

Revisiting *in-vivo* and *in-vitro* diagnostic approaches for respiratory allergy

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Abstract

The management of allergic diseases relies on an accurate diagnosis of causative agent. The probability of a correct diagnosis can be increased using a combination of diagnostic *in-vivo* and *in-vitro* tests with due consideration to the history of patients. To improve the reproducibility of the tests, it is important that proficiency testing be instituted for both skin and specific IgE estimation so that the coefficient of variance can be determined. This is already done for the latter and needs to be performed for skin tests as well. It is important to develop solid supports (antigen coated)/ allergen discs and reference sera using indigenous reagents and allergen sources for specific IgE estimation. In case of IgE determination with imported kits, one should ensure the relevance of allergen on solid support with exposure of patient. Appropriate positive and negative controls are required to be run with each set of immunoassay.

Keywords: Asthma, Rhinitis, Skin tests, RAST/ELISA, Allergens, Immunocap, Microarray.

INTRODUCTION

Allergy and asthma together afflict >20% of the world population¹. Around 70% cases of asthma and rhinitis are of atopic origin, where IgE plays central role in pathogenesis of disease^{2,3}. The discovery of IgE in 1964 has enabled to an understanding of the mechanism of Type I allergy. It has also helped in the development of diagnostic tools for allergies and standardization of allergen extracts. It is now possible to measure allergenic proteins (content) and also the

allergenicity in terms of the specific IgE antibodies in the sera of patients.

Allergens are derived from diverse sources such as pollens, animal proteins, molds, foods, insects venom, foreign serum proteins, industrial chemicals, and drugs. Most natural allergens are proteins or glycoproteins of molecular size 10,000 to 200,000 Daltons⁴. Chemicals or drugs of lower molecular weight (haptens) first bind to the body's own proteins (carriers) in order to become fully effective as allergens⁵. For the development of the hypersensitivity state underlying clinical allergies, repeated contact with the allergen is required. Duration of the sensitization period is usually dependent upon the sensitizing strength of the allergen and the intensity of exposure. The allergens such as saliva, urine, and hair proteins of domestic animals are more effective

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IJAAI, 2007, XXI (2) p 95-102.

sensitizers than others. Several annual seasonal exposures to grass pollens or ragweed pollen usually occur before an overt manifestation of hay fever (seasonal allergic rhinitis). On the other hand, allergy to cow's milk in infants can develop within a few weeks⁶. When previous contacts with allergens have not been apparent e.g., antibiotics in food, an allergy may clinically manifest even upon the first conscious encounter with the offending substance.

The inhalant allergens play an important role in stimulating specific IgE response in predisposed subjects inducing allergic manifestation⁷. Allergy diagnosis can help to determine the allergen responsible for patient's respiratory illness⁸. With the knowledge of offending allergen(s), the physician can select appropriate therapeutic interventions, such as allergen avoidance, medication, and or specific allergen immunotherapy. Diagnosis of allergic diseases encompasses several facets. Since many clinical manifestations of allergy are mimicked by nonallergic mechanisms, it is usually necessary to use appropriate diagnostic procedures to ascertain whether the person has developed an IgE mediated immune response toward the incriminated allergen(s). Such procedures primarily consist of skin tests, in which a small amount of allergen is applied on or injected into the skin⁹. If the individual is sensitized, a local immediate reaction ensues, taking the form of a wheal (for IgE-mediated reactions), or swelling and redness occur after several hours (for delayed hypersensitivity reactions). The blood can be analyzed for IgE and IgG antibodies by serological assays and or sensitized lymphocytes can be investigated by culturing them with the allergen for histamine release. Several unconventional methods are said to be useful for the diagnostic work up of respiratory allergy or even superior to conventional methods¹⁰⁻¹². The present article has been aimed to review *in-vivo* and *in-vitro* procedures for the diagnosis of immunoglobulin (Ig) E-mediated respiratory allergy and also assess their scope and limitations.

IN-VIVO TESTS FOR TYPE I ALLERGY DIAGNOSIS

Type I allergies (IgE mediated) are diagnosed with skin prick testing and/or with intradermal testing, employed as a follow-up to a negative skin prick test.

