

Can early asthma be confirmed by laboratory tests?

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We examined the performance of a number of laboratory tests in 23 patients who had had symptoms suggesting asthma, such as cough, sputum secretion, and chest tightness with wheezing, for less than a year. Even the best test, histamine challenge, had a sensitivity of only 48%. When more tests were added, sensitivity rose: with peak expiratory flow added, the sensitivity was 65%; with sputum eosinophil cationic protein (ECP) added, to 74%; and with serum ECP added, to 78%. Thus, a combination of tests measuring lung function and activation of eosinophils yielded fair, if not good, results. It appears that sensitivity could be increased further through the development of improved sputum tests.

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Asthma – including early asthma – is characterized by an inflammation of the bronchial mucosa, which is infiltrated by inflammatory cells, in particular, eosinophilic leukocytes (1). The eosinophils become activated and secrete characteristic proteins such as eosinophil cationic protein (ECP), eosinophil protein X (EPX) – also known as eosinophil-derived neurotoxin (EDN) – eosinophil peroxidase (EPO), and major basic protein (MBP). Increased levels of eosinophilic proteins have been found in the serum (2), bronchoalveolar lavage (BAL) fluid (3), and sputum (4) of asthma patients. Neutrophilic leukocytes probably also become activated, but the evidence is contradictory (5, 6). Activated neutrophils secrete potent enzymes and mediators including myeloperoxidase (MPO) (7).

The diagnosis of early, mild asthma can be difficult, as lung function measurements may remain normal. The inflammation present in the bronchial mucosa can be verified with bronchial biopsy or BAL fluid specimens, but these investigations are invasive and laborious and can therefore rarely be used. Other laboratory tests employed include measurements of blood eosinophil numbers and serum ECP levels; unfortunately, these methods are insensitive. Sputum may be a better sample material than blood or serum; the secretion comes from the bronchial mucosa, the actual site of the disease, and sputum specimens are obtained in a noninvasive manner (4, 8, 9).

We have studied sputum cytology and determined the leukocyte activation markers ECP, EPX, and MPO in the sputum, serum, and urine of patients with early or chronic asthma, as well as of control subjects. In addition, we used traditional lung function tests. The aim was to identify the laboratory tests most useful in the diagnosis of early or suspected asthma.

Material and methods

Patients and controls

Early or suspected asthma. The group consisted of 23 outpatients. The patients had had one or more of the following asthma symptoms for less than a year: cough, sputum secretion, and chest tightness with wheezing. In addition, they needed asthma medication. One patient had received anti-inflammatory medication for a short period before the study. Patients with chronic bronchitis respiratory infection, or prolonged undefined coughing were excluded.

Chronic asthma. Nineteen patients had had chronic asthma with a duration of 2–52 years (mean 13 years). All except one had received anti-inflammatory medication. At the time of the study, 11 patients suffered from exacerbation of symptoms and needed more efficient medication; in eight, the asthma was in a stable phase.

Table 1. Clinical characteristics of patients and controls

Characteristic	Early or suspected asthma	Chronic asthma	Control subjects
Number	23	19	10
Females/males	15/8	14/5	10/0
Mean age (years)	35.0	46.4	43.4
Age range (years)	17–61	20–81	23–55
Duration of symptoms (years)	<1	2–52	
Nonsmokers/smokers	20/3	19/0	10/0
Nonatopics/atopics	15/8	8/11	10/0
FEV ₁ *	94.2 ± 11.1	89.8 ± 10.6	ND
PEF**	93.6 ± 8.4	67.3 ± 24.1	ND

* % of predicted FEV₁ value; mean ± standard deviation. ** % of predicted value; mean ± standard deviation. PEF expressed as morning PEF values. ND = not done.

Control subjects. The group included 10 healthy, nonatopic, nonsmoking volunteers with no past or current symptoms of asthma.

The group with chronic asthma and the healthy subjects served as reference populations with which the patients with early or suspected asthma were compared. The groups are described further in Table 1.

Lung function measurements

Patients in both groups were subjected to flow volume spirometry (Vitalograph, Vitalograph Ltd, Buckingham, UK) and follow-up with peak expiratory flow (PEF) measurements (Spira Peak Flow, Hengityshoitokeskus, Hämeenlinna, Finland). Patients measured their PEF values for 1–2 weeks twice a day before and after two puffs of inhaled β_2 -agonist (0.5 mg terbutaline per dose). PEF values were expressed as a percentage of the patient's predicted value. Reversible bronchial obstruction was considered to be present if PEF values increased by 15% after the β_2 -agonist or fluctuated 20% from day to day.

Bronchial responsiveness was tested by the histamine challenge test. Results were considered positive if the provocative dose (PD₁₅) causing a 15% reduction in forced expiratory volume in 1 s (FEV₁) was less than or equal to 1.6 mg histamine (10).

