

Provocation testing with recombinant allergens

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Abstract

In the past few decades, DNA technology has enabled the production of defined recombinant allergen molecules for diagnostic and therapeutic purposes. Recombinant allergens containing most of the relevant IgE epitopes present in natural allergen sources are now available and allergen proteins can be produced that are identical, without biological or batch-to-batch variation. A great advantage of recombinant allergens is that they can be used for component-resolved diagnostics, which makes it possible to establish the patient's individual IgE reactivity profile before therapy is selected. However, before recombinant allergens can be applied in clinical practice their biological activity has to be carefully investigated *in vivo*. We here describe the most commonly used provocation methods (skin tests (prick and intradermal), nasal, bronchial, and conjunctival provocations) and how they can be performed. We also discuss the results so far obtained with *in vivo* testing using recombinant allergens and envisage their future use for immunotherapy.

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1. Introduction

The quality of allergens used for both diagnosis and treatment of IgE-mediated allergy is critical [1]. So far, the allergen extracts used for these purposes have been derived from natural allergen sources. However, these extracts have several disadvantages. They contain both allergenic and non-allergenic components and the amount of allergen can vary markedly [1]. Furthermore, when administered for immunotherapy they may cause local and systemic side effects and even new sensitisations [2,3]. DNA technology offered a way to overcome these problems and the last decade has seen the production of recombinant allergen molecules for diagnostic and therapeutic purposes. An ever-growing number of allergens have been cloned, sequenced, and produced identically without biological or batch-to-batch variation of the protein [4,5]. Recombinant allergens from the clinically most relevant allergen sources are available for diagnosis and therapy. Panels of re-

combinant allergens which correspond in epitope complexity to traditional extracts have been produced [5–11]. In addition, cocktails of recombinant allergens can be formulated with predetermined and uniform allergen levels, and can replace natural allergens for allergy diagnosis. Moreover, spiking crude allergen extracts, lacking sufficient amounts of allergen/s, with recombinant allergen/s has been shown to improve the sensitivity of extract-based diagnostic tests [12]. The availability of recombinant allergens also enables refined allergy diagnosis [13]. The patient's IgE-reactivity profile can now be identified precisely [14]. This has a major impact on therapy, since optimal forms of treatment can be selected on the basis of the allergen/s that elicit the disease. Furthermore, the advent of recombinant allergens has offered exciting new options for treatment. Peptide chemistry and recombinant DNA technology have allowed the production of T-cell and B-cell epitope-containing peptides, recombinant allergen isoforms, mutants, fragments, and recombinant allergen oligomers with reduced allergenic activity [15–18].

But before recombinant allergens can be used in clinical practice they have to be carefully evaluated in

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vitro and in vivo. Their IgE-binding capacity has to be tested with sera from a variety of populations. To elucidate their biological activity in vivo, the only definitive measure is provocation testing, which also allows the study of the allergens' potency to induce immediate and late phase reactions. The biological activity of several recombinant allergens has already been evaluated among allergic and non-allergic subjects using various challenge methods [5,19–23]. The aim of this review was to illustrate the most commonly used provocation methods (skin tests, bronchial, nasal, and conjunctival provocations) and how they can be performed and assist the clinician in the diagnosis of IgE-mediated allergic disease [24].

2. Allergen solutions

The allergen to be used in provocation testing is generally given in an aqueous solution. Because the potency of an aqueous solution often decreases rapidly, freshly reconstituted solutions should be made on the day of the test, preferably from freeze-dried lyophilised material. Preservatives such as glycerol, phenol or benzalkonium chloride ought to be avoided, as they can induce non-specific reactions [25]. If dilutions of recombinant allergens are to be made, they should be prepared in buffers that contain human serum albumin to prevent adsorption of diluted allergen to the tubes. In provocation testing, the diluent has to be included as control. Furthermore, temperature, pH, and osmolarity of the allergen solution should be checked. Nasal, bronchial, or conjunctival provocation with recombinant seasonal allergens should be performed outside the season, since there is a risk of increased responsiveness during the actual allergy season. If several recombinant allergens are going to be tested, it is advisable to let at least two weeks pass between each challenge. Recombinant allergens used in larger studies should preferably be manufactured according to GMP (Good Manufacturing Practice) guidelines to ensure their quality and stability over time.

