

Mediators of Hypersensitivity and “Fog”-Induced Asthma

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Seven asthmatic and five normal subjects inhaled increasing amounts of nebulized water (“fog”). Neutrophil chemotactic activity (NCA), histamine and FEV₁ measurements were undertaken before and at time intervals after challenge. In asthmatics, the mean maximal reduction in FEV₁ (± 1 SD) was $46.6\% \pm 11.5$; whereas, in normal subjects, the reductions were less than 20% of pre-challenge values after the inhalation of 33 ml of water. There were no significant differences in the pre-challenge values for NCA between the asthmatics and the normal controls. When the highest values for NCA during the 30 min after challenge in the asthmatics were compared with controls there was a significant increase ($P < 0.02$). The percentage change in NCA was also significantly greater in the asthmatics compared with the controls at 10 min after challenge ($P < 0.05$). Fog-induced NCA was shown to be associated with proteins with approximate molecular weight of 600,000 daltons (as assessed by gel filtration chromatography on Sephacryl-S400). There was an increase in plasma histamine in the asthmatics after challenge but this was not significantly greater than the controls. These findings support the view that mediators might be involved in fog-induced asthma, possibly as a result of mast cell degranulation by “osmotic shock”.

Key words: asthma; bronchial provocation tests; chemotactic factors; chemotaxis; diamine oxidase; fog; histamine; inhalation provocation tests.

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The inhalation of ultrasonically nebulized distilled water (UNDW, “fog”) has been shown to be a potent cause of bronchoconstriction in asthmatic patients when compared with normal subjects (1, 2, 7, 16). This method of bronchoprovocation is safe, inexpensive and simple to perform and has potential as a useful alternative means of assessing bronchial hyperreactivity.

The mechanism by which UNDW causes bronchoconstriction is unclear. However, the presence of a refractory period after challenge and the knowledge that the provoked airway narrowing is inhibitable by DSCG (2, 7) support the view that mediators of hypersensitivity may be involved.

The aim of the present study was to evaluate the possible role of mediators of hypersensitivity in “fog”-induced asthma. The peripheral venous concentrations of histamine, neutrophil chemotactic activity (NCA) and diamine oxidase (DAO) were measured before and after bronchoprovocation with UNDW in a group of asthmatic subjects, and compared with normal controls.

PATIENTS AND METHODS

Seven asthmatic patients aged 23–49 years (3 females, 4 males) and five normal non-asthmatic subjects ages 24–43 years (2 females, 3 males) participated in the study.

All the patients were receiving bronchodilator therapy for treatment of their asthma but were asked to refrain from taking any medication for 8 h prior to the study.

Forced expiratory volume in 1 s (FEV_1), forced expiratory flow rate over the middle half of the vital capacity (FEF_{25-75}) and the flow rate at 50% of the vital capacity (FEF_{50}) were measured using a Cavitron SC-20 spirometric computer (Anaheim, Ca., U.S.A.). Measurements were made at rest, during, and for 90 min after an inhalational challenge with ultrasonically nebulized distilled water (MistO₂gen EN 143a, Ca., U.S.A.). The nebulizer delivered approximately 1 ml of water/10 l inhaled aerosol, and the particle size ranged between 2–10 microns.

For the challenge each person breathed with a normal tidal volume (usually 500–1000 ml) through a two-way valve (Hans-Rudolph No. 2700, Kansas, U.S.A.). Initially 5–10 l of the aerosol was inhaled and 30 s later measurements of FEV_1 and FEF_{50} were made. If, after this initial challenge, a fall of more than 10% of the pre-challenge value was recorded, a further 5 to 10 l of aerosol were administered. When the fall was less than 10%, the volumes used in subsequent tests were 20 l, 40 l, 80 l and 80 l, until a fall of at least 20% in FEV_1 was recorded, or 320 l had been inhaled.

The total dose of water delivered for each challenge was determined by recording the expired ventilation and using a regression equation for the output of the nebulizer (2). A dose response curve was constructed for each subject relating the dose of water inhaled to the reduction in FEV_1 , FEF_{50} and FEF_{25-75} . The dose of water in ml which provoked the maximum reduction in lung function from the pre-challenge values was recorded.