Advantages of skin testing include rapid results, good sensitivity, and the ability to test various antigens within a few minutes. Disadvantages include the discomfort inherent in the procedure, possible danger of anaphylaxis, the contraindicating effects of medications such as anti-histamines, decongestants, beta blockers, bronchodilators and theophylline, and occasional interference from skin conditions namely eczema, dermatographism etc. Studies suggest that patients should be advised not to take long acting antihistamines 45 days before, whereas other drugs may be avoided 4 h to 1 day prior to skin tests¹³. Since skin testing only reflects IgE-mediated reaction, it cannot inform clinicians as to the potential for the delayed hypersensitivity reaction responsible for such a wide range of symptoms seen clinically.

1. Skin tests: Skin tests are generally safe, however the intradermal test has slightly higher risk of a severe reaction than others. Skin tests are used to detect immediate (antibody mediated) or delayed (cell mediated) sensitivity in patients. Three types of skin tests are commonly followed for allergy diagnosis, to detect the sensitization against allergen(s).

1.1. Skin prick test (SPT): The prick method is the most specific and safe skin test. But this may result into negative test due to low sensitivity of allergen extract/patients clinical reactivity and amount administered may be low due to false technique. SPT is preferred over intradermal test because it causes little discomfort to the patient. A drop of 50% glycerinated allergen extract is placed on the skin may be on the back or arm of patient and a needle is pierced through the extract to make a tiny puncture in the skin. Each test is to be placed about two inches apart and thus many allergens can be tested at the same time. In patients having IgE specific for the allergen, a wheal-and-flare response will develop at the site within 15-20 minutes. The area of wheal (edema) is then measured and the results are reported as wheal size in millimeters, identified on an arbitrary scale of 1+ to 4+, correlating to the size of the wheal. Histamine di-phosphate or histamine di-hydrochloride 5 mg/ml is used as a positive control. The reaction with wheal size > 3mm and or equal to positive control is graded as positive reactions^{14,15}. The reaction with wheal size 4-6 mm dia had shown good correlation with positive double blind placebo controlled food

challenge¹⁶. Various devices (needles/Lancet) have been used to carry out SPTs. Studies suggest that most of the devices yield similar SPT results^{17,18}.

1.2. Prick to prick test: Prick to prick method is mostly used for juicy fruits and other similar source of allergens. A needle is pricked in the allergen source and then immediately pricked into the skin. If the patient has IgE antibodies to the allergen, a wheal will appear at the site within 15 minutes. Skin reactions are graded in comparison to positive control as described for SPT.

1.3. Intradermal test: The intradermal test is most sensitive allergy diagnostic test but may yield false positive result. Intradermal tests are conducted on the volar aspect of forearm, lateral aspect of the upper arm or back of the patient. A syringe is used to inject allergen extract intradermally into the top layer of skin resulting in a small 2 mm bleb on the surface. Attempt is required to make same wheal size for each antigen while ID testing. In patients having IgE specific for the allergen, a wheal-and-flare response will develop at the site within 15-20 minutes. The wheal with redness (erythema) appears as a result of antigen-antibody (IgE) interaction on mast/basophils. The area of wheal (edema) is measured and the results are often reported as wheal size in millimeters, identified on an arbitrary scale of 1 to 4+. Histamine di-hydrochloride or histamine diphosphate (100 µg/ml) is used as a positive control. The ID reactions are to be graded based on wheal size of positive control i.e, wheal size equal to histamine is positive reaction (2+), more than histamine is 3+ and reaction with multiple pseudopodia is 4+¹⁹⁻²¹.

Factors affecting results of skin tests: Poor circulation under the skin may blunt the results in the scratch, prick, and puncture tests. Antihistamines, tricyclic antidepressants, phenothiazines, and other drugs may block allergic reactions. Reactions on the back, particularly the upper back, may be stronger than those on the arm. Dialysis or widespread cancer may blunt the allergic response. Redness and wheals can also be caused by skin irritation and not allergy.

2. Bronchial challenge test: Bronchial challenge test can be used to detect specific allergen responsible for allergic asthma, but the test can be done during the non symptomatic days or after controlling the

symptoms of patient. This test is particularly used to diagnose occupational asthma with offending allergen. It can help to evaluate the immune response to workplace allergens such as industrial gas, vapor, and fumes in factories or flour in bakeries etc directly into the exposed workers²². If allergic asthma is present, the exposure to the allergen will cause constriction of the bronchial tubes in testing. The resulting decrease in lung function is measured with a spirometer. Methacholine and/or histamine are mediators of allergic reaction. Airway hyper-reactivity can be measured after/before challenge with any of these mediator(s) to assess the disease state or benefit of drug/immunotherapy²³.