3. Skin testing

Skin prick tests (SPT) and intracutaneous or intradermal skin tests are easy to perform and allow a visualisation of sensitisation within 10 min at most. They have been the primary diagnostic tools in allergy since their introduction by Blackley in 1865 [26]. The basic principle of skin testing is the introduction of a small amount of allergen into the epidermis. If there are allergen-specific IgE molecules on the surface of the mast cells (between 5000 and 12,000 mast cells/mm³ located close to blood vessels and nerves), the allergenic mole-

cules crosslink the IgE molecules. The first step involves the binding of the Fc portion of allergen-specific IgE to high affinity (FcεRI) receptors leading to a signal which triggers histamine release and generates other mediators. The mediators cause vasodilatation and increase vascular permeability, which in turn leads to tissue oedema and the development of a weal. Histamine triggers the release of the neuromediator substance P by an axon reflex, which causes the surrounding skin to flush. This classical weal-and-flare response was first described by Lewis in 1907 [27]. The skin reaction begins 5 min after the allergen injection and is in sometimes followed by a late phase reaction, which starts between 1 and 2 h later, peaks at 6–12 h and resolves in approximately 24–48 h. The kinetics of histamine release during the immediate reaction has been studied by the skin microdialysis technique. The maximum histamine concentration is observed 4–8 min after intradermal injection of allergen and slightly later after skin prick tests [28]; a clear dose-response relationship is observed in subjects undergoing intracutaneous tests. Several mediators have been recovered from skin blister fluids during the late phase reaction, including histamine, kallikrein, thromboxane, prostaglandin D₂, and leucotriene C₄ [29].

There is no clear correlation between the concentration of allergen-specific IgE and skin prick test responses. The magnitude of the skin response is actually determined by the affinity between receptor-bound IgE and the allergen. By using purified Amb a 1 and Der p 1, Pierson-Mullany and colleagues defined the minimum capacity necessary to produce a measurable skin test reading [30]. The main limitation of skin tests is that a positive reaction does not necessarily mean that the symptoms are due to allergen-specific IgE and, conversely, symptom-free subjects may have allergen-specific IgE.

3.1. Methods of skin tests

3.1.1. Prick-puncture tests

A small drop of about 30 µl of serial dilutions of the recombinant allergen is placed on the volar surface of the forearm or occasionally on the back; the test sites are at least 2 cm apart to avoid false-positive reactions. A disposable hypodermic needle (25 or 26 gauge) is passed through the drop and inserted into the epidermal surface at a low angle; the needle tip is then gently lifted upward to elevate a small portion of the epidermis without inducing bleeding. The needle is then withdrawn and the solution is wiped away with a paper tissue approximately 1 min later. A new needle must be used for each test to avoid mixing of solutions [31,32]. There are currently several needles on the market that work according to the Morrow-Brown principle, where the needle is perpendicularly inserted to the skin (i.e., Stallerpoint), giving highly reproducible results [33,34]

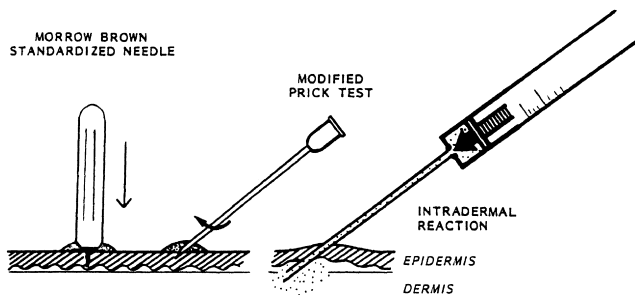


Fig. 1. Usual methods of skin testing.

(Fig. 1). Systemic reactions are very rare, but have been observed with natural allergen extracts [35]. Negative and positive controls have to be included in every recombinant skin test study. For a negative control, the diluent is usually physiological saline solution. The skin reaction to the trauma of prick testing may approach a 3 mm diameter depending on the devices used and the technique of the investigator. The positive control solutions detect suppression of cutaneous reactivity by medications (mainly antihistaminic drugs) and also if patients react poorly to histamine. The usual positive control is histamine (1 mg/ml, which induces a weal ranging from 2 to 7 mm or 10 mg/ml inducing a weal of 5–8 mm) [36]. Codeine phosphate (9% solution), a mastocyte secretagogue, can also be used.

3.1.2. Measurement of SPT

The size of a positive SPT should be recorded after 15 min (the weal expands progressively between 5 and 15 min), by measuring the largest diameter of the weal (D) and the orthogonal diameter (d). In scientific studies, areas of weal and flare reactions recorded after 15 min are scanned and calculated (with a Software program); a weal size of 7 mm² or greater has been regarded as positive [36]. Some authors score SPT as a percentage of the positive control, knowing that inter-individual variation in the skin response to histamine is quite large. In studies performed to evaluate biological activity of recombinant birch pollen allergens (wild-type or modified), the prick test was considered positive when the mean diameter $[D + d]/2$ of the weal induced by the recombinant allergen was at least 75% of the mean diameter of the weal induced by histamine control (10 mg/ml) [37]. Pepys, who contributed to the widespread use of SPT, considered weals as small as 1 or 2 mm positive if the negative control was completely negative [38]. However, usually only skin prick test reactions over 3 mm in weal diameter or over 10 mm in flare diameter are considered relevant for clinical allergy.

