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MAST CELLS AND PULMONARY FIBROSIS

Identification of a Histamine Releasing Factor in Bronchoalveolar Lavage Fluid[1, 2]

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As elevated bronchoalveolar lavage (BAL) fluid histamine levels are noted in patients with pulmonary fibrosis (PF), we assayed BAL fluid from 16 patients with PF for the presence of a histamine releasing factor (HRF). HRF activity was assayed by measuring release of the preformed mast cell-derived mediators, histamine, or β-hexosaminidase (β-hex) from a purified population of IL-3 dependent mouse bone marrow derived mast cells (MBMMC) or human blood basophils. Mean BAL cell free histamine levels in the patients with PF was 1226 ± 1349 pg/ml, whereas BAL histamine levels in a comparison group of six non-PF patients was 118 ± 60 pg/ml. HRF was significantly elevated in BAL fluid of patients with PF (mean β-hex release 24.5 ± 12.9%; range 6.8 to 52.4%) compared to the non-PF group of patients (mean β-hex release 7.9 ± 7.7%; range 1.8 to 20.7%). The PF HRF not only degranulated MBMMC, but also induced the generation of the arachidonic acid metabolite leukotriene C_4 from MBMMC (24.6 ± 4.2 ng leukotriene $C_4/10^6$ MBMMC). The PF HRF did not appear to be a cytokine previously identified in BAL fluid of patients with PF (i.e., platelet derived growth factor or insulin growth factor-1) or a human cytokine able to degranulate human basophils (i.e., IL-1, or granulocyte-macrophage-CSF) as these recombinant human cytokines did not induce MBMMC β-hex release. Physicochemical characterization of the HRF revealed that it was relatively heat stable, pronase sensitive and on Sephadex G-75 and G-200 column chromatography had an apparent molecular mass of 30 to 50 kDa. The ability of PF BAL to induce β-hex release from MBMMC was not dependent on IgE as unsensitized or lactic acid treated MBMMC release similar amounts of β-hex compared to MBMMC sensitized with IgE. Thus, BAL fluid of patients with PF contains an HRF that induces β-hex release from MBMMC via an IgE-independent mechanism. The presence of the HRF could explain elevated BAL histamine levels in patients with PF.

The association of mast cells and pulmonary fibrosis has been noted in animal models of pulmonary fibrosis (1–3) as well as in humans with PF[4] (4–8). In a study comparing the number of mast cells in lung biopsy specimens of patients with PF and control subjects, increased numbers of pulmonary mast cells were noted in the thickened fibrotic alveolar septa and within the alveolar lumen of the subjects with PF (8). Increased numbers of mast cells are also found in rodent models of pulmonary fibrosis produced by a diverse group of etiologic agents including bleomycin (3), radiation (1), and asbestos (2). The presence of elevated cell free BAL histamine levels in patients with pulmonary fibrosis (4–7) suggests that the increased number of mast cells have been activated to release histamine from preformed cytoplasmic granules. Additional support for the presence of activated pulmonary mast cells in patients with PF comes from electron microscopy studies showing evidence of partial mast cell degranulation in the areas of pulmonary fibrosis (8). As mast cells (9) and basophils (9, 10) degranulate not only in response to allergen/IgE but also to cytokines (IL-1, IL-3, GM-CSF) and HRF (11–22), we have sought in the BAL fluid from patients with PF a cytokine or HRF capable of inducing release of preformed (histamine, β-hexosaminidase) and newly generated (LTC_4) mast cell derived mediators.

MATERIALS AND METHODS

Study Subjects

Patients were diagnosed as having PF ($n = 16$) by history, physical examination, pulmonary function studies, chest x-ray, and lung biopsy (23, 24). Pulmonary function studied in the patients with PF, revealed that their mean total lung capacity was 77 ± 11% of predicted, whereas the mean single-breath carbon monoxide diffusing capacity was 46 ± 9% of predicted. Four PF patients were on prednisone therapy at the time of study. Patients undergoing bronchoscopy and BAL for a suspected malignancy or atelectasis served as a non-PF comparison control group ($n = 6$). The use of the bronchoalveolar lavage fluid in these studies was approved by the UCSD Human Subjects Committee.

Bronchoalveolar Lavage

Topical anesthesia of the upper and lower airways was achieved with a total dose of less than 30 ml of 0.45% tetracaine. A total

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[2] Presented in part at the meeting of the American Federation for Clinical Research, Washington, DC, April 29, 1989 (*Clin. Res. 37:239A, 1989*), as well as at the 7th International Congress of Immunology, Berlin, July 31, 1989.
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[4] Abbreviations used in this paper: PF, pulmonary fibrosis; MBMMC, mouse bone marow derived mast cells; LT, leukotriene; PDGF, platelet derived growth factor; IGF-1, insulin-like growth factor-1; BAL, bronchoalveolar lavage fluid; HRF, histamine releasing factor; GM-CSF, granulocyte macrophage CSF.

volume of 240 ml sterile 37°C normal saline in 4 × 60 ml aliquots was used to lavage the right lower lobe using a Pentax flexible fibreoptic bronchoscope (model 19D, Pentax, Orangeburg, NY). In the non-PF comparison group the right lower lobe was lavaged, unless it was involved in the disease process, in which case lavage of the clinically uninvolved left lower lobe was performed. BAL fluid was aspirated with gentle suction using a hand held syringe. The initial first aliquot lavage volume was recorded and then discarded. Recovered lavage aliquots 2 through 4 were immediately pooled and centrifuged at 300 × *g*. Aliquots of the cell free lavage fluid were stored at −70°C before mediator analysis.