The patient is made to inhale vapors of a neutral (saline) solution through the mouthpiece, and spirometry is repeated. If no decrease in the lung function is observed, the testing continues. Vapors (aerosol) of an allergen extract are introduced into the air of the spirometer, and airflow through lungs is evaluated again. If the flow drops too sharply, testing is discontinued immediately. Lung functions before and after exposure to the allergen is compared. A drop in lung function signals an allergic reaction and possibly allergic asthma. It is advised to avoid smoking for 12 hours, coffee, tea, cola, or chocolate for six hours, significant exercise for two hours, exposure with cold air for 2 hours and inhalers to facilitate breathing and certain other medications for six hours prior to bronchial challenge test.

Bronchial challenge test has some limitations. Subjects are exposed to a single high dose of an allergen corresponding to months or years of natural exposure. The bronchial challenge test has severe chances of anaphylaxis in highly sensitive patients. False-negative results may occur on exposure to non specific irritants or emotional/ physical stress. Once the test is positive, further testing with other allergen cannot be done unless patient becomes normal again.

3. Nasal challenge test or Rhinomanometry: Nasal challenge test is performed to confirm an allergic reaction, particularly when there is a discrepancy between skin and blood tests for selection of allergen for immunotherapy. Histamine released during an allergic reaction causes inflammation of the tissue lining the nasal passages, which impedes airflow.

A rhinomanometer is used to measure airflow and air pressure in the nose²⁴.

A control test is performed with a neutral spray (Saline) containing no potential allergens, and the passage of air through the airways is assessed with a rhinomanometer. One to two hours later, the suspected allergen is sprayed into the nose or introduced as a powder or coated on a paper disc or polymer film. Airway resistance is measured before and after allergen challenge^{24,25}. Symptoms produced by the challenge, such as sneezing, runny nose, and nasal blockage, are recorded. For Interpretation of result, an increase in pressure and a decrease of at least 40% in airflow in one of the nasal passages after exposure to an allergen indicate the positive challenge test. There are some factors such as medication that block an allergic reaction, the presence of viral infections and other nasal disorders may affect results of nasal challenge test. In pollen allergic individuals, the presence of same pollen in the air during testing can alter the outcome of test. Also, there are some limitations of this test e.g., it is time-consuming, unpleasant, and expensive. It may not correspond to real-life conditions because the amount of allergen in the spray is greater than in natural surroundings. If one test is positive, further tests cannot be carried out unless the patient becomes normal again.

IN-VITRO TESTS FOR TYPE I ALLERGY DIAGNOSIS

In-vitro tests offer the advantages over *in-vivo* tests in terms of safety (no risk of anaphylaxis), lack of interference by antihistamines or skin condition, and good reproducibility²⁶. Unlike *in-vivo* tests, they can be performed during the symptomatic days of patients. *In-vitro* tests also allow the use of parallel controls with each run. A positive reaction in an *in-vitro* test signifies sensitization to the allergen in that individual. For the “global reactor,” *in-vitro* testing can provide a starting place for trial elimination programs. As a screen for asymptomatic individual, *in-vitro* testing may reveal food allergies which are currently being effectively managed by the immune system (hence, no symptoms), but which could manifest symptomatically in the future, in the event of increased immune burden. Occasionally, a test will exhibit “across-the-board” low-level IgG reactivity for an individual. Clinical observation has suggested the

possibility of a chronically “leaky gut” in such situations, and the concomitant immune reaction to a large number of absorbed antigens. All *in-vitro* tests have some limitations and the results should be interpreted in the light of symptoms and clinical history²⁷⁻³¹.

In-vitro tests require efficient and relevant solid phase system (antigen coated) containing sufficient amount of allergen to bind all specific IgE present in the test sample. The system should be stable in terms of IgE binding capacity and low non specific interference. The assay system should be able to measure both ends of IgE molecule i.e, allergen specific site and isotype specific site. Among the various solid supports available for antigen coating, polystyrene microtiter plates and nitrocellulose paper discs are most popular. Both have different capacity of antigen coating in the range of 300 ng/cm² to 100 µg/cm².