Reproducibility of single-allergen skin prick tests is not very good, especially when the weal diameter is below 5 mm [32]. A patient with a weal diameter of 5 mm will show weals averaging 5 mm with a 95% confidence interval of 3.1–8 mm [39]. If the test is done by

inexperienced investigators, the coefficient of variation for repeated tests can rise by 40% or more, whereas this coefficient is only 15% for expert investigators. For this reason, duplicate or quadruplicate testing is recommended in scientific studies using recombinant allergens. When a patient's reactivity to recombinant allergens is evaluated in different seasons, variations may be observed; previous studies have shown increased cutaneous reactivity associated with seasonal exposure in pollen-sensitive patients [40]. Epidemiological studies frequently use skin prick tests for evaluation of atopy; here results can vary owing to the use of allergens from different manufacturers or different methods of application [41].

Doses: when recombinant allergens are used for skin testing, the starting doses must always be low for safety reasons, but because recombinant allergens are pure proteins, they can be applied at precisely calculated concentrations. In the more than 25 studies published to date, allergen concentrations range between 1 and 100 µg/ml, although concentrations up to 1000 µg/ml have been used occasionally (e.g., for latex recombinant allergens Hev b 2, 5, and 7 [42] and for celery recombinant allergen Api g 1 [43]). For Bet v 1, our two groups used serial dilutions from 1 to 100 µg/ml [37,44,45] and among 51 patients with clinically relevant birch allergy (tested by SPT at 3, 10, and 50 µg/ml), 27 had a significant positive SPT at 3 µg/ml and 20 at 10 µg/ml [37]. Several other recombinant allergens usually gave positive SPT at concentrations of 10 µg/ml, e.g., recombinant allergens of grasses [46], olive pollen rOle 1 [47], the mite *Dermatophagoides pteronyssinus* rDer p 2 [48], the storage mite *Lepidoglyphus destructor* rLep d 2 [49], bovine dander allergens rBos d 2 [22], and the cherry allergens rPru av 1, rPru av 4, and rPru av 3 [50].

3.1.3. Intradermal tests

Intradermal skin tests have largely been used for the biological evaluation of recombinant allergens and for validation of genetically engineered hypoallergenic derivatives. The recombinant allergen solution is injected intracutaneously from a 0.5 or 1.0 ml plastic syringe through a 26-gauge needle. Before injection, all bubbles are carefully eliminated to avoid "splash" reaction that could lead to misinterpretation. The syringe is placed at a 45° angle to the skin (Fig. 1). Between 0.02 and 0.04 ml of the allergen solution is injected into the skin to produce an intradermal bleb approximately 3 mm in diameter. If the solution is accidentally injected subcutaneously, no bleb is formed and this can give false-negative tests. Weal reactions less than 5 mm are regarded as negative. Some North American authorities recommend measuring the sum of perpendicular diameters of erythema rather than weal to determine positivity. In experienced hands, the intradermal skin test is more reproducible than SPT, but a higher level of

technical skill is required [51,52]. Intradermal tests can elicit large immediate or late local reactions as well as systemic reactions to usual commercial allergen extracts; however, the latter are rare. A few fatalities have been reported during skin testing with whole allergen extracts [53]. No such severe complications have been reported in studies which used recombinant allergens for intradermal tests.

3.2. Maximal doses and starting doses

The maximal doses used for intracutaneous tests performed with recombinant extract range between 10^{-2} and $10 \mu\text{g/ml}$ in published data. In several studies, serial dilutions were tested to determine the threshold dilution giving a positive response (endpoint titration). Two studies of recombinant allergens from mite rDer p 5 [54] and rDer f 11 [55] used a single concentration ($1 \mu\text{g/ml}$). The recombinant form of Bet v 1, the major allergen from birch pollen, is the recombinant allergen that has been studied most. Serial dilutions of rBet v 1 were used especially to compare its biological activity with those of hypoallergenic molecules: either isoforms [20] or fragments and polymers [44,45]. The maximal doses used ranged from 10^{-1} to $10 \mu\text{g/ml}$.

Among fungal allergens, the recombinant allergens of *Aspergillus fumigatus* were tested intradermally in several studies. Positive reactions occurred with very low dilutions ($10^{-5} \mu\text{g/ml}$) and although the maximum dose varied, it did not exceed $10 \mu\text{g/ml}$: i.e., for rAsp 1: $10^{-2} \mu\text{g/ml}$ [56] and $1 \mu\text{g/ml}$ [57]; for rAsp f 3: $10 \mu\text{g/ml}$ [58], and for rAsp f 4: $10 \mu\text{g/ml}$ [59]. In bee venom allergy, the maximum dose reached was $10^{-2} \mu\text{g/ml}$ [60,61].

Considering that in several studies prick tests with recombinant allergens are positive at $10 \mu\text{g}$ or less, and that the concentration required to elicit a positive reaction with intradermal testing is 100–3000 times lower, it is reasonable to choose starting doses of $10^{-2} \mu\text{g}$ or less for intradermal tests with recombinant allergens.