HRF assay

Two assays were used to detect HRF.

1. *Human basophil histamine release assay.* Basophils were isolated from heparinized venous blood of healthy laboratory volunteers using Ficoll-Hypaque (Ficoll 400, Pharmacia Fine Chemicals, Piscataway, NJ; 50% Hypaque, sodium; Winthrop, New York, NY). Ten ml of Ficoll-Hypaque (15.8 g Ficoll in 200 ml H_2O and 50 ml 50% Hypaque) were layered to the bottom of a 50 ml polypropylene conical tube containing 25 ml blood and 15 ml HBSS and centrifuged for 30 min at 12,000 rpm. The upper two-thirds of the plasma layer was aspirated and discarded. The mononuclear/polymorphonuclear cell layer (containing basophils), present below the discarded upper two-thirds of plasma layer was recovered and washed three times in HBSS. Basophils comprised 0.5 to 2.0% of the cells present in these preparations as assessed by using an alcian blue stain. The basophil histamine release assay was performed in triplicate with 2.5×10^6 cells (approximately 2.5×10^4 basophils) in a final volume of 250 μl containing 2 mM calcium chloride and 1 mM magnesium chloride. The cells were suspended in 125 μl HBSS and challenged with either 125 μl of BAL medium (negative control), anti-IgE (positive control) (Meloy Laboratories, Inc., Springfield, VA), PF BAL fluid, or non-PF BAL fluid, for 45 min at 37°C. The reaction was stopped by adding 2.75 ml of cold HBSS to each tube. Cell pellets and supernatants were separated by centrifugation at 4°C for 5 min at 1000 rpm. The cell pellets were resuspended in 3 ml HBSS and sonicated to release cellular histamine. The disrupted cells pellets and supernatants were stored overnight at −70°C and histamine assayed the next day using a fluorometric assay (25). Unstimulated histanine release was generally less than 10%. Results are expressed as net percent histamine release corrected for both unstimulated histamine release and histamine present in BAL fluid added to the basophil histamine release assay.

2. *MBMMC β-hexosaminidase release assay.* A purified population of IL-3-dependent MBMMC (more than 99% mast cells) were cultured in vitro as previously described (26). Duplicate samples of 3×10^5 MBMMC were suspended in complete Tyrode's buffer (containing 1.8 mM calcium chloride and 1 mM magnesium chloride) in a final volume of 300 μl and challenged individually for 45 min at 37°C with either 200 μl PF BAL fluid dilutions, non-PF BAL fluid, recombinant human cytokines (PDGF, IGF-1 IL-1, GM-CSF, TNF), gibbon lymphosarcoma cell line-(UCDE-144-MLA) derived IL-3 supernatant (gibbon IL-3 is 96% homologous with human IL-3 (27) kindly provided by Dr. Kim Barrett, UCSD), BAL fluid lavage medium (negative control) or 200 ng DNP-BSA Ag (positive control) (a kind gift from Dr. F. T. Liu, La Jolla, CA). Recombinant human cytokines were obtained from Amgen, Thousand Oaks, CA. As FCS, present in the gibbon IL-3 supernatant, influences the results obtained in the β-hexosaminidase assay, histamine release (measured with an [^{125}I] histamine RIA), instead of β-hexosaminidase release was measured as an index of HRF activity in the MBMMC experiments using gibbon IL-3. In experiments using DNP-BSA Ag as a secretagogue, MBMMC were first passively sensitized with anti-DNP IgE (1 μg/10^6 cells) (a kind gift from Dr. F-T Liu, La Jolla, CA) at 37°C for 30 min before challenge with DNP-BSA Ag. Reactions were terminated by centrifugation at 200 × *g* for 10 min. β-Hexosaminidase activity in supernatants and cell pellets were assayed by quantitative spectrophotometry (change in absorbance at 410 nm) that measures the release of 4-*p*-nitrophenol due to cleavage of the synthetic substrate *p*-nitrophenol-β-D-2-acetamino-2-deoxyglucopyranoside (Sigma Chemical Co., St. Louis, MO) (28). β-Hexosaminidase is a preformed mast cell cytoplasmic granule mediator whose release parallels that of histamine (28). The results are expressed as the net percentage of β-hexosaminidase release from MBMMC according to the formula: net % release β-hexosaminidase = % stimulated β-hexosaminidase release − % control unstimulated β-hexosaminidase release, where stimulated release is calculated from the following formula:

$$\frac{\text{corrected* supernatant } \beta\text{-hexosaminidase}}{\text{corrected* supernatant } \beta\text{-hexosaminidase} + \text{pellet } \beta\text{-hexosaminidase}} \times 100$$

* The supernatant β-hexosaminidase is corrected for β-hexosaminidase present in BAL fluid added to the assay.