Skin prick test

Skin prick tests were performed with a panel of 11 common environmental allergens (Soluprick SQ, 10 HEP, ALK, Denmark). A patient was defined as atopic if one or more allergens gave a wheal equal to or larger than 3 mm (11).

Sample collection and assays

Sputum, blood, and urine samples were collected at the same visit. Samples were kept at –20° until analyzed. For study of reproducibility, samples from patients with early or suspected asthma were collected on two occasions with a 2-week interval.

Serum was separated from blood under standardized conditions permitting release of leukocyte activation markers: blood was collected by venipuncture and allowed to clot at room temperature for 50–70 min. Samples were then centrifuged twice at 1350 g for 10 min to separate serum.

To induce sputum production, subjects inhaled nebulized hypertonic saline from an ultrasonic nebulizer (Spira Ultra, Hengityshoitokeskus, Hämeenlinna, Finland) until sputum was obtained or 15 min had passed. Before inhalation, subjects rinsed their mouths with water. The nebulizer was on maximum settings and was charged with 4 ml 5% NaCl. Most particles generated had a diameter of 1.1–2.2 μ m.

Native sputum was spread thinly on two microscope slides. The slides were air-dried and stained with eosin and methylene blue. Slides were examined under a microscope to determine the number of eosinophils and neutrophils in a semiquantitative manner. Results were graded as 0, 0.5, 1, 2, 3, or 4 for each type of cell (12, 13). To standardize interpretation, the analyst used a set of drawings showing typical microscope fields.

For liquefaction of sputum, the sample was diluted with an equal volume of a solution of 6.5 mM dithiothreitol (Sputolysin, CalbioChem, LaJolla, CA, USA) and incubated for 15 min at room temperature. The sample was centrifuged, and the pellet material was used for cytology, and the supernatant for ECP, EPX, and MPO assays.

Slides with pellet material were fixed with ethanol and stained by Papanicolaou. If examination of the slides revealed macrophages or ciliated epithelial cells as prominent cell types, the sputum sample was considered to be of bronchial origin; if not, the sample was rejected.

Blood total eosinophil counts were obtained with a Technicon model H2 analyzer. For the assay of ECP, EPX, and MPO in serum, sputum, and/or urine, radioimmunoassay research kits from Pharmacia Diagnostics (Uppsala, Sweden) were used according to the manufacturer's instructions. To adjust for variable urine water content, urinary EPX results are given as EPX/creatinine (μ g/mmol) ratios.

Statistical analysis

Analysis of variance with Tukey's studentized range test for pairwise comparisons was used. Spearman's

Table 2. Laboratory results

Test	Unit	Reference range (Normal values)	Source of reference range	Log-normal distribution*	Early or suspected asthma		Chronic asthma		Control subjects	
					n	Mean	n	Mean	n	Mean
PEF	% of predicted	> 80	Reference 17	No	20	94	14	67		
Histamine challenge**	PD ₁₅ , mg histamine	> 1.60	Reference 10	Yes	23					
Blood eosinophils	10 ⁹ /l	0–0.43	Own laboratory	Yes	23	0.20	19	0.22	10	0.13
Sputum eosinophils	(arbitrary)	0–0.5	Own laboratory	No	22	0.4	16	0.7	8	0
Serum ECP	µg/l	2.3–16.0	Manufacturer	Yes	23	8.9	19	8.9	10	6.4
Serum EPX	µg/l	8.5–37.5	Manufacturer	Yes	23	25.5	19	34.8	10	19.6
Sputum ECP	µg/l	0.0–26.0	Control group	Yes	22	13.0	16	73.0	8	2.7
Sputum EPX	µg/l	0.0–129	Control group	Yes	22	56.9	16	258.8	8	14.6
Urine EPX/creatinine	µg/mmol	11–68	Control group	Yes	23	56.8	17	52.2	9	34.2
Serum MPO	µg/l	257–682	Control group	Yes	23	354	18	475	10	443
Sputum MPO	µg/l	0–1545	Control group	Yes	22	247	15	466	8	54

* When log-normal distribution was assumed, logarithmic values were used for statistical calculations, and geometric means are given. ** Results for histamine challenge test in Table 3.

rank correlation was used to test for relationships and Wilcoxon's signed rank test to test for differences between visits. As the distribution of results for several assays (blood eosinophils, all ECP, EPX, and MPO assays) was roughly log-normal, logarithmic values were used in statistical evaluations (Table 2); e.g., geometric means were compared. Comparisons between atopic and nonatopic patients in the group with early or suspected asthma were performed by Student's *t*-test.

