

23896

ALLERGENS AND ATOPIC DISEASES

C. PINI, R. TINGHINO, F. SALLUSTO and G. DI FELICE

Laboratorio di Immunologia, Istituto Superiore di Sanità, Rome, Italy

Summary. - Allergens are molecules normally present in the environment, responsible of inducing the so called atopic diseases. These diseases are characterized by the high production of allergen-specific IgE antibodies, which bind to mast-cells and basophils. The cross-linking of the cell-bound IgE induces the production and/or release of various inflammatory mediators (histamine, leukotrienes, platelet-activating-factor, etc.), which trigger a cascade of events resulting in the allergic syndromes. Once the diagnosis of allergy has been made, two different therapeutic approaches can be followed: a) the pharmacologic management, aimed mainly at preventing or controlling the symptoms resulting from the release of mediators by effector cells; b) the specific immunotherapy, consisting in the administration to the patient of increasing amount of the relevant allergens, opportunely prepared and eventually chemically modified. In order to have the latter approach correctly applied it is necessary to gain a wider knowledge on both regulation of IgE synthesis and allergens characteristics.

KEY WORDS: IgE, allergens, atopy, characterization, purification, standardization.

Riassunto (Gli allergeni e le malattie atopiche). - Gli allergeni sono costituiti da molecole normalmente presenti nell'ambiente e responsabili delle cosiddette malattie atopiche. Queste malattie sono caratterizzate dalla produzione di elevati livelli di anticorpi IgE, capaci di legarsi a mastociti e basofili. L'interazione tra l'allergene e le IgE legate alle membrane cellulari induce la produzione e/o il rilascio di vari mediatori dell'infiammazione (istamina, leucotrieni, fattore attivante le piastrine, ecc.), che attivano una serie di eventi a cascata responsabili della patologia allergica. Dopo che la diagnosi di patologia allergica è stata posta, due differenti approcci terapeutici vengono in genere seguiti: a) la terapia farmacologica, il cui scopo fondamentale è la prevenzione o la riduzione dei sintomi risultanti dal rilascio di mediatori dalle cellule effettrici; b) la immunoterapia specifica, che consiste nella somministrazione al paziente di dosi crescenti degli allergeni

verso i quali è sensibilizzato, opportunamente preparati ed eventualmente modificati chimicamente. Al fine di applicare correttamente questa seconda strategia terapeutica è indispensabile acquisire sempre più ampie conoscenze sia sui meccanismi che regolano la produzione di IgE sia sulle caratteristiche degli allergeni.

PAROLE CHIAVE: IgE, allergeni, atopia, caratterizzazione, purificazione, standardizzazione.

Introduction

Allergens are molecules, normally present in the environment, which are responsible of inducing the so called atopic diseases. These diseases are characterized by a high production of allergen-specific IgE antibodies, which bind to high-affinity (10^{-9} - 10^{-10} M) $Fc_\epsilon R$ ($Fc_\epsilon RI$) on mast-cells and basophils [1]. The IgE production, together with the release by T and B lymphocytes of regulatory factors, is not only dependent on the presence of allergens, but it also requires a defined genetic background of the patient, since only particular subjects are able to mount an allergic response to such very common molecules.

The reintroduction of the allergen, which cross-links $Fc_\epsilon R$ -bound IgE, induces the production and/or release of various inflammatory mediators (histamine, leukotrienes, platelet-activating-factor, etc.), which trigger a cascade of events resulting in the allergic syndromes, such as oculorhinitis, asthma, etc. Among the above mentioned mediators, histamine has been considered to play a major role. This bioactive amine (MW 111) causes smooth muscle contraction of human bronchioles and small blood vessels, increased permeability of capillaries, and increased secretion by nasal and bronchial mucous glands. Since its maximal reaction occurs in 1-2 minutes, in humans it can be considered responsible for the symptoms of hay fever, urticaria, angioedema, etc.

Once the diagnosis of allergy has been made, two different therapeutic approaches can be followed: a) the pharmacologic management, aimed mainly at preventing

or controlling the symptoms resulting from the release of mediators by effector cells, is based essentially on the administration of three classes of drugs: antihistamines, which resemble histamine chemical structure and block histamine receptors on shock organ cells; corticosteroids, which are able to inhibit histidine decarboxylase, an enzyme that converts histidine into histamine; cromolyn sodium and related compounds, which probably acts by stabilizing the lysosomal membranes containing the chemical mediators; b) the specific immunotherapy (SIT), consisting in the administration to the patient of increasing amount of the relevant allergens, opportunely prepared and eventually chemically modified; the mechanisms underlying the well known clinical efficacy of SIT are still unclear, although significant changes in serological parameters (such as the increase of the specific IgG level and/or the reduction of the specific IgE level) have been associated in the literature to the positive outcome of SIT [2, 3]. For decades it has been postulated that allergen-specific IgG present in serum and secretions after IT may block allergens before their interaction with cell-bound IgE. However, this blocking activity has some relevance in the protective effect observed after venom IT, and it is unlikely to act as a major mechanism with inhalant allergens. On the other hand, the reduced level of specific IgE found after IT can be ascribed to the induction of specific suppressor T cells [4].

It is evident, therefore, that the development of improved strategies to the diagnosis and treatment of atopic diseases requires a wider knowledge on both allergens characteristics (this point being essentially related to the problem of the standardization of the preparations to be used for the diagnosis and the therapy) and regulation of IgE synthesis.

