

RESPONSIVENESS TO HUMAN MONONUCLEAR CELL-DERIVED HISTAMINE-RELEASING FACTOR

Studies of Allergic Status and the Role of IgE

MARIA LUISA BAEZA, MARY HAAK-FRENDSCHO, STEVEN SATNICK, AND ALLEN P. KAPLAN¹

From the Division of Allergy, Rheumatology, and Clinical Immunology, Department of Medicine, SUNY-Stony Brook, Health Sciences Center, Stony Brook, New York 11794

Human mononuclear cell-derived histamine-releasing factor (MNC-HRF) has m.w. of 29,000 and 23,000 and two charge species isoelectric at pH 6.9 and 7.3. Minor forms of HRF are also seen at m.w. of 12,000 to 15,000 and 60,000 to 80,000. In this manuscript we have further defined the response of human basophils to MNC-HRF in terms of atopic status of cell donors and the role of IgE antibody. We found that basophils of allergic persons, nonallergic controls, or donors who have positive skin tests but no symptoms, cannot be distinguished based upon responsiveness to HRF. Further, the response of basophils that have been desensitized to anti-IgE is retained when they are subsequently stimulated with HRF derived from human MNC. Thus an interaction with cell-surface IgE to cause histamine release is not evident. Nevertheless, one of two IgE myeloma proteins tested were capable of binding HRF and such binding could be utilized as a purification step. We conclude that MNC-HRF is heterogeneous in terms of size, charge, and affinity for a subpopulation of IgE. Histamine release is more prominent in atopic individuals but does not appear to require an interaction with IgE.

Human HRF² is a cytokine which causes histamine release from basophils (1) and, to a lesser degree, from mast cells (2). It can be produced from a variety of sources including T lymphocytes (3, 4), monocytes, alveolar macrophages (5), and platelets (6) and can be derived from nasal washings or neutrophils without stimulation (7, 8). There is general agreement that basophil responsiveness to HRF is not universal and a variety of studies have tried to define parameters that can predict which subgroups are responders and nonresponders and what cell surface property defines responsiveness. Prior studies have demonstrated that human basophil responsiveness to HRF derived from alveolar macrophages is dependent upon IgE antibody (7, 9); the response is independent of the

quantity of cell surface IgE and reflects heterogeneity of the IgE molecule, i.e., some IgE responds to HRF and some does not (7). Further, nonresponder basophils can be stripped of their IgE and made responsive by reconstitution with responder IgE (7). Implicit in this is the notion that HRF binds to IgE, but this has never been shown. Another approach is to look at allergic (atopic) vs nonatopic patients and define responsiveness. When this was examined, by using platelet-derived HRF or nasal washings, nonatopics were nonresponders, whereas about 50% of atopics responded (10, 11). Thus it appeared that the responder IgE is restricted to a subgroup of allergic individuals.

We have partially characterized MNC-HRF and found a major form at a m.w. of 30,000, and two charge forms isoelectric at pH 6.9 and 7.3 (1). Responsiveness to HRF appeared to vary with the ability of donor basophils to respond to anti-IgE (12). However, we considered the possibility that this is a property intrinsic to donor basophils in terms of responsiveness to many secretagogues, rather than necessarily indicating a dependence on IgE. In this study we have reexamined three aspects of the problem. We define HRF responsiveness in terms of allergic vs nonallergic patients, we examine the ability of HRF to bind to two IgE myeloma proteins available to us, and we report results germane to the role of cell surface-bound IgE and in vitro responsiveness to HRF.