Neutralizing Antibodies to Human IL-1 GM-CSF, and TNF

The ability of antibodies to either human IL-1, GM-CSF, or TNF to inhibit the HRF activity in BAL fluid was assessed by preincubating PF BAL fluid for 2 h at 37°C with the respective antibody. The antibody treated or untreated BAL fluid was then assessed for HRF activity in the MBMMC β-hexosaminidase release assay. None of the antibodies alone influenced the MBMMC β-hexosaminidase release assay. The polyclonal rabbit anti-human GM-CSF antibody (80% IgG, 20% IgM) (Genzyme Corp., Boston, MA) neutralizes the bioactivity of human GM-CSF, but not granulocyte-CSF or macrophage-CSF, in the human bone marrow colony formation assay (Genzyme product information). The polyvalent rabbit anti-human TNF-α IgG antibody (Endogen, Boston, MA) neutralizes the bioactivity of human rTNF using a standard L929 cytotoxicity assay (Endogen product information). This observation has been confirmed in our laboratory. The polyclonal rabbit anti-human IL-1 beta IgG antibody (Collaborative Research, Bedford MA) neutralizes the bioactivity of recombinant and natural human IL-1β in cultures of mouse thymocytes (Collaborative Research product information).

Leukotriene C4 Assay

The generation of the sulfidopeptide leukotriene LTC_4 was quantified with an RIA (Amersham, Arlington Heights, IL) as previously described (29). The concentration of LTC_4 in the sample was determined from a standard curve of known LTC_4 standards (0.25 ng to 1.6 ng LTC_4) and corrected for LTC_4 present in the BAL fluid. The LTC_4 RIA has a 55% cross-reactivity with LTD_4 and 8% cross-reactivity with LTE_4 but does not cross react with LTB_4 or PGD_2 (29).

Histamine Assay

BAL fluid from patients with PF or controls were assayed for histamine using a sensitive [^{125}I] histamine RIA (AMAC Inc. Westbrook ME). The histamine determination makes use of the binding competition between the acylated test sample or standard histamine and [^{125}I] acylated histamine to a mouse antihistamine mAb-coated test tube. Results are expressed as ng/ml histamine and extrapolated from the histamine standard curve. The assay has a sensitivity of 0.1 nM histamine an intra-assay coefficient of variation of 8.0 ± 0.4% and an interassay coefficient of variation of 9.9 ± 0.9%. The assay is also specific as cross-reactions with t-methylhistamine or histidine are very low ($<10^{-4}$).

Physicochemical Characterization of HRF

Heat Stability. Aliquots of PF BAL fluid were placed in either a 37, 56, or 100°C water bath for 30 min and their subsequent ability to induce MBMMC β-hexosaminidase release assessed.

Enzyme Sensitivity. The ability of pronase (0.1 mg/ml), DNAse (0.5 mg/ml) (Cal-Biochem-Behring, La Jolla, CA) or RNAase (1 mg/ml) (Sigma) to influence HRF activity in PF BAL fluid was assessed by adding pronase, DNAse or RNAase to PF BAL fluid for 30 min at 37°C. The subsequent ability of enzyme-treated or -untreated BAL fluid to induce β-hexosaminidase release from MBMMC was assessed. Pronase alone induced 11 ± 5% β-hexosaminidase release from MBMMC, whereas DNAse or RNAse alone induced <1% β-hexosaminidase release from MBMMC.

Partial Purification of PF BAL HRF. HRF was partially purified from Amicon Ultrafiltration and Sephadex G-75 or G-200 chromatography.

1. Amicon ultrafiltration: a 10-ml aliquot of PF BAL fluid was further concentrated to 1 ml under pressure using an Amicon stirred cell (model 12) containing an Amicon UM2 diaflo ultrafiltration membrane (m.w. cutoff 1000).

2. Sephadex G-75 or G-200 chromatography: the Amicon ultrafiltration concentrate was applied to a calibrated column (1.6 × 90.5 cm) of Sephadex G-200 (Pharmacia) equilibrated in 0.01 M Tris/0.15 M NaCl, pH 7.4. The column was eluted at a flow rate of 0.5 ml/min. Fractions of 1.5 ml volume were monitored for their absorbance at 280 nM as well as their ability to induce β-hexosaminidase release from MBMMC. Molecular weight standards used to calibrate the G-200 column included blue dextran (m.w. 2,000,000), aldolase (m.w. 158,000), BSA (m.w. 67,000), OVA (m.w. 43,000), and chymotryp-

