

Further characterization of histamine releasing chemokines present in fractionated supernatants derived from human mononuclear cells

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Summary

Background Histamine releasing factors (HRF) are members of the β chemokine family of cytokines and have been characterized using recombinant proteins. Mononuclear cell and/or platelet supernatants have been shown to contain HRF and the initial void peak obtained using Mono Q anion exchange chromatography possesses such activity, as do two later peaks eluted from the column.

Objective We wished to further characterize the activity present in the void peak and determine which of the chemokines present are responsible for the activity measured.

Methods We fractionated the void peak obtained from Mono Q chromatography on Mono S. The elution profile of individual chemokines was determined and the fractions were assayed for histamine releasing capability. We used monospecific antisera to inhibit the activity and quantitate the contribution of each protein.

Results The fractions contained MCP-1/MCAF, CTAPIII/NAP-2, IL8, and a small quantity of RANTES. About 90–95% of the total histamine containing capability was attributable to MCP-1/MCAF. There was a small contribution by CTAPIII/NAP-2, and RANTES, and no activity associated with IL8.

Conclusion MCP-1/MCAF is the critical HRF present in the initial void peak obtained by anion exchange chromatography of supernatants derived from human mononuclear cells and platelets. The α chemokine CTAPIII/NAP-2 has relatively weak activity and IL8 has none although they are prominent in this fraction and overlap with MCP-1/MCAF. RANTES makes a minor contribution but most of it is eluted in a later peak.

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Introduction

We have previously shown that human histamine releasing factors (HRF) can be fractionated by anion exchange chromatography to yield three separate fractions which activate basophils to release histamine [1]. Soon thereafter we identified CTAP III/NAP-2 as one constituent

possessing HRF activity [2], and localized it within the initial peak (void peak) which does not adhere either to QMA-Accell or a Mono Q column. These data also suggested that HRF may be related to members of the chemokine group of cytokine-like molecules and subsequently MCAF/MCP-1 [3,4], RANTES [5], and MIP-1 α and β [6,7] were reported to possess HRF activity. MCAF/MCP-1 was shown to be the most potent [7]. In this manuscript we have further fractionated HRF within the initial void peak by Mono S cation exchange chromatography and have further separated the various forms of HRF contained therein. We then employed monospecific inhibitory

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monoclonal and polyclonal antibodies to demonstrate the functional contributions of MCP-1/MCAF, CTAP III/NAP-2, IL-8, and RANTES to the activity seen and have accounted for virtually all of the histamine releasing capability of this peak.

Materials and methods

Materials

Chemicals used in the production of HRF and the histamine release assay were purchased as previously described [7]. Recombinant human MCAF/MCP-1 (rh-MCAF/MCP-1) (maximal chemotactic activity on human monocytes at 20 ng/mL) and rh-endothelial IL-8 (maximal chemotactic activity on human neutrophils at 5 ng/mL) were purchased from Pepro Tech Inc., Rocky Hill, NJ, USA; goat anti-IL-8 neutralizing antibody and IL-8 enzyme linked immunosorbent assay (ELISA) were from R&D System, Minneapolis, MN, USA; purified goat anti-mouse and rabbit anti-goat IgG conjugated to alkaline phosphatase were purchased from Jackson ImmunoResearch Lab., West Grove, PA, USA. Spectrapor dialysing tubing was from Spectrum Medical Industries, Los Angeles, CA, USA; tissue culture flask from Corning Glass Works, Corning, NY, USA. Percoll, Mono Q FPLC and Mono S FPLC columns and protein G-Sepharose HiTrap columns were purchased from Pharmacia, Uppsala, Sweden; microtitre plates (immulon-2 and immunlon-4) were purchased from Dynatech Laboratories Inc., Alexandria, VA, USA; tressyl activated agarose AffinicaTM gel was from Schleicher and Schuell Inc., Keene, NH, USA; monoclonal antibody (MoAB) #18-1 to CTAP III/NAP-2 was prepared as described [2].

