# Relationship of One Form of Human Histamine-releasing Factor to Connective Tissue Activating Peptide-III

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## Abstract

**We have previously reported purification of three forms of histamine-releasing factors (HRFs) from mixtures of streptokinase-streptodornase stimulated human mononuclear cells and platelets with apparent molecular masses of 10–12, 15–17, and 40–41 kD (1989. *J. Clin. Invest.* 83:1204–1210). We have also prepared mouse MAbs against the 10–12-kD HRF (1989. *J. Allergy Clin. Immunol.* 83:281). Affinity-purified 10–12-kD HRF appears as a broad band upon polyacrylamide gel electrophoresis in the presence of SDS. We determined the $NH_2$-terminal amino acid sequence of the top and bottom halves of this broad band. Sequence analysis revealed striking homology between this HRF and connective tissue activating peptide-III (CTAP-III), a platelet-derived 8–10-kD protein known to cause mitogenesis and extracellular matrix formation in fibroblast cultures. 19 of 21 $NH_2$-terminal residues in the top half of the HRF band were identical to the $NH_2$-terminal sequence of CTAP-III. 20 of 21 $NH_2$-terminal residues in the bottom half were identical to the $NH_2$-terminal sequence of neutrophil-activating peptide-2, which is derived from CTAP-III by proteolytic cleavage between residues 15 and 16. Purified CTAP-III also released histamine from basophils. Rabbit antiserum raised against either native or recombinant CTAP-III recognized affinity-purified HRF in immunodot blot assays, and MAb against HRF recognized CTAP-III in both dot blot and microtiter plate based immunoassays. These data demonstrate the first structural, functional, and immunologic relationship between one form of human HRF and a previously described cell product. (*J. Clin. Invest.* 1990. 85:1516–1521.) basophil secretion • histamine release • histamine-releasing factor purification**

## Introduction

Histamine-releasing factors (HRFs)[1] are defined as secreted cellular products that interact with basophils and/or mast cells to release histamine. Originally described as products of human mononuclear cells (MNC) (1, 2), factors having this property have been found as products of alveolar macrophages (3), platelets (4), neutrophils (5), and nasal washings (6). Most attention has been directed to the products of human MNC (T lymphocytes, B lymphocytes, monocytes), and there is evidence that each of these may be capable of producing HRF (7–9), although the products of B lymphocytes may predominate when mixtures are assessed (8). Although human IL-3 (10), granulocyte-macrophage colony-stimulating factor (10), and, to a lesser degree, IL-1 (11) can cause basophil histamine release, the major MNC-derived HRF(s) appear distinct from these and other known cytokines (12, 13).

We have developed methods for purification of human HRF that is derived from a mixture of streptokinase/streptodornase (SK/SD)-stimulated MNC and platelets. We have isolated molecules of molecular masses 10–12, 15–17, and 40–41 kD, each of which possess histamine-releasing activity (13, 14). We have also prepared a MAb to the 10–12-kD molecular weight form, which can be used to purify it by affinity chromatography (15). We now report partial amino acid sequence data of the $NH_2$-terminal region of the 10–12-kD HRF which was found to be strikingly homologous with human connective tissue activating peptide-III (CTAP-III) (16). A close relationship between this HRF and CTAP-III was confirmed both functionally and immunologically.

## Methods

*Cell source.* Donor lymphopheresis packs were obtained from two sources: normal volunteers after informed consent, or patients undergoing cytopheresis according to an experimental protocol for the treatment of multiple sclerosis. The molecular characterization of the HRF described herein did not differ according to source, and the yields of activity appear comparable (14).

*Preparations of cell supernatants.* MNC supernatants were prepared as previously described (13, 14). Briefly, lymphopheresis packs of ~ 200 ml were diluted to 360 ml in Dulbecco's PBS (Gibco Laboratories, Grand Island, NY). 30 ml of cellular suspension was layered over 15 ml of Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) in 50-ml polypropylene tubes (Falcon Plastics, Cockeysville, MD). The suspension was centrifuged for 15 min at 1,000 *g*. The interphase was aspirated, pooled, washed with PBS, and centrifuged at 400 *g* for 12 min. After a second wash the mixture was centrifuged at 150 *g* for 7 min to deplete platelets. This protocol achieved 90–99% platelet depletion. Nevertheless, the stained cell pellet consisted of MNC essentially devoid of other leukocytes, but with significant numbers of platelets such that the platelet/MNC ratio varied from 1:1 to 10:1. The pellet was

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1. *Abbreviations used in this paper:* CTAP-III, connective tissue activating factor; HBS, Hepes-buffered saline; HRF, histamine-releasing factor; IL-8/MDNCF, IL-8/monocyte-derived neutrophil chemotactic peptide; MNC, mononuclear cell; NAP-2, neutrophil-activating peptide-2; SK/SD, streptokinase/streptodornase.