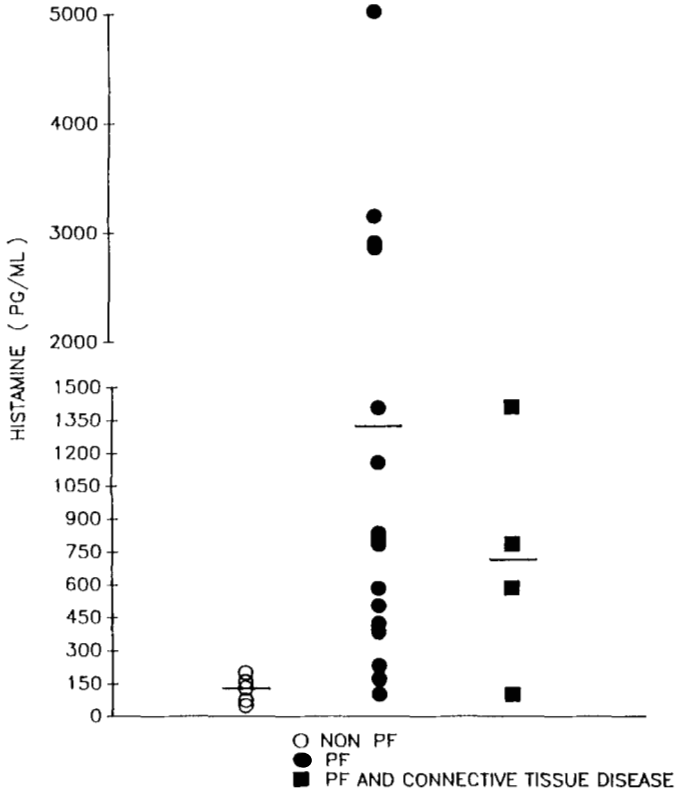

Figure 1. BAL histamine levels. BAL histamine levels from patients with PF (n = 16), PF and associated connective tissue disease (n = 4), and non-PF controls (n = 6) were assayed using an [^{125}I]histamine RIA. Results expressed as mean ± SD.

sinogen (m.w. 25,000), whereas m.w. standards used to calibrate the G-75 column included blue dextran, BSA, OVA, chymotrypsinogen, and ribonuclease A (m.w. 13,700).

Interaction of HRF and MBMMC

IgE dependence. In experiments to assess the IgE dependence of PF BAL HRF, MBMMC were either passively sensitized with anti-DNP IgE (1 μg/10^6 cells) or incubated in the absence of anti-DNP IgE at 37°C for 30 min before challenge with PF BAL fluid or 200 ng DNP-BSA Ag.

Additional experiments with lactic acid to elute IgE from MBMMC before challenge with PF BAL fluid were also performed. MBMMC (± IgE sensitization) were washed in unbuffered normal saline and incubated in the presence or absence of 5 ml of 0.01 M lactic acid, pH 3.9, for 3.5 min at 23°C (20). The reaction was stopped by the addition of 30 ml of Tyrodes buffer, pH 7.4. MBMMC were washed twice before use in the beta hex release assay. In these experiments anti-IgE (0.1 to 100 μg/ml) (kindly provided by Dr. F-T. Liu, Molecular Biology Institute, La Jolla) was used in addition to PF BAL fluid as a secretagogue stimulus.

PF BAL HRF dose response. Serial twofold dilutions of PF BAL fluid were used in studies to determine the optimum dose response of PF BAL fluid in inducing β-hexosaminidase release.

Statistical Analysis

Results are expressed as mean ± SE. Statistical analysis was performed using the Student's two-tailed t-test.

RESULTS

PF subjects. The PF study was comprised of nine males and seven females with a mean age of 51 ± 9 y. Four of the PF subjects had an associated connective tissue disease (one SLE, one rheumatoid arthritis, one sjogrens syndrome, one polymyositis). In this subset of patients with PF and associated connective tissue disease, levels of histamine (715 ± 538 pg/ml) and histamine releasing factor (24.3 ± 0.6% MBMMC β-hexosaminidase release) were not significantly different from the PF patients without associated connective tissue disease (Figs. 1 and 2).

BAL histamine levels. PF BAL fluid histamine levels (1226 ± 1349 pg/ml) (n = 16) were significantly elevated compared to the non-PF comparison group of patients (118 ± 60 pg/ml) (n = 6) (Fig. 1) (p = 0.03). The PF BAL fluid histamine levels ranged from 100 to 5000 pg/ml, with all but one of the 16 PF BAL fluid histamine levels being more than the mean histamine level in the non-PF comparison group (Fig. 1).

PF HRF. HRF activity, as assessed by the MBMMC β-hexosaminidase release assay, was significantly elevated in patients with PF (mean β-hexosaminidase release 24.5 ± 12.9%, range 6.8 to 52.4%) compared to the non-PF patients (mean β-hexosaminidase release 7.9 ± 7.7%, with a range of 1.8 to 20.7%) (Fig. 2) (p = 0.004). The negative control BAL medium induced 1.5 ± 0.6% β-hexosaminidase release, whereas the antigen positive control (200 ng DNP-BSA) induced 33.7 ± 4.2% β-hexosaminidase release.

PF HRF is also able to induce release of the newly generated arachidonic acid metabolite LTC_4 from MBMMC (24.6 ± 4.2 ng LTC_4/10^6 MBMMC, range 7.9 to 76.6 ng LTC_4/10^6 MBMMC) (n = 3) (Fig. 3). In these

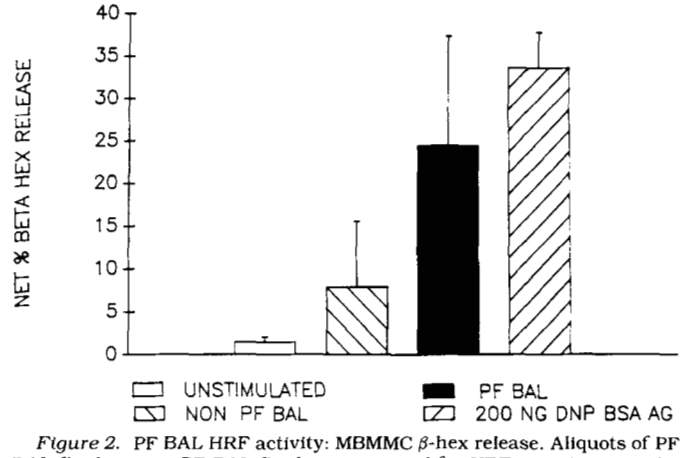

Figure 2. PF BAL HRF activity: MBMMC β-hex release. Aliquots of PF BAL fluid, or non-PF BAL fluid were assayed for HRF activity using the MBMMC β-hexosaminidase release assay. Unstimulated MBMMC β-hexosaminidase release was 1.5 ± 0.6%, whereas 200 ng DNP-BSA Ag induced 33.7 ± 4.2% β-hexoaminidase release. Results expressed as mean ± SE (n = 3).

