

Comparison of Mononuclear Cell and B-Lymphoblastoid Histamine-Releasing Factor and Their Distinction from an IgE-Binding Factor

MARY HAAK-FRENDTSCHO,* MARIE SARFATI,† GUY DELESPESE,† AND ALLEN P. KAPLAN*,¹

*The *Division of Allergy, Rheumatology, and Clinical Immunology, Department of Medicine, State University of New York, Health Sciences Center, Stony Brook, New York, and the †University of Montreal, Notre Dame Hospital Research Center, 1560 E. Sherbrooke Street, Montreal, Quebec, Canada H2L 4M1*

We have characterized the histamine-releasing factors (HRF) from a B-lymphoblastoid cell line (RPMI 8866), compared it to mononuclear cell-derived HRF, and distinguished these from the IgE-binding factor produced by RPMI 8866. The B-cell-derived HRF fractionates at molecular weights of 90,000, 70,000, and 12-15,000 while mononuclear cell HRF has a major component at 24-26,000. The isoelectric points for B-cell HRF are 6.2-6.3 and 6.6-6.8 in contrast to 6.9 and 7.3 for mononuclear cell-derived HRF. The kinetics of histamine release by either HRF was the same, with half-maximal release in 5-10 min, unlike the rapid release caused by anti-IgE. Since HRF has been reported to be an IgE-binding factor, we screened column fractions for the IgE-binding factor secreted by RPMI 8866; this is known to be a shed low affinity IgE receptor. The chromatographic pattern and isoelectric point of this IgE-binding factor does not correspond to HRF; the purified IgE-binding factor had no significant histamine releasing activity on human basophils, and neither source of HRF was reactive in a radioimmunoassay to the IgE-binding factor. Our data suggest that HRF is quite heterogeneous and varies in physiochemical properties depending upon the cell source. The molecular relatedness (or nonrelatedness) of those HRFs is unclear; however, our data indicate that although HRF or fractions therefrom may bind to IgE as has been reported, it is unrelated to the low affinity IgE receptor. © 1988 Academic Press, Inc.

INTRODUCTION

Histamine-releasing factor(s) (HRF) has been shown to be released upon activation of human T cells (1, 2), alveolar macrophages (3), and mixtures of mononuclear cells (4-6). Constitutive release of HRF has been reported from human platelets (7) and neutrophils (8, 9). Since these factors have not yet been purified to homogeneity, nor are antisera available, it is unclear whether the various differences in physiochemical properties reported are due to heterogeneity of a single gene product, or represent multiple proteins which share common bioactivity. HRF derived from alveolar macrophages has been reported to function by virtue of its ability to bind IgE (10); for example, cell responsiveness can be inhibited by desensitization with anti-IgE or removal of cell-surface IgE with acid treatment. Yet only some basophils respond to HRF even though they release with anti-IgE (10), and some form of IgE heterogeneity has been suggested as the explanation.

¹ To whom correspondence should be addressed.

In this manuscript we compare preparations of HRF derived from human mononuclear cells with HRF secreted by the B cell line RPMI 8866 and demonstrate that their size and charge properties appear to be different. Further, RPMI 8866 is a source of IgE-binding factor which can modulate IgE synthesis (11). We have compared the properties of both sources of HRF with this IgE-binding factor and demonstrate that they are distinguishable functionally and immunologically.

MATERIALS

Piperazine-*N,N*-bis[2-ethanesulfonic acid]-1,4-piperazinediethanesulfonic acid (Pipes) buffer, Hepes buffer, *N*-methyl histamine, histamine dihydrochloride, disodium ethylene diamine tetraacetic acid (EDTA), polyethylene glycol (PEG) 8000, dextran, and glucose (Sigma Chemical Co., St. Louis, MO); chloroform, 10 *N* NaOH, and perchloric acid (Fisher Scientific Co., Springfield, NJ); RPMI 1640, fetal calf serum (FCS), and L-glutamine (GIBCO, Grand Island, NY); TSK 3000 GF-HPLC column (Varian, CA); 1.5 ml microfuge tubes and polypropylene screw cap tubes (Sarstedt, Princeton, NJ); tritiated [³H]histamine, *S*-[¹⁴C]adenosyl methionine and Aquasol scintillation counting fluid (New England Nuclear Corp., Boston, MA); and YM 5 membranes (Amicon Corp., Danvers, MA) were purchased as indicated.