A dwell-cath (Tuta No. 17) was inserted under local anaesthesia into an antecubital fossa in. The catheter was kept patent using an infusion of normal saline. No anti-coagulants were used during the study. Blood was collected (20 ml), without the use of a tourniquet. Samples were taken in duplicate at rest, at an interval of 10 min. Further samples were collected immediately after the water challenge, 5,

10, 15, 30 and 60 min later and again 30 min after the inhalation of 5 mg terbutaline sulphate delivered as an aerosol.

Each sample of blood was divided into two aliquots. One aliquot was allowed to clot on glass at 4°C for 3 h and then centrifuged for 10 min at 2,000 rpm. Approximately 0.5 ml of serum was stored in a plastic bottle and immediately frozen for subsequent analysis of diamine oxidase. The rest of the serum was transferred to another bottle and the sample heated at 56°C for 30 min. This serum was then further divided into four 0.6 ml aliquots and frozen at –80°C for 2–7 days. The samples were then transported in dry ice to London for measurements of NCA. The second aliquot of blood was transferred to a 10 ml plastic tube containing 75 µl tetra-sodium EDTA to prevent clotting. The samples were centrifuged at 1800 rpm for 8–10 min at 4°C. When removing the plasma, care was taken to leave at least 1 ml above the buffy coat to ensure that there was no contamination with white cells. Duplicate samples of 0.4 ml of plasma were frozen and subsequently transported to Auckland, New Zealand, for measurements of histamine.

Neutrophil chemotactic activity assay

The assay of neutrophil chemotactic activity was performed using the modified Boyden technique as previously described (10). 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 a proportion of 2.3:1. After centrifugation at $200 \times g$ for 40 min at 20°C the cell-rich pellet was retained, washed in lysis buffer (ammonium chloride 0.82%, potassium bicarbonate 0.1%, pH 7.35) further centrifuged and washed twice in Hank's balanced salt solution. The cells were resuspended in Hank's solution containing 0.4% ovalbumin. This procedure routinely gave neutrophil purity greater than 95% and the cell counts were adjusted to 2×10^6 /ml. Samples (800 µl) of either 20% serum, diluted with Hank's solution or Sephacryl S400 fractions in

phosphate buffered saline were placed in the lower compartment. After a 90-min incubation the microphore filters (Sartorius membrane filters, 8 μ l pore size, 34 Gottingen, W. Germany) were removed, fixed and stained, as previously described, and the number of cells that had migrated through the entire thickness of the filter were counted. The results were expressed as the total number of cells in 10 random high power fields. The samples were assayed in duplicate and coded in such a way that the investigators did not have prior knowledge of the protocol. The values of each duplicate filter did not differ by more than 20%. In this assay, buffer alone gave cell count of 0–20 cells/10 high power fields and zymosan-activated serum (diluted 2.5% in Hank's solution), 400–800 cells/high power fields.

Gel filtration

Gel filtration chromatography was performed on columns of Sephacryl S400 (2.2 $\times$ 90 cm) with phosphate buffered saline (pH 7.35). The columns were operated at a flow rate of 10 ml/h. The serum samples were heated at 56°C for 30 min before being applied to the column. A 1 ml fraction of the pre-challenge serum was applied first, and after thorough washing, the post-challenge serum with the peak NCA was subjected to the same procedure. Fractions of 1 ml were collected and stored at –20°C prior to the neutrophil locomotion assay. Serum samples from three separate patients were examined.

Histamine assay

The method for histamine assay was modified from the microassay procedure described by Taylor et al. (17). Briefly, histamine methyl-transferase (HMT) solution was freshly prepared each day using an extract of lyophilised rat kidney previously purified by ammonium sulphate precipitation (15). HMT/S-adenosyl-L-(methyl-³H) methionine (³H-SAMe) reactant solution was prepared by adding 10 μ l ³H-SAMe (10 Ci/mmol NEN, Boston, U.S.A.) to 1.0 ml HMT immediately before use in the assay. Then each of five plasma samples (400 μ l) was in-

cubated with 50 μ l HMT-³H-SAMe reactant solution at 37°C for 1 h. An internal standard of one ng histamine was added to two samples. The reaction was stopped by addition of 20 μ l 5 M NaOH containing methylhistamine (10 μ g ml⁻¹). The ³H-methylhistamine formed was extracted into chloroform (3 ml) and the radioactivity in organic phase was determined by liquid scintillation spectrometry. With each assay, blanks and external standard standards of histamine were performed in triplicate.

