

Identification and partial characterization of an exercise-induced neutrophil chemotactic factor in bronchial asthma.

T H Lee, ... , M J Walport, A B Kay

J Clin Invest. 1982;69(4):889-899. <https://doi.org/10.1172/JCI110528>.

Research Article

A heat-stable neutrophil chemotactic factor (NCF) has been identified in the serum of 13 atopic asthmatic subjects after treadmill exercise. Peak activity was detected at 10 min and returned to prechallenge values by 1 h. No NCF activity was detected in the sera of three nonasthmatic atopic or four normal nonatopic individuals performing the same task. NCF produced by exercise (NCFEX) had a similar time-course of release to NCF provoked by specific antigen (NCFAG). The appearance of circulating NCFEX and NCFAG closely paralleled the fall in peak expiratory flow rate/forced expiratory volume in 1 s (PEFR/FEV1). Histamine challenge in atopic asthmatics at concentrations giving a comparable change in PEFR/FEV1 to that evoked by exercise or inhaled antigen was not associated with the appearance of circulating NCF. In seven subjects NCFEX release was inhibited by prior administration of disodium cromoglycate. NCFEX and NCFAG eluted as single peaks of activity when applied separately to columns of Sephadex G-200, and both were an estimated 750,000 daltons. NCFEX and NCFAG also eluted as single peaks of activity, at between 0.15 and 0.30 M NaCl, following anion exchange chromatography on DEAE-Sephacel (pH 7.8). The isoelectric points of NCFEX and NCFAG were virtually identical (between pH 6.0 and 6.5) as determined by chromatofocusing on Polybuffer Exchanger 94. The activities of NCFEX and NCFAG were substantially reduced, [...]

Find the latest version:

<https://jci.me/110528/pdf>



Identification and Partial Characterization of an Exercise-induced Neutrophil Chemotactic Factor in Bronchial Asthma

T. H. LEE, L. NAGY, T. NAGAKURA, M. J. WALPORT, and A. B. KAY, *Department of Allergy and Clinical Immunology, Cardiothoracic Institute, Brompton Hospital, Fulham Road, London, SW3 6HP, England*

ABSTRACT A heat-stable neutrophil chemotactic factor (NCF) has been identified in the serum of 13 atopic asthmatic subjects after treadmill exercise. Peak activity was detected at 10 min and returned to pre-challenge values by 1 h. No NCF activity was detected in the sera of three nonasthmatic atopic or four normal nonatopic individuals performing the same task. NCF produced by exercise (NCF^{EX}) had a similar time-course of release to NCF provoked by specific antigen (NCF^{AC}). The appearance of circulating NCF^{EX} and NCF^{AC} closely paralleled the fall in peak expiratory flow rate/forced expiratory volume in 1 s (PEFR/FEV₁). Histamine challenge in atopic asthmatics at concentrations giving a comparable change in PEFR/FEV₁ to that evoked by exercise or inhaled antigen was not associated with the appearance of circulating NCF. In seven subjects NCF^{EX} release was inhibited by prior administration of disodium cromoglycate. NCF^{EX} and NCF^{AC} eluted as single peaks of activity when applied separately to columns of Sephadex G-200, and both were an estimated 750,000 daltons. NCF^{EX} and NCF^{AC} also eluted as single peaks of activity, at between 0.15 and 0.30 M NaCl, following anion exchange chromatography on DEAE-Sephacel (pH 7.8). The isoelectric points of NCF^{EX} and NCF^{AC} were virtually identical (between pH 6.0 and 6.5) as determined by chromatofocusing on Polybuffer Exchanger 94. The activities of NCF^{EX} and NCF^{AC} were substantially reduced, in both a time- and dose-dependent fashion, after incubation with trypsin and chymotrypsin. Partially purified NCF^{EX} and NCF^{AC} promoted both stimulated random migration (chemokinesis) as well as directional migration (chemotaxis).

These experiments indicate that NCF^{EX} and NCF^{AC} might be identical substances and raise the possibility

that mediators of hypersensitivity are released during exercise-induced asthma in atopic subjects.

INTRODUCTION

In 1977 Atkins et al. (1) described a high molecular weight, heat-stable neutrophil chemotactic factor (NCF)¹ detectable in the circulation of patients with bronchial asthma after inhalation of specific antigen (1). Comparable NCF have been identified in the sera from cold (2), cholinergic (3), and solar (4) urticaria after the appropriate challenge. There is circumstantial evidence to suggest that NCF might be derived from the mast cell. NCF release after challenge in cold urticaria closely paralleled the appearance of recognized mast cell mediators such as histamine and the eosinophil chemotactic factor of anaphylaxis (ECF-A) (2). Bronchial challenge in asthmatics with nonspecific agents such as methacholine was not associated with the release of NCF, whereas prior administration of disodium cromoglycate to asthmatics challenged with specific antigen inhibited both the increase in airways obstruction and the appearance of NCF into the circulation (5, 6).

It is well recognized that exercise provokes airways obstruction in most patients with bronchial asthma and that in many instances exercise-induced asthma (EIA) is also inhibited by disodium cromoglycate (DSCG). For these reasons we have attempted to establish whether an NCF is released after exercise challenge in asthmatics and whether it has similar properties to NCF produced by specific antigen.

¹ *Abbreviations used in this paper:* DSCG, disodium cromoglycate; ECF-A, eosinophil chemotactic factor of anaphylaxis; EIA, exercise-induced asthma; NCF, neutrophil chemotactic factor; NCF^{AC}, NCF provoked by specific antigen; NCF^{EX}, NCF produced by exercise; PBS, phosphate-buffered saline; PEFR/FEV₁; peak expiratory flow rate/forced expiratory volume in 1 s.

Received for publication 6 March 1981 and in revised form 15 December 1981.

METHODS

26 nonsmoking atopic asthmatic volunteers were studied. They were defined by (a) previously documented airways obstruction that reversed by >20% of their predicted values, either spontaneously or as the result of treatment, (b) a positive skin-prick test (wheal > 5 mm) to at least two of the following common allergens: mixed grass pollens, cat fur, dog hair, house dust, and the house dust mite, *Dermatophagoides farinae*, (Bencard, Brentford, Middlesex, England), (3) a raised total IgE (measured by the Phadebas IgE PRIST radioimmunoassay, Pharmacia Diagnostics, Div. of Pharmacia, Inc., Uppsala, Sweden). Each subject gave informed consent and the study was approved by the Brompton Hospital Ethical Committee. 13 patients were subjected to an exercise task, 11 were challenged by inhalation of antigen and 6 to bronchoprovocation with histamine. Two patients had all three challenges. Their ages ranged from 16 to 38 yr and there was no difference in the mean age between the group that performed the exercise task (25 yr) with that undergoing antigen (29.9 yr) and histamine (28.5 yr) provocation. The total immunoglobulin E (IgE) (PRIST) was raised in each of the asthmatic groups: exercise 620 ± 172.3 (KU/liter \pm SEM); antigen 272 ± 62.8 (KU/liter \pm SEM); histamine 408 ± 211.4 (KU/liter \pm SEM). Seven normal subjects also underwent the exercise procedure and of these, three were atopic as defined by positive skin-prick tests (see above) and four were nonatopic. There was no difference in their mean ages as compared to the asthmatic subjects studied [(atopic normal: 27.5 yr; nonatopic: 30.3 yr), and their total IgE was lower than the asthmatics (atopic normal: 11.7 ± 0.6 (KU/liter \pm SEM); nonatopic normal: 11.0 ± 4.8 (KU/liter \pm SEM)]. Medication was discontinued in all subjects for at least 24 h before the tests, and none had taken DSCG during the previous 28 d.