Preparation of HRF containing supernatants

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from normal blood bank donors. Leukopheresis packs of approximately 200 mL were diluted to 360 mL in Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium containing 4 mM ethylenediamine tetra-acetic acid (EDTA) (PBS-EDTA). Thirty millilitres of cellular suspension were layered over 15 mL Ficoll-PAQUE in 50 mL polypropylene tubes. The suspension was centrifuged for 35 min. The interface was collected, washed with PBS-EDTA, and centrifuged at $400 \times g$ for 10 min. The cell pellet was collected and washed three times with PBS. The cells were resuspended at a concentration of 6×10^6 cells/mL in RPMI 1640 with L-glutamine, 0.03% radioimmunoassay (RIA) grade bovine serum albumin (BSA), penicillin 100 µg/mL, streptomycin 100 µg/mL and streptokinase/streptodornase (SK/SD) at 13 units/ 10^6

cells. The major contaminant and the source of CTAPIII/NAP-2 is platelets as noted previously. The cells were then cultured for 24 h at 37°C with 5% CO₂ in 75 cm² tissue culture flasks. At the end of the culture period the conditioned media were pooled, centrifuged at $400 \times g$ for 15 min and clarified at $38\,000 \times g$ for 20 min. PMSF and EDTA were added to a final concentration of 0.5 mM and 10 mM respectively, the supernatant was sterilized by filtration through 0.22 µm pore sized membranes, and concentrated 50-fold using YM-3 membranes. Concentrated conditioned media were dialysed (molecular weight cut-off, 3500 kDa) against PBS, filtered through 0.22 µm filters and stored at -85°C. Cell viability at the end of the culture was >95% by trypan blue exclusion.

Fractionation of HRF

Crude HRF was further concentrated, 150 mg of protein was dialysed against 10 mM Tris-HCl, pH 8.0, and applied in a volume of 7.0 mL to a 1.0×10.0 cm Mono Q (anion exchange) FPLC column equilibrated with the dialysis buffer. The column was washed at a flow rate of 2.0 mL/min with the equilibration buffer until the $A_{280\text{ nm}}$ of the wash fractions returned to baseline. The protein peak from the wash fraction was collected, lyophilized, reconstituted in distilled H₂O and dialysed into 10 mM sodium acetate buffer pH 6.0. This material was stored in -85°C and used as a source of HRF void peak. Protein concentration were determined by the Coomassie dye binding method of Bradford [8].

Purification of CTAP III/NAP-2

CTAP-III/NAP-2 were purified according to the procedure described by Reddigari *et al.* [9]. Briefly, platelets were isolated from normal human donors, suspended in PBS containing 1 mM EDTA, 5 mM glucose and 0.1 mM PMSF, and stimulated with 20 µM calcium ionophore A-23187, at 37°C for 30 min according to Coller *et al.* [10]. MoAb #18-1 to CTAP III was coupled to tressyl activated agarose (10 mg IgG/mL of AffinicaTM gel, Schleicher and Schuell Inc.) in 0.2 M NaHCO₃, 0.5 M NaCl pH 8.5 according to the manufacturer's recommendations. The gel was equilibrated with 0.01 M sodium phosphate buffer, pH 7.5 containing 0.5 M NaCl, 0.1 mM PMSF, 0.1 mM EDTA and 0.02% azide (equilibration buffer). Platelet releasate was applied to the column and washed with the equilibration buffer until the $A_{280\text{ nm}}$ of the wash fractions returned to the baseline. 0.5 mL fractions were then eluted with 3.0 M potassium thiocyanate and peak protein fractions were pooled, dialysed against PBS (pH 7.4) containing 0.1 mM PMSF, and stored at -70°C. Purity of CTAP III was greater than 90% as determined by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the remainder was accounted for by NAP-2.

Histamine release from basophils

Leucocytes obtained from atopic subjects were prepared by dextran sedimentation (0.6% dextran, 0.6% glucose, 0.02 M EDTA), the basophil containing layer was washed twice with HEPES-buffered saline containing 0.3% HSA (HBS-HSA), and the cells were resuspended in HBS-HSA containing 2 mM CaCl_2 and 2 mM MgCl_2 . The cell count was 1×10^6 cells/mL. Fifty to 100 μL of sample was incubated with 100 μL of leucocyte suspension (prewarmed to 37°C) for 45 min at 37°C. After incubation the supernatants were separated by centrifugation at $700 \times g$ for 5 min at 4°C and histamine release determined. Two replicate aliquots of cells were boiled to determine total basophil histamine content. All histamine release experiments were done in duplicate and the results expressed as a percentage of total histamine content. Spontaneous histamine release from the cells was less than 5% and was subtracted for the induced histamine release values.