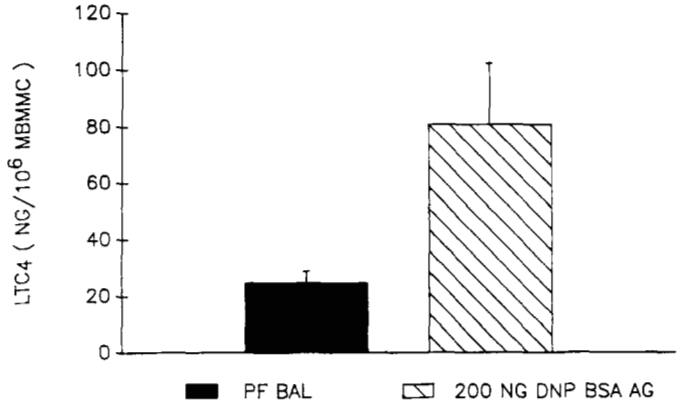

Figure 3. PF BAL-induced MBMMC LTC_4 release. Supernatants from MBMMC stimulated with either PF BAL or 200 ng DNP BSA Ag for 45 min at 37°C were assayed for LTC_4 generation using a [^{3}H] LTC_4 RIA. Non-PF BAL fluid stimulated MBMMC release 3 ± 2 ng LTC_4/10^6 MBMMC.

experiments 200 ng DNP-BSA Ag induced release of 80.9 ± 21.4 ng $LTC_4/10^6$ IgE-sensitized MBMMC. PF HRF is not species specific as it is able to induce β-hexosaminidase release from mouse mast cells (MBMMC) as well as histamine release from human basophils (mean histamine release 15.4 ± 3.1%, range 10.3 to 25.7%) (Fig. 4). PF HRF induces release of β-hexosaminidase from MBMMC via an IgE-independent mechanism (mean PF HRF β-hexosaminidase release from IgE-sensitized MBMMC 31.7 ± 5.3%, compared to 29.3 ± 7.4% release from MBMMC not sensitized with IgE) (Table I). In addition, PF HRF is able to induce β-hexosaminidase release from either IgE^+ (23.0 ± 2.3% β-hexosaminidase release) or IgE^- (21.3 ± 2.4% β-hexosaminidase release) MBMMC treated with lactic acid (Table II). In contrast, anti-IgE (10 μg/ml) induces significant β-hexosaminidase release from MBMMC sensitized with IgE (23.6 ± 2.7% β-hexosaminidase release), but not from either MBMMC cultured in vitro in the absence of IgE (1.3 ± 0.2% β-hexosaminidase release), or from IgE^+ MBMMC treated with lactic acid (4.3 ± 1.8% β-hexosaminidase release) (Table II).

PF study subject 1, whose chest x-ray demonstrated bibasilar interstitial infiltrates, provided us the opportunity to measure levels of histamine and HRF from areas of the lung with radiographically evident PF (lower lobes) and compare these to levels in areas of the lung (upper lobe) radiographically uninvolved with PF. In this patient levels of HRF, as assessed by MBMMC β-hexosaminidase release, in the radiographically uninvolved upper lobe were 12.9 ± 1.8% compared to 43.4 ± 3.7% HRF activity in the radiographically involved lower lobe. Similarly, histamine levels in the upper lobe (380 pg/ml) were less than those detected in the lower lobe (500 pg/ml). A year after the initial BAL study (BAL 1) study subject 1 had a repeat BAL (BAL 2). At this time her clinical symptoms, chest x-ray, total lung capacity, and single-breath carbon monoxide diffusing capacity were essentially unchanged. Levels of HRF (43.4 ± 3.7%, BAL 1; 33.8 ± 1.7%, BAL 2 and histamine (500 pg/ml, BAL 1; 320 pg/ml, BAL 2) remained elevated although at slightly reduced levels from the initial BAL.

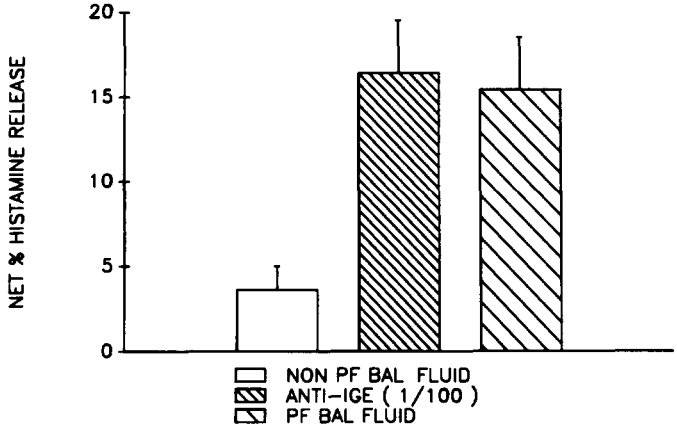

Figure 4. Aliquots of BAL fluid (from PF and non-PF study subjects) (n = 6) were assayed for their ability to induce histamine release from human blood basophils. Results expressed as mean ± SE (n = 3).

