

Recombinant allergens for analysing T-cell responses

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Abstract

T-cell responses constitute a central element of allergic disease and a model for studying Th1 and Th2 cytokine pathways. Most studies to date have used extracts of allergens which contain variable quantities of different allergens and non-allergenic antigens. Recombinant allergens provide the tools for studying the responses to allergens in a reproducible and dose-dependent manner and the different T-cell responses of allergic and non-allergic subjects provide a method for verifying the responses and their relationship to allergic sensitisation. Most allergies show dominant responses to one or a few major allergens. These allergens have been described for the common allergies and have been produced as recombinant allergens. A particular problem for allergens is that many are mixtures of proteins from multi-gene families or are highly polymorphic. Information now exists so the sequence variation can be represented. Purified recombinant allergens produced by standard expression systems stimulate the expected T-cell responses from the peripheral blood of allergic and non-allergics to allergen extracts. Although stimulation with recombinant allergens which are not produced with a natural IgE binding activity can provide a measure of allergenicity, the altered tertiary structure can reduce Th2 responses. The sequence information now available provides the means to use PCR to produce cDNA for the production of recombinant allergens from readily available sources. The production of the highly reactive recombinant Der p 2 allergen of house dust mite from natural sources is described.

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1. T-cell allergen interactions

T-cell responses to allergens are the key regulators of diseases such as asthma and hayfever, as well as allergic responses to insect stings and the ingestion of food [1,2]. The disease of atopic dermatitis is more complex and probably consists of allergic responses to inhaled allergen which are enhanced by the presence of responses elicited in the skin by bacterial products [3]. It is, however, accompanied by very high levels of T cell and IgE reactivity to allergens, usually the house dust mite. The most frequent allergic responses and the ones which will be dealt with here are the type I hypersensitivities associated with Th2 cytokine responses. To date, most investigations of T cells in allergic sensitisation have been performed with allergen extracts which are mixtures of a large number of allergenic and non-allergenic

proteins which vary in concentration from batch to batch. Given that T-cell responses are highly dose dependent and that doses can affect cytokine profiles [4], the need for more standardised allergens is evident.

The different allergens and antigens in allergen extracts can also have different immunostimulatory activities. The experiments of Wurtzen [5], for example, showed that while grass pollen extracts stimulated the production of IFN- γ from T cells the purified major allergen did not. In another example, the less important group 7 house dust mite allergens were found to be the most potent inducers of T-cell IFN- γ release while the major group 1 allergens induced comparatively low levels of cytokine release in allergic and non-allergic subjects [6]. The study of stimulation induced by extracts therefore does not represent the responses to the major allergens. Recombinant allergens provide an avenue for producing the purified allergens in the concentration required, and as will be described below, with a defined amino acid sequence.

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The responses to allergens also provide a model system for studying many aspects of T-cell activation and the development of immunological memory and effector function. The prevalence of the allergy is very high so patients and allergic volunteers can be readily recruited and Th2 cytokine pattern provides a signature for allergen-specific T cells. Unlike infectious disease, the exposure to allergen is regular and predictable so the antigenic stimulation can be better defined. Pollen and house dust mite allergens provide the most prevalent source of sensitisation. Most people are exposed to house dust mite allergens from birth and inhale the allergens every day. T-cell responses to purified major group 1 and 2 allergens can be readily detected in infancy [7] and throughout adulthood [6,8], providing a readily accessible response to well-defined antigens. Responses can be induced in T cells from peripheral blood mononuclear cells (PBMC) from both allergic and non-allergic subjects [6] and T-cell clones can be produced [9]. The pollen allergens can also be readily used to study T-cell responses and are an important cause of allergic disease. The temperate grasses such as rye grass and timothy are abundant in many temperate areas of the world and there are other well-documented pollens such as ragweed, birch, and olive, which have a more limited (but still considerable) geographical distribution. Exposure to pollen allergens is seasonal, which is a factor that could influence the design of T-cell studies. The T-cell responses have been found to increase after the pollen season but responses of PBMC can be detected all year [10,11]. Another allergen used for study has been the major phospholipase A2 protein from bee venom [12]. Exposure to the cat allergen is perennial and almost universal but the studies have been limited.