RPMI 8866 cell line

The RPMI 8866 cell line was obtained from Dr. D. J. Volkman (SUNY, Stony Brook, NY). The line was maintained in RPMI 1640 tissue culture medium containing 10% FCS and L-glutamine. The cells were seeded at $0.2\text{--}0.4 \times 10^6$ cells/ml in the absence of FCS when cultured for HRF. The cells were cultured at 37°C with 5% CO₂ in Falcon 75-cm² tissue culture flasks at all times. Cell viability was $\geq 98\%$ by trypan blue exclusion in the cultures with FCS and $\geq 85\%$ viable in the serum-free cultures when the supernatants were harvested for HRF. The doubling time was 18–20 hr with FCS and 36 hr without.

Preparation of RPMI 8866 supernatants

RPMI 8866 cells were resuspended at $0.2\text{--}0.4 \times 10^6$ cells/ml in Falcon 75-cm² tissue culture flasks containing 50–75 ml/flask. The serum-free supernatants were usually cultured for approximately 72 hr. The conditioned medium was harvested by centrifugation at 500g for 20–30 min and then clarified by superspeed centrifugation at 40,000g for 15 min. The pooled supernatants were dialyzed overnight in Spectaphor 3 dialysis tubing at 4°C against one change of Hepes-buffered saline, pH 7.4. The supernatants were then concentrated by ultrafiltration using Amicon YM5 membranes.

Preparation of mononuclear cell supernatants

Leukapheresis packs of approximately 200 ml were diluted 1:1 in Hepes-buffered saline (HBS) (4 mM Hepes in 0.15 M sodium chloride). Twenty-five milliliters of cellular suspension was layered over 15 ml of Ficoll-Hypaque in Falcon 50-ml polypropylene tubes. The suspension was centrifuged for 15 min at

1000g. The interface was aspirated, pooled, washed two times with HBS, and centrifuged at 400g for 12 min. After a second wash, the mixture was centrifuged at 150g for 5 min to deplete platelets. The remaining mononuclear cell pellet was resuspended at a concentration of 5×10^6 cells/ml in RPMI 1640 tissue culture medium containing penicillin (100 U/ml) and streptomycin (50 μ g/ml). Cell viability was 98% by trypan blue exclusion. SK/SD was added to the cell cultures at $13 \text{ U}/10^6$ cells. The cells were then cultured 15 to 17 hr at 37°C with 5% CO_2 in Falcon 75-cm² tissue culture flasks. This time was optimal when a time course was performed between 6 and 72 hr. At the end of the culture period, the conditioned media was pooled and centrifuged at 400g for 15 min. The supernatant was dialyzed for 24 hr at 4°C against one change of HBS and was concentrated by ultrafiltration (Amicon YM5 membranes; Amicon Corp.).

Basophil isolation and histamine release

Venous blood from normal healthy donors was anticoagulated with 0.2 M EDTA and then diluted 5:1 with a 3% dextran–3% glucose phosphate-buffered saline solution. The cells were allowed to sediment for 40 min at 37°C in Falcon 50-ml polypropylene conical tubes. The supernatant was aspirated and washed twice, with Hepes-buffered saline containing 0.3% human serum albumin (HBS–HSA), by centrifugation at 300g for 10 min. The cell pellets were resuspended in HBS–HSA made up in 50% D_2O containing 2 mM CaCl_2 and 2 mM MgCl_2 at 30×10^6 cells/ml. Cell viability was $\geq 98\%$ by trypan blue exclusion. Three million cells were added to 1.5-ml microfuge tubes containing 180 μ l HBS–HSA in 50% D_2O with Ca^{2+} and Mg^{2+} and 20 μ l of the test sample. The microfuge tubes were incubated for 40 min in a 37°C waterbath. Boiling cells for 10 min was used to determine total histamine content in the basophils. The background spontaneous release was determined by adding 20 μ l of HBS–HSA in place of the test sample. Tubes were centrifuged at 650g for 5 min and the cell-free supernatants were collected. Samples that were not immediately assayed for histamine content were stored at -20°C and assayed within a week.