Results obtained with the different recombinant allergens, tested intradermally, are consistent with a large inter-individual variation. For rBet v 1, it ranged from 10^{-4} to $1 \mu\text{g/ml}$ [44]; this is in accordance with a previous study using intradermal testing in 87 patients sensitive to ragweed pollen extract in whom sensitivities ranged from 10^{-5} to $10^{-1} \mu\text{g/ml}$ [62]. The high biologic activity of recombinant proteins is clear from the very low amount of recombinant allergen (less than 2 pmol), which is able to elicit a positive reaction in highly sensitised subjects [63].

Both for SPT and intradermal tests, only large differences in allergen potency will be detected by using a fixed concentration of allergen. This renders the approach unsuitable when the allergenicity of genetically engineered new hypoallergenic recombinant molecules

has to be evaluated by in vivo methods. Much more information is obtained if skin tests are performed by employing 3- or 10-fold dilution series. The size of the weal for a single recombinant allergen dose may be identical despite a 100-fold variation in potency, emphasising the necessity of using methods based on end-point titrations [64].

In conclusion, skin tests with a single recombinant allergen are extremely sensitive, especially in intradermal testing [64]. However, with recombinant allergens, prick tests will gain full acceptance in routine clinical practice, especially as the problem with the variable potency of natural allergen extracts is avoided. For scientific studies, intradermal tests are still recommended. Relative advantages of prick and intradermal testing are summarised in Table 1.

3.3. Specificity and sensitivity of recombinant allergens in skin testing

Specificity and sensitivity have been defined in populations with a positive skin test to the commercial extracts and in controls with a negative skin test. Schmitt-Gendelmeier and Cramer compiled the available studies for recombinant mite, pollen, fungal, bee venom, latex, and celery allergens [65]. The number of subjects included in the different studies is highly variable and the number of control subjects is sometimes very low (less than 22 in 14 of the reported studies), and controls were even lacking in a few studies. Only one false positive reaction was observed. The specificity was very high and often reached 100%. In contrast, the sensitivity was generally lower and was evaluated for different recombinant allergens in populations that differed greatly in number. For Bet v 1, the reactions of 343 subjects to commercial extracts were tested and compared with their reactions to the major rBet v 1 allergen whereas only 33 patients were tested with rOle 1, the major

Table 1
Relative advantages of skin prick tests and intradermal testing adapted from [31]

	Prick	Intradermal test
Simplicity	++++	++
Speed	Fast	Less fast
Technical skill required	++	+++
Discomfort	Painless	Painful
Interpretation of positive and negative reactions	+++	+++
False positive reaction	Rare	Possible
False negative reaction	Possible	Rare
Reproducibility	++	++++
Safety	++++	++
Testing in children	Easy	Difficult
Sensitivity	+++	++++
Specificity	++++	+++

allergen of olive pollen. The sensitivity is greater if there is a high incidence of sensitisation to a recombinant allergen in a given population. For instance, sensitivity of the cutaneous tests performed with rBet v 1 reached 90% in populations from northern and eastern Europe, but it is only 40% in a Mediterranean population [66]. If all allergens present in a given extract can be produced as recombinant allergens and can be tested simultaneously, the sensitivity of the diagnosis will approach 100%. This has been shown for sensitisation to birch with rBet v 1 and rBet v 2 [37], and for sensitisation to grass pollen with rPhl p 1, rPhl p 2, and rPhl p 5 [46]. In the future, the use of recombinant allergens will allow diagnosis of individual sensitisations on a molecular basis. A cocktail of selected recombinant allergens (containing both very specific recombinant allergens as well as recombinant panallergens (e.g., calcium-binding allergenic protein, profilin)) can be sufficient to detect sensitisation to a given allergen with high specificity and sensitivity.

However, when sensitisations are detected, it is often difficult to ascertain their clinical relevance. The degree of an in vivo response, allergic asthma for instance, may be predicted at least in part by an equation incorporating non-specific hyperresponsiveness and the end-point skin tests [67]. It would be interesting to know the clinically relevant cut-off values for skin test performed with recombinant allergens. At the present time, this is not feasible because recombinant allergens are not yet approved for clinical use. However, some authors have used in vitro analysis and cutaneous tests to determine which of the recombinant allergens of *Aspergillus fumigatus* was specific for allergic bronchopulmonary aspergillosis. In contrast to fungal extracts, rAsp f 4 and rAsp f 6 allowed discrimination between allergic bronchopulmonary aspergillosis and sensitisation to *Aspergillus fumigatus* [59]. The positive and negative predictive values of cutaneous tests with recombinant allergens (which is the likelihood that a positive—or negative—cutaneous test reflects the presence—or absence—of respiratory or oral allergic symptoms) have not yet been studied enough. In one of our own studies including 47 subjects, sensitisation to rBet v 1 was encountered in patients with clinical birch pollinosis, but also in 9 patients sensitised to birch without clinical expression of this allergy [37]. Sometimes the predictive value can be quite good; for instance, the negative predictive value of a negative cutaneous test to rBet v 1 is very high for apple intolerance [68]. However, although a positive cutaneous test to rBet v 1 is a hallmark of the birch apple-syndrome, the positive predictive value is rather low (29 out of 47 subjects sensitised to rBet v 1 presented this syndrome in our study) [37]. In the case of cherry allergy, Ballmer-Weber and colleagues [50] used skin prick responses obtained with rPru av 1 (homologous to Bet v 1), rPru av 4 (homologous to Bet v 2), and rPru av 3 (cherry lipid transfer protein) to assess the