Inhalation challenge. Bronchial challenge was performed as described (7). The antigens were freeze-dried and reconstituted in sterile Coca's solution. Four subjects were challenged with an extract of *Dermatophagoides farinae* (Bencard), five with mixed grass pollens (Bencard), one with a partially refined extract of rat urine (kindly given by Dr. Joan Longbottom, Cardiothoracic Institute, London), and one with a purified extract of cat fur (Dr. Joan Longbottom). The initial antigen concentration used in the challenge procedure was one tenth of the smallest concentration that gave a 3-mm wheal on skin-prick testing. The concentrations were increased in logarithmic increments up to a maximum of 10 mg/ml. A positive response was defined as a fall in the forced expiratory volume in 1 s FEV₁ of 20%. Prechallenge and postchallenge measurements at time intervals up to 60 min were performed as indicated. The same procedure was used for histamine provocation using concentrations between 0.25 and 8.0 mg/ml.

Exercise testing. The exercise task was performed on a treadmill at a speed of 4 mph at a 10–15% incline for 6 min (8). All procedures were performed at ambient room temperature. The peak expiratory flow rate (PEFR) was measured before and after exercise for up to 60 min. In seven individuals the exercise task was performed before and after 60 mg of DSCG administered by a Wright nebulizer. DSCG was given 10 min before the commencement of exercise. Exercise tests undertaken before and after administration of DSCG were spaced by an interval of at least 4 h, during which time the patients' pulmonary function had returned to base line.

Serum samples. Venous blood was withdrawn through a 19-gauge indwelling needle at a site below the antecubital

fossa. No anticoagulants were used; the patency of the catheter was maintained at frequent intervals by small infusions of 0.9% saline. The blood was allowed to clot on glass at 4°C for 3 h and the serum was separated, divided into portions, and stored at –20°C until use. Before use in the neutrophil locomotion assay the sera were thawed and heated at 56°C for 30 min.

Neutrophil locomotion. The preparation of neutrophils for use in modified Boyden technique was performed as described (9). Leucocyte-rich plasma, obtained by dextran sedimentation of heparinized blood from normal, healthy donors, was applied to a density gradient of 9% Ficoll solution and sodium diatrizoate (density 1.140) in the proportions 2.4:1. After centrifugation at 200 g for 40 min at 20°C the cell-rich pellet was retained, washed in Hanks' balanced salt solution, and resuspended in Hanks' solution containing 0.4% ovalbumin. This procedure routinely gave neutrophil purity of >96%, and the cell counts were adjusted to 2×10^6 /ml. 800 μ l of either 20% serum, dilutions of Sephadex G-200 fractions, or diluent controls were placed in the lower compartment. Serum was diluted to 20% serum with Hanks' solution. Fractions from the various columns were assayed in phosphate-buffered saline (PBS). Following a 90-min incubation, the micropore filters (Sartorius-membrane filters, 8- μ m pore size, 34 Göttingen, W. Germany) were removed, fixed, and stained as previously described (9), and the number of cells that had migrated through the entire thickness of the filter was counted and the results were expressed as the total number of cells in 10 random high-power fields. Counts obtained with buffer alone were subtracted from those obtained with the test solutions. On 11 separate occasions the lower test compartments were examined for the presence of cells that might have become detached from the lower surface of the filter. In no instance was this observed. The samples were assayed in duplicate and coded in such a way that the investigator did not have prior knowledge of the protocol. The values of each duplicate filter did not differ by >20%.

Gel filtration. Gel filtration chromatography was performed on columns of Sephadex G-200 (2.2 \times 90 cm) using PBS, pH 7.35. The columns were operated at 4°C using a flow rate of 15 ml/h. 2 ml of the prechallenge sample was applied first and after thorough washing the postchallenge sample was subjected to the same procedure. 2-ml fractions were collected. One column was used for NCF^{EX} samples and one for NCF^{AG}. Alternate undiluted fractions were tested for NCF. Dilutions of the fractions giving peak activity were tested in the dose-response studies. In the chemokinetic/chemotactic experiments the fractions containing peak NCF activity were pooled.

Anion exchange chromatography. Anion exchange chromatography was performed on columns of DEAE-Sephacel (Pharmacia Fine Chemicals) (1.8 \times 30 cm) at 4°C. The columns were equilibrated using 0.0035 M sodium phosphate buffer, pH 7.8. The fractions having peak chemotactic activity after Sephadex G-200 chromatography were pooled and concentrated to 10 ml by ultrafiltration (XM 300 Amicon Corp. Scientific Sys. Div., Lexington, MA). The equivalent fractions of the prechallenge sera after G-200 gel filtration were similarly processed. The pooled samples were dialysed at 4°C overnight against one change of 100 \times volumes 0.0035 M phosphate buffer. These were then applied separately to the column of DEAE-Sephacel, which was washed with two bed volumes of buffer before elution with a linear salt gradient of 320 ml to 0.5 M NaCl, using a flow rate of 24 ml/h. 4-ml fractions were collected. In all instances the prechallenge sample was applied before the postchallenge. Re-

generation of the column with 0.1 M NaOH and reequilibration with 0.0035 M phosphate buffer was performed before each experiment and separate columns were used for the exercise and antigen experiments. Each third fraction following anion exchange chromatography was dialysed at 4°C overnight against one change of 100 × volumes PBS, pH 7.35. 0.8 ml of each of these dialysed fractions were assayed undiluted for chemotactic activity.

Chromatofocusing. Samples were dialysed overnight at 4°C against 0.025-M tris-CH₃COOH buffer, pH 8.3 and applied to separate columns of Polybuffer Exchanger 94 (1.8 × 30 cm) (Pharmacia), which had previously been equilibrated with the same buffer. A solution of Polybuffer 96 (30%) and Polybuffer 74 (70%)—CH₃COOH, pH 5.0 (Pharmacia), was used for elution using a flow rate of 35 ml/h. A linear pH gradient of 500 ml from pH 8.0 to 5.0 was generated and 8-ml fractions were collected. Alternate fractions were dialysed overnight against one change of 100 × volumes PBS, pH 7.35 and 0.8 ml subsequently tested undiluted for chemotactic activity. The column was regenerated with 1 M NaCl and reequilibrated with the running buffer before each experiment. Separate columns were used for the exercise and antigen experiments.