Histamine assay

The radioenzyme assay for histamine was performed using a modification of the method of Beaven *et al.* (12). Supernatants from the basophil-release assays were added to 15 ml polypropylene screw cap tubes at 50 μ l/tube with 300 μ l 0.025 M Pipes buffer containing 1 mM EDTA. Next 10 μ l of [³H]histamine (18,000–20,000 cpm) and 100 μ l Pipes buffer containing S-[¹⁴C]adenosyl methionine (110,000–120,000 cpm) and 3 μ l rat kidney histamine N-methyl transferase were added. The samples were centrifuged at 300g for 3–5 min to bring all components into solution and then incubated for 90 min in a 37°C waterbath. The reaction was stopped by either freezing or by addition of 200 μ l of freshly made 1.0 M N-methylhistamine in 0.4 M perchloric acid. Next, 0.2 ml of 10 N NaOH and 4 ml of chloroform were added to each tube. The tubes were vortexed for 5 min, spun at 175g for 5 min, and the aqueous phase was aspirated. A second extraction was performed in a like manner using 3.3 N NaOH. After complete evaporation, the samples were counted in 8 ml of Aquasol using a Beckman LS 7500 beta counter programmed

to calculate dpm ratios of the ^3H and ^{14}C windows. The unknown samples were assayed in duplicate and computed using a standard curve of known histamine values.

Gel filtration chromatography

Concentrated supernatants were applied to a column (1 cm $\times$ 120 cm) of Sephadex G-75 Superfine and eluted with phosphate-buffered saline (PBS), pH 7.4. One milliliter fractions were collected. The column was calibrated with ovalbumin (43,000 mol wt), chymotrypsin (25,700 mol wt), and lysozyme (14,300 mol wt). This procedure was always done at 4°C.

Protein determinations

Protein not determined directly by optical density at 280 nm was determined by a modification of the Bradford reaction (13). The microBradford was performed using 5–50 μl of sample with 100 μl PBS and 200 μl Bradford reagent in each microtiter plate well. The microtiter plates were read at $R = 1$ (405 nm) and $T = 4$ (540 nm) with a Dynatech MR580 ELISA reader. Bovine serum albumin (BSA) was used as the protein standard.

GF-HPLC

The operating parameters were controlled using a Waters HPLC system with detection of proteins by absorbance at 280 nm. The separations were carried out at an ambient temperature on a TSK 3000 column. The isocratic runs had a constant flow rate of 0.5 ml/min using a mobile phase of PBS with 0.3% PEG 8000, pH 7.4. The column was calibrated prior to each run using proteins of known molecular weights.

Isoelectric focusing

Concentrated supernatants (50–100 μl) were applied to 3 mm $\times$ 10 cm urea tube gels containing ampholines. The gels were focused overnight for a total of 10,000 V-hr, then sliced into 4-mm sections, eluted with 1 ml H_2O for 2 hr, and the pH was determined using a PHM62 standard pH meter. The eluates were then dialyzed in Spectraphor 3 dialysis tubing against 10 liter of 0.1 M ammonium bicarbonate at 4°C overnight. The dialyzed material was collected into individual polypropylene tubes, frozen to -70°C , and lyophilized. The freeze-dried material was resuspended into HBS-HSA for use in the basophil release assay.

Purified IgE-BF

The IgE-BF was purified by affinity chromatography, DEAE-ion exchange, reverse-phase HPLC, and chromatofocusing as previously described (14).

Monoclonal antibody

The Mab specific to human lymphocyte IgE receptors (FcER) has been described earlier (15). The antibody employed in this study was MabER 135, an IgG1 K derived from clone 208.25.A.4.3/135. It was purified from ascites fluid by salt

precipitation, ion exchange, and gel filtration. The purity was assessed by polyacrylamide gel electrophoresis and Coomassie blue staining.

Radioimmunoassay for the detection of IgE-BFs

Polyvinyl microtiter plates were incubated overnight with 0–10 ml of MabER 176 (10 $\mu\text{g/ml}$ in 0.01 M bicarbonate buffer, pH 9); the wells were blocked with H-FCS Hepes-buffered salt solution containing 10% fetal calf serum (H-FCS) (buffered with Tris-HCl, pH 7.2, and containing 0.1% sodium azide) for 2 hr at room temperature and finally washed with PBS. One hundred microliters of test sample (diluted in H-FCS, when indicated) was added to the wells and after 1 hr at room temperature these were washed and supplemented with 100 μl ^{125}I -MabER 135 ($2.5\text{--}3 \times 10^5$ cpm, in 100 μl of H-FCS). After overnight incubation, wells were washed and counted in a gamma counter. MabER was radioiodinated by the chloramine T method to a specific activity of 15,000–20,000 cpm/ng; the labeled material was passed through a 0.22 μm Millipore filter before the assay. Samples were tested in duplicate unless otherwise indicated.