The precision of blood and sputum eosinophil, and of ECP, EPX, and MPO results was examined by comparing results obtained 2 weeks apart for patients with early or suspected asthma. No significant differences were observed with Wilcoxon's signed ranks test (*P* values from 0.27 to 0.95), nor were there any significant differences in the geometric means for the two visits.

To examine the sensitivity and specificity of various tests, one needs reference (normal) ranges. For some of the assays, 95% (mean $\pm$ 2 SD) ranges were obtained from the control group. In other cases, reference ranges previously determined in our laboratory or obtained from the reagent manufacturer or from literature were considered more dependable, as the number of subjects in the control group was small (Table 2).

Results

As we were trying to find a sensitive laboratory test with which to confirm early asthma, attention was focused on patients with early or suspected asthma and on the use of sputum as sample material. A sputum sample which by cytologic criteria was of truly bronchial origin was obtained from 96% of subjects in the early or suspected asthma, 84%

of subjects with chronic asthma, and 80% of controls. When sensitivities of sputum assays are examined below, only those subjects from whom samples could be obtained are considered.

Reference ranges (normal values) for tests used were obtained from several sources. For five tests, the source was the control group of the study, i.e., a group with only 10 subjects, eight of whom could produce a sputum sample for the assays; the reference ranges in question should be regarded as provisional (Table 2). For the control group, all test results were within the reference ranges; thus, specificity for all tests was 100%.

Lung function measurements

The PEF follow-up suggested reversible bronchial obstruction in 39% of the patients with early or suspected asthma. In two (9%) patients, mild obstruction could be observed with spirometry. The histamine challenge test, which is generally considered a good diagnostic aid, had a sensitivity of 48%, the increase in bronchial responsiveness being mild in seven patients, moderate in three patients, and severe in one patient (Table 3).

Table 3. Bronchial hyperreactivity of 23 patients with early or suspected asthma as measured with histamine challenge test

Hyperreactivity	PD ₁₅ (mg histamine diphosphate)	Patients	
		n	%
Severe	< 0.10	1	4
Moderate	0.10–0.39	3	13
Mild	0.40–1.60	7	31
None	> 1.60	12	52

Eosinophils, ECP, and EPX

When eosinophils, ECP, and EPX were assayed, better sensitivity was attained with sputum than with blood or serum as sample material. The sensitivities of sputum eosinophil, ECP, and EPX assays were roughly similar, 35–39% in the early or suspected asthma group and a somewhat more variable 44–76% in chronic asthma. Urine EPX/creatinine

Table 4. Sensitivity of laboratory tests and combinations of laboratory tests when two patient groups were studied

Test or combination of tests	Sensitivity (%)	
	Early or suspected asthma	Chronic asthma
PEF*	39	100
Histamine challenge	48	n.a.
Blood eosinophils	26	24
Sputum eosinophils	35	44
Serum ECP	30	32
Serum EPX	26	32
Sputum ECP	35	76
Sputum EPX	39	50
Urine EPX/creatinine	39	24
Serum MPO	9	17
Sputum MPO	13	13
Histamine challenge + PEF	65	
Histamine challenge + PEF + sputum eosinophils	70	
Histamine challenge + PEF + sputum ECP	74	
Histamine challenge + PEF + sputum ECP + serum ECP	78	
All tests	83	
Sputum eosinophils + sputum ECP	43	88
Histamine challenge + sputum eosinophils + sputum ECP	70	

* Reversibility in PEF; positive bronchodilation test or diurnal variation. n.a. = not available.

also exhibited similar sensitivity, 39%, in early or suspected asthma, but an unexpectedly poor sensitivity, 24%, in chronic asthma (Table 4). A close correlation ($r=0.95$) existed between patients' ECP and EPX levels; this was true both for sputum (Fig. 1) and serum (not shown).

MPO

Increased levels of the neutrophil activation marker MPO were occasionally found in the sputum or serum of patients in both asthma groups. Sensitivity was low (Table 4).

Combinations of tests

In early or suspected asthma, various combinations of tests exhibited higher sensitivities than single tests. The use of two lung function tests (PEF and histamine challenge) gave a 65% sensitivity; supplementation of this with a sputum examination (eosinophils and ECP) gave 78%. If all other tests were added, one additional patient was detected, and the sensitivity reached 83% (Table 4).