Histamine assay

The radioenzyme assay for histamine was performed in duplicate for each sample by using a modification of the method of Beaven *et al.* [11]. Supernatants from the basophil release experiments were added to 15 mL polypropylene screw-cap tubes at 50 μL /tube with 300 μL 0.025 M PIPES buffer containing 1 mM EDTA. Then 100 μL Piperazine-N, N'-bis[2-ethanesulfonic acid] buffer containing ^{14}C -S-adenosyl methionine (100 000 cpm), [^3H]histamine (100 000 cpm) as internal standard, and 0.5 μg histamine N-methyltransferase were added. Histamine N-methyltransferase was purified from rat kidney according to the method described by Bowsher *et al.* [12] and pretested for optimal concentration in our assay. The tubes were mixed, centrifuged at $700 \times g$ for 3 min, and incubated for 90 min at 37°C.

Two-tenths millilitres 10 N NaOH plus 4 mL chloroform were added to each tube and the mixture was vortexed vigorously for 5 min. The aqueous phase was discarded after 5 min and centrifugation at $175 \times g$. A second extraction was performed by using 1.0 mL of 3.3 N NaOH per tube. The chloroform extracts were transferred to scintillation vials, and allowed to evaporate to dryness. Ten millilitres of Ecolume were introduced into the vials and the radioactivity measured on an LKB-Wallac Rac Beta liquid scintillation counter.

Preparation of monoclonal anti-MCAF antibodies

BALB/c mice were immunized with purified human

recombinant MCAF/MCP-1. The presence of MCAF/MCP-1 specific antibody in serum was determined by indirect ELISA and Western blot. The lymphocytes were fused with P3X63-Ag 8.653 mouse myeloma cell line using a standard fusion protocol. The initial clones and subsequent subclones selection was done on the basis of reaction with human recombinant MCAF/MCP-1 by ELISA and Western blotting. The subclone that yielded the strongest signal termed (#40-2) was grown in serum-free RPMI 1640 medium. Cells were removed and the supernatant concentrated. IgG was purified using a protein G Sepharose HiTrap column. An SDS-PAGE was performed to assess purity, more than 99% of the total protein migrated as IgG. Antibody class and subclass as determined by ELISA was IgG_{2b/k}. It was specific and did not react with RANTES, HuMIP-1 α , and - β , HC-14, I-309, IP-10, IL-8, CTAP III/NAP-2 and platelet factor 4 (PF4).

Anti-RANTES and anti-MCAF antibody preparation

Polyclonal anti-RANTES antibody was raised in guinea-pigs immunized with purified recombinant human RANTES. The antibody was purified using a protein A sepharose column and adjusted to a concentration of 2 mg/mL. We used guinea-pig preimmune IgG purified on protein A sepharose as a control. A polyclonal anti-MCAF antibody was raised in a rabbit. Recombinant human MCAF/MCP-1 100 μg in 200 μL of PBS was diluted in 1.0 mL of 1:4 mixture of Freund's complete and incomplete adjuvant and injected at three sites: one subcutaneous and two deep intramuscular. Booster injections were given as above every 2 weeks, however, the third and all subsequent injections were given in incomplete adjuvant. After 3 months the rabbit was bled, antiserum collected, and assayed by ELISA against purified recombinant human MCAF/MCP-1, IL-8, RANTES, HuMIP-1 α , HuMIP-1 β , I-309, IP-10, CTAP III and PF4. The antiserum was specific and reacted only with MCAF/MCP-1. The antiserum and the preimmune rabbit serum were made 45% in ammonium sulfate, centrifuged at $10\,000 \times g$ for 15 min, the supernatant was discarded, and the precipitate was dissolved in PBS and dialyzed against PBS. IgG was purified using a protein G Seraphose HiTrap column. The purified protein migrated as IgG on SDS-PAGE. Rabbit anti-MCAF polyclonal antibody at a concentration of 100 $\mu\text{g}/\text{mL}$ inhibited basophil histamine release induced by 10^{-7} M MCAF/MCP-1 by 50%.