Diamine oxidase assay

Frozen serum was thawed and the diamine oxidase (DAO) concentration determined by the radiobiochemical method of Tufvesson & Tryding (19) using ¹⁴C-putrescine as a substrate. Prior to the assay, it was found necessary to heat the radiolabelled substrate (0.5 mM putrescine dihydrochloride, 1.5 mCi/mmol) at 100°C for 30 min to restore any activity lost through storage. The mean values of duplicate analyses were recorded as nano-units of DAO/l plasma, where one unit is equivalent to a substrate consumption of 1 mol/s.

Statistical analysis

Statistical analysis comparing the changes in the absolute values of the various parameters at different times for the same patients, was performed using the non-parametric Wilcoxon matched-pairs signed-rank test. When the normal and asthmatic groups were compared using the results expressed as the percentage change from pre-challenge value, the Mann-Whitney U test was used.

RESULTS

Individual values for FEV₁ measured immediately prior to challenge are given in Table 1. There was a wide variation in the degree of airways obstruction present at rest, with only three of the seven subjects having values within the predicted normal range (80–120% predicted).

The asthmatic patients were extremely sensitive to the inhalation of UNDW. After challenge the seven patients had a reduction in FEF_{25-75} and FEF_{50} greater than 40% of the pre-challenge level. One additional patient, who had entered the study, had a reduction in FEV_1 of 30% after challenge but only small falls in FEF_{25-50} and FEF_{50} and for this reason was excluded.

The changes in FEV_1 expressed as a percentage of the predicted value are illustrated for both the asthmatic and normal subjects in Fig. 1. The dose of water required to induce the maximum reduction recorded for FEV_1 is also given in Table 1, together with the values for the provoking dose of water required to induce a 20% reduction in each of the measurements of lung function. The values for PD_{20} were obtained from the dose response curve con-

structed for each patient as previously described (2).

The normal subjects inhaled 32 ml of water without registering a change of greater than 20% for FEV_1 and FEF_{25-75} although one normal did have a reduction in FEF_{50} of 20.7% after 23 ml of water had been inhaled.

Neutrophil chemotactic activity

There was no significant difference between the values for number of neutrophils/10 high powered fields (N/10 HPF) observed on the two occasions prior to challenge and the coefficient of variation for the resting levels was 27%. The mean of the two resting values has been used in Fig. 1, and the maximum change in N/10 HPF is shown. For the asthmatics there was an increase in N/10 HPF of 186 ± 48 (SEM) after challenge;

Table 1

Values for forced expiratory volume in 1 sec (FEV_1) expressed as a percentage of the predicted normal value (R%P). The provoking dose of water for the maximum fall (PD_{max}) observed for FEV_1 , forced expiratory flow in the middle half of the vital capacity (FEF_{25-75}) and the flow rate at 50% of the vital capacity (FEF_{50}) and the PD for a 20% reduction in FEV_1 , FEF_{25-75} , and FEF_{50} . The maximum values for the reduction in FEV_1 , FEF_{25-75} , and FEF_{50} observed after challenge are expressed as a percentage of the pre-challenge level