Enzyme experiments. Insoluble trypsin ("Enzygel," Boehringer, Mannheim) and insoluble chymotrypsin (Sigma Chemical Co., St. Louis, MO) were used. The experiments were performed on the NCF^{EX} and NCF^{AG} that had been sequentially and partially purified by gel filtration, anion exchange chromatography, and chromatofocusing. The enzymes were added to either NCF^{EX} or NCF^{AG} to achieve a final concentration of 0.4%. The chemotactic activity was tested immediately after the addition of the enzyme and at 15-min intervals (during incubation at 37°C with constant shaking) for 1 h thereafter. The insoluble enzyme was separated from the NCF, before testing for chemotactic activity by centrifugation at 4,000 g, 4°C for 10 min. Control experiments were performed using (a) NCF alone, without the

addition of enzyme; (b) enzyme control: enzyme was added to NCF but was immediately separated by centrifugation at 4°C before the incubation at 37°C. The effect of different concentrations of these proteolytic enzymes on NCF^{EX} and NCF^{AG} was assessed as indicated (Table I). The incubation time for these experiments was 45 min.

RESULTS

The appearance of neutrophil chemotactic activity after exercise and inhalation challenge in asthmatic subjects

The pre- and postchallenge values for NCF are shown in Fig. 1. In asthmatics exercise produced a marked increase in NCF activity that was not found when the same treadmill task was performed by the normal control subjects. The increase in NCF after exercise in asthmatics was similar to that observed in asthmatics who inhaled specific antigen. Histamine challenge in asthmatics was not associated with an increase in NCF activity. The differences between the pre- and postchallenge values in asthmatics challenged with either exercise or antigen were highly statistically significant ($P < 0.0005$). Although the total IgE serum concentration of the asthmatics subjected to exercise was considerably higher than the group challenged with histamine or antigen (Fig. 1), there was no correlation between the percentage of change in NCF activity and the total IgE concentration, either in the three asthma groups or the seven normal control subjects.

TABLE I
Effect of Trypsin and Chymotrypsin on NCF^{EX} and NCF^{AG} Activity

	Concentration of enzyme, %	Trypsin				Chymotrypsin			
		0.2	0.4	0.8	1.6	0.1	0.2	0.4	0.8
Inhibition, %									
NCF ^{EX}	Experiment 1	29.2	66.3	87.6	82.4	0	47.2	61.6	85.7
	Experiment 2	0	27	50.1	85.7	0	60.7	52.1	75.7
	Experiment 3	35	75.1	80.1	77.5	5	65.2	81.5	79.1
	Mean	21.4	56.1	72.6	81.8	1.6	57.7	65	80.1
Inhibition, %									
NCF ^{AG}	Experiment 1	8	60.6	21.3	0	0	37.2	69.6	41.8
	Experiment 2	43.1	40.9	53.1	59.9	20.6	27.5	69.7	57.2
	Experiment 3	21	62	59	51	0	10	45.3	16.5
	Mean	24	54.5	44.5	36.9	6.9	24.9	61.5	38.5

The experiments were performed on NCF that had been partially purified by Sephadex G-200 gel filtration, anion exchange chromatography on DEAE-Sephacel, and chromatofocusing. NCF was incubated with the enzyme for 45 min before testing for chemotactic activity.

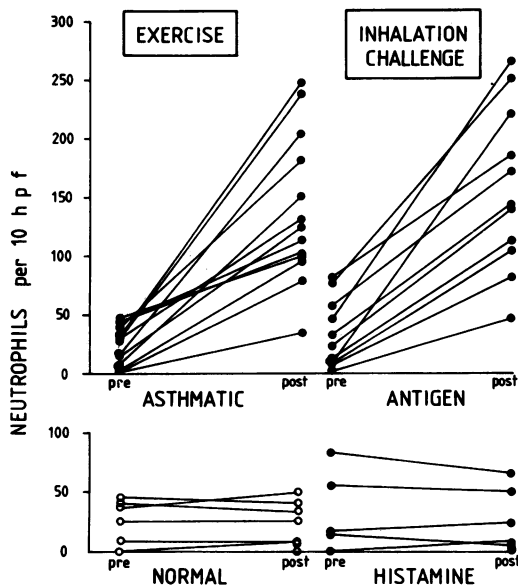


FIGURE 1 The release of NCF after exercise or inhalation challenge with antigen or histamine. The asthmatic subjects (closed circles) and the four nonatopic and three atopic normal controls (open circles) were challenged as indicated. The postchallenge samples were those giving peak NCF activity, which in all instances was either the 5- or 10-min sample. There was a statistically significant difference (calculated by the Student's *t* test) between the pre- and postchallenge NCF concentrations in asthmatics challenged with exercise or antigen ($P < 0.0005$). There was no significant difference between the pre- and postchallenge samples in the normal controls subjected to the same exercise task or the asthmatics challenged with histamine.

The time-course of release of NCF into the circulation and its relation to changes in airways obstruction are shown in Fig. 2. Asthmatics challenged with exercise or antigen gave a peak of NCF activity at 10 min and this corresponded to the time of maximal fall in FEV₁ or PEFR. In both exercise and antigen challenge NCF activity returned to base-line values by 60 min, whereas the PEFR or FEV₁ was still decreased by ~15% at the end of the observation period. Normal subjects subjected to the same exercise procedure did not produce circulating NCF and had no fall in their FEV₁. Asthmatics challenged with histamine also failed to produce detectable NCF but the change in airway resistance was comparable in magnitude to that produced by exercise or antigen in the asthmatic subjects.

In two asthmatic individuals it was possible to perform all three maneuvers, i.e. exercise, histamine, and antigen inhalation (Fig. 3). The results were similar to those shown in Fig. 2, i.e. a fall in FEV₁ or PEFR with each procedure with NCF release occurring after exercise and antigen challenge but not after histamine inhalation.

In seven individuals NCF release and changes in the

PEFR were observed before and after the administration of DSCG. DSCG inhibited both the change in PEFR and the appearance of NCF into the circulation. There were clear differences in NCF and PEFR between the pre- and post-DSCG measurements as shown in Fig. 4.

Partial purification and characterisation of neutrophil chemotactic factors

Gel filtration. Pre- and postchallenge samples induced by exercise (NCF^{EX}) or antigen (NCF^{AG}) were applied to columns of Sephadex G-200 (Fig. 5). NCF^{EX} and NCF^{AG} eluted as a single peak and had an estimated molecular size of 750,000. In both instances there was a small amount of NCF activity in the prechallenge sera that eluted at the same bed volume as the postchallenge sera. These experiments were performed three times with both the pre- and postchallenge samples from exercise and antigen stimulation, i.e. a total of 12 chromatographic procedures. The fractions from Sephadex G-200 containing maximal NCF activity were assayed in a dose-response fashion (Fig. 6). NCF^{EX} and NCF^{AG} (G-200) both gave a dose-dependent increase in neutrophil chemotactic activity. The postchallenge samples, at the highest concentrations, were approximately three times more active in neutrophil locomotion than the prechallenge samples.

Anion exchange chromatography. The NCF^{EX} and NCF^{AG} were further characterised by anion exchange chromatography. The pooled, concentrated, and dialysed pre- and postchallenge Sephadex G-200 fractions containing NCF^{EX} and NCF^{AG} were applied separately to columns of DEAE-Sephacel. A single peak of chemotactic activity eluting between 0.15 and 0.30 M NaCl was consistently observed from all the postchallenge samples. There was minimal activity under these conditions in the prechallenge fractions. These experiments were performed on three separate occasions with both the pre- and postchallenge samples (exercise and antigen). Representative examples are shown in Fig. 7. A second peak of activity eluting between 0.35 and 0.40 M NaCl was also observed in 10 out of the 12 chromatographic procedures. The degree of activity present in this small peak was virtually the same between the pre- and postchallenge samples and for this reason further purification of this second peak was not undertaken.