2. The test subjects: T-cell responses from allergic and non-allergic populations

People responding to allergens can be divided into allergic and non-allergic subjects, although there are gradations of sensitisation and people allergic to more than one source of allergen produce higher T-cell responses [8,13]. The existence of the different types of responses provides a number of avenues to test the veracity of the allergenic stimulation. From 20 to 30% of most populations are allergic to a common allergen and are aware of their clinical history. They can be expected to show T-cell responses to the sensitising allergens and furthermore the responses will be characterised by the production of Th2 cytokines. Ideally, the Th2 cytokines are best represented by IL-4, which is required for B cells to produce IgE antibodies and drives the T cells towards the Th2 differentiation pathway [1]. Other Th2 cytokines are IL-5 which is required for eosinophilia and IL-13 which provides help for IgE antibody production

and has pleomorphic inflammatory properties, acting on both lymphoid and non-lymphoid cells [1]. The production of the Th2 cytokines by cells from allergic subjects will therefore provide a confirmation of the specificity of the T-cell stimulation. In some cases, the diagnosis of allergy may need to be verified. ELISA and other solid-phase assays have now been well established for measuring serum IgE antibodies to allergens. The assay can be conducted by directly coating the allergen on the plate and using commercially available anti-IgE or biotinylated anti-IgE to provide the readout. For the common allergens such as mite and pollen, the extracts sold by pharmaceutical manufacturers for diagnosis are suitable plating reagents and purified or recombinant allergens can be used. Only small quantities (1–5 µg/ml) are required for coating wells and the IgE assays tend to be very specific because the amount of IgE in serum is low and most of it is directed to specific allergens. It is therefore possible to identify a substantial population of allergic subjects who can be expected to respond to recombinant allergens with a predictable cytokine pattern.

Allergens can also induce responses from the T cells of non-allergic subjects. In some instances including the authors' studies of house dust mite allergy, the proliferative responses of non-allergic subjects have been just as large as the responses induced in the cells of allergic subjects [6,14,15]. Other investigations have, however, reported that the T cells from non-allergic subjects respond less. In some cases, fewer subjects show proliferation [8,16,17] or in others the cells from most non-allergic subjects have not been stimulated at all [18]. There are therefore some unidentified variables. Some may be associated with the efficiency of the culture system. The first report of T-cell responses to the purified major mite allergen could only detect responses from subjects with atopic dermatitis [19]. Cells from subjects with asthma and allergic rhinitis did not respond nor did the cells from normal subjects. The follow-up studies by O'Brien et al. found responses from the cells of subjects with asthma and lower responses from the cells of non-allergic subjects. Only about 50% of the allergic people gave substantial responses and the level of response was higher in multi-allergic people [8]. In contrast, many recent reports from reputable laboratories have described responses, which can readily be induced in the cells of almost 100% of subjects with and without allergy [6,7,15]. A similar situation exists for pollen allergy. T-cell responses have been demonstrated for non-allergic subjects [5,7] but as shown for birch [20], grass [21], and olive pollen [22], allergic subjects are more responsive. For birch pollen, although the PBMC response has been shown to be higher in allergic subjects, T-cell clones have been grown from non-allergic subjects [23]. In summary, T-cell responses of non-allergic subjects can be measured with efficient cultures systems although they can be less than the responses of

cell, from allergic subjects. These responses probably reflect the immunological events, which protect from the development of allergy and provide a useful comparison for evaluating the usefulness of different allergen preparations. As will be described below, this is particularly evident for cytokine profiles.

3. The readout: cytokine and proliferative responses

The release of human IL-2 can be measured by ELISA or by the CTLL mouse cell indicator assay [24]. The sensitivity of the ELISA and the CTLL assay is similar at about 4 pg/ml [25], although other factors such as IL-15 can read out in the bioassay. The preferred surrogate marker for IL-2 has, however, been T-cell proliferation measured by the incorporation of tritiated thymidine. This can readily be detected in cells which release minimal quantities of measurable IL-2 [18] and the utilisation of the IL-2 by the stimulated cells is a problem. The release of the Th2 type cytokines IL-4, IL-5, and IL-13 provides measures of allergenicity. IL-5 and IL-13 release by PBMC of allergic subjects can be easily detected by commercially available ELISAs [16,18,26,27]. The latter references [26,27] describe the release induced by recombinant Der p 2. The IL-5 release assay is particularly useful because it is essentially only released by the cells of allergic subjects. IL-13 is not such a strict Th2 cytokine [28] but it is produced in higher titres from the cells of allergic subjects. The levels of IL-13 are usually easy to measure so it provides a backup for the IL-5 production when cytokine secretion is low, for example in the responses of infants. The detection of IL-4 is difficult and cannot usually be accomplished for primary cultures of PBMC. It is the key cytokine for regulating the Th2 response and inhibiting the Th1 response so in many cases it is important that some measure of the IL-4 is made. The easiest method is to measure mRNA. Most laboratories now have access to real-time PCR technology, which can permit quantitative RT-PCR. Other methods have used T-cell lines [29] or have stimulated short-term T-cell lines, first with antigen to expand the cells and then with a polyvalent mitogen to induce cytokine release [30]. The latter procedure should be viewed with some concern both for the specificity of the assay and for the relevance of cytokine release which cannot be induced by the allergen. A more recent assay has been to measure intracellular cytokine by immunostaining and fluorescence flow cytometry [31]. This can be achieved with short-term cultures amplified and induced with allergen.