MATERIALS AND METHODS

Polypropylene tubes, ammonium acetate (HPLC grade), ammonium hydroxide, sodium hydroxide, and propanol (Fischer Scientific, Springfield, NJ); Ficoll-Hypaque, Sepharose 6B beads (Pharmacia Uppsala, Sweden); SK/SD (Lederle Laboratories, Pearl River, NY); RPMI 1640 with L-glutamine, Dulbecco's PBS (hereafter referred to as PBS) (GIBCO, Grand Island, NY); Pipes, D₂O, human serum albumin, EDTA, sodium phosphate monobasic, sodium chloride, human IgG, potassium thiocyanate, histamine hydrochloride, Tris hydrochloride, HEPES (Sigma, St Louis, MO); Si 300 polyol gel filtration-HPLC columns (7.1 × 500 mm) with 5 μm particle size (Serva Fine Biochemicals, Westbury, NY); 0.75 × 60 cm TSK 3000 SW Gel Filtration Column (Variant, Sugar Land, TX); YM 100 and YM5 membranes (Amicon Corp., Danvers, MA); skin test allergens (Greer Lab., Lenoir, NC), and goat anti-human IgE (Kirkegaard & Perry Lab, Gaithersburg, MD) were purchased as indicated. The IgE myeloma PS serum was kindly provided by Dr. K. Ishizaka (Baltimore, MD) and IgE myeloma ADZ was provided by Drs. Guy Delespesse and Sarfati, Montreal, Canada.

The human rIL-3 and GM-CSF culture supernatants from COS 7 cells, harvested 4 days after transfection, were the generous gift of Drs. Ken-ichi and Naoko Arai (DNAX, Palo Alto, CA).

Cell source. Donors of leukopheresis packs were obtained from two sources; either normal volunteers after informed consent or patients undergoing cytopheresis according to an experimental protocol for the treatment of multiple sclerosis. The molecular charac-

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¹ To whom correspondence and reprint requests should be addressed.

² Abbreviations used in this paper: HRF, histamine-releasing factor; SK/SD, streptokinase/streptodornase; hrGM-CSF, human recombinant granulocyte-macrophage CSF; MNC-HRF, mononuclear cell-derived histamine-releasing factor; D₂O, deuterium oxide; HBS-HSA, HEPES-buffered saline-human serum albumin.

terization of the HRF described herein did not differ according to the source and the yields of activity were comparable.

Preparation of MNC supernatants. Leukopheresis packs of approximately 200 ml were diluted to 360 ml in PBS. Thirty milliliter of cellular suspension were layered over 15 ml of Ficoll-Hypaque in Falcon 50-ml polypropylene tubes. The suspension was centrifuged for 15 min at $1000 \times g$. The interface was aspirated, pooled, the cells washed twice by resuspension in PBS followed by centrifugation at $400 \times g$ for 12 min. After the second wash, the cells were resuspended in PBS and platelets depleted by centrifugation for an additional 7 min at $150 \times g$. The remaining leukocyte pellet was resuspended at a concentration of 25 to 45×10^6 cells/ml with RPMI 1640 tissue culture medium and 13 U of SK/SD per 10^6 cells. The cells were then cultured 16 to 19 h at 37°C with 5% CO_2 in Falcon 50-ml conical polypropylene tubes. At the end of the culture period the conditioned media were pooled, centrifuged at $400 \times g$ for 15 min, and then clarified by centrifugation at $38,000 \times g$ for 20 min. The supernatants were ultrafiltered by using an Amicon YM100 membrane and then concentrated 50-fold with an Amicon YM5 membrane.

Basophil isolation and histamine release. After informed consent of the subject, peripheral blood was obtained by venipuncture. The cells were prepared by dextran sedimentation as described previously (13). The leukocyte-containing plasma layer was washed twice with HBS-HSA and resuspended in HBS-HSA containing 2 mM CaCl_2 , 2 mM MgCl_2 , and 50% D_2O . One hundred microliters of cell suspension were incubated with 170 μl of the aforementioned buffer and 30 μl of the sample to be tested for HR activity for 40 min at 37°C and assayed for histamine as described below.

Histamine assay. The radioenzyme assay for histamine was performed by using a modification of the method of Beaven et al. (14) as described by Kaplan et al. (1). Standard curves for each assay were constructed by linear regression. The assay results were quantitated from a standard curve by using a computer program written by Dr. M. Silverberg (SUNY, Stony Brook, NY) with an Apple IIe.

Skin test. Healthy volunteers, after informed consent, were given a prick test on the forearm. The allergens used were orchard grass, ragweed mix, oak, poplar, alternaria, penicillium, horradendrum, dust, dust mite, and cat. Normal saline was used as a negative control and histamine (10 $\mu\text{g}/\text{ml}$) as a positive control. The wheal and flare response was assessed on a five-point scale of zero and $1+$ to $4+$ after 15 min. All allergens were tested at $1:20$ w/v.