Chromatofocusing. NCF^{EX} and NCF^{AG} that had been partially purified by gel filtration and anion exchange chromatography were subjected to chromatofocusing, which separates proteins according to their isoelectric points. A single peak of chemotactic activity was consistently observed that was maximal between pH 6.0 and 6.5. This procedure was performed on three separate occasions using NCF^{EX} and NCF^{AG} prep-

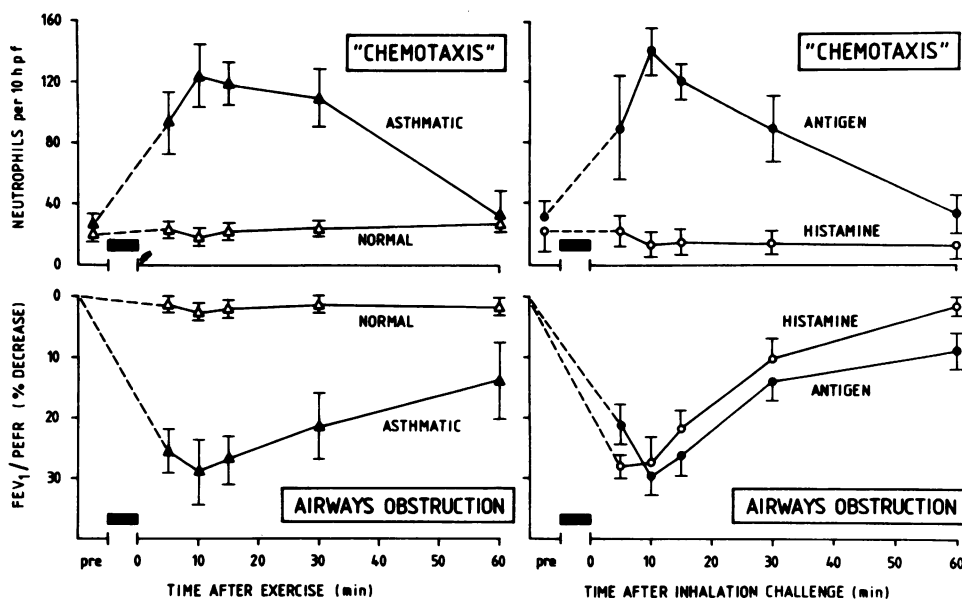


FIGURE 2 The time-course of release of NCF and the changes in $FEV_1/PEFR$ after exercise or inhalation challenge with antigen or histamine. The points represent the mean (\pm SEM) of 13 atopic asthmatics (closed triangles) and 7 normal controls (open triangles) who performed the exercise task, and 11 atopic asthmatics challenged with antigen (closed circles) and 7 atopic asthmatics who inhaled histamine (open circles). There were statistically significant differences in NCF activity at 5, 10, 15, and 30 min between asthmatics challenged with exercise and the normal controls who underwent the same procedure. Using the Student's *t* test, these were $P < 0.025$, $P < 0.0005$, $P < 0.025$, and $P < 0.025$, respectively. There were also statistically significant differences in NCF release between atopic asthmatics challenged with antigen and asthmatics challenged with histamine. At 5, 10, 15, and 30 min these were $P < 0.0125$, $P < 0.0005$, $P < 0.0125$, and $P < 0.01$, respectively. The period of challenge (either exercise, antigen, or histamine) is indicated by the solid bar.

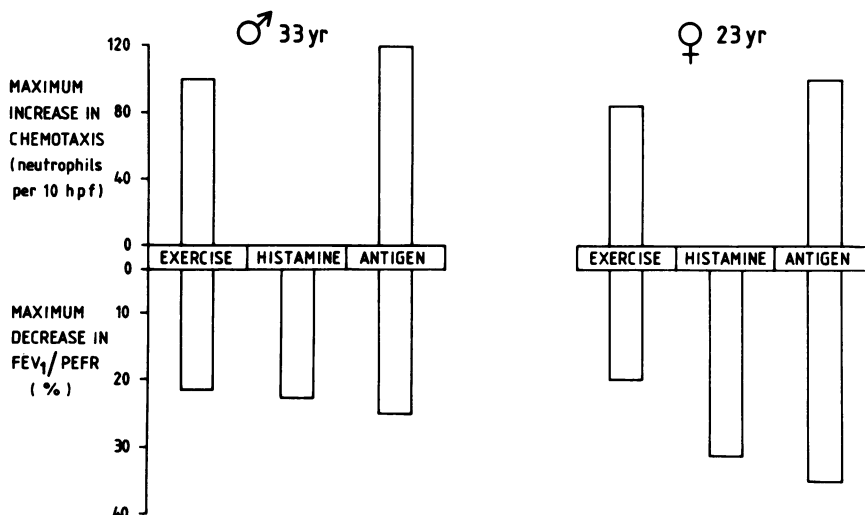


FIGURE 3 The release of NCF and the changes in $FEV_1/PEFR$ in two atopic asthmatic subjects after exercise or inhalation challenge with antigen or histamine. The two individuals were subjected either to treadmill exercise or inhalation of histamine or antigen on three separate occasions. The values for NCF are those that gave maximal neutrophil chemotaxis at the time intervals described in Fig. 2. In each individual this was either the 5- or 10-min sample. Similarly, the decrease in FEV_1 (for histamine and antigen) and $PEFR$ (for exercise) was measured over the same time period with the maximum decrease observed being at 5 or 10 min.

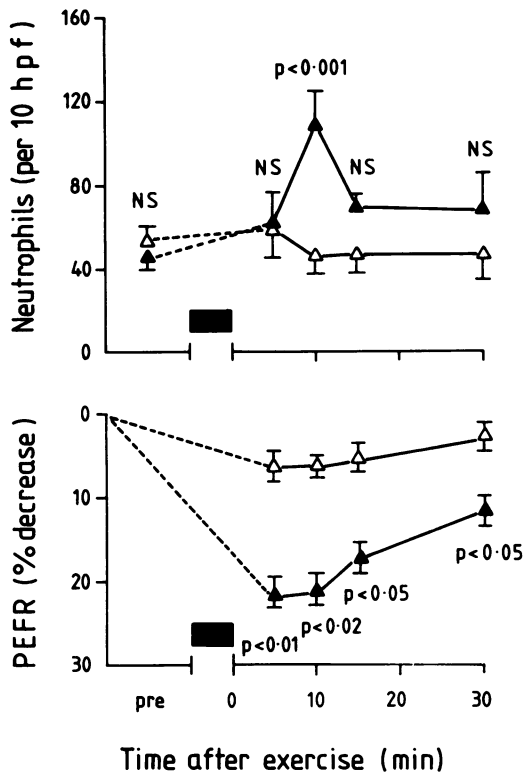


FIGURE 4 The effect of disodium cromoglycate on NCF and PEFR before and after treadmill exercise. The points represent the mean \pm 1 SEM from seven individuals. The solid bar represents the exercise challenge. Post-DSCG measurements (open triangles) were performed at least 4 h after the pre-DSCG measurements (closed triangles). The statistics were performed by the Student's *t* test and represent the differences between the pre- and post-DSCG values at the time intervals indicated. DSCG: \blacktriangle , pre; \triangle , post.

arations obtained from six separate asthmatic subjects. Representative examples are shown in Fig. 8.