TABLE I

Interaction of PF BAL HRF with MBMMC[a]
MBMMC β-Hexosaminidase Release

IgE	PF BAL Fluid	DNP-BSA Ag
IgE^+	31.7 ± 5.3%	25.0 ± 3.7%
IgE^-	29.3 ± 7.4%	0.2 ± 0.1%

[a] Aliquots of pooled PF BAL fluid (derived from five PF patients) or 200 ng DNP-BSA Ag were tested for their ability to induce β-hexosaminidase release from IgE sensitized (IgE^+) or unsensitized (IgE^-) MBMMC. Results expressed as mean ± SE (n = 3).

TABLE II

Interaction of PF BAL HRF with lactic acid-treated MBMMC[a]

		MBMMC β-Hexosaminidase Release (Net %)	
	IgE	Lactic $acid^+$	Lactic $acid^-$
PF BAL fluid	+	23.0 ± 2.3%	26.3 ± 3.1%
	−	21.3 ± 2.4%	22.5 ± 2.0%
Anti-IgE (10 μg/ml)	+	4.3 ± 1.8%	23.6 ± 2.7%
	−	0.9 ± 0.2%	1.3 ± 0.2%

[a] Results are expressed as mean ± SE (n = 3). IgE sensitized (IgE^+) or unsensitized (IgE^-) MBMMC were incubated in the presence (lactic $acid^+$) or absence (lactic $acid^-$) of 0.01 M lactic acid, pH 3.9, for 3.5 min at 23°C. Aliquots of pooled PF BAL fluid (derived from five PF patients) or anti-IgE (10 μg/ml) were then tested for their ability to induce β-hex release from the lactic acid-treated or -untreated MBMMC.

Physicochemical characterization of HRF. PF HRF is inactivated by pronase (0.1 mg/ml), but not by RNAse, or DNAse. Heating of PF HRF to 100°C reduces HRF activity 57% whereas heating of PF HRF to 56° has minimal effect on the resultant HRF activity (Table III). The HRF in PF BAL fluid induces a dose-dependent release of β-hexosaminidase from MBMMC (Fig. 5).

Comparison of BAL HRF with recombinant human cytokines and growth factors. As PF has been associated with increased production of PDGF (30) and IGF-1 (31),

TABLE III

Physicochemical characterization of PF BAL HRF[a]

PF BAL Fluid	MBMMC β-Hexosaminidase Release (Net %)
Untreated	28.3 ± 7.1%
Heat 100°C	12.2 ± 6.1%
Heat 56°C	26.3 ± 4.8%
Pronase + PF BAL fluid	3.2 ± 0.8%
DNAse + PF BAL fluid	28.0 ± 5.0%
RNAse + PF BAL fluid	26.1 ± 3.4%

[a] Aliquots of PF BAL fluid were heated for 30 min (at 56 or 100°C) or treated with either pronase (0.1 mg/ml), DNAse (0.5 mg/ml), or RNAse (1.0 mg/ml) for 30 min. The treated or untreated PF BAL fluid was then assayed for HRF activity using the MBMMC β-hexosaminidase release assay. Results expressed as mean ± SE (n = 3) corrected for spontaneous or enzyme-treated MBMMC β-hexosaminidase release. Addition of pronase (0.1 mg/ml) to MBMMC induced 11 ± 5% MBMMC β-hexosaminidase release, whereas DNAse or RNAse alone induced <1% β-hexosaminidase release.

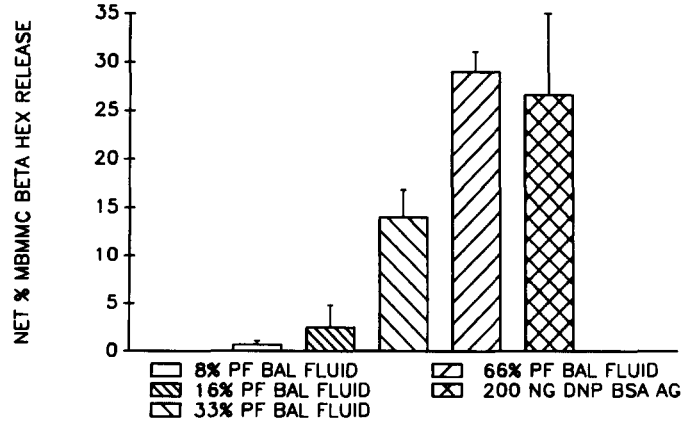

Figure 5. Dilutions of PF BAL fluid (8 to 66%) were assessed for their ability to induce MBMMC β-hexosaminidase release. Results expressed as mean ± SE (n = 3).

we assayed the effect of these growth factors on MBMMC β-hexosaminidase release. Neither of these recombinant human growth factors (PDGF 0 to 100 ng/ml, IGF-1 0 to 200 ng/ml) were able to induce β-hexosaminidase release from MBMMC. In addition as human cytokines rGM-CSF (10), IL-3 (10), and IL-1 (9) have been demonstrated to degranulate human basophils, we also compared the ability of these recombinant human cytokines to activate MBMMC. None of the human cytokines tested (GM-CSF, IL-1, TNF) or gibbon IL-3 were able to activate MBMMC in the dose range tested (0.01 to 100 ng/ml). Moreover, incubation of BAL fluid with antibodies to human GM-CSF, IL-1β, and TNF-α did not influence the PF HRF activity (Table IV).