Electrophoresis, gel staining, and immunoblotting

SDS-PAGE was performed according to the method of Laemmli [13]. After electrophoresis, the protein bands were blotted onto polyvinylidene difluoride membranes

according to the method of Towbin *et al.* [14]. After the transfer, unoccupied sites were blocked with BLOTTO [15] for 1 h at room temperature, and treated with 5 µg/mL of MoAb to MCAF/MCP-1 or CTAP-III/NAP-2, or polyclonal antibodies to IL-8 in BLOTTO for 2 h. The membranes were then washed four times, treated with goat anti-mouse or Rabbit anti-goat IgG-alkaline phosphate conjugate, at dilutions of 1:2000 in BLOTTO, and washed with BLOTTO four times (15 min each wash). Bands were developed with a substrate kit specific for alkaline phosphatase.

ELISA for MCAF/MCP-1 and RANTES

Enzyme-linked immunosorbent assays were performed by standard procedures with 96-well microtitre plates (Immulon-4). The wells were coated with 5 µg/mL of the rabbit anti-MCAF/MCP-1 IgG in 100 µL of 0.05 M NaHCO₃ buffer (pH 9.6) for 24 h at 4°C. The wells were washed and unbound sites were blocked by incubation for 60 min at 37°C with 1% non-fat milk in PBS. After washing, triplicate samples of 100 µL MCAF/MCP-1 standards or unknowns (Mono Q HPLC fractions, HRF crude, HRF void peak) at dilution of 1:100 in PBS containing 0.05% Tween were added and incubated for 2 h at 37°C. The plates were washed again with PBS/Tween, filled with 100 µL/well of anti-MCAF 42-2 MoAB at a concentration 10 µg/mL, and incubated for 2 h at 37°C. The wells were washed and goat anti-mouse IgG conjugated to alkaline phosphatase, diluted 1:2000 in PBS/Tween were added, and incubated for 2 h at 37°C. After washing, 100 µL of p-nitrophenyl phosphate (1 mg/mL) in 10% diethanolamine-HCL buffer (pH 9.6) was added to the wells. The absorbance was read after 30 min at 405 nm on a Thermo max microplate reader from Molecular Device. RANTES concentrations were measured by an analogous sandwich ELISA [16]. The assay employs two anti-RANTES MoAbs that recognize different, noncompeting determinants. The specificity of the ELISA was tested with other chemokines of the C-X-C and C-C family, none of which were shown to crossreact in the assay. All experiments were repeated twice. Using this procedure we were able to quantitatively measure human MCAF/MCP-1 and RANTES at concentrations of 100 pg/mL and higher.

Results

Analysis of HRF using Mono S cation exchange chromatography

HRF void peak obtained by fractionation of a mononuclear cell supernatant by Mono Q anion exchange chromatography was dialysed into 10 mM sodium acetate buffer pH 6.0 and applied to a Mono S column equilibrated with the same buffer. Twenty-two milligram of protein was applied to the

column and stepwise gradients were sequentially applied from 0.01 M to 0.25 M NaCl and from 0.25 M NaCl to 1.0 M NaCl over 140 min. One millilitre fractions were collected every 2 min. Fifty microlitre of each fraction were passed through desalting Sephadex G-25 minicolumns equilibrated with PBS as described by Baeza *et al.* [1], and tested for histamine releasing activity. As shown in Fig. 1(a), a major protein peak is found between fractions 2–9 and a lesser peak at fractions 13–23; a major peak of HRF activity was found in fractions 42–49 at the start of the 1.0 M salt gradient, preceded by a small peak between 30–34 and an even smaller one at tubes 5–9. The fractions were dialysed against 10 mM phosphate pH 7.4 containing 50 mM NaCl, lyophilized, and reconstituted with 0.3 mL of H₂O. The HRF activity profile was the same using leucocytes of two atopic subjects.

Since fractions 42–49 possessed the main peak of histamine releasing activity, we examined each one by SDS gel