	Maximum							
	R%P	PD	FEV ₁	Per cent Fall	FEF ₅₀	PD for 20% Fall in		
	FEV ₁	Max		FEF ₂₅₋₇₅		FEV ₁	FEF ₂₅₋₇₅	FEF ₅₀
Asthmatics								
A	117.6	13.73	25.0	42.7	48.0	5.94	3.53	2.47
C	92.5	6.31	31.9	51.4	50.7	2.09	1.27	1.60
D	58.1	1.21	56.0	42.1	47.7	<0.75	<0.75	<0.75
F	73.6	0.75	49.6	50.0	60.0	<0.28	<0.28	<0.28
G	68.3	9.55	38.9	44.5	47.4	1.44	0.60	0.69
P	45.0	1.21	33.6	42.1	44.2	0.42	0.32	0.31
Q	99.3	10.48	56.3	52.2	59.1	5.01	5.24	4.45
Mean	80.3	6.18	41.6	40.7	51.0	2.28	1.71	1.51
SD	23.6	5.26	12.4	17.3	6.1	2.28	1.92	1.51
SEM	8.9	1.98	4.7	6.5	2.3	0.86	0.73	0.57
Normals								
E	123	32	0	0	2.9	>32	>32	>32
B	87	32	0	9.7	1.3	>32	>32	>32
J	104	32	0.9	0	8.1	>32	>32	>32
K	80	32	0	5.1	1.4	>32	>32	>32
M	127	32	6.6	8.4	20.7	>32	>32	23
Mean	104	32	1.5	4.6	6.9			
SD	19	0	2.6	4.1	7.3			
SEM	8.4	0	1.2	0.5	3.3			

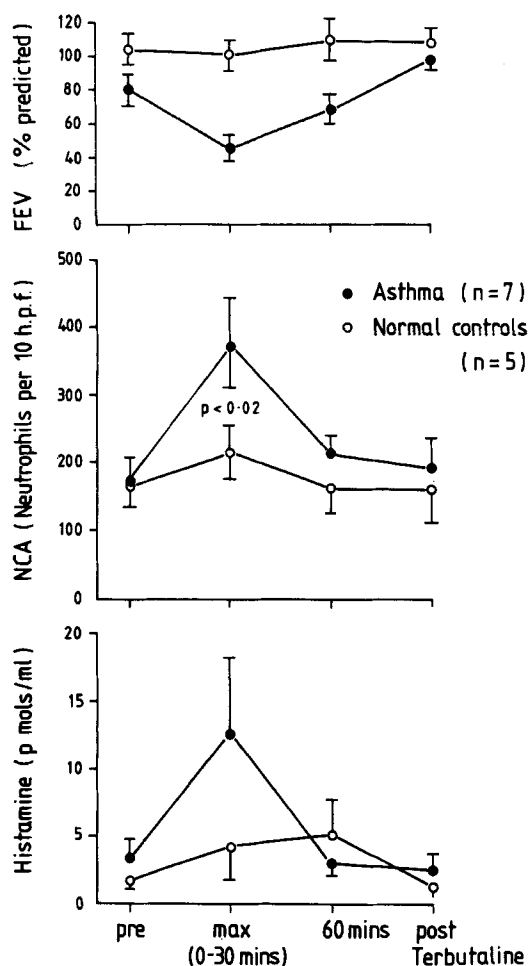


Fig. 1. Changes in serum NCA, plasma histamine and FEV₁ expressed as a percent of predicted after inhalation of ultrasonically nebulized distilled water.

The points represent the mean $\pm$ 1 SEM of seven asthmatic patients (closed circles) and five normal subjects (open circles). There was a significant increase in NCA in the asthmatic patients after inhalation, $P < 0.05$ (Wilcoxon matched pairs signed rank test). This increase in NCA was significantly greater than that in the normal subjects, $P < 0.02$ (Mann-Whitney U test).

(Table 3). The maximum change in histamine response to challenge (highest post-challenge value minus mean of pre-challenge values) was significant ($P < 0.05$) for the asthmatics but not the normal subjects. However, the increase was not significantly greater in the asthmatic than the control subjects. Six of the seven asthmatic subjects had an elevation in plasma histamine