The Th1 cytokine interferon- γ provides a convenient T-cell assay for the non-allergic response and even detects responses in cultures where proliferative responses are low [18]. The commercial ELISAs for IFN- γ are reliable and the cytokine is usually produced in

high amounts and is easy to detect. Although much of the secondary literature emphasises the presence of a protective Th1 response mounted by non-allergic subjects, the reality is somewhat different. Many of the conclusions were based on T-cell lines and ignored the very strong polarising effect of the Th1 and Th2 cytokines released into the culture, especially IL-4. For house dust mite allergy, it is now known that PBMC from allergic subjects produce at least as much Th1 cytokines as cells from non-allergic subjects and often more [6,18,32–34]. For pollen allergy, the work of Imada et al. reported a preference for non-allergic subjects to produce more IFN- γ than the cells from allergic subjects [35], although PBMC from many allergic subjects also released high titres of IFN- γ . The more recent papers have used Th2/Th1 ratios as a measure of the allergic response [36].

The decision of whether to perform the allergen stimulation with PBMC or with T-cell clones depends on the nature of the experimental questions. The cloning can increase the cytokine titre and thus provide a means of measuring IL-4 production. It does, however, distort the cytokine profile by the negative and positive feedback of IL-4 and IL-12 and the selective pressure of the culture on the clones may be significant. Different clones from the same individuals have a markedly different cytokine profile [37,38]. The lines and clones do provide an extra element of specificity in that they would only be expected to only respond to the one allergen and they have been very useful in examining the diversity of epitopes recognised by allergen-specific T cells. The lifespan of T-cell lines is limited so they do not provide an infinite source of a standard T-cell clone, which can readily be exchanged between laboratories [39]. In the initial establishment of the clones, there is a need for a ready supply of antigen-presenting cells from the donor. While this can eventually be replaced by EBV transformed cells or transfectants expressing human MHC molecules, the logistics can limit the number of individuals which can be examined with T-cell clones.

4. Major allergens

One of the major reasons for the use of recombinant allergens is to provide a standard and well-characterised preparation of allergen to replace the use of extracts. The allergen extracts, however, contain a large number of different proteins and many are allergenic to some degree. At least 19 different allergens have been described for mites, 14 for the common temperate grasses, 10 for the Fagales tree pollens which includes birch, and seven for ragweed. An updated list of the allergens can be found on the website of the allergen nomenclature committee www.allergen.org. Studies with IgE binding have established that major allergens exist for each

source and these dominant specificities often comprise a high percentage of the IgE binding activity. The major allergens can be used as markers for the allergic response.

The major specificities for house dust mites are the group 1 and group 2 allergens. For the most abundant species *Dermatophagoides pteronyssinus*, the allergens are called Der p 1 and Der p 2 and for the species *Dermatophagoides farinae* which predominates in drier regions the allergens are Der f 1 and Der f 2. It should be noted that although *D. farinae* has been called the American mite, most house dust mites on this continent are *D. pteronyssinus*. Of the eight locations studied by Arlian et al. [40] only one, Cincinnati Ohio, had a higher abundance of *D. farinae* with 72% of homes being infested with this species and 27% with *D. pteronyssinus*. The infestations in Western Europe are also predominantly with *D. pteronyssinus* [41,42]. Thus, although local knowledge is important the choice of allergens would generally be *D. pteronyssinus*. The purified allergens from each species also cross-react extensively in T-cell assays [14]. Double site ELISAs with monoclonal antibodies can be used to determine the species of mite and are available in kit form (Indoor Biotechnologies, Cardiff, UK, www.inbio.com). Separate homes tend to be infested by only one species [40]. Recombinant mite allergens are available commercially (Indoor Biotechnologies, Cardiff, UK).