RESULTS

A time course of production of HRF from RPMI 8866 cells was performed in the absence of serum to facilitate purification of the factor. Flasks of cells were cocultured and harvested in pairs after 4, 24, 48, 72, and 96 hr of incubation. The cell-free supernatants were concentrated 15-fold and assayed for histamine-releasing activity as shown in Fig. 1. The results shown are the mean of duplicates for each time point. HRF is first detectable by 24 hr, peaks at 72 hr, and declines thereafter. This was consistent in three separate experiments although the mag-

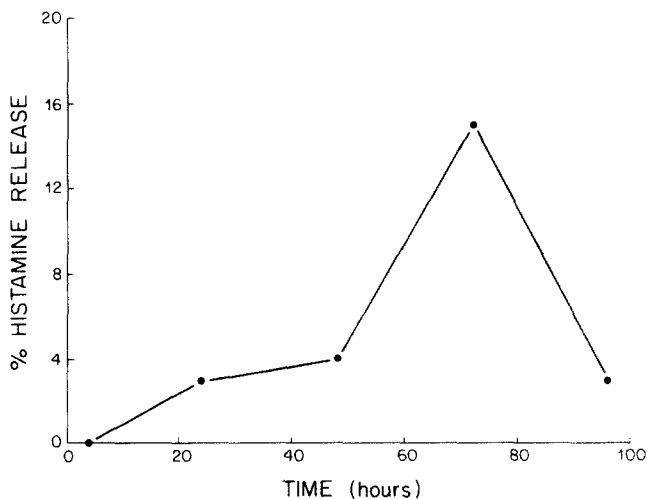


FIG. 1. Time course of HRF production by the cell line RPMI 8866 in serum-free conditions. The average of duplicate flask following 4, 24, 48, 72, and 96 hr in culture demonstrate a peak of activity at 72 hr and declining thereafter.

nitude of histamine release obtained was variable. When serum is present (data not shown), the peak is at 24–36 hr.

The concentrated conditioned medium was then subjected to gel filtration on Sephadex G-75 Superfine and assayed for HRF as illustrated in Fig. 2. Fractions 26 and 28 were positive (27 was zero) suggesting two close peaks of activity whose molecular weights were estimated to be 70,000–100,000. A third major peak was seen in fractions 83 and 84 at molecular weights of 12,000–15,000. The same column fractions were assayed for IgE–BF which is known to be constitutively produced by RPMI 8866 (11). The radioimmunoassay revealed at least four separate areas of activity all but one of which were totally separate from the elution profile of HRF. The first IgE–BF peak overlaps the second HRF peak but there was no detectable IgE–BF in the first and last HRF peak.

In an attempt to achieve better separation of the first two HRF peaks, concentrated supernatant from RPMI 8866 was loaded onto a TSK 3000 GF–HPLC column. HRF was clearly resolved into two discrete peaks at molecular weights 90,000–100,000 and 70,000. The lower molecular weight form was confirmed at 13,000 Da. The IgE–BF RIA of the GF–HPLC again revealed areas of overlap of the two activities as seen in Fig. 3a. Nevertheless, the profile of the two activities are completely different, thus they are likely to be two different gene products. We also wished to test mononuclear cell-derived HRF in a similar fashion. This result is shown in Fig. 3b. A major peak of activity is seen at molecular weight 24,000–26,000 and a small peak of activity is seen at the void volume. The amount