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resuspended at a concentration of 25–45 × $10^6$ cells/ml in RPMI 1640 tissue culture medium (Gibco Laboratories). Cell viability was > 98% by trypan blue exclusion. SK/SD was added to the cell cultures at 13 U/$10^6$ cells. The cells were then cultured for 16–19 h at 37°C with 5% $CO_2$ in 50-ml conical polypropylene tubes (Falcon Plastics). At the end of the culture period the conditioned media were pooled, centrifuged at 400 *g* for 15 min, and clarified at 38,000 *g* for 20 min. The supernatant was filtered through YM100 membranes (Amicon Corp., Danvers, MA) and concentrated 50-fold using YM5 membranes (Amicon Corp.). PMSF was added to a final concentration of 0.1 mM before freezing and the material was stored at −20°C. In separate experiments we demonstrated that this concentration of PMSF had no effect on the release of histamine by HRF.

*Purification of HRF.* The three identified forms of HRF were purified by a combination of HPLC gel filtration, HPLC anion exchange chromatography, and preparative SDS-PAGE as previously described (14).

*MAbs.* MAb 18-1 to the 10–12-kD component of HRF was prepared (15) according to the procedure of Lipsich et al. (17). Mice were immunized with purified HRF species obtained by electroelution after SDS-PAGE (14) of concentrated crude MNC supernatant. The 10–12-kD antigen used for screening was prepared by ion exchange HPLC and electroelution from SDS gels as described previously (14). The initial clone and subsequent subclone selection was done on the basis of reaction with the same antigen by ELISA and Western blots. Antibody class and subclass were determined by ELISA using a commercial kit (Southern Biotechnology Associates, Birmingham, AL). The antibody was $IgG_{1\ kappa}$. The subclone that yielded the strongest signal (termed MAb 18-1) was grown in RPMI 1640 medium. After the cells were removed by centrifugation at 1,000 *g* for 15 min, the supernatant from this culture was clarified and concentrated and an SDS-PAGE was performed to assess purity. > 90% of the total protein migrated as IgG and this was directly used in the preparation of affinity columns.

*Affinity chromatography.* MAb 18-1 was used to isolate this form of HRF in a single step as follows: 6.5 mg of anti-HRF MAb 18-1 was coupled to 2 ml of Affigel-10 (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions in 0.1 M $NaHCO_3$ buffer (pH 8.5). Chromatography was performed in a 3-ml Flex column (Kontes Co., Vineland, NJ) at 25°C. Unoccupied active sites on Affigel were blocked with 0.1 M sodium glycinate, pH 9.0, and the column was then equilibrated with PBS containing 0.1 mM PMSF, 0.1 mM EDTA, and 0.02% azide (equilibration buffer). All other buffers in this procedure also contained these additives. The same procedure was followed to prepare a precolumn in which the antibody was omitted. Both columns, arranged in tandem, were washed with 100 ml of equilibration buffer before sample application. 1.5 ml of crude MNC supernatant was applied to the precolumn and its effluent was allowed to pass directly through the MAb 18-1 column. Both columns were washed with the equilibration buffer in tandem until the protein concentration returned to baseline. The columns were then separated, washed with equilibration buffer containing 1.0 M NaCl, and eluted with 3 M KSCN. 1-ml fractions were collected. Peak fractions were pooled, dialyzed against 0.05 M ammonium acetate, lyophilized, and resuspended in 400 μl of PBS (pH 7.4) containing 0.1 mM PMSF.

*Purification of CTAP-III.* CTAP-III was isolated from human platelets by acid-ethanol extraction, precipitation with cold acetone, and chromatography over Sephacryl S-200. Final purification was accomplished by immunoaffinity chromatography using monospecific anti-CTAP-III antisera (16).