Chromatography of MNC-HRF. Concentrated crude supernatant of SK/SD stimulated MNC was applied to a Serva 7.1 mm $\times$ 500 mm SI 300 polyol gel filtration column (Westbury, NY). The buffer system was 0.3 M ammonium acetate in 5% propanol adjusted with concentrated ammonium hydroxide to pH 7.4 . The flow rate was 0.5 ml/min. One-half-milliliter fractions were collected, lyophilized, and resuspended in 100 μl of PBS, pH 7.4 . Twenty microliters of each fraction were brought to a total volume of 300 μl of cell suspension when assayed for histamine-releasing activity.

Affinity chromatography studies. The IgE myelomas were purified by DE-52 anion exchange chromatography and HPLC gel filtration as follows. Lyophilized material from 1.3 ml of IgE myeloma serum was reconstituted in 10 mM Tris-HCl (pH 7.85) and applied to a 25 ml DE-52 anion exchange column equilibrated with the same buffer. After washing with 70 ml of equilibrating buffer, a linear salt gradient was applied from 10 mM NaCl (200 ml) to 100 mM (200 ml) in 10 mM Tris-HCl (pH 7.85), and 6 -ml aliquots were collected. The eluate was monitored by absorbance at 280 nm and the peaks were pooled, dialyzed against PBS, and concentrated with an Amicon YM5 membrane. An Ouchterlony test was performed with anti-human IgE, and the positive fractions were pooled and applied to a $.75 \times 60$ cm) TSK 3000 SW gel filtration HPLC column (Variant) equilibrated with PBS. The flow rate was 0.5 ml/min and 1 -ml fractions were collected. An aliquot of each tube was subjected to SDS-PAGE to confirm purity. Purified myeloma IgE was pooled and used to make an affinity column by the cyanogen bromide method (13). Briefly, the sample was extensively dialyzed against 0.1 M sodium bicarbonate (pH 9.0) and coupled to 2 ml of CNBr activated Sepharose-6B. The mixture was shaken for 16 h at 4°C , poured into a column, unreacted sites blocked with glycine, and washed extensively with PBS. The beads were then tested for their binding capacity with mouse antihuman IgE. The column (2 ml) prepared with myeloma ADZ had a 250 μg capacity, whereas the column prepared with myeloma PS had a capacity of 1.3 mg.

Concentrated MNC culture supernatants were applied to each column. The columns were washed with 14 ml PBS followed by 8 ml PBS containing 1 M NaCl to minimize nonspecific binding and eluted with 8 ml of 3 M potassium thiocyanate (KSCN). The eluate, effluent, and 1 M NaCl wash were dialyzed against 0.05 M ammonium acetate (pH 7.4), lyophilized, and resuspended in PBS. Fifty microliters of each sample were assayed for histamine-releasing activity. The

elution buffer and the starting HRF were processed identically but without column fractionation and served as negative and positive controls, respectively.

SDS-PAGE. The fractions from the ADZ IgE affinity column were analyzed by SDS-PAGE (12% acrylamide) under nonreducing conditions following the procedure of Laemmli (15). After the electrophoresis the gel was fixed in 25% isopropanol- 10% acetic acid for 1 h, treated with 10% ethanol- 5% acetic acid for 10 to 15 min, and fixed again with 10% glutaraldehyde for another 30 min. The gel was then washed extensively with water, and treated with 5 $\mu\text{g}/\text{ml}$ dithiothreitol for 30 min. The gels were shaken in 0.2% silver nitrate for 1 h. After rapid rinsing, the bands were developed with 0.28 M sodium carbonate and 0.02% formaldehyde for 4 to 5 min and the reaction was stopped with 3% acetic acid (16).