1. Radioallergosorbent Test (RAST): RAST is performed to detect specific IgE in serum against environmental allergens, such as, pollens, house dust mites, molds, insects or animal dander^{29,31}. Among the *in-vitro* tests, RAST is most sensitive test and can confirm sensitization against offending allergen. It can be used in subjects with eczema, dermatitis and or dermographism who cannot undergo skin testing. In this procedure, allergen coated paper discs are incubated with patient serum containing specific IgE to that allergen. After washing, allergen-antibody complex is incubated with radiolabelled secondary antibody. For comparison, test is also done with normal human serum (NHS). The RAST ratio of 2 or more is taken to grade as positive test²⁰. Variation in the RAST result may occur due to variations in the solid phase to which the allergen is attached, the method by which antibodies are counted (counts per minute) and quality of reagents. The RAST outcome can be derived by the equations given below. RAST is considered positive with RAST ratio of >2.

$$\% \text{ CPM} = \frac{\text{Total count bound to the disc} \times 100}{\text{Total counts added}}$$

$$\text{RAST ratio} = \frac{\% \text{ CPM with patient's sera}}{\% \text{ CPM with NHS}}$$

RAST results show the amount of antibodies in a milliliter of serum. If the subject is allergic, specific

IgE to the tested allergen will be elevated in serum. However, RAST result can be affected by the presence of radioactive substances in blood (from other procedures in the previous few days). Presence of diseases such as atopic dermatitis and parasitic infestation can cause increased IgE levels even if there is no IgE mediated sensitization to the substance being tested by RAST. A positive response to an allergen indicates only a potential allergic reaction that may not be the cause of symptoms. Cost wise RAST is twice more expensive than skin testing and the results are not immediately available. It may produce negative results for drugs or insect venom when in fact an allergy exists with them. In addition, only a few properly standardized allergens extracts (solid phase) are available for this test. RAST set up requires the licensing of laboratory and facility for use of radioactive reagents and their appropriate disposal after the test.

2. Enzyme linked immunosorbent assay (ELISA):

This test measures the amount of specific IgE, circulating in the blood that the immune system has produced against a offending allergen²⁶. The test is carried out on a small sample of blood (sera) causing minimal discomfort to patients. The serum sample is processed in laboratory for specific IgE estimation against suspected allergen(s) by ELISA. The test is particularly useful when, 1) the patient is highly sensitive and has a risk of an anaphylactic (shock) reaction on skin testing, 2) when extensive eczema makes skin prick testing impractical, 3) when antihistamine medication cannot be stopped because of severe symptoms and 4) no skin testing facilities are available.

For ELISA, allergen/antigen is coated on the solid support, often the wells of a microtiter plate (~2 µg/100µl/ well). The antigen is fixed to the surface to render it immobile²⁶. The antigen concentration may vary depending upon the type and quality of antigen. The antigen coated wells are then incubated with patient's serum sample of unknown antibody concentration, usually diluted in phosphate buffer saline (PBS). The plate is washed with PBS-Tween 20 (0.1 %) to remove unbound antibodies. and the antigen-antibody complexes remain attached to the well. The secondary antibodies, which bind to antigen-antibody complex, are added to the wells. These

secondary antibodies are coupled to the substrate-modifying enzyme. The plate is washed to remove excess of unbound antibodies with PBS- Tween 20. A substrate specific to enzyme is added to elicit a chromogenic or fluorescent signal. The results are quantified using a spectrophotometer or ELISA reader. The enzyme acts as an amplifier even if only few enzyme-linked antibodies are bound and the enzyme molecules produce many signal molecules.

ELISA/dot immunoassay may be run in a qualitative or quantitative format depending upon the substrate. For qualitative results insoluble substrate is used and the test provides a simple positive or negative result for a serum sample³². The cutoff between positive and negative is determined by the analyst and may be statistical. In quantitative ELISA, the optical density or fluorescent units of the sample is interpolated into a standard curve, which is typically a serial dilution of the highly positive serum raised against the target antigen. Mean +2 or 3 SD of NHS can be taken as cut off to grade the ELISA positive results²⁶. According to Mcsharry et al (1993), 4 PRU/mL i.e 9.6 ng/ml specific IgE can be considered as ELISA positive²⁸.