*IL-8/monocyte-derived neutrophil chemotactic peptide (IL-8/MDNCF).* MDNCF was obtained from Dr. E. J. Leonard of the National Cancer Institute, Frederick, MD.

*Basophil histamine release.* The procedures for basophil isolation and histamine release have been described in detail (13). Briefly, the cells were prepared by dextran sedimentation (0.6% dextran, 0.6% glucose, 0.02% EDTA), the basophil-containing layer was washed twice with Hepes-buffered saline (HBS)-BSA, and the cells were resuspended in HBS-BSA containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 50% $D_2O$. 20–50 μl of each sample or PBS control were incubated with 3 × $10^6$ cells in a total volume of 300 μl of HBS-HSA containing 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 50% $D_2O$ for 40 min at 37°C and centrifuged at 1,000 *g* for 5 min. The total basophil histamine content was determined by boiling one of the aliquots. The presence of $D_2O$ had no effect on the baseline histamine release and augmented the stimulated histamine release 2–2.5-fold. Therefore it was routinely used to facilitate that assay and conserve reagents.

*Histamine assay.* The radio enzyme assay for histamine was performed as described by Beaven et al. (18).

*ELISA.* ELISA were performed by standard procedures with 96-well microtiter plates (strip 8; Costar, Cambridge, MA). Wells were coated with either 100 ng of affinity-purified HRF or CTAP-III in 100 μl of PBS (pH 7.4) for 24–48 h at room temperature. Unbound sites were blocked by incubation for 60 min at 37°C with 3% BSA in PBS. Wells were then incubated with 1 μg/ml murine MAb to HRF in PBS + 0.05% Tween-20 for 2 h, followed by incubation with the secondary antibody (goat anti-mouse IgG and M antibody conjugated to alkaline phosphatase [Jackson Immunoresearch Laboratories Inc., West Grove, PA]) at 1:1,000 dilution for 2 h. After washing, 100 μl of *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine-HCl buffer (pH 9.6) was added to the wells and the absorbance read at 405 nm on an ELISA reader (Dynatech Laboratories, Inc., Alexandria, VA).

*Gel electrophoresis.* SDS-PAGE was performed under nonreducing conditions according to Laemmli (19).

*Preparation of antisera to CTAP-III.* Rabbit antisera to native and recombinant CTAP-III were raised as described previously (20).

*Dot blot.* Dot blots of HRF and CTAP-III were carried out using anti-native CTAP-III, anti-recombinant CTAP-III, and monoclonal anti-HRF antibodies (18-1) according to a procedure supplied by Bio-Rad Laboratories along with their immunoblot assay kit.

*Microsequencing.* The protein contained in the eluate fraction of the affinity column underwent SDS-PAGE as described above and was electroblotted onto Immobilon polyvinylidene difluoride (Millipore/Continental Water Systems, Bedford, MA) according to Towbin et al. (21). The transblot was stained with amido black to mark the protein bands and then destained with several changes of methanol, followed

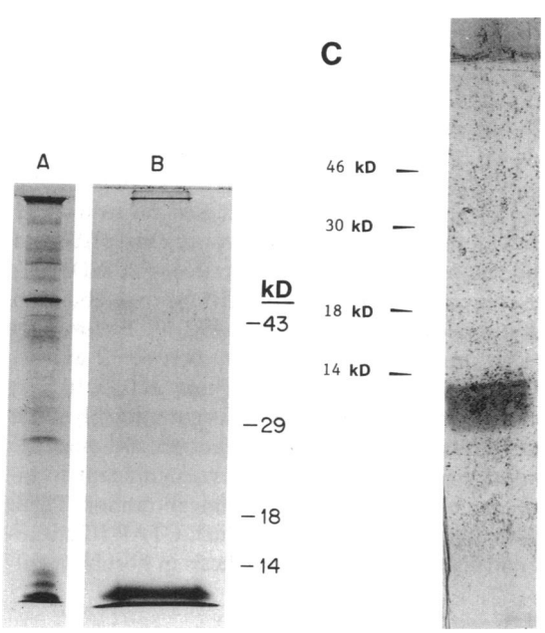


*Figure 1.* SDS-PAGE of HRF. Unfractionated (*A*), affinity-purified (*B, C*) HRF was subjected to SDS-PAGE on 12 (*A, B*) or 15% (*C*) acrylamide gels as described in Methods and stained with Coomassie blue R-250.

by rinsing with deionized water. The transblot was air-dried on filter paper under a fume hood and the stained spots were excised and placed in Eppendorf tubes (22). Amino-terminal sequences were determined using a gas phase microsequencer (model 470A; Applied Biosystems Inc., Foster City, CA) coupled to an on-line high performance liquid chromatograph (model 120A; Applied Biosystems Inc.).