specificity and the positive and negative predictive values of skin prick tests. In 79 individuals (among them 24 with history of allergic reactions to cherry), they could demonstrate that skin prick tests do not indicate whether sensitisation to cherry in patients allergic to birch might represent clinically manifest allergy or clinically irrelevant sensitisation. Moreover, there was no threshold weal area of the prick test that predicted food allergy in individual subjects. So at the moment we do not have sufficient data to assert that clinically relevant sensitisation detected by recombinant allergens, is reflected in the degree of cutaneous responses.

In conclusion, skin tests performed with recombinant allergens from various pollens, moulds, mite, bee venom, latex, celery, and cherry are highly specific and safe. The diagnostic sensitivity is variable according to the prevalence of sensitisations among the population, but a positive reaction against a recombinant given allergen can be detected in the picomole range, permitting us to classify certain rare allergens as relevant allergens. The diagnostic sensitivity can also be increased by using panels of recombinant allergen (cocktail) covering the most important allergen structures present in a given allergen extract. Moreover, the use of recombinant allergens for skin tests does not lead to an increased risk of side effects compared with commercial extracts as shown in a thorough compilation of studies, where more than 1600 allergic individuals were tested with 21 different recombinant allergens [65]. In scientific studies, comparisons of recombinant wild-type allergens and recombinant allergen derivatives with reduced anaphylactic potential have been taken as prerequisites for the evaluation of recombinant hypoallergen molecules for future immunotherapy trials. Several studies performed in a dose-dependent manner showed that hypoallergenic recombinant molecules were at least 100 times less liable to cause positive skin prick test reactions than wild type recombinant allergens [18,44,45, 69–71].

4. Nasal provocation tests with recombinant allergens

The first allergen provocation was performed already in 1873 by Blackely [26]. He was able to show that application of fresh pollen to the membranes lining his nostril provoked itching and sneezing. Unlike the lower airways, the nose provides an ideal site for allergen provocation. It is readily accessible and allergen can be applied to the mucosa with a high degree of accuracy. The challenge procedure, which should reflect natural exposure, is today above all used in research and to a lesser extent in clinical practice.

Up to now only recombinant allergens from birch and bovine dander have been used in nasal provocation

tests. Godnic-Cvar and colleagues carried out a study among ten birch pollen allergic patients to compare the potency of rBet v 1 with that of natural (n)Bet v 1 [19]. Recombinant Bet v 1 induced an equally extensive reaction to that of nBet v 1 in five subjects, a more pronounced reaction in one, and a less extensive reaction in four. The results were specific, as none of the 10 control subjects reacted to either rBet v 1 or nBet v 1. Ruoppi and colleagues compared the *in vivo* response to rBos d 2 with those to rBos d 2 fragments (1–131 and 81–172) among ten farmers with cow-induced allergic rhinitis [22]. All subjects were additionally challenged with natural Bos d 2. The nasal provocation tests revealed that rBos d 2 had nearly the same allergenic activity as nBos d 2 (9/10 vs. 10/10), but the reactivity to the fragments was lower (five to fragment 81–172 and 3 to fragment 1–131). No late phase reactions were recorded. The authors concluded that the fragments might be better tolerated in immunotherapy than the complete allergen. We have recently evaluated the nasal reactivity to rBet v 1 derivatives and compared it with the reactivity to rBet v 1 wild-type as the basis for new therapeutic strategies for birch pollen allergy [23]. Ten patients with birch pollen allergy were included in a randomised, double-blind, and cross-over study. All patients tolerated the highest accumulated dose, 8.124 µg, when challenged with rBet v 1 trimer, and eight tolerated rBet v 1 fragments, but only one when challenged with rBet v 1 wild-type. No significant difference in symptom scores was recorded between the two derivatives or between each of the derivatives and the diluent. Furthermore, no late phase reactions were observed. In the nasal lavage fluid, we could show that the rBet v 1 derivatives induced lower mast cell- and eosinophil activation than rBet v 1. The derivatives could in the future be candidates for immunotherapy based on mucosal tolerance induction.