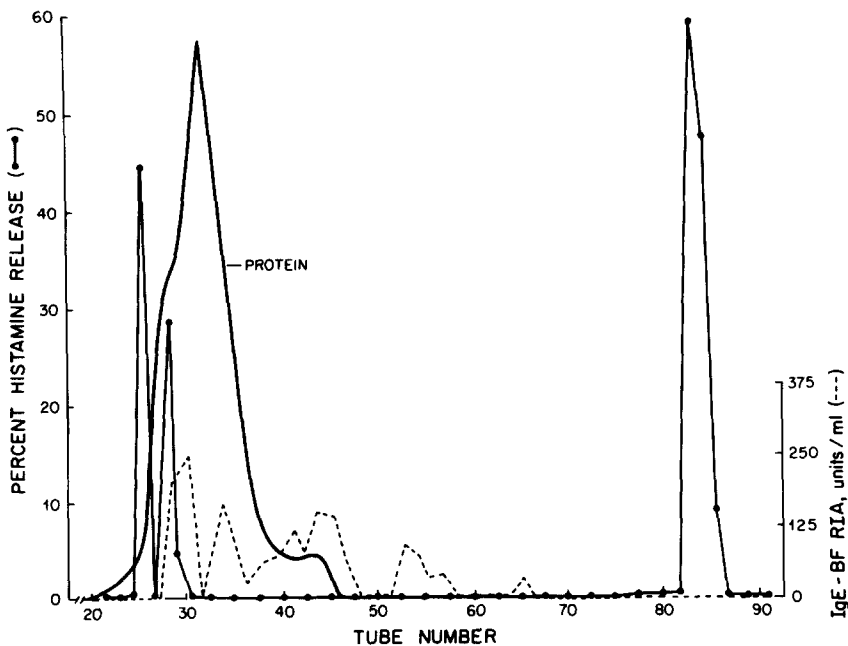


FIG. 2. G-75 Superfine column of concentrated RPMI 8866 conditioned medium. The protein elution profile is represented by the solid line, IgE–BF by the continuous hatched line, and HRF activity is expressed as the percentage of histamine release (●).

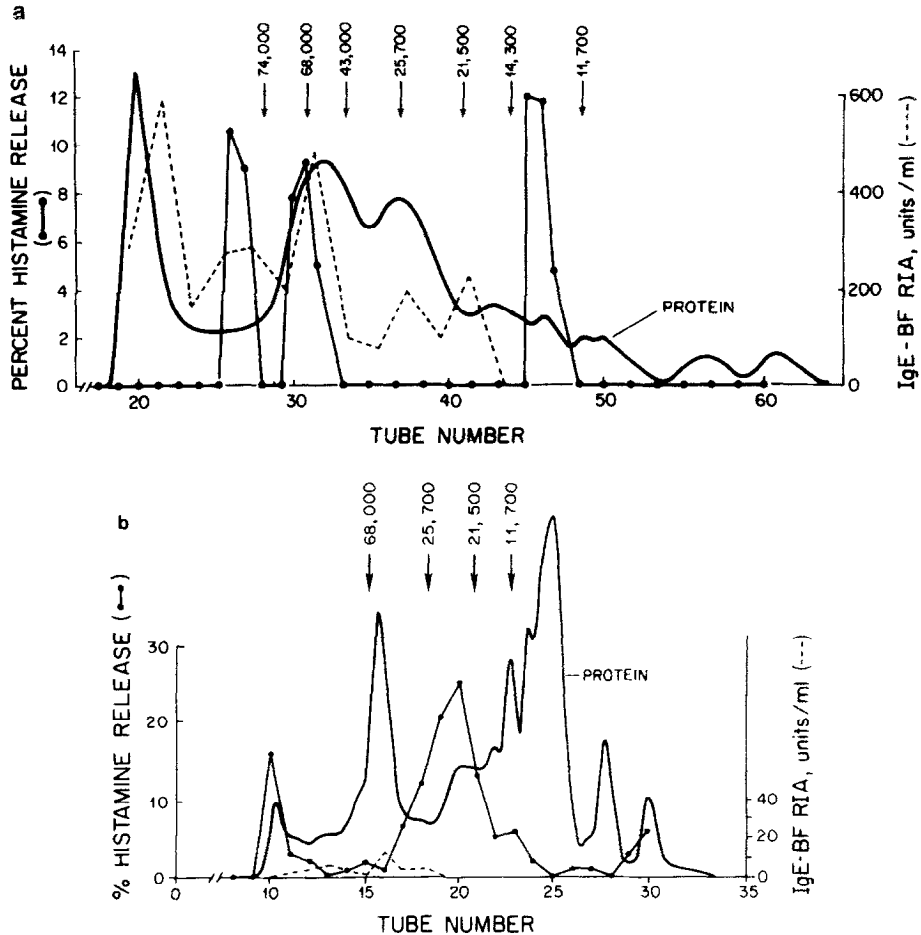


FIG. 3. (a) GF-HPLC chromatograph of concentrated RPMI 8866 supernatant. Protein was continuously monitored at 280 nm shown by the solid line. The dashed line represents IgE-BF, and HRF activity is shown as the percentage of histamine release (●). (b) Mononuclear cell HRF GF-HPLC. The elution profile of IgE-BF is clearly distinguishable from HRF.