The important pollen allergens depend on the different climatic conditions and the aerobiological information is readily available in the literature, including maps with the regional distribution of pollen in Europe [43] and North America [44]. For tree pollen, the birch allergen is very important in Europe and olive and cedar allergens are a major cause of sensitisation. The major allergen of birch is unquestionably Bet v 1 and the response of many birch-allergic people is almost completely directed to this protein [45,46]. There are large numbers of other Fagales trees such as hornbeam, chestnut, and hazel which have the homologous allergens to Bet v 1 and Bet v 1-related molecules are important food allergens found in many species [47]. There are also a wide range of birch species but the allergen from *Betula verrucosa* has been used as a prototype and Bet v 1 has been successfully employed for many T-cell studies. Recombinant Bet v 1 is available commercially (Pharmacia Diagnostics AB, Uppsala, Sweden).

The major species used in T-cell studies of grass allergy has been the timothy grass *Phleum pratense*, which is widely used in Europe and most other countries as a meadow grass. The other most studied grass is ryegrass. The major grass allergens are the group 1 and group 5, which account for almost all the IgE binding activity of pollen extracts [48]. Recombinant Phl p 1 and Phl p 5 are available commercially (Pharmacia Diagnostics AB, Uppsala, Sweden).

5. Allergen sequence variation: the selection of recombinant allergens

One of the practical difficulties for using recombinant allergens is that most major allergens show sequence variation either as a result of the existence of multi-gene families or because of allelic variation. Many pollen allergens are a mixture of products from gene families. Direct evidence for this has been presented for ragweed [49] and birch allergens [50]. The cDNA sequences of the group 1 ragweed allergen Amb a 1 showed that the allergen was a mixture of three different isoforms which, depending on the comparison made, had 15–20% sequence divergence [49]. The PBMC from all individuals examined showed T-cell proliferation to all three isoforms but Amb a 1.2 was less stimulatory [51]. While there was a general concordance of the response induced by Amb a 1.1 and Amb a 1.3 and natural Amb a 1, there were also considerable differences in the responses of cells from some individuals. The allergen Amb a 2 was also found to be a similar molecule to Amb a 1 with 35% sequence divergence [52]. Bet v 1 has been shown to have at least 11 isoforms which have from 5 to 16% sequence divergence [53]. A comparative analysis of the proliferative response of T-cell clones from a number of individuals has been reported. All subjects had at least one clone which proliferated in response to the archetypal recombinant Bet v 1 a [54]. At the clonal level, however, there was considerable variability. Some subjects had a majority of clones which responded to Bet v 1b and Bet v 1d while others had more clones which were reactive with Bet v 1a. Within individuals there was considerable variation amongst the clones. Often over 50% of clones would be considered to be non-reactive based on their response to Bet v 1a but responded to other isoforms. A mixture of three isoforms (a, b, and d or l), however, would have stimulated all clones. With respect to abundance, Bet v 1 a and b-like sequences represent 35% of the allergen found in pollen and d and f each represent 10%. The study of Sparholt et al. [55] found that 30% of T-cell clones which reacted to Bet v 1 did not react to the cloned recombinant Bet v 1a. This agrees with the results of Ferreira et al. and demonstrates further that the inclusion of several isoforms would improve the recombinant preparations.

The group 5 grass allergens consist of a series of about four proteins which can show less than 70% sequence identity within the species [56]. The response of T-cell clones and lines to two isoforms of the timothy grass allergen Phl p 5 has been examined. The T-cell clones usually responded only to recombinant Phl p 5a or Phl p 5b but rarely to both [37]. The different recognition was related to their specificity for different epitopes as shown by their response to truncated recombinant polypeptides [57]. A similar result was obtained by Muller et al. [58] who described a large

number of T-cell clones which only proliferated to Phl p 5a or Phl p 5b. In this case, many clones which were stimulated with natural Phl p 5 did not show a significant response to either isoform. Responses to other isoforms of this allergen must be implicated and would be expected.