Sephadex G-200 chromatography. Sephadex G-200 gel filtration of a 10-fold concentrated pool of PF BAL fluid from five study subjects revealed one significant peak of HRF activity with an apparent m.w. of 30,000 to 50,000 (Fig. 6). A similar peak, with m.w. of 30,000 to 50,000 was observed when gel filtration utilizing Sephadex G-75 was performed on concentrated PF BAL fluid (data not shown).

TABLE IV

Effect of neutralizing antibodies on PF HRF-induced MBMMC β-hexosaminidase release[a]

	MBMMC β-Hex Release (Net %)
1. PF BAL fluid	28.4 ± 3.0%
2. Anti-human GM-CSF	
Anti-GM-CSF (1/100)	0.7 ± 0.3%
Anti-GM-CSF (1/100) + PF BAL fluid	26.3 ± 4.0%
3. Anti-human TNF-α	
Anti-TNFα (1/100)	0.7 ± 0.3%
Anti-TNFα (1/100) + PF BAL fluid	25.5 ± 4.9%
4. Anti-human IL-1-β	
Anti-IL-1-β (1/100)	0.8 ± 0.3%
Anti-IL-1-β (1/100) + PF BAL fluid	27.0 ± 2.8%

[a] Aliquots of PF BAL fluid were preincubated for 2 h at 37°C in the presence or absence of a 1/100 dilution of the neutralizing antibody to be tested (anti-human GM-CSF, anti-human TNF-α or anti-human IL-1-β). The neutralizing antibody treated or untreated PF BAL fluid was then assayed for HRF activity using the MBMMC β-hexosaminidase release assay. The effect of each neutralizing antibody alone on MBMMC β-hexosaminidase release is also indicated. Results expressed as mean ± SE ($n = 3$). Similar results were obtained with a 1/50 dilution of neutralizing antibody.

DISCUSSION

HRF have previously been identified in biologic fluids derived by nasal lavage (20) and bronchoalveolar lavage (21, 22) in normal and allergic individuals. The HRF we have identified and partially characterized in PF BAL fluid is relatively heat stable, pronase sensitive, and has an apparent molecular mass of 30 to 50 kDa. This HRF is able to induce preformed granule mediator release from MBMMC and human basophils. This lack of species-specific activity is similar to that reported for an HRF derived from human neutrophils (17). Some cytokines such as TGF-β, are highly conserved from mouse to man (share 99% amino acid homology) (26) and as one might predict are bioactive across species barriers. In contrast, mouse and human IL-3 have only 29% amino acid homology and do not demonstrate cross species bioactivity (27, 29). The cross-species bioactivity of the HRF in PF BAL fluid allowed us to use two assays with differing target cell purity for its detection and characterization. In the human basophil histamine release assay (using a target cell population of 1 to 2% basophils) PF HRF could either be acting directly on the target basophil, or indirectly via a contaminating cell population to produce a second mediator/cytokine to act on the target basophil. However, an advantage of the MBMMC β-hexosaminidase release assay is that the purified population of MBMMC (more than 99% mast cells) allows one to exclude such an indirect effect. Using the MBMMC β-hexosaminidase release assay, we have also been able to standardize the assay and test all supernatants on the same MBMMC target cells and thus exclude the donor variability noted in assays using human blood basophils (32).

In contrast to nasal lavage HRF that induces histamine release from basophils via an IgE-dependent mechanism (20), PF HRF is not dependent on IgE for histamine release. In this respect, PF HRF is similar to the HRF derived from neutrophils (17) and mononuclear cells (32). The difference in the IgE dependence of the HRF-induced

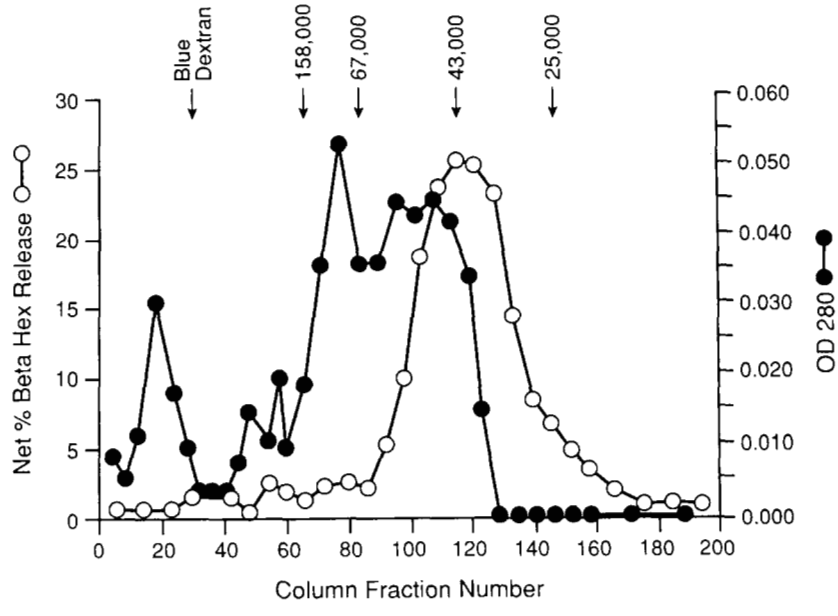

Figure 6. Sephadex G-200 chromatography of PF HRF. PF BAL fluid (10 ml) was concentrated to 1 ml using an Amicon UM2 Diaflo ultrafiltration membrane (m.w. cutoff 1000) before passage over a Sephadex G-200 column. Fractions of 1.5 ml were collected and assayed for their absorbance at 280 nm (●) and HRF activity (○) (assessed by MBMMC β-hexosaminidase release).