of IgE-BF secreted by mononuclear cells is far less than that seen with RPMI 8866 and only trace quantities of IgE-BF were detected. Here too, the elution profile has no relationship to the peaks of HRF activity and indicate that IgE-BF is not responsible for the histamine-release activity. In addition, we determined the histamine-releasing ability of IgE-BF which had been purified to homogeneity from RPMI 8866. Serial dilutions (15-fold) were assayed using basophils from four separate donors. There was a mean of 7% histamine release with the highest concentrate tested ($0.05 \mu\text{g/ml} = 115,000$ units IgE-BF) and all other dilutions were negative. The highest unit of IgE-BF in the mononuclear cell HRF chromatograms (Fig. 3b) is 20 units. Thus IgE-BF cannot account for the histamine-releasing activity seen. We also attempted to inhibit the ability of HRF to cause basophil histamine release by a monoclonal antibody to IgE-BF, Mab 135. As

shown in Table 1, this antibody did not itself cause histamine release and it had no effect upon basophil histamine release by Con A, *f*-Met-Leu-Phe, or HRF. Further, no inhibition of HRF was detected whether the antibody was first incubated with factor or cells.

Figures 3a and b suggested that the various forms of HRF obtained from RPMI 8866 have a different molecular weight than HRF derived from mononuclear cells. We next contrasted the isoelectric points of these two sources of HRF as shown in Figs. 4a and b. Equal HRF activity was applied in each instance. The RPMI 8866-derived HRF had isoelectric points at 6.2–6.3 and 6.6–6.8 while mononuclear cell HRF was found at 6.9 and 7.3 (6). It should be noted that B cell HRF was less stable than mononuclear cell HRF and was denatured by isoelectric focusing. Thus a low yield of activity was obtained. Nevertheless the above *pI*'s were reproducible in three separate experiments while in two others, no activity was recovered. Further, the *pI*'s of either HRF are very different than that of IgE–BF which is pH 4.4–5.0 (15, 16).

DISCUSSION

Histamine-releasing factors have been described from human T cells (1, 2), alveolar macrophages (3), platelets (7), and neutrophils (8, 9). It is not known whether these HRFs are the same gene products or represent multiple molecules that share a function. We have previously partially purified an HRF from human peripheral blood mononuclear cells; it had a molecular weight of 28,000–30,000 and two charge forms isoelectric at 6.9 and 7.3 (6). The cell source(s) of this HRF was not specified. In the course of these experiments, a variety of cell lines were

TABLE 1
MONOCLONAL ANTIBODY TO IgE–BF, MabER 135, HAS NO EFFECT ON HISTAMINE RELEASE

			Percentage release
HRF + cells			24
Mab 135 10 μ g/ml + cells			1
1 μ g/ml + cells			1
0.1 μ g/ml + cells			0
Mab 135 10 μ g/ml + cells	Con A 1 ng/ml		82
	Con A 1 ng/ml		85
	Con A 1 ng/ml		86
Mab 135 10 μ g/ml + cells	fmlp 2×10^{-4} M		17
	fmlp 2×10^{-4} M		21
	fmlp 2×10^{-4} M		10
Mab 135 10 μ g/ml + cells	HRF		32
	HRF		34
	HRF		30
	HRF		33
Mab 135 10 μ g/ml + HRF	cells		23
	cells		18
	cells		24

tested for constitutive and induced (with SK/SD, PHA, or Con A) production of HRF. Cell line RPMI 8866 was positive for constitutive production although the quantity made is variable even when optimal culture conditions are maintained. Nevertheless it was of interest to characterize this HRF and compare it to the mononuclear cell-derived product. It is clear that these differ in physicochemical properties including molecular weights (Figs. 3a, b) and isoelectric points (Figs. 4a, b).

