7), implying, but not establishing, a mast cell origin for this mediator. Chemotactic activity preferential for neutrophils and greater than 30,000 molecular weight has been noted in extracts of human leukemic basophils (29) and lung fragments (30), but this activity has not been further characterized. The appearance of a factor that augmented neutrophil migration in the serum of sensitized asthmatic patients after inhalation challenge with specific antigen has been reported (31), but the active principle has not been characterized physicochemically or functionally. Neutrophils have been observed, along with other cell types, in experimental cutaneous lesions in humans in which the mast cell has been implicated. Thus, in a unique patient with cold- and trauma-induced cutaneous necrotizing venulitis and hypocomplementemia but without cryoglobulins, mast cell degranulation has been observed to precede the ingress of neutrophils into the lesional site (32). Moreover, the delayed cutaneous in-

flammatory reaction, mediated by IgE-dependent mechanisms, is characterized not only by mast cell degranulation but also by infiltration of diverse cell types including neutrophils (33, 34). These observations suggest that the mast cell may be involved in neutrophil chemotaxis. The recognition of high molecular-weight neutrophil chemotactic factor in cold urticaria, an IgE-mast cell-dependent disease, provides a mechanism by which the mast cell may affect the responsiveness of neutrophils and thereby modulate neutrophil-dependent inflammatory events.

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