RAST/ELISA are confirmatory *in-vitro* tests and the titer of antibodies measured correlate well with the wheal/flare reactions produced by skin tests with pollen and fungal allergens³³⁻³⁵. Also studies have shown significant correlations in specific IgE values determined by RAST and ELISA^{36,37}. But ELISA is now preferred over RAST, since it does not require the use of radiolabelled secondary antibody³⁸. In India, RAST/ELISA can be carried out using imported kits. ELISA can be developed using indigenous reagents, except secondary antibody is required to be imported. For performing, *in-vitro* IgE estimation with commercial kits it is important to ensure the relevance of allergen (solid phase) as per the exposure of patients in the country.

TYPES OF ELISA

2.1 Indirect ELISA: The indirect ELISA utilizes an unlabeled primary antibody in conjunction with a labelled secondary antibody. Since the labeled secondary antibody is directed against all antibodies of a given species (e.g. anti-mouse), it can be used with

a wide variety of primary antibodies (e.g. all mouse monoclonal antibodies). The use of secondary antibody also provides an additional step for signal amplification, increasing the overall sensitivity of the assay. The indirect ELISA can be used for measuring specific IgE/IgG against antigen/allergen coated on the solid support³⁹. Indirect ELISA has some limitations, as cross-reactivity may occur with the secondary antibody, resulting in a nonspecific signal. An extra incubation step is required in this procedure.

2.2 Direct ELISA: The direct ELISA uses the method of directly labeling the antibody itself⁴⁰. Microwell plates are coated with the target antigen, and the binding of labelled antibody is quantitated by a colorimetric, chemiluminescent, and or fluorescent end-point. Since the secondary antibody step is omitted, the direct ELISA is relatively quick, and avoids potential problems of cross-reactivity of the secondary antibody with components in the antigen sample. But, the direct ELISA requires the labeling of every antibody to be used, which can be a time-consuming and expensive proposition. In addition, certain antibodies may be unsuitable for direct labeling. Direct ELISA also lack the additional signal amplification that can be achieved with the use of a secondary antibody. The direct ELISA can be used for measuring total serum IgE by coating anti-IgE on the solid support as a first step. However, direct ELISA has some limitations since the immunoreactivity of the primary antibody may be reduced as a result of labeling. There is no flexibility in choice of primary antibody label from one experiment to another.

2.3 Competitive ELISA: This is used to find out the potency or cross reactivity of an allergen with other species^{41,42}. Here the serum is preincubated with the antigens used as inhibitor. The antigen to which the cross reactivity or potency is to be found out is coated on the solid phase. The remaining procedure is same as described for ELISA.

3. Immunocap: Immunoassay should be independent of time, person and allergen used. The assays conducted manually suffer with certain drawbacks e.g, time consuming, require large quantity of serum, labour intensive and costly due to more number of allergens/multiple sensitization in patient. Advances in

technology has provided new laboratory tools for measurement of allergen specific IgE in serum⁴³⁻⁴⁵. Among two automated systems namely Immunocap and Microarray, Immunocap (Unicap) is already marketed and in use for detection of specific IgE against aeroallergens. This system is considered as gold standard for *in-vitro* diagnosis due to consistency of results with various allergen extracts⁴⁶.

4. Microarray: Microarray technology potentially offers advantages in diagnostic applications such as allergy testing because of the little amount of reagents required, and thus the cost per assay, is greatly reduced. This approach has been difficult to introduce in practice because the extremely small volumes (~0.5–5 nL) of sample used to create spots on these microarrays require extremely sensitive methods of analyte detection. This method also requires purified antigens (protein/peptide) for component resolve diagnosis of allergy. Microarray technology has offered a extremely sensitive detection system like immunofluorescence. However, it requires defined allergen molecule i.e., purified/recombinant allergenic protein/peptide component. Simultaneous, multiallergen testing is possible using this method which mimics natural allergen exposure. Studies by Tae-Eun Kim et al. (2002) with *Dermatophagoides pteronyssinus*, egg, milk, soybean, wheat and human serum albumin (-ve control) using microarrays demonstrated comparable results with Unicap⁴⁷. The sensitivity of chip was found at level of 1 IU/mL of IgE. This system can be used successfully for serum IgE estimation in a clinical laboratory for diagnosis of allergy, autoimmune and infectious diseases.

CONCLUSION

Skin tests are the easiest tool to obtain quick and reliable information for diagnosis of respiratory allergic diseases³⁰. Measurement of serum IgE antibodies may give complimentary information and can be employed in clinical and epidemiological settings. Immunoassays and skin tests are not entirely interchangeable and neither will replace the other in appropriate circumstances²⁹.

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