histamine release reported in these studies could be due to the use of different target cell populations, or different cytokines being responsible for histamine release in the respective studies. The PF HRF induces not only preformed MBMMC mediator release but also release of the newly generated arachidonic acid metabolite LTC_4. In analogous studies, human rIL-3 has been shown to induce the release of 78 to 326 ng $LTC_4/10^6$ human basophils (25 to 98% basophil purity) (33). The HRF we have identified is unlikely to be IL-3 as IL-3 is not bioactive across species barriers (27, 29) and gibbon IL-3 did not induce mediator release from MBMMC.

The cellular sources of known histamine releasing factors include lung macrophages (12, 13), neutrophils (17), lymphocytes (14, 15, 19), mononuclear cells (18), and platelets (16). Current theories of PF suggest that immune complexes (23, 24, 34) activate alveolar macrophages to produce polypeptide growth factors (35) that stimulate fibroblast proliferation. Alveolar macrophages from patients with PF have been shown to spontaneously produce polypeptide growth factors including PDGF (30), macrophage-derived growth factor (36), and IGF-1 (31). The HRF we have identified in PF BAL fluid is unlikely to be IGF-1 or PDGF as neither of these recombinant human cytokines induced MBMMC β-hexosaminidase release. Additionally antibodies to human GM-CSF, IL-1β, and TNF-α did not influence the HRF activity in PF BAL fluid, suggesting that they are not responsible for the HRF activity found in patients with PF. Lung macrophages derived from surgical specimens of lung tissue from patients with lung carcinoma have been shown to spontaneously secrete HRF with m.w. of <2,000 (12) and 18,000 (13). In contrast to PF HRF these lung macrophage derived HRF are dependent on IgE for histamine release. Macrophage-CSF is a cytokine whose molecular mass (47 to 76 kDa) is similar in range to that of the HRF we have partially characterized in PF BAL fluid. However, human M-CSF does not induce histamine release from human basophils (37), whereas PF HRF does. In addition, the PF HRF we have identified (m.w. 30,000 to 50,000) does not appear to be similar in size to HRF previously described such as neutrophil derived HRF (m.w. 1400 to 2300) (38) or monocyte derived HRF (m.w. 23,000 and 29,000) (32). However, it is of interest that when the biochemically best characterized HRF (monocyte derived HRF), is purified by sequential gel filtration HPLC, Accell-QMA anion exchange and SDS gel electrophoresis, homogenous bands of two purified HRF (17 and 41 kDa) are apparent (39). Whether the 30 to 50 kDa HRF we have identified is analogous to the 41-kDa monocyte derived HRF (39) will require further biochemical and molecular characterization of the PF HRF.

The functional significance of the PF HRF to pulmonary fibrosis will also require further study. It is possible that the presence of PF HRF is merely an epiphenomenon indicating activation of an airway inflammatory cell (macrophage, neutrophil, or other airway cell) and that the HRF-induced mast cell mediator release does not influence the ensuing pulmonary fibrosis. However, it is also possible that the mast cell-derived mediators could play a role in either exacerbating or ameliorating the extent of the macrophage and neutrophil-induced pulmonary fibrosis. In this regard histamine present in the PF BAL fluid could increase alveolar epithelial cell permeability and allow access of alveolar macrophage and neutrophil-generated cytokines/mediators to the lung interstitial space (23, 24). In addition, histamine stimulates fibroblast proliferation (40), and mast cell cytoplasmic granule-derived tryptase activates collagenases that degrade type III collagen (41) (type I and type III collagen are the major secretory products of lung fibroblasts) (42). The newly generated mast cell-derived mediator LTC_4 has been shown to stimulate normal human skin fibroblast proliferation when endogenous synthesis of fibroblast-generated PG are blocked (43). As LTC_4 is not only generated by MBMMC but also by human lung mast cells this may be pertinent to the pathogenesis of PF. The functional significance of mast cells to fibrosis is also suggested from coculture experiments of MBMMC and mouse fibroblasts, in which the cocultured fibroblasts have an increased proliferative capacity and synthesize increased amounts of collagen and proteoglycan compared to fibroblast cultures not exposed to mast cells (44).

Although the above mentioned mast cell-derived mediators might exacerbate the extent of the pulmonary fibrosis, the ability of another mast cell product, heparin, to suppress murine fibroblast proliferation (45), if confirmed in human lung fibroblasts, would suggest a role for the mast cell in ameliorating the extent of the pulmonary fibrosis. Human lung mast cells contain approximately 5 μg of heparin per 10^6 mast cells, stored preformed in mast cell cytoplasmic granules, and released upon mast cell activation (46). Thus, several potential mechanisms can be postulated to explain a role for the mast cell in fibrosis. The identification of the PF HRF will allow further studies to characterize the molecular structure and cellular sources of this HRF. In addition the use of PF HRF levels as a marker of disease activity will need to be addressed in prospective studies of patients with PF.

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