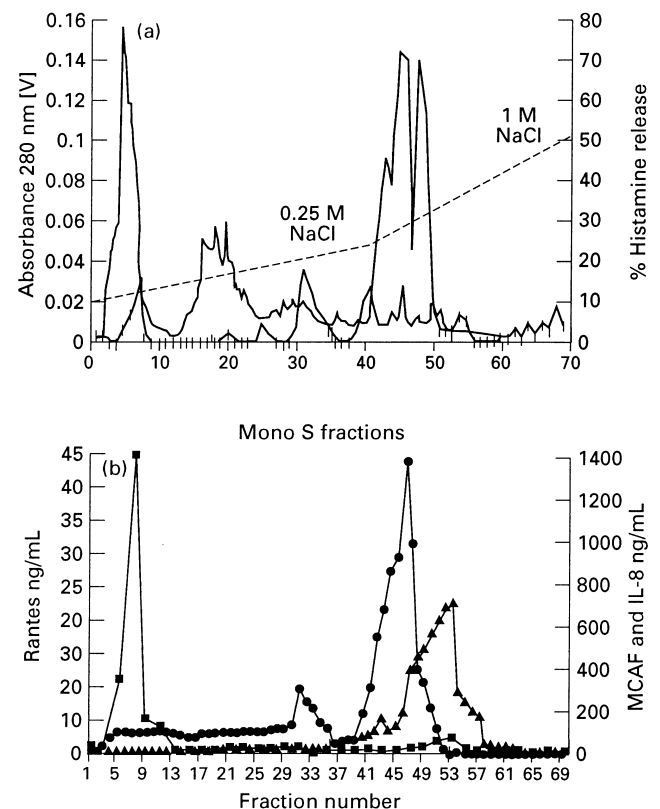


Fig. 1. Mono chromatography of HRF void peak material derived from a Mono Q column. Sequential gradients from 0 to 0.25 M NaCl and 0.25 M NaCl to 1.0 M NaCl were applied and 2.0 mL fractions collected. The protein profile and histamine releasing activity of each fraction are shown (a). Assay of column fractions for RANTES (■), MCAF/MCP-1 (●), and IL-8 (▲) was performed by ELISA. The protein profile of each chemokine is shown. Note the much lower scale (left) for RANTES (b).

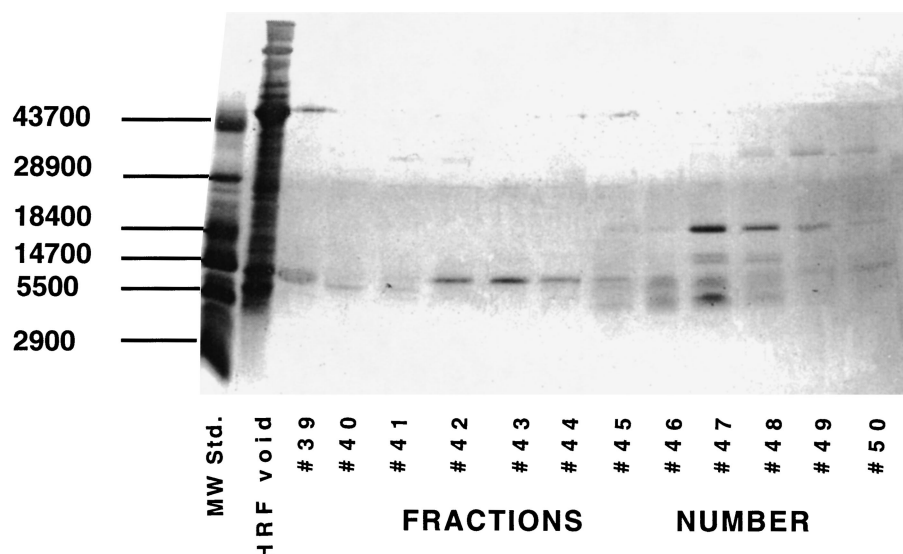


Fig. 2. SDS gel electrophoresis (10–25% gradient gel) of fractions 39 to 50 obtained by Mono S cation exchange chromatography. Molecular weight standard and the starting material (HRF void peak) are shown.

electrophoresis as shown in Fig. 2 and compared the pattern to that of the starting material. As suggested by the protein profile of void peak, considerable further purification is obtained in a single step. A band at molecular weight 8000 Da is seen between fractions 40–44 followed by additional bands in fractions 45–50 most of which range from 5 kDa to 15 kDa. The fractions from 42 through 58 were next

subjected to Western blots using monoclonal antibodies to MCAF/MCP-1 and CTAP III/NAP-2 and a polyclonal antibody to IL-8. As shown in Fig. 3 MCAF/MCP-1 was found throughout the HRF activity peak from fractions 41 to 51; it was not visible beyond fraction 52 (not shown). CTAP III was not clearly separable from MCAF/MCP-1 but the peak fractions were 46–48. Interleukin 8 was found between