The major house dust mite allergens are the products of single genes but they show a high degree of allelic polymorphism [59]. Recombinant Der p 1 has been difficult to produce so it has not been possible to compare the stimulatory activity of recombinant variants. Studies on peptides have, however, indicated that it would be best to use two molecules which represent each form of a common allelic variation at position 124 and to use variants which contain the tyrosine at position 50 [59]. The most stimulatory epitope of the highly responding H-2^b strains preferentially necessitates to the alanine variant [60]. The polymorphism at position 50 is important because all environmental sequences of Der p 1 have tyrosine for this residue. Although there are variations in other amino acids, the changes are sporadic and are unlikely to be important for overall responses. The polymorphism of Der p 2 is different to Der p 1 in that a set of allelic substitutions representing an evolutionary divergence is evident [59]. The amino acid substitution at positions 40, 47, 111, and 114 tends to be inherited together and form a cluster in one of the loop regions of the immunoglobulin-like fold. A recent analysis of the T-cell responses to polymorphisms has shown that variants which represent the two extremes of substitutions at these regions (Der p 2. 0101 and Der p 2. 0104) were equally stimulatory and induced a similar profile of cytokine release from PBMC [27]. Although variants with changes in less substituted positions were stimulatory, they induced different cytokine profiles. One such variant induced the release of large amounts of IL-10 and reduced levels of the Th1 and Th2 cytokines while another stimulated a pattern which failed to induce significant Th2 polarisation.

6. Production of recombinant allergens

A wide variety of methods have been used to produce recombinant allergens for T-cell stimulation. The general methods used to produce some of the more ubiquitous major allergens from cDNA clones are described below. For T-cell stimulation, it is important that the allergens be purified from the proteins of the host organism and the amount of allergen be measured for dose–response comparisons. The typical doses required for T-cell stimulation (detailed below) range from 10 to 30 µg/ml so with replicate T-cell cultures 1–2 mg of recombinant allergen is required for a small study.

The Bet v 1 allergen used in T-cell studies has been produced in an untagged or unfused form in inclusion

bodies in *Escherichia coli*, then dissolved in urea, and renatured by dialysis against a physiological buffer. This was then followed by chromatofocussing and reverse-phase HPLC [54]. Other groups have produced Bet v 1 with its natural sequence in *E. coli* and purified the soluble protein from the cytoplasmic fractions with a monoclonal antibody column [55]. The allergens prepared either way showed natural IgE antibody binding activity and stimulated T cells. The cytokine profile of T-cell clones produced by stimulation of cells from allergic donors with recombinant Bet v 1 has been Th2 or Th0 [61].

Most T-cell studies with recombinant grass allergens have examined responses to the major group 5 allergen of timothy grass Phl p 5. Isoforms of this allergen have been expressed in *E. coli* as an N-terminal hexahistidine tagged protein [57,62] or a maltose fusion [63]. The protein has been purified by nickel chelate chromatography or by amylose affinity depending on the tag. The proteins have been used with or without further chromatographic purification and were shown to stimulate T-cell clones. The T-cell clones were found to have a Th0 or Th2 cytokine pattern as expected from allergic subjects [57]. The group 1 allergens have not been used as frequently as the group 5 but the stimulation of T cells with recombinant Phl p 1 has been reported [64]. The allergen was produced in *E. coli* without a fusion tag. Allergen in inclusion bodies was dissolved in 8 M urea, renatured, and purified by DEAE chromatography [65].

The recombinant house dust mite allergens used to stimulate T cells have either been produced as fusion polypeptides with glutathione-S-transferase or as hexahistidine tagged proteins. In the case of the glutathione-S-transferase fusions, the proteins can be isolated from soluble fractions of *E. coli* cytoplasm. Successful T-cell stimulation has been achieved with glutathione-S-transferase fusions of the group 1 and 2 allergens [66,67] as well as with less characterised allergens [67]. The fusion polypeptides have been reported to stimulate cells from allergic subjects to a higher degree than the cells of non-allergic subjects [67]. The pGEX system of cloning glutathione-S-transferase fusions has the advantage of producing a good yield of most recombinant proteins, which can be affinity purified by affinity chromatography on reduced glutathione–agarose in a highly effective one-step purification procedure. The glutathione-S-transferase which is from *Schistosoma japonicum* is rarely stimulatory by itself and the authors, unpublished observations show that its presence makes no difference to the cytokine response to the Der p 2 allergen. The production of unfused recombinant proteins or with small tags, however, does eliminate this variable. T-cell stimulation with recombinant Der p 2 produced with a hexahistidine tag has now been extensively used. The polypeptide was isolated from inclusion bodies

solubilised in guanidine and isolated by Ni-chelating affinity chromatography followed by size exclusion chromatography [26,27]. The recombinant allergen stimulated PBMC from allergic and non-allergic subjects and induced the characteristic release of Th2 cytokines from the cells of allergic subjects.