## Results

Fig. 1 shows the SDS gel electrophoresis of unfractionated MNC supernatant (Fig. 1 *A*) and affinity-purified HRF (Fig. 1 *B*) using a 12% (T) gel. This shows a sharp band close to the dye front. The same preparation, shown in Fig. 1 *C*, results in a better resolution and yields a broader band on a 15% gel. The average molecular weight of the preparation was the same on both gels. An identical gel (15%) was subjected to electrophoretic transfer to PVDF membrane and the membrane stained with amido black. The stained band was cut in half and the $NH_2$-terminal sequence of the upper and lower portions were determined separately (Table I). The upper band yielded the first 18 residues and the 21st residue of a peptide which revealed identity with CTAP III based on a computer search. Residues 19 and 20 could not be identified. The lower half of the band yielded a different sequence for the $NH_2$-terminal 20 residues, which was also homologous with CTAP-III if the sequence alignment began with residue number 16. It thus appears likely that the lower half of the HRF band represents the proteolytic product of the upper protein band. Thus the epitopes recognized by our MAb may be located towards the COOH-terminal side of the cleavage site.

Striking homology over 19 of 21 (upper band) and 20 of 21 residues (lower band) between HRF and CTAP-III suggested that immunologic crossreactivity should be demonstrable. We therefore performed a dot blot of HRF, recombinant CTAP-III, and native CTAP-III with monospecific antibodies to each antigen. All antigens reacted with MAb 18-1 and polyclonal antisera to both native and recombinant CTAP-III recognized HRF as well as CTAP-III (Fig. 2). We also examined this crossreactivity by ELISA using the MAb 18-1, and assessed its reactivity to HRF, CTAP-III, and a related cytokine, IL-8/MDNCF. Each protein was tested at equal concentrations. As shown in Fig. 3, MAb 18-1 recognized HRF and CTAP-III, but not IL-8/MDNCF. Similar results were obtained when the concentration of antigens used was increased up to fivefold (data not shown); the absorbance at 405 nm against HRF was > 1.2 U, while no reactivity was demonstrable with MDNCF.

Next, we compared HRF and CTAP-III for their ability to cause histamine release from basophils (Fig. 4). Preliminary results for each protein indicated activity between 2 and 15 μg/ml. Therefore, we performed an experiment at 0, 5, 10, and 20 μg/ml of HRF and CTAP-III. HRF was considerably more active than CTAP-III at 5- and 10-μg/ml doses, although the 20-μg/ml point was similar. Nevertheless, significant histamine release was observed at all three concentrations. These concentrations are similar to those at which CTAP-III stimulated DNA and glycosaminoglycan synthesis in fibroblast cultures (16).

## Discussion

CTAP-III is one of a series of polypeptides that were originally isolated from platelets and were found to activate fibroblasts with resultant mitogenesis and extracellular matrix formation.

*Table I. $NH_2$-terminal Sequence Comparison of CTAP-III and HRF*

| Residue No. | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 |
|---|---|
| CTAP-III: | ASN-LEU-ALA-LYS-GLY-LYS-GLU-GLU-SER-LEU-ASP-SER-ASP-LEU-TYR-ALA-GLU-LEU-ARG-CYS-MET-CYS-ILE-LYS-THR-THR-SER-GLY-ILE-HIS-PRO-LYS-ASN-ILE-GLN-SER-LEU-GLU-VAL-ILE |
| HRF-1: | ASN-LEU-ALA-LYS-GLY-LYS-GLU-GLU-SER-LEU-ASP-SER-ASP-LEU-TYR-ALA-GLU-LEU——————MET |
| HRF-2: | ALA-GLU-LEU-ARG——MET-CYS-ILE-LYS-THR-THR-SER-GLY-ILE-HIS-PRO-LYS-ASN-ILE-GLN-SER |