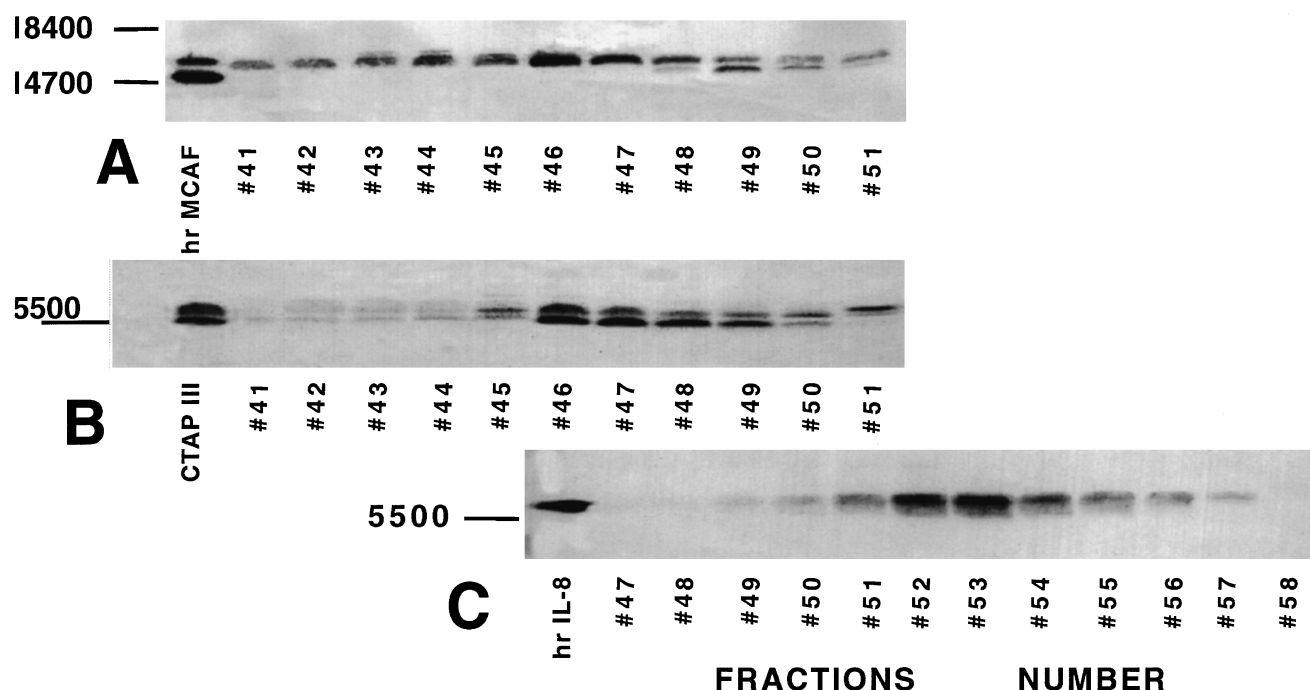


Fig. 3. Western blots of selected fractions obtained by Mono S chromatography assessed using monoclonal antibodies to MCAF/MCP-1 (a) and CTAP III/NAP-2 (b) and polyclonal antibodies to IL-8 (c). The molecular weights are shown on the left followed by a positive control consisting of recombinant cytokines.

fractions 50–57 and did not correspond to a peak of histamine releasing activity (Fig. 1 panel a).

We next analysed all the fractions by ELISA for MCAF, IL-8, and RANTES (Fig. 1(b)) which gives a more quantitative elution profile of MCAF and IL-8. In general, the chromatographic profile of each chemokine is coincident with its assessment by SDS gel electrophoresis. We also found a small amount of RANTES in tubes 5 to 9. This corresponds to the smallest peak with HRF activity and was not pursued further.

Inhibition of various forms of HRF by specific antisera

Fractions 45–47, containing a mixture of MCAF/MCP-1 and CTAP III/NAP-2, were incubated either with individual antibodies or a mixture of antibodies, and their residual histamine releasing activity determined. The same procedure was used to assess recombinant MCAF/MCP-1 and CTAP III purified from platelet releasate. As shown in Fig. 4 anti-MCAF/MCP-1 inhibited the activity in the Mono S column by 85% and anti-CTAP III inhibited much of the remaining activity. When tested with recombinant proteins anti-MCAF/MCP-1 inhibited MCAF/MCP-1 (80%) while anti-CTAP III/NAP-2 had no effect. Conversely the activity of CTAP-III/NAP-2 was inhibited by anti-CTAP III/NAP-2 by 50% while anti-MCAF/MCP-1 had no effect. A mixture of MCAF/MCP-1 and CTAP III/NAP-2 was inhibited 30% with anti-CTAP III/NAP-2, and 60% with anti-MCAF/MCP-1 and 90% with a mixture of antibodies. The addition of anti-CTAP III/NAP-2 to recombinant MCAF/MCP-1 appeared to augment its activity but this was not statistically significant and the antibody possessed no histamine releasing ability when tested separately.