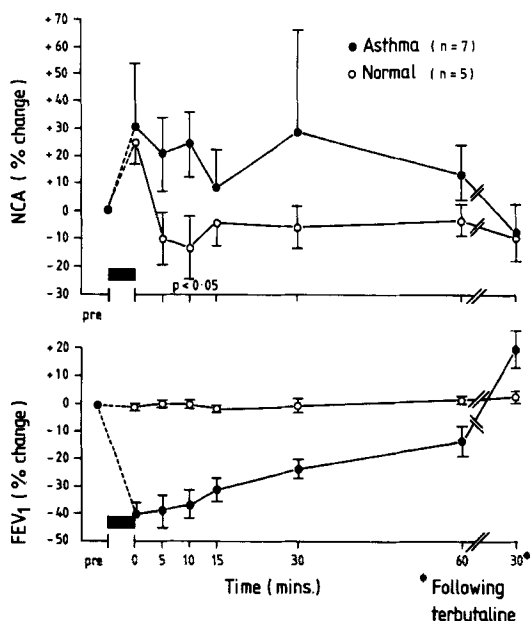


Fig. 2. Time course of NCA after inhalation of ultrasonically nebulized distilled water.

The points represent the mean $\pm$ 1 SEM of seven asthmatic patients (closed circles) and five normal subjects (open circles). The challenge period is represented by the solid bar. Both NCA and FEV₁ were expressed as percentage change from the mean of two pre-challenge values. There was a significant increase in NCA in the asthmatic patients compared with the normal subjects at 10 min after challenge, $P < 0.05$ (Mann-Whitney U test).

level by 100% within 30 min of challenge. This was only found in two of the control subjects. The change in histamine levels in relation to changes in NCA and airway function are illustrated in Fig. 1.

Diamine oxidase

Individual results for the values measured for diamine oxidase at rest, after challenge and after the administration of terbutaline are given in Table 4. The values at rest were within normal limits in the asthmatics and on the upper limit of normal (less than 130 nano-units ml⁻¹) for the normal subjects. The levels of diamine oxidase tended to be lower in the asthmatics compared with the normals but there was a wide variation within each group.

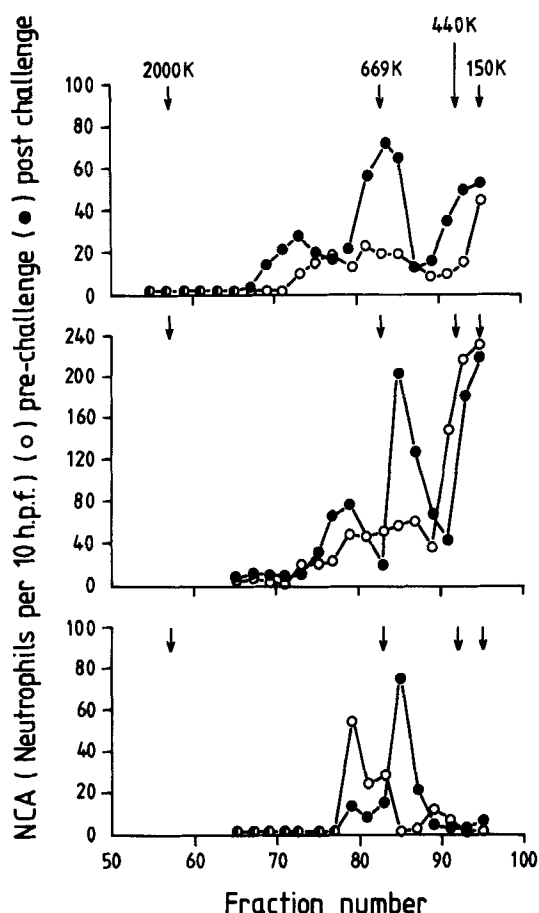


Fig. 3. Sephacryl S400 gel filtration of serum before and after the inhalation of ultrasonically nebulized distilled water. The symbols represent pre-challenge (open circles) and post-challenge (closed circles) serum samples from three individuals. The elution profiles of the molecular markers Blue Dextran (2000K), Thyroglobulin (669K), Ferritin (440K) and Immunoglobulin G (150K) are shown.

There was a small increase in the levels for DAO 60 min after challenge in the asthmatics but this was not statistically significant. There was no correlation between the maximum change observed in DAO levels and plasma histamine in the asthmatic subjects.