4.1. Methods

The tests should be performed by well-trained personnel under standard conditions and in a well-equipped unit. Written consent should be obtained from the patient. Different methods for the provocation and measurement of nasal responses have been used. For experimental research purposes, techniques for quantitative measurements with high reproducibility are important [72]. Recently, Bousquet and the ARIA Workshop Group have presented a document on allergic rhinitis and its impact on asthma [73]. They have put forward guidelines from a subcommittee of the “International Committee on Objective Assessment of the Nasal Airways” for nasal provocation tests concerning indications, techniques, and evaluation of the tests [74]. We here briefly describe the performance of nasal challenge tests based on these documents [72–74].

4.1.1. Deposition of the allergen

The solutions of allergen can be delivered from a meter-dose pump spray or on paper disks. Micropipettes have also been used to apply small and exact volumes of allergens onto the nasal mucosa. However, only the nasal pump spray delivers the solution over a large area of the nasal mucosa. The recently introduced “nasal pool” device may be a further improvement of the provocation technique [75]. The nasal pool device is a compressible plastic container equipped with a nasal adapter. This novel technique brings known concentrations of agents into contact with a large and defined area of the nasal mucosal surface for extended periods of time. However, prior to the challenge a rhinoscopic examination should be performed to disclose any structural pathology that may influence the results [72]. The challenge procedure should always start with the negative control (diluent) to rule out false positive reactions to the diluent *per se*. The doses of the recombinant allergens can thereafter be increased, for instance, every 10 min and symptoms can be registered until the subject shows a positive (immediate) nasal reaction.

4.1.2. Recommended periods for withholding medication

For adrenergic drugs (nasal spray): 24 h; for antihistamine (oral and nasal spray) 5 days; for disodium cromoglycate (nasal spray) 3 days, for inhaled corticosteroids (nasal spray) 2 weeks, and for oral corticosteroids at least 2 weeks.

4.1.3. Measurements of response

Different methods have been used to measure the nasal response to the allergen [72–74]. Recording of symptoms produced after provocation is the easiest method. Symptoms of nasal blockage, secretion, and itching can be rated and the number of sneezes counted. It is advisable to use a combination of symptoms since patients may react with different symptoms, on different test days [76]. These parameters should preferably be combined with objective measures such as evaluation of nasal patency, airflow or airflow resistance. The most common techniques for these purposes are: rhinomanometry, acoustic rhinometry, rhinostereometry, nasal inspiration or expiratory peak flow, of which rhinomanometry is the best evaluated and standardised technique [73]. Nasal inspiration or expiratory peak flow measurements are the simplest techniques. They are inexpensive, quick, and easy to perform. Comparisons between symptom scores, acoustic rhinometry, nasal inspiration or expiratory peak flow and rhinomanometry during histamine challenge showed that there was no difference in the mucosal reactivity regardless of the method used, but nasal peak inspiration flow or nasal peak expiratory flow is more sensitive to mucosal changes than other methods studied [77].

4.2. Assessment of the inflammatory response

Nasal provocation offers a good tool for studying the inflammatory reaction induced by allergens. In this context, both biopsies and nasal lavages have been used. Since nasal biopsies may cause trauma and bleeding, a mild technique should be used to obtain high-quality specimens of nasal mucosa [78]. The method allows analyses of the deeper layers of the mucosa. However, anaesthesia is needed before any biopsy can be taken and it may affect the results. Furthermore, a vasoconstrictor is usually required to reduce the risk of bleeding. Other less common methods used for collecting cells are nasal scrapings and nasal brushes [72].

If nasal lavages are to be performed, the technique should be well controlled and reproducible with regard to the distribution of the lavage fluid, mucosal surface area in contact with the same fluid, and duration of mucosal exposure to the lavage fluid. Furthermore, the techniques should be non-injurious, the recovery of the lavage fluids should be quantitative, and the employment of the techniques should be repeatable without undue effects on nasal mucosal physiology. The “nasal pool” device, which was recently introduced for lavages, meets the above requirements of being well controlled and highly reproducible [75]. The nasal pool fluid is instilled in one of the nasal cavities and is maintained in contact with a large and reproducible area of the mucosal surface for a determined period of time. When the pressure on the device is released the fluid quantitatively returns into the container. An advantage of this method is that the patients themselves can lavage their noses.

5. Bronchial provocation tests with recombinant allergens

Since the 1950s bronchial allergen provocation tests have been widely used to evaluate the relevance of a specific allergen in patients suspected or known to suffer from extrinsic asthma, although the test in itself does not diagnose asthma [79–81]. In recent years, bronchial challenges have been employed to assess the complex molecular and cellular mechanisms involved in the pathogenesis of asthma as well as the efficacy of the drugs intended to combat asthma and allergy [82]. However, inhaled allergen challenges are model situations whose artificial aspect has been brought into light by our better understanding of how respiratory allergens are distributed in the airways; we still need to go deeper into the question of the “natural” doses able to provoke bronchial hyperreactivity and specific broncho-obstruction [83]. Very few studies are focused on the impact of allergen-bearing particle sizes, which can greatly influence the quantitative bronchial response to a specific allergen [84].