7. Quality of recombinant allergens for T-cell stimulation

A frequent concern with allergens produced from *E. coli* is the presence of endotoxin, which can have polyclonal T-cell stimulating activity and also bias responses to the Th1 cytokine by stimulating IL-12 release from macrophages. The experience with recombinant allergens purified by the Ni-chelating chromatography [27] or glutathione-*S*-transferase fusions purified on glutathione-agarose [68] is that the endotoxin levels are low with levels of less than 0.125 Eu/ml being achieved with routine purifications. This is below the level which affects cytokine activation in cell culture [69] and was below the level found in allergen extracts and purified natural allergens. Recombinant preparations can also be inexpensively cleaned of endotoxin by immobilised polymyxin, for example B detoxi-Gel (Pierce Endogen, Woburn, MA, USA). This tends to non-specifically bind proteins so a 50% reduction in yield should be anticipated. The measurement of endotoxin can be accomplished by the Limulus coagulation test (Sigma, St Louis, MI, USA) with a strict adherence to manufacturer's protocols.

Although some recombinant allergens are produced as an excellent representation of the natural protein, others are not folded in their natural conformation. In some circumstances, T-cell stimulation can be used to test allergenicity when recombinant proteins cannot be synthesised with a natural IgE binding activity. The proliferation can be measured to test the degree of sensitisation and the release of Th2 cytokines can be a measure of allergenicity. This has been used to examine a fragment of the group 14 house dust mite allergen, a large protein which cannot be readily produced [70]. While this may be possible for some polypeptides, it has also been reported that poorly folded recombinant polypeptides tend to stimulate the release of Th1 cytokines, even from the cells of allergic subjects. This would be consistent with stimulation by antigen taken up by macrophages activated to produce IL-12. The bias to Th1 cytokine production has been demonstrated by recombinant allergens deliberately modified to alter the presentation. For honey bee phospholipase A2 this was accomplished by the use of unfolded aggregates [71] and for Bet v 1 by the use of a genetically engineered trimeric allergen [72]. The trimers were found to have a reduced anaphylactic activity and stimulated normal IFN- γ release with a reduced release of IL-5 and IL-13. Okanao

et al. [73] have reported that deglycosylation of Cry j 1 from Japanese cedar pollen decreased the ability of this allergen to induce Th2 cytokines but did not affect the production of IFN- γ . Therefore, Th2 responses may be reduced if recombinant proteins are not glycosylated or glycosylated correctly. Another phenomenon which could be relevant is that antibodies can assist in the capture of allergens by antigen-presenting cells. Thus good serological activity would be advantageous for recombinant allergens [74]. It, however, should be noted that this was demonstrated for responses to low doses of allergen, and allergens mutated to remove antibody binding activity retain their ability to induce T-cell proliferation [63,75].

8. cDNA for recombinant allergens

With the advent of high fidelity PCR techniques it is now technically easy to use the published sequence information to produce cDNA by reverse transcriptase PCR (RT-PCR). As with RT-PCR used for other purposes such as the quantitation of mRNA the first strand of the mRNA is best produced by priming the reverse transcriptase reaction with an oligo(dT) primer and the allergen cDNA can then be amplified with specific primers encoding the N- and C-termini.

8.1. Pollen allergens

Pollen can be purchased from standard chemical suppliers (Sigma, St Louis, MI, USA), and from allergen suppliers (Allergon AB, Angelholm, Sweden). The method of RNA extraction has been based on the standard Chomczynski and Sacchi guanidinium thiocyanate method as, for example, described by Suphioglu and Singh [56].

8.2. *Der p 2*: a recombinant mite allergen

The *Der p 2* allergen from the house dust mite (or *Der f 2* in the geographical regions where *D. farinae* predominates) provides a convenient system for studying responses to recombinant allergens. Mites are perennial allergens which are abundant in most geographical regions. The group 2 allergens are major specificities which regularly induce high responses and bind IgE to high titre. The recombinant group 2 proteins are easy to produce and bind IgE to the same degree as the natural allergens. cDNA can readily be prepared from house dust using the following method. It is necessary to use live house dust mites to extract the mRNA.