DISCUSSION

In this study, bronchoprovocation with ultrasonically nebulized distilled water provided good discrimination between asthmatic and

non-asthmatic subjects. All the asthmatic patients had a fall in FEV₁ of greater than 20% after less than 6 ml of water, whereas the control subjects were administered over 32 ml with no similar changes in FEV₁. This supports the view that this test, which is easy to perform with no discomfort to the patient, may have a place in the routine diagnosis of asthma.

The pre-challenge NCA was similar in both asthmatic and control groups. The mean increase in NCA in the asthmatic subjects was over 90% of the baseline value. This was statistically greater than that found in the normal subjects. When the mean values of NCA at each time point after challenge were compared (Fig. 2) the difference between asthmatics and control subjects was not so marked as when maximum values were contrasted (Fig. 1). These values were statistically different only at the 10 min time point after challenge ($P < 0.05$). This may be explained by the differing duration of challenge procedure in each individual, with consequent variation in the time of maximum NCA release. Four of the five normal subjects had an increase in NCA greater than 20% at the end of the challenge, but at no other time did any of the normal subjects have NCA values more than 20% above baseline.

The demonstration that the NCA was of high molecular weight using S400 gel filtration confirmed that it was similar to the NCA found after both antigen and exercise challenge (10, 11, 14).

There are well-known difficulties with measurements of plasma histamine. In order to improve the accuracy, all assays were repeated between 4 and 6 times. From the mean and standard deviation of these results (Table 3), the average coefficient of variation of the assays was calculated to be 1.2 pmol ml⁻¹. The recovery of synthetic histamine added to the plasma at the time of sampling was 73 to 85%. The baseline values tended to be higher in the asthmatic than the control subjects and, although not statistically significant, is in keeping with other studies (3). There was an elevation in the plasma histamine concentration in all the asthmatic patients within 30 min of the challenge. In three of the control subjects, the

plasma histamine concentrations fell but the overall difference between the groups was not statistically significant.

In an attempt to assess the contribution of degradation to the plasma histamine concentration and thus improve the interpretation of the changes in plasma histamine, circulating DAO, the main degradation enzyme of histamine, was measured. However, there was wide variation in the resting values within each group. The higher resting values in the normal subjects compared to the asthmatic patients, although of interest, are unexplained. There was no change in DAO after challenge. Thus in this study serum DAO measurement was of no value in corroborating changes in plasma histamine concentration, and would appear to be a poor marker of histamine release.

In this study, on a limited number of patients, the increase in NCA and the trend towards a rise in plasma histamine in the asthmatic patients when compared with the control subjects supports the view that mediators may

be involved in "fog"-induced asthma. The changes seen were more modest than those found after exercise challenge (9). This may be accounted for by the failure of mediators to diffuse rapidly into the circulation in the absence of a raised cardiac output, as has been suggested to explain the modest rises in mediators after cold air isocapnic hyperventilation (13).

Previous studies in asthmatic patients using different challenge procedures have shown release of the mediators NCA and histamine, during the early and late responses to both antigen bronchoprovocation and exercise tests (5, 9, 12). This suggests that in addition to IgE-mediated stimuli, which are presumed to act directly on the mast cell, other non-specific stimuli may trigger mediator release. In this study similar mediator release was found in association with another non-specific stimulus, the inhalation of nebulized water. Previous studies have shown that in addition to distilled water, inhalation of 3.6% saline or 20% dex-

Table 3

The mean of 4-6 measurements of venous plasma histamine $\pm$ ISD expressed in pmol ml⁻¹ in samples from the asthmatics and non-asthmatic subjects, collected before and after challenge, and following administration of terbutaline