Correlation of MCAF/MCP-1 protein with HRF activity

We compared the histamine releasing activity of serial dilution of HRF void peak (containing $\approx 10^{-6}$ M MCAF/MCP-1, as determined by the sandwich ELISA) with that of recombinant MCAF/MCP-1 (10^{-7} – 10^{-10} M). Very similar curves were obtained (Fig. 5). We then related the per cent histamine release of HRF void peak to that of MCAF/MCP-1 (Fig. 5 insert) and calculated the correlation coefficient of the resultant line. The correlation was 0.995 which indicated that the major form of HRF within void peak is MCAF/MCP-1 and that CTAP III/NAP-2 and RANTES make only minor contribution. IL-8 is clearly negative.

Discussion

Most forms of human HRF (MCAF/MCP-1, RANTES and MIP-1 alpha and beta) have been identified by assessment of the functional activity of recombinant proteins [3–7]. Their

relative activities were then compared on a molar basis in order to assess potency [7]. On the other hand, the HRF activity present in a mononuclear cell supernatant is dependent on the relative concentration of protein possessing HRF activity, as well as any inhibitory protein present, and there may be a difference between the native factor and recombinant material even when derived from an eukaryotic cell. CTAP III/NAP-2 is the exception in that it was purified directly from a mononuclear cell supernatant and identified by amino acid sequence [2,9]. It is the most prevalent of the chemokines, accounting for its ready isolation, but it is far less potent than MCAF/MCP-1 [7]. In this manuscript we demonstrate definitively that 90–95% of the activity within this initial chromatographic fraction is due to MCAF/MCP-1 and that CTAP III/NAP-2 and to even lesser degree RANTES, are minor contributors. Recently, MCP-2 has been characterized as a weak basophil agonist while MCP-3 is a potent basophil activator which also acts upon eosinophils. These were not examined in this study and

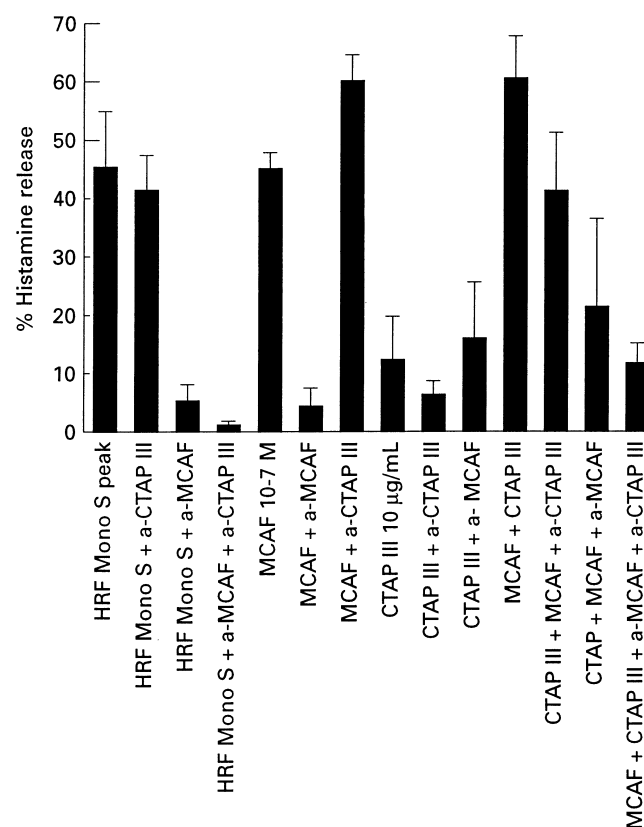


Fig. 4. Inhibition of HRF peak tubes 45–47 obtained by Mono S chromatography employing antisera to MCAF/MCP-1, CTAP III/NAP-2, or a mixture of antisera. For comparison, each antibody alone or in combination is tested against recombinant MCAF (10^{-7} M), purified CTAP III/NAP-2 (10 µg/mL) or a mixture of MCAF/MCP-1 and CTAP III/NAP-2.