Despite the widespread use of bronchial provocation tests with allergen, it is only over the last few decades

that the procedures have been standardised and their reproducibility determined [85,86]. The methods of allergen inhalation challenge do not differ from those used for methacholine and histamine tests, for which standard procedures are well established [87]. Guidelines for the standardisation of bronchial provocation tests with allergens have been given in several reports [88–91]. It became clear early on that clinical accuracy and reliability as well as clinical safety of allergen bronchial provocation tests depend to a great part on standardisation of allergen extracts [92]. Recombinant allergens will overcome this problem, which is especially acute for comparative studies. However, the performance of recombinant allergens needs to be assessed individually when applied to new investigations. So far, bronchial provocation tests with recombinant allergens have only been reported for Bet v 1, the major birch pollen allergen. Ten individuals allergic to birch pollen inhaled nBet v 1 and rBet v 1 and the results were compared. Seven out of 10 patients had an equally extensive early response to the two allergens, whereas 3 out of 10 patients reacted more strongly to rBet v 1. The allergen provocation caused a significant change in non-specific bronchial reactivity in 6 subjects. The inhalation tests with recombinant allergen were specific, as 10 control subjects reacted neither to rBet v 1 nor to nBet v 1. In 5 patients, late phase reactions to both rBet v 1 and nBet v 1 were observed [19].

5.1. Methods

Bronchial provocation should be performed under the direction of a physician trained in pulmonary function testing and experienced in bronchial provocation. Written consent should be obtained from the patient.

5.1.1. Aerosol delivery systems and inhalation methods

A high quality nebuliser with consistent output should be used to produce the aerosol. A breath-activated dosimeter can be attached to the nebuliser [93]. The same nebuliser should be used for every concentration delivered to a given patient, and the particle sizes should have a mass median aerodynamic diameter (MMAD) of 2–5 μm .

5.1.2. Recommended periods for withholding medication before bronchial challenges with allergen

For β -adrenergic drugs: 8 h for inhaled short-acting, 12 h for inhaled long-acting, and 12 h for oral preparations. For theophylline 48 h, for anticholinergic drugs 12–24 h, for disodium cromoglycate and nedocromil sodium 12–24 h, and for antihistamines 3–5 days. For corticosteroids: oral corticosteroids inhibit late responses and should be decreased to a minimum level 2–3 weeks before the tests. Inhaled corticosteroids can prevent early responses when administered at adequate

dosage for as much as a week before the challenge [67] and should therefore be withheld at least that long before challenge.

5.1.3. Starting and maximum doses for recombinant allergen inhalation challenges

The starting dose of recombinant allergen should be related to the dilution that provokes a positive reaction in skin tests. Cockcroft showed that the concentration that in a prick test elicits a 2 mm weal diameter is generally less than that which induces a significant allergic broncho-obstruction [67]. When the specific starting dose for a given patient is 10–100 times less than that causing a significant positive prick test, there is but a small risk of inducing unwanted side effects even in sensitive patients. The subsequent allergen inhalations are given in doubling doses up to the top dose, which should be sufficiently potent to exclude false negative reactions in patients with clinical allergy, but not so high as to cause false positive reactions. Further studies are required to determine the triggering doses for each recombinant allergen, knowing that non-specific bronchial reactivity also plays a role and that the level of allergen-specific IgE may be predictive, in part at least, of the degree of the bronchial responses [94].

5.1.4. Measurement of the response

The forced expiratory volume during one second (FEV₁) is the primary variable to be monitored (reproducibility within $\pm 5\%$ for each set of manoeuvres associated with a given dose). A 20% fall in FEV₁ is considered positive, though some investigators consider a fall of 15% sufficient. Clinical symptoms such as cough, breathlessness, and changes in auscultation sounds have to be considered in patient evaluation and test interpretation [95]. Dose response curves should be drawn. Early responses occur within 20 min and are reversed after 120 min, whereas late responses occur, up to 8 h later. In subjects with negative early responses, late reactions can still occur, and it is recommended to record lung function parameters at least over 8 h (some physicians prefer 12 h). Non-specific bronchial responsiveness can be enhanced by a positive bronchial allergen challenge and may increase the risk of asthma exacerbations in the following days [96]. In the case of positive responses, bronchodilator may be administered to speed up recovery. When several tests have to be compared in a given subject, medications, time of day, and nebuliser employed should be taken into consideration.

5.2. Assessment of the inflammatory response

Numerous techniques have been used after bronchial allergen challenges to study the complex cellular and molecular mechanisms of eosinophil influx, which has been associated with the late asthmatic response [97].