	1.	Rest 2.	Mean	Post challenge Highest	Time	Post terbutaline
Asthmatics						
A		2.16 $\pm$ 2.21	2.16	5.14 $\pm$ 4.54	15	0.004 $\pm$ 0.008
C	2.86 $\pm$ 4.05	0.89 $\pm$ 0.65	1.87	17.02 $\pm$ 4.28	10	1.44 $\pm$ 1.78
D	19.67 $\pm$ 3.96	3.86 $\pm$ 1.50	11.76	45.02 $\pm$ 4.73	5	2.27 $\pm$ 2.03
F	1.60 $\pm$ 0.25	2.12 $\pm$ 1.06	1.86	4.87 $\pm$ 1.83	30	9.76 $\pm$ 2.25
G	0.11 $\pm$ 0.14	0.10 $\pm$ 1.23	1.10	4.92 $\pm$ 2.12	30	0.00 $\pm$ 0.00
P	4.36 $\pm$ 2.96	5.20 $\pm$ 3.82	4.78	5.97 $\pm$ 5.48	30	3.61 $\pm$ 3.21
Q	2.10 $\pm$ 0.76	0.68 $\pm$ 0.65	1.39	4.91 $\pm$ 1.77	5	0.10 $\pm$ 0.17
Mean			3.56	12.56		2.45
SD			3.81	15.00		3.50
SEM			1.44	5.66		1.32
Normals						
E	3.05 $\pm$ 1.7	2.05 $\pm$ 1.61	2.55	14.39 $\pm$ 10.71	60	2.19 $\pm$ 2.31
B	4.11 $\pm$ 0.65	1.46 $\pm$ 3.11	2.78	2.52 $\pm$ 1.72	60	0.05 $\pm$ 0.12
J	2.74 $\pm$ 3.32	2.07 $\pm$ 0.88	4.81	1.23 $\pm$ 2.74	end	0.33 $\pm$ 0.52
K	0.77 $\pm$ 0.73	1.16 $\pm$ 1.40	0.96	1.09 $\pm$ 2.67	60	0.30 $\pm$ 0.38
M	0.00 $\pm$ 0.00	0.06 $\pm$ 0.15	0.03	7.25 $\pm$ 5.49	60	3.13 $\pm$ 0.91
Mean			1.75	5.30		1.20
SD			1.32	5.67		1.38
SEM			0.59	2.54		0.62

trose but not normal saline, causes similar diminution of FEV_1 (16). These responses were not related to temperature, as they were reproducible when the temperature of the aerosol was increased to 36°C. This implies that it is the change in the osmotic pressure on the surface of the bronchi which initiates the bronchoconstriction. Human bronchi have mast cells in the lumen (18), and further support for the involvement of mediators comes from *in vitro* experiments on rat pleural and peritoneal cells which have shown that mast cells release their contents in response to hypotonic stimulus (4, 8).

The physiological importance of NCA and histamine is uncertain but they serve as markers for a plethora of mediators which are thought to be released by lung mast cells. These mediators, such as histamine and the sulphidopeptide leukotrienes may act directly on the airways, whilst other mediators such as NCA and leukotriene B_4 may act by recruitment of white cells to augment the response. These mediators of hypersensitivity may possibly also stimulate,

or be released in response to stimulation from, autonomic nerves. There is some evidence of involvement of neurological pathways in UNDW-induced bronchoconstriction as high doses of anti-cholinergic agents may inhibit the response (6), but other studies using smaller doses have found a more variable or absent effect (1, 2).

In this study bronchoprovocation with ultrasonically nebulized distilled water has been found useful in discriminating between asthmatic and normal individuals. The elevation of NCA and the similar trend in the plasma histamine concentrations support the view that mediators of hypersensitivity may be involved in bronchoconstriction after this form of challenge.

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Table 4

Values for diamine oxidase levels measured as nano-units per ml at rest, after challenge with distilled water and after the administration of 5 mg terbutaline sulphate by aerosol

	1.	Rest 2.	Mean	Post challenge		Post terbutaline 10 min
				10 min	60 min	
Asthmatics						
A	8	6	7	12	17	16
C	0	2	1	4	6	4
D	50	59	54	88	84	73
F	0	0	0	43	34	33
G	66	64	65	70	219	54
P	101	103	102	74	82	—
Q	37	34	35	51	38	53
Mean			38	49	67	
SD			38	32	77	
SEM			14.4	12.1	29.1	
Normals						
F	230	197	214	227	—	213
B	10	7	9	0	11	18
J	106	91	98	138	120	63
K	83	75	79	69	72	59
M	302	302	302	310	300	327
Mean			140	140		130
SD			117	123		136
SEM			52	55		61

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