IgE desensitization experiments. Basophils were desensitized by incubation with 0.001 mg/ml anti-IgE for 45 min at 37°C . The cells were then washed twice in 50 ml HBS-HSA and centrifuged at $1000 \times g$ for 10 min. The cell pellets were resuspended in HBS-HSA made 50% in D_2O , 2 mM CaCl_2 and 2 mM MgCl_2 so that 270 μl per sample could be used in the histamine release assay. Thirty microliters of agonist was added (anti-IgE, HRF), incubated for 40 min at 37°C , and assayed for histamine content.

RESULTS

Basophil reactivity to crude HRF. The ability of human MNC-HRF preparations to stimulate histamine release from allergic and nonallergic patients was assessed as indicated in Table I. In each instance the response to anti-IgE is shown for comparison and the mean and SE is shown for each group. It is clear that nonatopic persons (A), skin test positive persons lacking symptoms (B), or patients with significant allergic symptoms (C), all responded equally well to anti-IgE. The MNC-HRF was active in all three groups but the atopic population appears to be more responsive. The use of D_2O augmented histamine release in all groups but was not requisite for a positive response; in its absence values decline about 2 - to 2.5 -fold. The MNC-HRF was concentrated and then fractionated by gel filtration HPLC as shown in Figure 1. The solid continuous line tracing represents the protein elution profile (OD 280 nm) and above that is shown the basophil histamine release by each fraction. Two major peaks of activity are seen; these correspond to apparent

TABLE I
Donor variability in histamine response to anti-IgE vs HRF preparations

	Donor	Percent Histamine Release to	
		Anti-IgE	MNC-HRF
A ^a	1	65	9
	2	39	4
	3	42	48
	4	82	19
	5	80	67
		62 ± 8	29 ± 11
B	6	6	7
	7	94	72
	8	97	45
	9	60	39
	10	46	13
	11	12	5
		53 ± 15	30 ± 10
C	12	69	50
	13	62	77
	14	4	9
	15	68	16
	16	56	28
	17	94	61
		59 ± 11	40 ± 10

^a Basophil histamine release in response to 0.001 mg/ml anti-IgE and five times concentrated HRF made from mononuclear cells. The histamine release was assessed by using basophils derived from A, nonatopic donors; B, skin test-positive donors having no clinical symptoms; and C, atopic donors. Group cumulative values are mean $\pm$ SEM.

m.w. of 29,000 and 23,000. The basophil donor chosen for this assay was a nonatopic person.

Interaction of HRF with IgE myeloma proteins. Because HRF has been reported to require interaction with cell-surface IgE to stimulate basophils, we next evaluated the ability of HRF to interact with affinity columns prepared with IgE myeloma proteins. Myeloma PS was assessed first. Three milligrams of protein were coupled to activated Sepharose-6B and a column prepared. Crude concentrated MNC supernatant was applied to the column and washed with PBS and then 1 M NaCl in phosphate buffer. The OD of the effluent was monitored and the effluent peak dialyzed against 0.02 M ammonium acetate (pH 7.4), lyophilized, and resuspended in PBS to the original volume. No protein peaks were observed when the column was washed with 1 M NaCl, and this fraction was added to the effluent. Then the column was eluted with 3 M KSCN and the eluate dialyzed, lyophilized, and resuspended in PBS. Serial dilutions of both effluent and eluate were prepared and each was tested for HRF activity. As seen in Figure 2, there was significant HRF activity seen in the undiluted and 1/2 dilution of the effluent, whereas the undiluted eluate gave a borderline positive value (5% histamine release). The same procedure was followed by using an affinity column prepared with E-myeloma ADZ. The results are shown in Figure 3, A to E. In Figure 3A is shown the dose response with the starting material, the dialysis buffer control (which is negative), and the effluent and eluate. Both effluent and eluate had significant activity. We reasoned that the