Discussion

Detecting early asthma is difficult as no sensitive, routine laboratory method is available to confirm or rule out the diagnosis. Lung function tests remain the mainstay, although, as shown, 35% (8/23) patients with early or suspected asthma had neither increased bronchial responsiveness nor fluctuating PEF (Table 4). The histamine sensitivity of

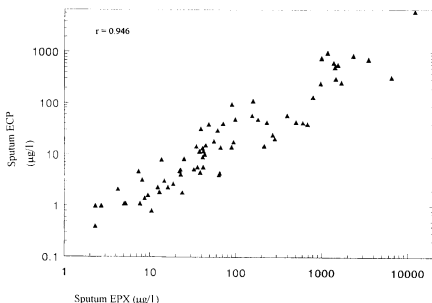


Fig. 1. Correlation between sputum ECP ($\mu\text{g/l}$) and sputum EPX ($\mu\text{g/l}$) in controls and all patients.

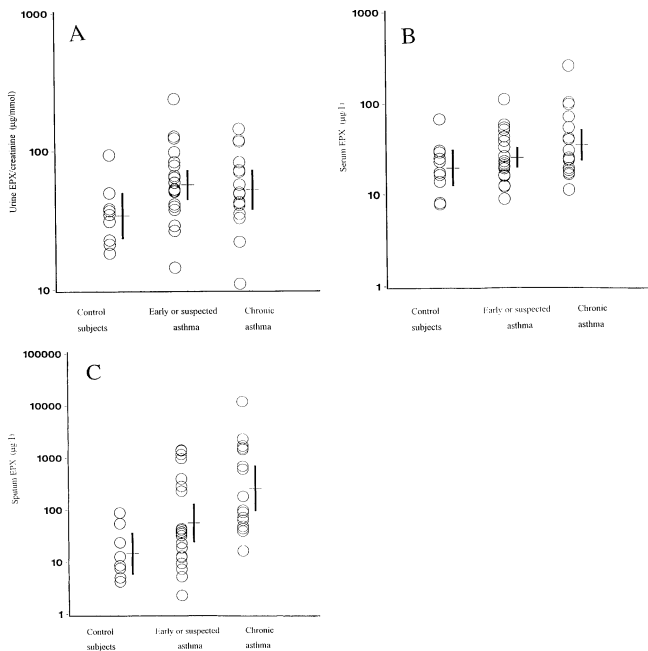


Fig. 2. Urine EPX/creatinine (A), serum EPX (B), and sputum EPX (C) levels in control subjects, and in patients with early or suspected asthma, and chronic asthma. Geometric means with 95% confidence interval.

patients with recently detected asthma is quite variable (10); frequently, it is mild or undetectable.

Sputum samples have long been used for the diagnosis of some lung diseases; e.g., tuberculosis. Sputum production can be induced by having the subject inhale nebulized hypertonic sodium chloride solution (8, 9). Thus provoked, even 80% of our healthy control subjects produced a sputum sample. The bronchial origin of the sample can be verified through cytologic examination (14); this is useful, as some samples contain only saliva.

The combined saline and dithiothreitol treatment,

to which sputum cells were subjected in our study, caused cellular disruption and release of intracellular inflammation markers in some samples. Cells also became difficult or impossible to identify, and we rejected the samples in question. Sputum induction and handling methods should be developed further to eliminate this source of error.

The histamine challenge test with its 48% sensitivity was better than any of the sputum tests (eosinophils, ECP, EPX, or MPO) that we tried in patients with early or suspected asthma. The three sputum eosinophil-related tests all had a similar sensitivity

of 35–39%. Serum and blood tests were poorer. Previously, it has been shown that sputum ECP levels correlate better than serum ECP levels with parameters of airflow obstruction (4).

In patients with early or suspected asthma, the 65% sensitivity obtained with the two lung function tests combined (PEF plus histamine challenge) could be raised to 78% by adding sputum and serum ECP. If rapid results are needed, histamine challenge, sputum eosinophils, and sputum ECP could be used together; the sensitivity was 70%.

Among our 23 patients with early or suspected asthma, there were four (17%) in whom none of the tests applied gave a positive result. The patients did have symptoms suggesting asthma, but the diagnosis could not be verified with laboratory methods; then, by a strict definition of the disease, they did not have asthma, and a longer follow-up is needed to evaluate such patients further.

The high prevalence of increased leukocyte activation marker (ECP, EPX, and MPO) concentrations in sputum and serum samples from patients with chronic asthma strengthens the assumption that some of the chronic mucosal inflammation usually remains even when medication is considered adequate.

No differences were seen between atopics and nonatopics in any variable examined. This agrees well with the lack of differences in variables measured in BAL fluid and biopsies of bronchial mucosa from patients with intrinsic and extrinsic types of asthma. T-lymphocyte activation and eosinophil infiltration are common features in both conditions, but a distinct pattern in cytokine production seems to exist (15, 16).

We conclude that, at present, the answer to the question, "Can early asthma be confirmed by laboratory tests?", is, "No, not always, but tests can be helpful." Further improvements in the techniques of sputum handling and the use of new inflammation and asthma markers could change the answer to "Yes, almost always."

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