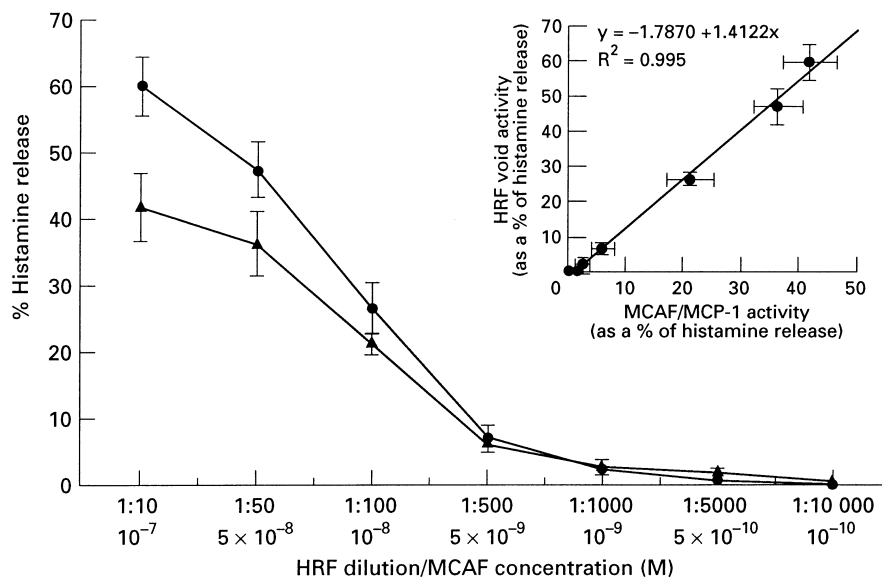


Fig. 5. Comparison of histamine releasing activity of serial dilutions of HRF void peak derived from Mono Q and recombinant MCAF/MCP-1. The insert shows the correlation of their histamine releasing capability.

monospecific antisera to them are not yet available [17,18]. Since the monoclonal antibodies to MCP-1 used in this study do not recognize MCP-2 and MCP-3 (data not shown), the HRF activity observed in fraction #40–50 is entirely due to the presence of MCP-1 and CTAP III. Interleukin-8 has been reported to cause basophils to secrete histamine [19,20], but very high concentrations were needed and it also possesses inhibitory activity [20,21]. Our results show that fraction #53, which contains 800 ng/ml of IL-8 did not have any HRF activity (Figs 1 and 3). This confirms our previous observation that human recombinant IL-8 does not release histamine from basophils [21]. On the other hand priming of basophils with IL-3, IL-5, or GM-CSF has been reported to render them responsive to IL-8 [22]. In this manuscript, we demonstrate no detectable HRF activity corresponding to the peak containing IL-8, even when tested using cells of atopic subjects whose basophils are probably already primed (Figs 1 and 3).

Interaction of IL-8 and MCAF/MCP-1 with their receptors on inflammatory cells is, however, very complex. Recently, we have reported the phenomenon of receptors class desensitization on human basophils for C-X-C and C-C chemokines, showing that low concentration of various chemokines desensitized basophils to subsequent challenge with an optimal concentration of MCAF/MCP-1. IL-8 is one of the most potent inhibitors of MCAF/MCP-1 induced histamine release among chemokines [23]. The identification of the substances in peaks 2 and 3 of our original report have not been definitively identified although some progress has been made. RANTES accounts for about half the activity of peak-2 [7], and MIP-1 alpha and beta are present in peaks 2 and 3 but their contribution to the activity

seen has not yet been determined. Perhaps MCP-2 and MCP-3 are there as well.

It is clear that chemokines of the B type (C-C family) are the major contributors to HRF activity while members of the A chemokines (C-X-C family) such as CTAP III, or its derivative NAP-2 are relatively weak by comparison [7,9]. Within HRF peak 1, MCAF/MCP-1 is clearly the predominant form of HRF. We believe this type of analysis is an important prelude to assessment of these factors in human disease since inhibitory antisera not only provide valuable reagents, but dissection of the prevalence and relative contribution of each chemokine to the activity present in activated supernatants or other biologic fluids, assures that the recombinant factors reported to induce histamine release are also the responsible factors *in vivo*.

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