activity in the effluent might be HRF that is incapable of binding to myeloma ADZ, or that the column has been overloaded so that potentially all the HRF could be bound, or that there is a binding fraction with low affinity. To test this hypothesis the effluent was dialyzed against 0.1 M ammonium bicarbonate, lyophilized, resuspended in PBS, and reapplied to the same column after it was stripped and reequilibrated. As can be seen in Figure 3B, the same result is obtained, although there is more apparent activity in the effluent. When this effluent was dialyzed, lyophilized, resuspended, and reapplied to the column (after reequilibration) (Fig. 3C), virtually all of the HRF was in the effluent and little could be bound. This is similar to the result obtained with a human IgG affinity column (Fig. 3D) where virtually all the HRF is found in the effluent and little activity is recovered in the eluate. The original ADZ-IgE myeloma affinity column was then used to assess a new preparation of crude MNC-HRF (Fig. 3E). As can be seen, the same initial result was obtained, i.e., some HRF is bound and eluted while another portion remains unbound and passes through as the effluent. This result suggests that HRF is heterogeneous and that some of it binds to some IgE proteins (but apparently not all) and a substantial portion of HRF appears nonreactive with IgE. A 12% SDS gel of the concentration fractions assayed in Figure 3E is shown as Figure 4. The starting material, effluent, and eluate were electrophoresed. There appears to be considerable purification of the bound HRF fraction (eluate) with major bands visualized at 24, 21, and 14 kDa in the lower region of the gel corresponding to the approximate size range shown in Figure 1.

Functional analysis of the interaction of HRF with basophil IgE antibody. We next examined the ability of basophils that have been desensitized with anti IgE to respond to a subsequent challenge with HRF. For this experiment, four different preparations of MNC-HRF

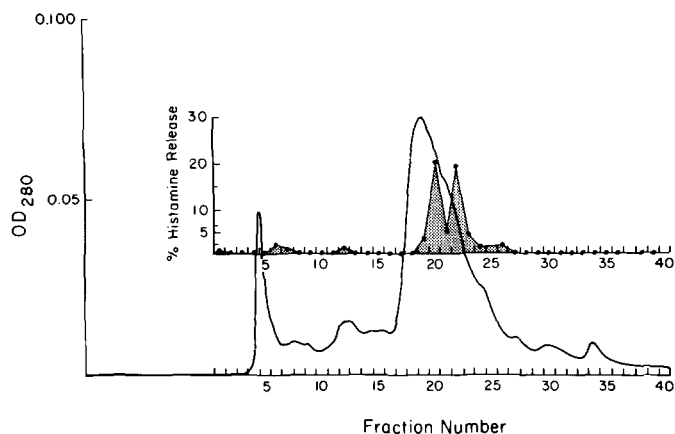


Figure 1. Gel filtration HPLC of concentrated supernatant derived from cultured human mononuclear cells. The protein profile at OD 280 nm is given by the continuous line at the bottom and the histamine-releasing activity shown above.

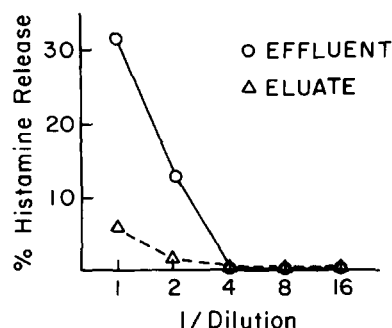


Figure 2. Histamine release of effluent and eluate fraction obtained by passage of mononuclear cell supernatant over an affinity column prepared with IgE myeloma protein PS.