Enzyme experiments. NCF^{EX} and NCF^{AG} prepared by Sephadex G-200 gel filtration, anion exchange chromatography, and chromatofocusing steps were treated with trypsin and chymotrypsin. Both enzymes inhibited the activity of the NCF in a time- (Fig. 9) and dose-dependent (Table I) fashion. The maximal inhibition observed using 0.4% enzymes was with an incubation time of 45 min, when it was possible to achieve an inhibition of NCF^{EX} activity of ~65%. Chymotrypsin and trypsin reduced the activity of NCF^{AG} by ~65 and 55%, respectively. In the case of NCF^{EX} it was possible to increase this inhibition to ~80% by using 0.8% enzyme. However, with NCF^{AG} there was evidence that the higher enzyme concentrations were less effective in inhibiting chemotactic activity, i.e. a high-dose inhibition effect.

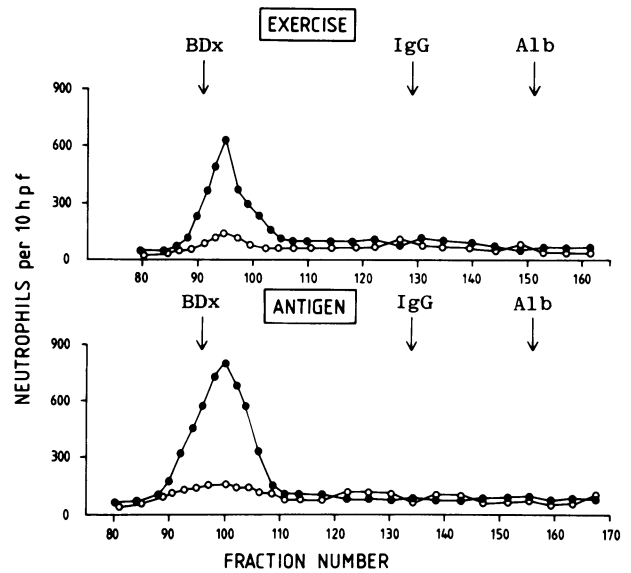


FIGURE 5 Sephadex G-200 chromatography of pre- and postexercise or antigen challenge serum from atopic asthmatics. The points are representative of one experiment. Each experiment was performed on three separate occasions, i.e. three prechallenge and three postchallenge after exercise, and three prechallenge and three postchallenge after antigen inhalation, i.e. a total of 12 column chromatographic procedures. The elution profile of the molecular markers [Blue Dextran (BDx), human IgG, and albumin (Alb)] is indicated. The open and closed circles represent the pre- and postchallenge samples, respectively.

Chemotaxis and chemokinesis

NCF^{EX} and NCF^{AG}, prepared by Sephadex G-200 chromatography, both promoted directional migration (chemotaxis) and stimulated random migration (chemokinesis) of neutrophil leucocytes (Table II). When NCF^{EX} was placed at the same concentration on either side of the micropore filter the numbers of cells migrating were approximately four times greater than those observed with cells in buffer alone. When the neutrophils migrated towards a gradient the difference was increased to approximately fivefold. Similarly, the numbers migrating towards a gradient of NCF^{AG} were higher than those observed with stimulated random migration, which in turn were more than the control containing Hanks' solution alone.

DISCUSSION

We have shown that after treadmill exercise or antigen challenge in asthmatics there was a three- to fivefold increase in heat-stable serum neutrophil chemotactic activity. The differences between pre- and postchallenge sera were highly significant (Figs. 1, 2), and for

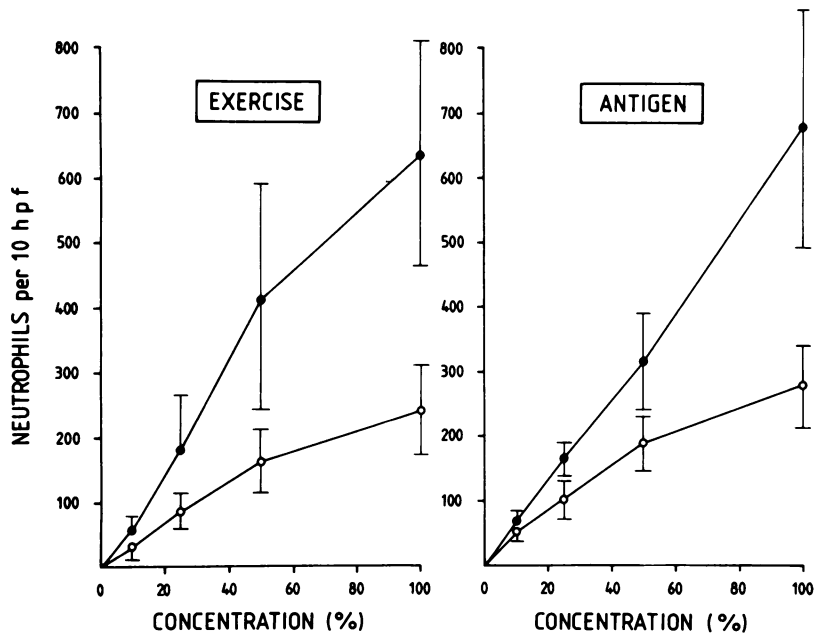


FIGURE 6 The effect of increasing concentrations of pre- and postchallenge NCF obtained by exercise or antigen inhalation. The material was partially purified by Sephadex G-200 chromatography. The points represent the mean \pm 1 SEM of three experiments. The concentration refers to the percentage of Sephadex G-200 fractions contained in the lower compartment of the chemotactic chamber. Samples were diluted with PBS.

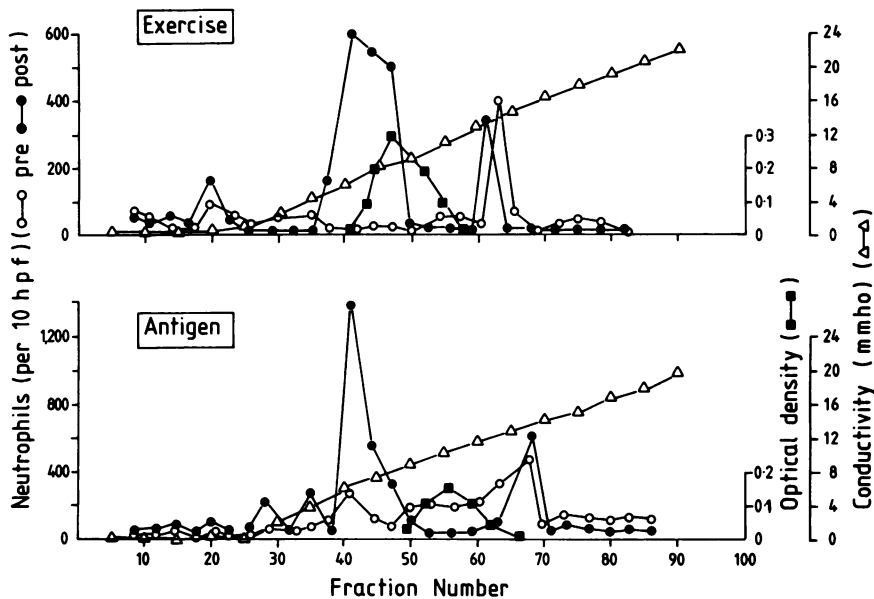


FIGURE 7 Anion exchange chromatography of pre- and postexercise or antigen challenge samples on DEAE-Sephacel. The points are representative of one experiment and each experiment was performed on three separate occasions. The samples subjected to this procedure were the pooled fractions having peak chemotactic activity following Sephadex G-200 gel filtration. These were concentrated to 10 ml by ultrafiltration (\times M300) and dialysed against 0.0035 M phosphate buffer (pH 7.8) before being applied to the column. The equivalent fractions of the prechallenge sera after G-200 chromatography were similarly processed.