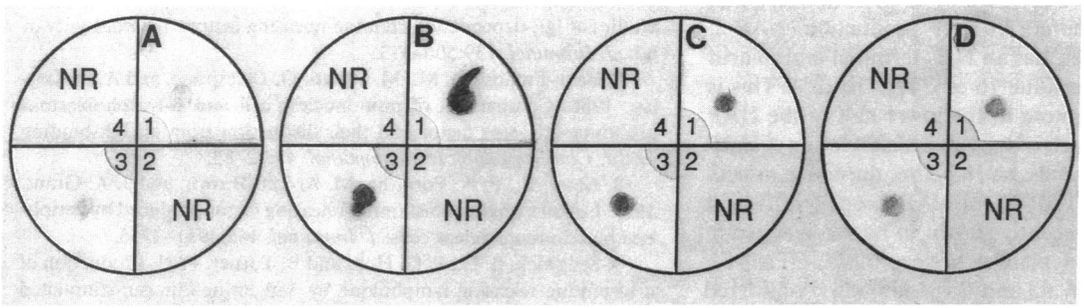


*Figure 2.* Immunodot blot assay of CTAP-III and HRF. 35 ng each of CTAP-III (*1*), human serum albumin (*2*), HRF (*3*), or BSA (*4*) were applied to nitrocellulose membranes as described in Methods. Membranes were then treated with a 1:50 dilution of preimmune rabbit serum (*A*), rabbit anti-recombinant CTAP-III serum (*B*), anti-native CTAP-III serum (*C*), and 0.8 μg/ml anti-HRF MAb (*D*). Dots were developed with horseradish peroxidase-labeled secondary antibodies.

CTAP-III has a molecular weight of 8,000–10,000 by SDS gel electrophoresis and 9,278 D by direct protein sequencing (16). Polyclonal antisera have been developed both to the purified protein and to a recombinant form expressed by *Escherichia coli* transfected with a synthetic gene for CTAP-III (23). Both antisera were used in this study and gave the same results. Further, several proteins have now been isolated that are structurally related to CTAP-III; these include β-thromboglobulin and neutrophil-activating peptide-2 (NAP-2), which are derived from platelet basic protein (24, 25); a monocyte-derived factor chemotactic for neutrophils (26); a derivative of Rous Sarcoma virus transformed chick embryo fibroblasts (27); a growth-regulated gene in transformed Chinese hamster fibroblasts and human cells (28); a melanoma growth-stimulating factor (29); and gene products derived from T and B lymphocytes whose function is as yet unknown (30). These appear to comprise a family of proteins with significant homology to CTAP-III. However, it is unclear which, if any, are sufficiently related to share epitopes and crossreact immunologically.

We isolated the 12-kD form of HRF using MAb 18-1 and obtained a broad band by SDS-gel electrophoresis. It seems likely that the lower portion of HRF is formed by specific proteolysis of the upper band, since our sequence analysis (when compared with that of CTAP-III) indicates loss of the first 15 amino acids from the upper portion of the band. There was 90 (upper band)–95% (lower band) sequence homology with CTAP-III and NAP-2, respectively. MAb to HRF clearly recognized CTAP-III both by dot blot assay and ELISA, and both polyclonal antisera to CTAP-III reacted with HRF.

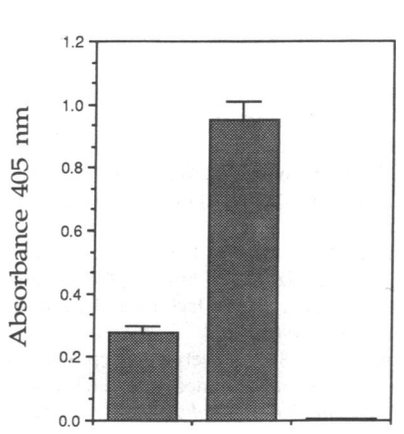


*Figure 3.* Anti-HRF MAb 18-1 recognizes CTAP-III. Microtiter plate wells were coated with 100 ng of affinity-purified HRF, CTAP-III, or MDNCF in triplicate, blocked with BSA, treated with anti-HRF MAb 18-1, and processed with alkaline phosphatase–labeled goat–anti mouse IgG as described in Methods. Bars indicate mean absorbance at 405 nm (±SD).