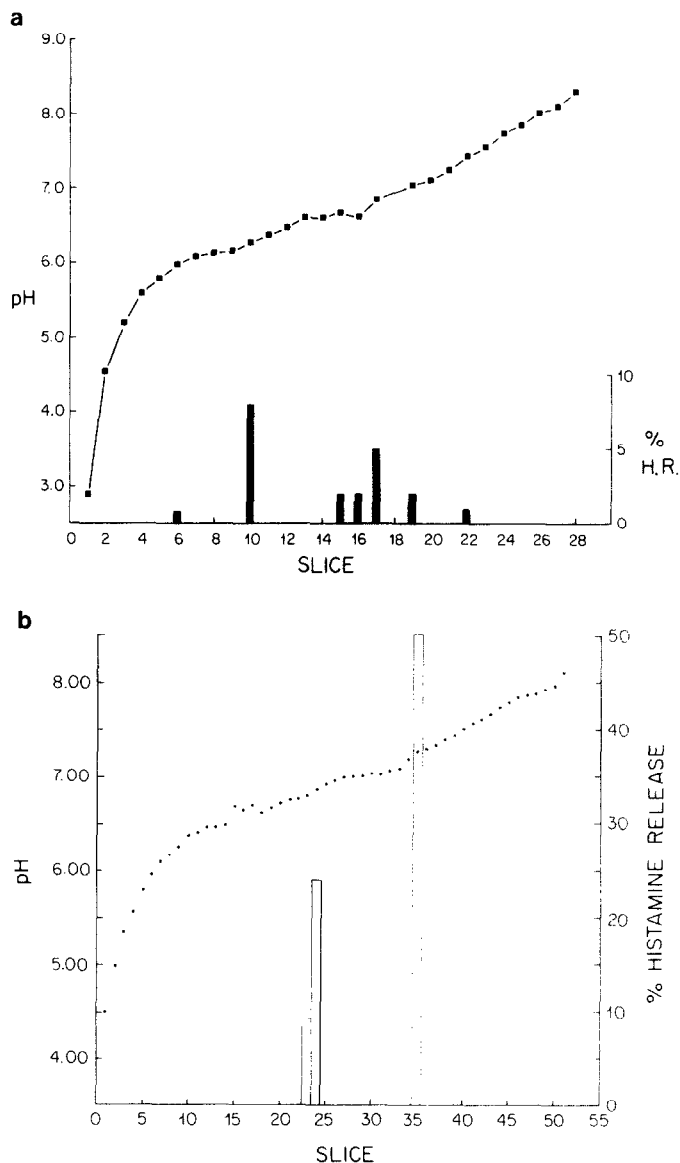


FIG. 4. (a) Two isoelectric points of RPMI 8866-derived HRF at pH 6.2 and 6.6–6.8 with poor recovery of the bioactivity. In contrast, nearly 100% of the mononuclear cell-derived HRF activity is recovered, as illustrated in (b) with pI 's of pH 6.9 and 7.3.

Other workers have demonstrated that HRF derived from alveolar macrophages is an IgE-binding factor (10) and may therefore act upon basophils and mast cells by cross-linking cell-surface IgE. Among the described IgE-binding factors are proteins which have been shown capable of regulating IgE synthesis (11); these have recently been shown to be low affinity IgE receptors that have shed from the cell surface. Cells possessing such low affinity IgE receptors include macrophages (16, 17), eosinophils (17, 18), B cells (16), platelets (18, 19), and subpopulations of T cells (16). The cell line RPMI 8866 is one of the major sources of human IgE-binding factor. The protein has been purified using IgE affinity chromatography, monoclonal antibody has been prepared, a radioimmunoassay has been developed, the gene has been cloned, and recombinant material is available. Using the RIA for the IgE-binding factor we have demonstrated that it is very prominent in the RPMI 8866 supernatants, but only trace quantities are in the mononuclear cell-derived supernatant. In either case, its elution profile by standard or HPLC gel filtration is clearly distinguishable from HRF. Further, little or no HRF-like activity was found using affinity purified or recombinant IgE-binding factor. This is consistent with a recent report that this factor can augment ongoing histamine release but cannot initiate it (20).

We have not addressed the question herein whether the mononuclear cell-derived HRF does or does not bind to IgE or act via an IgE-dependent mechanism. However we have distinguished it from one well-characterized human IgE-binding factor. Although HRF derived from mononuclear cells and RPMI 8866 appears to be different, monospecific antisera and, perhaps, amino acid sequence data will be needed to determine their relatedness in terms of antigenicity and as gene products.

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