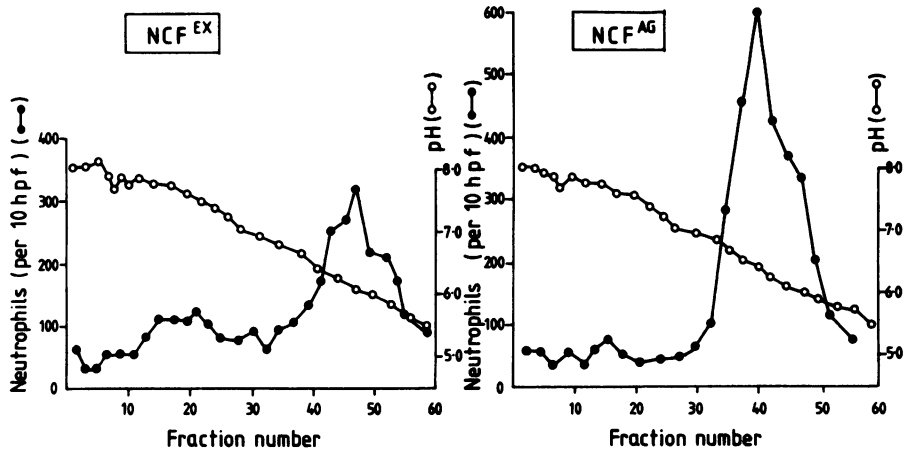


FIGURE 8 Chromatofocusing of the postexercise or antigen challenge samples. The points are representative of one experiment and each experiment was performed on three separate occasions. The samples subjected to this procedure had already been partially purified by Sephadex G-200 gel filtration and anion exchange chromatography (DEAE-Sephacel). The postchallenge fractions with peak chemotactic activity eluting between 0.15 and 0.30 M NaCl, pH 7.8, from DEAE-Sephacel were pooled and concentrated to 10 ml by ultrafiltration ($\times M300$). The sample was then dialysed against 0.025 M tris acetic acid buffer, pH 8.3, before being applied to the column.

this reason the results were expressed as the absolute numbers of migrating neutrophils rather than a "chemotactic index" used in a report (1) on antigen-induced NCF-A in bronchial asthma.

The magnitude of treadmill exercise undertaken by

the subjects in the present study was that which had previously been shown to regularly induce a fall in the PEFR of at least 10% in asthmatics (8). For convenience, the PEFR was measured after exercise, whereas FEV₁ was used after the inhalational challenges. It is

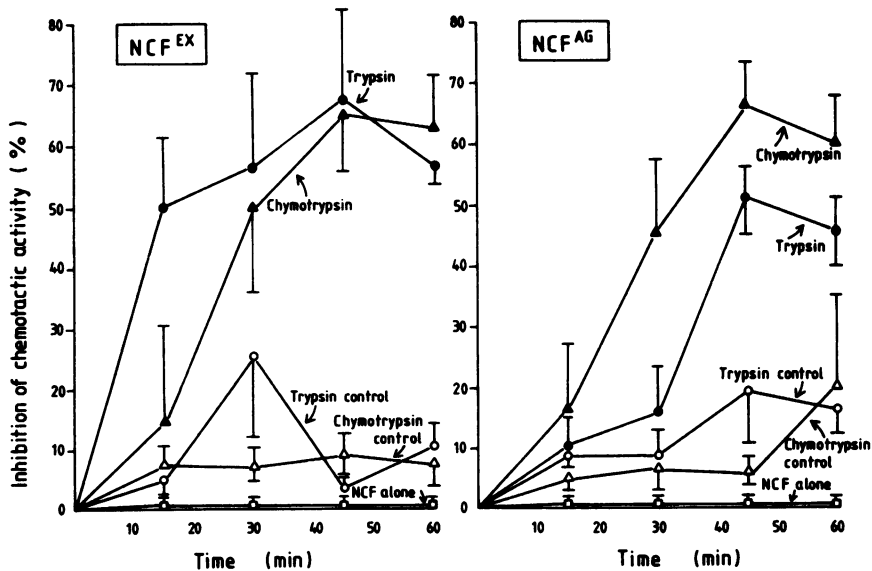


FIGURE 9 The effect of trypsin and chymotrypsin on the activity of NCF^{EX} and NCF^{AG}. The enzymes were used at a concentration of 0.4%. The NCF used in these experiments was prepared by Sephadex G-200 gel filtration, anion exchange chromatography (DEAE-Sephacel), and chromatofocusing. Each point is the mean \pm 1 SEM of three experiments. The time refers to the incubation period of NCF with the enzymes.

TABLE II
Chemotaxis and Chemokinests of NCF^{EX} and NCF^{AC}.

Upper chamber Lower chamber	Cells alone Buffer	Cells + NCF ^{EX} NCF ^{EX}	Cells alone NCF ^{EX}
Neutrophils per 10 hpf			
Experiment 1	41	144	212
Experiment 2	41	120	182
Experiment 3	42	186	228
Mean	41	150	207
Upper chamber Lower chamber	Cells alone Buffer	Cells + NCF ^{AC} NCF ^{AC}	Cells alone NCF ^{AC}
Neutrophils per 10 hpf			
Experiment 1	45	72	116
Experiment 2	62	82	142
Experiment 3	41	91	140
Mean	49	82	133

Undiluted NCF^{EX} and NCF^{AC}, partially purified by Sephadex G-200 chromatography, were placed in the lower or upper and lower compartments as indicated. In all experiments 0.4% ovalbumin was present on both sides of the filter.

now clear that the magnitude of the airways obstruction after exercise is directly related to the extent of heat and water loss from the respiratory tract and that similar pulmonary physiological changes can be produced by isocapnic hyperventilation (10–12). Although isocapnic hyperventilation can mimic the airflow obstruction after exercise in asthmatics, it did not evoke the release of detectable NCF or histamine (13). In contrast, our present findings and those of Barnes et al. who have demonstrated elevated plasma histamine concentrations during exercise-induced asthma but not after isocapnic hyperventilation (14, 15) indicate that there are differences in terms of the release of mediators of hypersensitivity, between an exercise task and isocapnic hyperventilation. The reason for this difference is not clear, but it is unlikely to be due to a fall in the arterial partial pressure of carbon dioxide (PaCO₂) on exercise, because there is good evidence to show that during and after exercise, the PaCO₂ of asthmatic and normal subjects does not change significantly from base line when work loads similar to that of the exercise task in the present study were used (16–20). Furthermore, there are other recognized differences between these two procedures. For instance, after exercise there was a lag period before the onset of airflow obstruction (21) and a refractory period during which the same manoeuvre does not cause an increase in airways resistance (22); neither of these occurs in isocapnic hyperventilation (23).