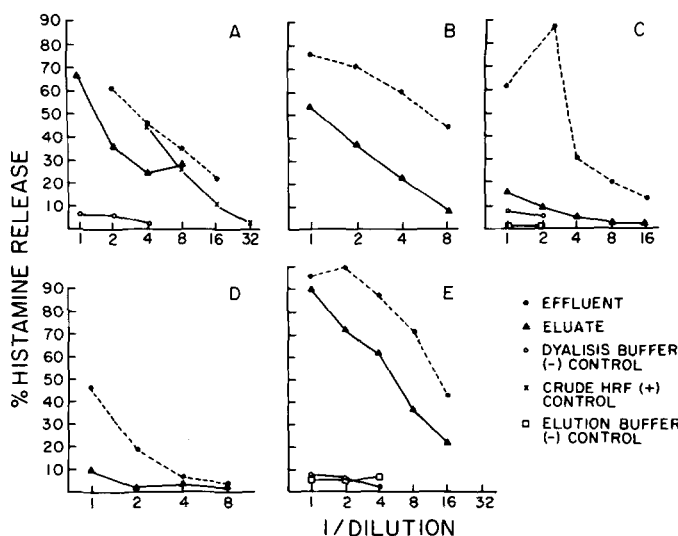


Figure 3. Histamine release of effluent and eluate fractions obtained by passage over mononuclear cell supernatant over an affinity column prepared with IgE myeloma protein ADZ (A). The effluent was dialyzed, lyophilized, resuspended in PBS, and recycled over the same column. The assay of this effluent and eluate is shown in B. The effluent was recycled once again and the result given in C. D shows the result obtained when a comparable supernatant was fractionated on an affinity column prepared with the same quantity of polyclonal IgG. E utilizes the same column as for the experiments shown in A thru C; however, a fresh mononuclear cell supernatant has been applied.

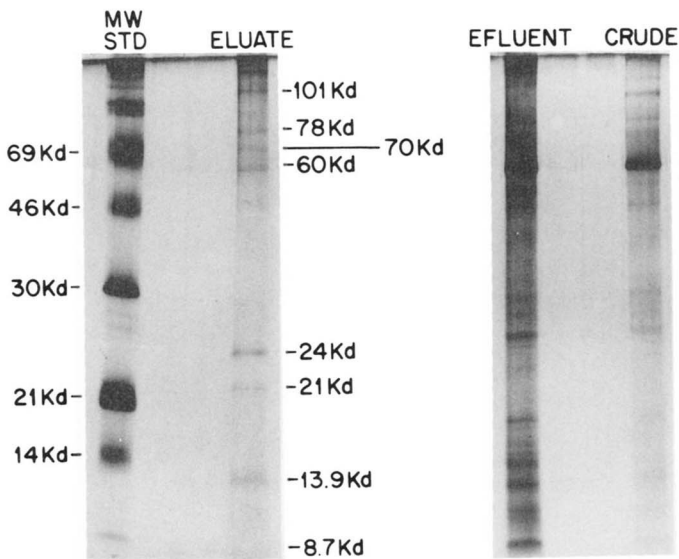


Figure 4. SDS gel electrophoresis of the concentrated mononuclear cell supernatant labeled "crude." At the right are the effluent and eluate obtained after affinity chromatography by using IgE myeloma ADZ. Molecular weight standards are shown on the left.

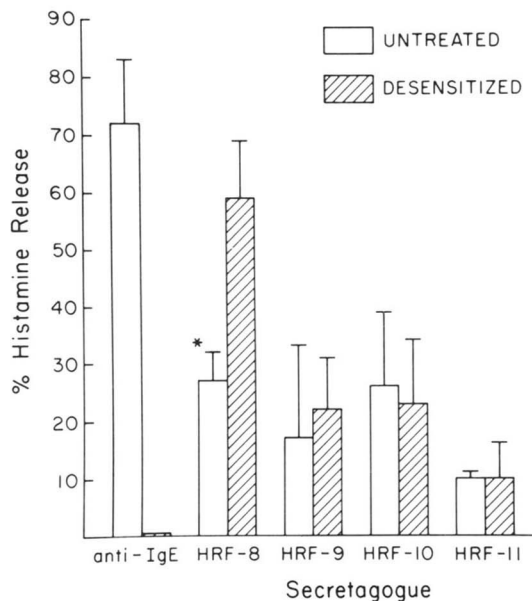


Figure 5. Response of basophils to mononuclear cell derived HRF after desensitization with anti IgE. The asterisk indicates a significant difference of $p < 0.05$.

were used, the results of which are shown in Figure 5. Anti-IgE gave 72% histamine release before desensitization and zero release when challenged after the desensitization protocol. When MNC-HRF was examined in the same fashion, the response to preparations numbered 9, 10, and 11 was unaltered in desensitized cells, whereas the response to preparation 8 was enhanced. We therefore do not observe a requirement for interaction with cell-surface IgE as a prerequisite for the function of MNC-HRF. This experiment was performed with a concentrated MNC supernatant. The peak material obtained by gel filtration (not shown) gives the same result. We have not compared the effluent or eluate obtained with myeloma ADZ in this fashion.