1. Collect a sample of dust by vacuuming a carpet. The dust can be collected by placing a cotton cloth at the connection of the hose of a standard vacuum cleaner and the nozzle. Mites are 200 μ m in diameter so a

- fine mesh is not required. Vacuuming about 2 m² with a bayonet-style or crevice attachment is adequate to capture 20–50 mites. It may be necessary to change the cloth during the process because the accumulation of dust will decrease the sucking power.
2. The dust is placed in a wire mesh cup-sized domestic sieve and this is placed over a funnel feeding into an Eppendorf tube at the collection end. To force the mites into the tube, a desk lamp is shone over the top of the sieve from a distance of a few centimetres. The mites move out of the dust to avoid the light and are collected in the tube at the end of the funnel (15–30 min).
 3. The yield of mites is then examined under a dissecting microscope. Mites are easily recognisable and it is not necessary to be able to identify the species. This can be determined by the subsequent sequence analysis. Some houses have low levels of mites so a few sources should be examined.
 4. From 5–10 mites are recommended for RNA purification [59]. They can be transferred to 25–50 ml of denaturing solution on the point of a needle and homogenized in an Eppendorf tube with a mini homogenizer. The totally RNA kit (Ambion Inc, Austin, Texas, USA) can be used. After precipitation with isopropanol and washing the RNA is air dried and suspended in 10 ml of DEPC-treated water.
 5. The 10 m sample can be used for first strand cDNA synthesis for example with Thermoscript (GIBCO Life technologies, Rockville, Maryland, USA).
 6. The PCR cycle protocol for Der p 2 is then 98 °C 30 s, 37 °C 30 s, and 72 °C 1 min for 5 cycles and then 98 °C 30 s, 50 °C 30 s, and 72 °C for 1 min for 35 cycles.
 7. The PCR primers which can be used are forward 5'-GCGGATCCTGATCAAGTCGATGTCA-3' and reverse 5'-TGGGATCCCTTGTGTTGATTTAATCGC-3'. These contain a *Bam*HI site for cloning (underlined) but *Eco*RI would also be suitable.
 8. The Der p 2 can conveniently be produced from vectors which contain an N-terminal hexa-histidine tag such as pET 19b (Novagen, Madison WI, USA) or pQE-80L (QiagenValencia, CA, USA) cloned into the *E. coli* strain BL21.pLysS (Novagen).
 9. The recombinant Der p 2 is produced in high yield as an insoluble inclusion body. The procedure adopted has been to dissolve pelleted inclusion bodies in 6 M guanidine, clarify by centrifugation 200,000g, 4 h, 4 °C, and then to renature by dialysis against several changes of 20 mM Tris-HCl, pH 8.5, 1 mM EDTA. A substantial precipitate normally forms and is removed by centrifugation, 50,000g 30 min, and discarded.
 10. Two final dialysis steps are performed. First into 50 mM NaH₂PO₄, pH 8.0, 600 mM NaCl and then into the same buffer containing 10 mM imidazole (imidazole buffer).
 11. The sample is then clarified by centrifugation at 50,000g for 30 min and applied to an equilibrated NiNTA column according to standard procedures of the manufacturer.
 12. After washing the column in 20 mM imidazole buffer, the Der p 2 is eluted with 500 mM imidazole.
 13. The Der p 2 isolated in this fashion is usually highly purified but contains aggregates of Der p 2 which are removed by size exclusion chromatography, for example with Sephadex G75.
 14. The extinction coefficient for Der p 2 at 280 nm is 6.6.
 15. The yields of protein are about 10 mg/litre of culture.

9. T-cell stimulation with Der p 2

The culture conditions for recombinant allergens are the same as standard T-cell stimulations. The use of the defined AIM-V medium which will be outlined here produces stimulations without the need to add human serum. Heparinised venous blood from volunteers is diluted with an equal volume of RPMI (Gibco, New York, NY, USA) and 10- to 15-ml aliquots were layered over 6 ml of Lymphoprep (Nycomed, Norway) in 20-ml tubes and centrifuged for 600 g for 30 min. The PBMC which accumulate at the interface are collected and washed twice in RPMI. A typical yield is 10⁶ PBMC per ml of blood. For proliferation assays, the cells are seeded in 96-well round-bottomed microtitre trays with 2 × 10⁵ cells per well in 200 µl AIM-V lymphocyte medium (Gibco, New York, USA) supplemented with 4 × 10⁻⁵ M of 2-mercaptoethanol and 1 mM L-glutamine. The cells are cultured with allergen for 5 days at 37 °C in 5% CO₂ and pulsed with [³H]thymidine (0.5 µCi) for 16–18 h. Thymidine uptake is determined by harvesting the cells onto glass fibre mats and measuring the level of incorporation using a Packard Matrix 9600 beta counter (Packard Instrument, Meriden, CT, USA). The optimal concentration of Der p 2 for stimulation is about 30 µg/ml, although it varies slightly with individuals. Other allergens including natural proteins require similar doses.