After exercise or antigen challenge, NCF concentrations returned to prechallenge values by ~60 min,

whereas the PEF_R and FEV₁ recovered more slowly and at 1 h were still 15% less than the base-line values. It is not possible to ascribe a role for NCF in causing airflow obstruction after treadmill exercise or antigen challenge even though the time-course of the changes in these variables was very similar (Fig. 2).

In the present study all asthmatic volunteers were atopic, as defined by the presence of positive skin-prick tests to common allergens and raised serum IgE concentrations. Mediator release in nonatopic individuals with exercise-induced asthma will be the subject of a separate study.

It has previously been shown (2–4) that NCF, histamine, and ECF-A were released into the circulation in cold, cholinergic, and solar urticaria. This would support the concept that mast cell degranulation can occur in response to physical stimuli. Thus exercise might be a comparable, nonimmunological stimulus for the release of mediators of hypersensitivity in atopic asthmatics. However, not all nonimmunological stimuli produce NCF because histamine challenge was not associated with detectable amounts of the mediator (Figs. 1, 2). It is important to appreciate that, by itself, the induction of airways obstruction does not lead to the release of circulating NCF, because neither histamine challenge (Figs. 1, 2) nor isocapnic hyperventilation (13) were associated with the appearance of detectable circulating mediators.

The gel filtration experiments confirm that NCF^{AC} is a macromolecule and indicate that NCF^{EX} has a similar molecular size. The elution profile after anion ex-

change chromatography was also identical, giving a consistent single peak of activity at 0.15–0.30 M NaCl. The presence of the later peak of activity at 0.40 M NaCl may be due to normal serum components that were not separated after the initial gel filtration step, as there was little difference between the pre- and postchallenge sera.

From the data we have presented it seems probable that NCF^{EX} and NCF^{AG} are identical because (a) their time-course of release was virtually the same (Fig. 2); (b) the appearance of NCF^{EX} was inhibited by the prior administration of DSCG (Fig. 4) in the same way as was reported for NCF^{AG} (5, 6); (c) both activities were associated with molecules of ~750,000 daltons (Fig. 5); (d) the NCF had chemokinetic and chemotactic properties (Table II); (e) NCF^{EX} and NCF^{AG}, partially purified by Sephadex G-200 chromatography, gave similar dose-response curves (Fig. 6); (f) they eluted as a single peak of activity after anion exchange chromatography (Fig. 7); (g) both had an estimated isoelectric point of between pH 6.0 and 6.5, as determined by chromatofocusing (Fig. 3); (h) the NCF were susceptible to tryptic and chymotryptic inhibition (Table I), suggesting that peptide bonds are important in the expression of their chemotactic activity. In addition we have shown in preliminary experiments (unpublished observations) that there is cross-deactivation between NCF^{EX} and NCF^{AG}, suggesting that they compete for the same chemotactic receptor site.

Chromatofocusing is a novel column chromatographic technique first proposed by Sluyterman and his co-workers (24–26) that separates proteins according to their isoelectric points. It is simple to operate and combines the high resolution obtainable by separations based on isoelectric focusing with the high capacity of ion exchange techniques. The isoelectric point of NCF determined by this method is very similar to that obtained previously using sucrose density gradient columns (2) and thin layer Sephadex G-75 gels (2, 27).

Although the role of NCF in bronchial asthma is unclear, the factor may participate in the early recruitment of neutrophils characteristic of immediate hypersensitivity tissue reactions (28–33). Infiltrating neutrophils, by releasing intracellular constituents, may contribute to bronchial hyperreactivity. The origin of NCF^{AG} is unknown, but its release appears to be closely dependent upon IgE/mast cell activation and as such may prove to be a useful marker for these events. It is important to note that mast cell dependency does not necessarily imply mast cell origin, because it is possible that NCF is released from another cell type after mast cell degranulation. It is reasonable to speculate that NCF^{EX} may also be mast cell-depen-

dent because (a) its release was inhibited by the prior administration of DSCG (Fig. 4), an agent thought to act predominantly on mast cells, and (b) the elevation in plasma histamine concentrations (using a modified radioenzymatic assay (14, 15)) after exercise or antigen challenge, closely paralleled the increase in NCF.² However, the possibility that NCF^{EX} may originate from circulating basophils that are known to increase after exercise (34, 35) cannot be excluded at this time.

The appearance of a single peak of activity from NCF^{EX} and NCF^{AG} after gel filtration, anion exchange chromatography, and an isoelectric point in the range of pH 6.0 to 6.5 are reminiscent of the NCF generated by cold challenge in cold urticaria (2) and from rat peritoneal mast cells by rabbit anti-rat F(ab)₂ antisera (36). Whether NCF^{EX} or NCF^{AG} are identical to the neutrophil chemotactic factor produced from basophil leukaemia cells (37) is yet to be determined.

ACKNOWLEDGMENTS

We are grateful to Miss Kaye Cussen for technical help.

Dr. Nagy is the recipient of a scholarship from the Hungarian Academy of Sciences. Dr. Nagakura is the recipient of a scholarship from the British Council.

REFERENCES

1. Atkins, P. C., M. Norman, H. Weiner, and B. Zweiman. 1977. Release of neutrophil chemotactic activity during immediate hypersensitivity reactions in humans. *Ann. Intern. Med.* **86**: 415–418.
2. Wasserman, S. I., N. A. Soter, D. M. Center, and K. F. Austen. 1977. Cold urticaria. Recognition and characterisation of a neutrophil chemotactic factor which appears in serum during experimental cold challenge. *J. Clin. Invest.* **60**: 189–196.
3. Soter, N. A., S. I. Wasserman, K. F. Austen, and E. R. McFadden, Jr. 1980. Release of mast cell mediators and alterations in lung function in patients with cholinergic urticaria. *New Engl. J. Med.* **302**: 604–608.
4. Soter, N. A., S. I. Wasserman, M. A. Pathak, J. A. Parrish, and K. F. Austen. 1979. Solar urticaria: release of mast cell mediators into the circulation after experimental challenge. *J. Invest. Dermatol.* **72**: 282. (Abstr.)
5. Atkins, P. C., M. Norman, and B. Zweiman. 1978. Antigen induced chemotactic activity in man, correlation with bronchospasm and inhibition by disodium cromoglycate. *J. Allergy Clin. Immunol.* **62**: 149–155.
6. Nagy, L. 1981. The effect of disodium cromoglycate on human serum neutrophil chemotactic activity in antigen induced bronchospasm. *Allerg. Immunol. (Leipz)*. **27**: 48–52.
7. Harries, M. G., P. S. Burge, and I. M. O'Brien. 1980. Occupational type bronchial provocation tests: testing with soluble antigen by inhalation. *Br. J. Ind. Med.* **37**: 248–252.