Such techniques include bronchoalveolar lavages, bronchial biopsies, and segmental washouts. However, the bronchial allergen response involves a much more complex interplay between leukocytes, numerous tissue cell types, cytokines, chemokines, and inflammatory mediators than was initially suggested [82]. Bronchial challenges with recombinant allergens, using pure allergenic proteins with constant potencies from batch, to batch will offer better reproducibility. This is of great interest knowing that bronchial allergen challenges are likely to remain at the forefront for assessment of new therapeutics in initial trials and for evaluation of new methods of immunotherapy.

6. Conjunctival provocation tests with recombinant allergens

Conjunctival allergen provocation has been performed since 1932 [98]. It represents a reproducible and safe method to verify local sensitisation and to induce experimentally an allergic reaction in the eye [90,99], and could be useful for evaluating the biological activity of new allergens. The method has also been used for testing patients' sensitivity to allergen during immunotherapy [100]. The provocation induces an IgE-dependent degranulation of mast cells, which gives rise to a typical allergic response, with itching, hyperaemia, tearing, chemosis, and eyelid swelling. The intensity of the reaction is related to allergen dose [101]. The late phase reaction is similar to that occurring in the skin or bronchi following specific allergen challenges [102] and is reported to be dose dependent [101]. Conjunctival provocation tests with recombinant allergens have hitherto only been performed with recombinant isoforms of Bet v 1 (rBet v 1a and rBet v 1d) [20]. Forty-eight birch pollen allergic patients were challenged with the two recombinant rBet v 1 isoforms to evaluate their *in vivo* allergenicity. None of the patients reacted to rBet v 1d, whereas a positive response to rBet v 1a was recorded in 33 of the 48 patients at the highest dose (10 $\mu\text{g}/\text{ml}$), in 10 at 1 $\mu\text{g}/\text{ml}$, and in none at 0.1 and 0.01 $\mu\text{g}/\text{ml}$. None of the patients in the two control groups showed a positive reaction. The authors concluded that rBet v 1d might be a promising candidate for use in immunotherapy of birch pollen allergic subjects.

6.1. Methods

The conjunctiva is well exposed for macroscopic clinical observation and is easily accessible for diagnostic and non-traumatic procedures.

6.1.1. Application of the allergen

The allergen should be topically applied to the conjunctiva with a pipette. A bilateral ocular allergen

challenge can be performed, where allergen and diluent are administered to one eye each. The challenge procedure should always start with the negative control (diluent) to rule out false positive reactions to the diluent per se.

6.1.2. Recommended periods for withholding medication

For adrenergic drugs (eye drops) 24 h; for antihistamine (oral and eye drops) 5 days; for disodium cromoglycate (eye drops) 3 days, for corticosteroids (eye drops) 2 weeks, and for oral corticosteroids at least 2 weeks.

6.1.3. Starting dose of recombinant allergen

In diagnostic practice, the starting concentration of allergen may be equal to the one used to obtain a positive skin prick test. However, it should be pointed out that the dose of crude allergen extract needed to elicit a conjunctival allergen reaction is reported to be higher than the dose needed to give rise to a positive skin reaction [90]. The conjunctiva thus seems less sensitive than skin for allergen provocation.

6.1.4. Measurements of response

Several methods exist for evaluating the conjunctival response. Assessment of symptoms (redness, swelling, tearing, and itching) by a scoring system [99] has been found to be highly reproducible by us [103,104] as well as by others [105].

6.2. Assessment of inflammatory response

The immune response to allergen can be measured in tear fluid [102–104], which can be collected in glass capillary tubes or on preweighed strips of filter paper. The tubes have to be gently placed at the ocular surface to avoid irritation, since it is known that enhanced tear flow rates reduce protein concentrations [106]. The inflammatory response can also be investigated in conjunctival specimens obtained by moderately invasive scraping or brush techniques [107,108]. Biopsies offer the best material for a comprehensive objective analysis of the inflammatory reaction in the conjunctiva [109]. If repeated sampling is necessary, as is the case in treatment evaluation studies, this technique is, however, less than ideal since previous excisions leave scars, which may render the interpretation of further samples difficult.

7. Conclusion

Recombinant allergens are highly pure reagents of stable quality, which can be produced in large quantities. They enable the identification of disease-eliciting allergen molecules, which will improve diagnosis and

treatment. Even though the diagnostic sensitivity of single recombinant allergens is generally lower than that obtained with natural allergen extracts, using panels of recombinant allergens covering the most important allergen molecules present in natural allergen extract can significantly increase the sensitivity. The biological activity of several recombinant allergens has already been evaluated using skin tests, nasal, bronchial or conjunctival provocation testing. Skin tests performed in a large number of studies have demonstrated highly reproducible results. Furthermore, provocation testing with recombinant allergens has shown that they are safe tools with the same immunological properties as their native counterparts. The advent of recombinant allergens has also offered exciting new options for treatment, where genetic engineering has led to molecules with reduced allergenicity but with retained immunogenicity. Taken together, recombinant allergens represent promising tools for refined diagnosis and improved therapy of IgE-mediated allergy.

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