DISCUSSION

HRF have been described as products of alveolar macrophages (5, 9), lymphocytes (3, 4), platelets (6, 11), neutrophils (8), and can be found in nasal washings (7, 10). It is unclear whether the products of these diverse sources are the same (or related) or whether the ability to cause histamine secretion from basophils or mast cells is a function common to a variety of different gene products. Some published characteristics appear to distinguish these factors. The products of nasal washings, macrophages, and platelets have been reported to require cell-surface IgE for their function. Further, basophils can be divided into responsive and nonresponsive, and non-responsive cells can be converted to responsive cells by passive sensitization with IgE derived from the responsive cells. Thus it was postulated that IgE is heterogeneous and that responsiveness to HRF can define the difference. Studies with platelets and nasal washings indicated that responders can also be subdivided by allergic (atopic) status, i.e., nonatopics are nonresponders while atopics are heterogeneous; about half respond and half do not. The atopic responders may therefore have the responder type of IgE.

Studies with other HRF preparations have given a different result. MNC-derived HRF reacts with both nonatopic and atopic basophils (Table I) and this is also true of neutrophil-derived HRF (17). Further, both neutrophil and MNC-derived HRF act best on basophils that respond well to anti-IgE (12) (Table I), but no *prima facie* evidence has been found to indicate that HRF reactivity with cell-bound IgE is required. As shown in Figure 5, desensitization to anti-IgE is dissociated from HRF responsiveness in this system. Attempts to remove cell-surface IgE by using lactic acid indicated that neutrophil-derived HRF retains reactivity with basophils (17). Taken in aggregate, the MNC-derived HRF appears to act independently of cell-bound IgE, the neutrophil-derived material appears similar, and the relationship of cell responsiveness to these agents and the cellular response to anti-IgE appears to be dependent on a general parameter of cell responsiveness that has not yet been defined biochemically, but is likely a postreceptor event.

Studies which have demonstrated IgE dependence or a relationship to atopic status of HRF have utilized crude supernatants or gel-filtered material that has not been highly purified. The studies reported herein have utilized material at a similar stage of purification. Clearly, this material is heterogeneous in terms of charge or size (1) (Fig. 1) and may also contain other histamine-releasing cytokines such as IL-3 or GM-CSF (18). We have also found heterogeneity of MNC-HRF in terms of its ability to bind to IgE myeloma proteins. A positive result was found using one of two IgE myelomas. Thus even though the mixture showed that responsiveness was not dependent on atopic status or cell-surface IgE, an IgE affinity column could be used to fractionate HRF and elution of the bound protein can be used as a purification step. It is important to note that the sample applied to the affinity column in Figure 3A contained 800 μg of which 40 μg were bound. Because the capacity of the column was 250 μg , it appeared that the capacity was sufficient to bind more. Thus we concluded that binding material present in the effluent of Figure 3A which bound when reappplied

(Fig. 3B) is indicative of a low affinity. The sample applied in Figure 3C was the nonbinding fraction. We have not attempted to distinguish the IgE binding or nonbinding fractions in terms of reactivity with atopic vs nonatopic basophils or by separate desensitization experiments with anti-IgE, and such studies are in progress. A similar analysis of IL-3 and GM-CSF is of interest in case some unsuspected reactivity with IgE (or some IgE) can be demonstrated. This reactivity with certain IgE myeloma proteins need not imply a requirement for IgE for the function of MNC-HRF (as our data suggests), but does not preclude an interaction with cell surface IgE that does not lead to histamine release. Binding of a univalent Ag would be an analogous example. Further, a requirement for cell-surface IgE that leads to histamine release has been reported using other sources of HRF, but may not relate to the IgE binding described herein. In both systems there is heterogeneity in terms of which IgE is reactive; this may indicate an interaction with carbohydrate or with idiotypic differences seen in myeloma proteins.

Clearly, each form of HRF from each cell source must be purified and characterized in terms of protein structure, function, and reaction with antisera, as well as characterization of the genes encoding such proteins. Only then will we be able to definitively distinguish the various types that appear to exist. A method for purification of two of the major forms of MNC-HRF has been reported (19) and the material has been subjected to amino acid analysis and N-terminal analysis (19).

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