² Lee, T. H., M. J. Brown, L. Nagy, R. Causon, M. J. Walport, and A. B. Kay. Submitted for publication.

8. Godfrey, S., M. Silverman, and S. D. Anderson. 1973. Problems of interpreting exercise induced asthma. *J. Allergy Clin. Immunol.* **52**: 199-209.
9. Turnbull, L. W., and A. B. Kay. 1976. Eosinophils and mediators of anaphylaxis. Histamine and imidazole acetic acid as chemotactic agents for human eosinophil leucocytes. *Immunology.* **31**: 797-802.
10. Strauss, R. H., E. R. McFadden, Jr., R. H. Ingram, Jr., E. C. Deal, Jr., and J. J. Jaeger. 1978. Influence of heat and humidity on the airway obstruction induced by exercise in asthma. *J. Clin. Invest.* **61**: 433-440.
11. Deal, E. C., Jr., E. R. McFadden, Jr., R. H. Ingram, Jr., R. H. Strauss, and J. J. Jaeger. 1979. Role of respiratory heat exchange in production of exercise-induced asthma. *J. Appl. Physiol. Respir. Environ. Exercise Physiol.* **46**: 467-475.
12. Deal, E. C., Jr., E. R. McFadden, Jr., R. H. Ingram, Jr., and J. J. Jaeger. 1979. Hyperpnoea and heat flux: initial reaction sequence in exercise induced asthma. *J. Appl. Physiol. Respir. Environ. Exercise Physiol.* **46**: 476-483.
13. Deal, E. C., Jr., S. I. Wasserman, N. A. Soter, R. H. Ingram, Jr., and E. R. McFadden, Jr. 1980. Evaluation of role played by mediators of immediate hypersensitivity in exercise-induced asthma. *J. Clin. Invest.* **49**: 659-665.
14. Brown, M. J., P. W. Ind, P. J. Barnes, D. A. Jenner, and C. T. Dollery. 1980. A sensitive and specific radiometric method for the measurement of plasma histamine in normal individuals. *Anal. Biochem.* **109**: 142-146.
15. Barnes, P. J., and M. J. Brown. 1981. Venous plasma histamine in exercise and hyperventilation induced asthma in man. *Clin. Sci. (Lond.)*. In press.
16. Lugliani, R., B. J. Whipp, C. Seard, and K. Wasserman. 1971. Effect of bilateral carotid-body resection on ventilatory control at rest and during exercise in man. *New Engl. J. Med.* **285**: 1105-1111.
17. Katz, R. H., B. J. Whipp, E. M. Heimlich, and K. Wasserman. 1971. Exercise-induced bronchospasm, ventilation, and blood gases in asthmatic children. *J. Allergy.* **47**: 148-158.
18. Vassallo, C. L., J. B. L. Gee, and W. M. Domm. 1972. Exercise-induced asthma. Observations regarding hypocapnia and acidosis. *Am. Rev. Respir. Dis.* **105**: 42-49.
19. Allen, T., W. Addington, T. Rosendal, and D. Cugell. 1973. Alveolar carbon dioxide and airway resistance in patients with post exercise bronchospasm. *Am. Rev. Respir. Dis.* **107**: 816-821.
20. Åstrand, P. O., and K. Rodahl. 1977. Respiration. In *Textbook of Work Physiology—physiological bases of exercise*. McGraw-Hill Book Company, New York. 251.
21. Jones, R. S., M. H. Buston, and M. J. Wharton. 1962. The effect of exercise on ventilatory function in the child with asthma. *Br. J. Dis. Chest* **56**: 78-86.
22. Edmunds, A. T., M. Tooley, and S. Godfrey. 1978. The refractory period after exercise induced asthma: its duration and relation to the severity of exercise. *Am. Rev. Resp. Dis.* **117**: 247-254.
23. Stearns, D. R., E. R. McFadden, F. J. Breslin, and R. H. Ingram. 1981. Reanalysis of the refractory period in exertional asthma. *J. Appl. Physiol. Respir. Environ. Exercise Physiol.* **50**: 503-508.
24. Sluyterman, L. A. AE., and J. Wijdenes. 1977. Chromatofocusing: isoelectric focusing on ion exchangers in the absence of an externally applied potential. In *Proc. Int. Symp. Electrofocusing and Isotachopheresis*. B. J. Radola and D. Graesslin, editors. de Gruyter, Berlin. 463-466.
25. Sluyterman, L. A. AE., and O. Elgersma. 1978. Chromatofocusing: isoelectric focusing on ion exchange columns. I. General principles. *J. Chromatog.* **150**: 17-30.
26. Sluyterman, L. A. AE., and J. Wijdenes. 1978. Chromatofocusing: isoelectric focusing on ion exchange columns. II. Experimental verification. *J. Chromatog.* **150**: 31-44.
27. Wasserman, S. I. 1981. Physicochemical and functional analysis of a high molecular weight neutrophil chemotactic factor (HMW-NCF) released into the circulation by antigen challenge in humans. *J. Allergy Clin. Immunol.* In press.
28. Kline, B. S., M. B. Cohen, and S. A. Rudolph. 1932. Histologic changes in allergic and non-allergic wheals. *J. Allergy.* **3**: 531-541.
29. Kay, A. B. 1970. Studies on eosinophil leucocyte migration. I. Eosinophil and neutrophil accumulation following antigen-antibody reactions in guinea-pig skin. *Clin. Exp. Immunol.* **6**: 75-86.
30. Dolovich, J., and D. C. Little. 1972. Correlation of skin test reactions to *Bacillus subtilis* enzyme preparations. *J. Allergy Clin. Immunol.* **49**: 43-53.
31. Atkins, P. C., G. R. Green, and B. Zweiman. 1973. Histologic studies of human skin test response to ragweed, compound 48/80 and histamine. *J. Allergy Clin. Immunol.* **51**: 263-273.
32. Dolovich, J., F. E. Hargreave, R. Chalmers, K. J. Shier, J. Gauldie, and J. Bienenstock. 1973. Late cutaneous allergic responses in isolated IgE-dependent reactions. *J. Allergy Clin. Immunol.* **52**: 38-46.
33. Solley, G. O., G. J. Gleich, R. E. Jordan, and A. L. Schroeter. 1976. The late phase of the immediate wheal and flare skin reaction. *J. Clin. Invest.* **58**: 408-420.
34. Harries, M. G., P. S. Burge, I. O'Brien, O. Cromwell, and J. Pepys. 1979. Blood histamine levels after exercise testing. *Clin. Allergy.* **9**: 437-441.
35. Duner, H., and B. Pernow. 1958. Histamine and leucocytes in blood during muscular work in man. *Scand. J. Clin. Lab. Invest.* **10**: 394-396.
36. Lewis, R. A., E. J. Goetzl, S. I. Wasserman, F. H. Valone, R. H. Rubin, and K. F. Austen. 1975. The release of four mediators of hypersensitivity from human leukemic basophils. *J. Immunol.* **114**: 87-92.
37. Center, D. M., N. A. Soter, S. I. Wasserman, and K. F. Austen. 1980. Characterisation of neutrophil specific chemotactic activity from rat mast cells. *Am. Rev. Resp. Dis.* **121**: 61. (Abstr.)