CTAP-III exhibited histamine-releasing activity similar to HRF, although HRF appeared to be relatively more active (Fig. 4). By contrast, our MAb did not recognize IL-8/MDNCF/NAP-1, which (*a*) has a molecular mass of 8 kD, which is in the same range as CTAP-III (25), (*b*) was shown to be structurally homologous to CTAP-III, and (*c*) has recently been reported to cause histamine release from basophils (31). Further, IL-8-dependent histamine release was augmented by prior exposure of basophils to IL-3 (32). It is important to point out that IL-1, IL-3, GM-CSF, and IL-8 have all been shown to cause histamine release from basophils in some, but not all, subjects. These studies were performed by testing previously characterized factors whose purification was based on lymphocyte stimulation or bone marrow colony stimulation, but not on HRF activity. Thus their contribution to the total HRF activity of an MNC and/or platelet preparation is unknown.

Since platelets are a source of HRF-like activity (4), it is likely that CTAP-III itself may be the responsible component; other possibilities are β-thromboglobulin and NAP-2, which are known degradation products of CTAP-III. Since most MNC preparations are contaminated with platelets (25, 33) we must consider the possibilities, (*a*) that this form of HRF is in fact a platelet product rather than an MNC product; (*b*) that both cell types produce similar forms of HRF; or (*c*) that both

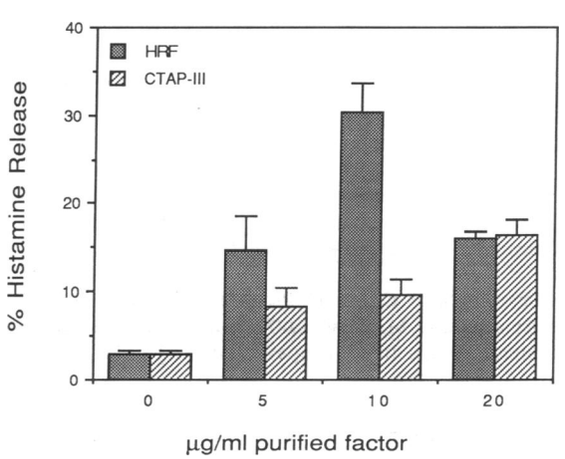


*Figure 4.* Histamine-releasing activity of HRF and CTAP-III. Indicated amounts of HRF or native CTAP-III were assayed for basophil histamine-releasing activity as described in Methods. Total histamine content of basophils in the assay mixture released upon cell lysis is taken as 100%.

MNC and platelets collaborate in HRF production. NAP-2, produced by MNC cultures, has an $NH_2$-terminal amino acid sequence beginning with residue 16 of CTAP-III (25). This is the same cleavage site we note in the lower half of the HRF band. We do not know which of the two isolated forms of HRF (Table I) is active on basophils; i.e., whether the active moiety more closely resembles CTAP-III, or NAP-2, or both. It is of interest that NAP-2 was recently shown to be more active in neutrophil activation than platelet basic protein, CTAP-III, or platelet factor 4 (34). Walz and Baggiolini also considered an MNC-platelet interaction to produce this peptide (25). Studies to distinguish these possibilities are in progress.

The 15–17- and 40–41-kD forms of MNC-derived HRF previously described by us do not react with our MAb 18-1 (15) and may represent separate gene products possessing HRF activity. Therefore, characterization of the functional and structural properties of MNC-derived HRFs may best be done by assessment of each purified component, since individual species of HRF may not share receptors, their mechanisms for signal transduction may differ, and HRF inhibitors (35) may be specific for individual components. Our purified factor released histamine in the microgram range, and this is consistent with the microgram quantities of CTAP-III that are required to stimulate DNA synthesis in fibroblast cultures. Since cytokines in general are active in nanogram or picogram quantities, more active histamine-releasing moieties (on a molar basis) may yet be identified. It is also possible that in crude supernatants different components may be acting synergistically, some actively releasing histamine and some others primarily augmenting cell responsiveness (12).

This study represents the first demonstration that one form of HRF is related to a previously described cell product. Since CTAP-III belongs to a family of proteins related at primary structure level, it will be of interest to determine whether other forms of HRF are homologous to known molecules. This will require not only further sequence analysis of the 12-kD moiety described herein, but a similar assessment of the other forms of MNC-derived HRF, direct isolation of HRF from purified platelets, and further characterization of HRF derived from other cell types.

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