For cytokine assays PBMC are seeded at 3 × 10⁶ cells per 24-well plate in 2 ml of AIM-V medium. The antigen is added at 30 µg/ml (or a titrated concentration) and the supernatants are harvested after 5–6 days. Positive controls should be included for both the proliferative and cytokine assays. Tuberculin purified protein derivative (PPD) and HDM extract are excellent controls and induce high levels of proliferation and cytokine release. The decision of when to harvest the supernatant may need to be altered depending on the dose and type of allergen used. The kinetics of cytokine release is variable

but typically our studies have found that Th2 cytokines like IL-5 and IL-13 are released before IFN- γ but all of these cytokines are optimally released at around day 5–6. Several commercial ELISAs are now available for human cytokines and IL-5, 10, 13, and IFN- γ can be readily measured. The ELISAs are usually conducted with neat supernatants and the releases interpolated from standard curves of the standards available at known concentrations. Another method of measuring cytokines is by the time-resolved fluoroimmunoassay (TRF), which is listed in more detail below. This technique gives a slightly higher sensitivity and a larger dynamic range by measuring fluorescence dissociation from Europium (Eu) instead of the colorimetric from the enzyme–substrate reaction. Both the ELISA and TRF can be measured with a Wallac 1420 Victor multilabel counter (Perkin–Elmer).

10. Time-resolved fluoroimmunoassay

The TRF is similar to a normal ELISA but the assay is developed by using Europium-conjugated streptavidin and DELFIA enhancement solution (Perkin–Elmer, Boston, MA, USA). The reagents are readily available at a cost similar to that of ELISA reagents. Below is a protocol for the measurement of cytokines where the dynamic range for detection extends from 1 to 10,000 pg/ml. This is not usually required for Th2 cytokines where concentrations are usually below 500 pg/ml but IFN- γ release can be high. Pairs of monoclonal antibodies for solid-phase sandwich assays are sold with a capture antibody to coat the plates and a biotinylated anti-cytokine antibody for the readout. The reagents from BD Pharmingen, San Diego, CA, USA, have the following sensitivities: IL-2, 5 pg/ml; IL-4, 0.1 pg/ml; IL-5, 5 pg/ml; IL-10, 1 pg/ml; IL-13, 2.5 pg/ml; and IFN- γ , 5 pg/ml.

1. The capture antibody is coated onto MaxiSorp (Nunc, Roskilde, Denmark) plates at 1 μ g/ml in carbonate coating buffer ($\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 9.6) at 100 μ l/well (i.e., 100 ng/well) and incubated at 4 °C overnight. For maximum sensitivity, the coating concentration can be titrated but it is similar for most antibodies.
2. The plate is washed five times in Tris–HCl, pH 7, 0.05% Tween 20 buffer.
3. The plate is then blocked with 200 μ l/well of 0.5% bovine serum albumin (in Tris–HCl, pH 7, 0.05% Tween 20 wash buffer) for an hour at room temperature (with shaking) before the samples or standard dilutions are added at 100 μ l/well.
4. After washing five times, the serial dilutions (2.5-fold gives a curve with a good distribution of points) of the cytokine standard (BD Pharmingen) are made from 10,000 to 1.05 pg/ml in DELFIA assay buffer

(Perkin–Elmer). Samples are usually added neat and can be diluted later in another assay if required. It is also feasible to extend the standard curve to 50,000 pg/ml without altering the sensitivity of the assay but most samples should fall under 10,000 pg/ml. The plates are incubated for 2–3 h at room temperature with shaking.

5. After washing five times, 100 μ l/well of biotinylated antibody (BD Pharmingen) diluted to 1 μ g/ml in assay buffer (Perkin–Elmer) is incubated for 1 h at room temperature with shaking.
6. Europium–streptavidin (Perkin–Elmer) diluted 1:1000 in assay buffer (Perkin–Elmer) is added at 100 μ l/well after washing the plate five times. The plate is incubated for 30 min at room temperature with shaking.
7. The assay is developed by washing the plate eight times and adding 100 μ l/well of enhancement solution. The plate can be read after 5 min and the fluorescent signals are measured on the Victor 2 multilabel counter. The concentrations of cytokine are then transformed from the standard curve by the Workout software (Workout, Perkin–Elmer).

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