Regulation of IgE response

Since the beginning of this century, atopy has been recognised as a peculiar disease, induced by several factors. It has been calculated that nowadays the percentage of people suffering from allergic diseases is around 20%, reaching higher values in those areas where pollution represents an important problem. As already outlined, the main feature of atopy is the high level of immunoglobulin E produced in response to environmental allergens. Since the discovery of this isotype, a large body of papers on regulation of the IgE synthesis have been published, and the results have been obtained both in the human model and in the murine model as well. As for the production of other Ig classes, IgE are produced by B lymphocytes and require the presence of antigen presenting cells (macrophages and B cells) able to process and present fragments of antigen to antigen-specific T cell. This antigen presentation is restricted to the major histocompatibility complex (MHC), in the sense that B cells, T cells and macrophages must have identical Class II molecules, produced by genes coded in a particular MHC region.

After the presentation has taken place, T cells will either stimulate (T helper) or suppress (T suppressor) the B cells to produce immunoglobulins. The important role played by T cells in the regulation of IgE production has been proved in the early 1970s by several experiments carried out in animal models, based on the selective depletion of T cells by neonatal thymectomy [5] or whole-body irradiation [6] and administration of immunosuppressive drugs [7], followed by passive transfer of syngeneic T cells. Interestingly, these experimental measures affected selectively IgE, but not IgG synthesis, indicating that these two isotypes are subject to different regulatory influences. In humans, the study of serum IgE levels in patients with primary immunodeficiency disorders [8] gave similar evidences.

In the murine model where a more detailed investigation has been carried out, it was possible to demonstrate that several IgE-specific factors are released by T cells upon optimal stimulation. Ishizaka's group isolated and characterized two IgE-binding factors, which were able to down-regulate (IgE-suppressive factor, IgE-SF) or up-regulate (IgE-potentiating factor, IgE-PF) the IgE production [9]. These factors, both displaying a molecular weight of 15,000 Da, are coded by the same gene, but they are different, and therefore behave differently, according to the presence of oligosaccharide residues: IgE-PF has both N-linked and O-linked oligosaccharides, while IgE-SF contains only O-linked oligosaccharides. The glycosylation of these factors, and therefore their biological activities, is controlled by two T cell factors, glycosylation-inhibiting factor (GIF) and glycosylation-enhancing factor (GEF), which regulate the post-transcriptional glycosylation processes of the IgE-binding factors peptide [9]. Therefore, the balance between GIF and GEF appears to determine the nature of IgE-binding factors produced by T cells and their effects on the IgE production.

IgE-binding factors of B cell origin have been also described. In humans, the attention has mainly been focused on the IgE-binding factor/Fcε RII. The low-affinity receptor for the Fc part of IgE (Fcε RII, 10^{-6} - 10^{-7} M) [10] was evidenced on B cells and on various other hemopoietic cells such as monocytes/macrophages, eosinophils and platelets, while the presence of Fcε RII on T lymphocytes remains controversial. It was then demonstrated the identity of Fcε RII with the CD23 surface marker of the B lymphocytes [11, 12]. Briefly, Fcε RII/CD23 is a transmembrane glycoprotein with a molecular weight of 45,000 Da, with a proteolytic cleavage site. A soluble fragment of Fcε RII with a MW of 37,000 Da is released from the cell surface; this fragment further degrades into 33,000 Da, 25,000 Da and finally 12,000 Da species. This family of soluble molecules, all retaining IgE-binding activity except the 12,000 Da species, constitutes the IgE-binding factors of B cell origin, termed sCD23. The cleavage of Fcε RII into sCD23 is inhibited by IgE, whose binding to the receptor stabilizes the molecule on the cell surface. The biological role of Fcε RII and of its soluble fragment in the regulation of IgE production is still unclear, although it has

been reported that soluble Fc_ε RII/sCD23 is able to potentiate human IgE synthesis [13], while the intact Fc_ε RII may be involved in the downregulation of IgE production, probably by cross-linking with IgE and formation of immune complexes [14].

The regulatory role played by T cells in the IgE synthesis is not restricted to the production of IgE-binding factors: a different pathway of IgE regulation essentially based on the reciprocal role of two T-cell derived lymphokines, interleukin-4 (IL-4) and interferon- γ (IFN- γ), has been recently disclosed [15].

The initial observations about the *in vitro* and *in vivo* inducing effect exerted by IL-4 on the IgE production by murine B cell blasts [16] were soon followed by reports in humans, all pointing to the key role played by IL-4 in the regulation of IgE synthesis. Recently, evidence was presented that IL-4 from helper T cells drives the B cell response toward the IgE isotype, acting as an IgE-switching factor [17]; furthermore, IL-4 is a potent inducer of Fc ϵ RII on both B cells and monocytes, and may contribute to the activation of B cells.

The IgE synthesis induced by IL-4 can be negatively regulated by IFN- γ . These two lymphokines can be produced by two different subsets of T helper cells (Th 1 and Th 2), as clearly demonstrated in the mouse model, but they can also be the product of the same T cell clone. Then, the balance between the two subsets of T helper cells, or between the amounts of lymphokines produced by the same clone, will determine the levels of IgE synthesis and more generally the isotype of the immune response. It seems that in humans the relative synthesis of IL-4 and IFN- γ that T cells are capable to produce is different in atopic and nonatopic individuals: in particular, the increased prevalence of T cell clones able to produce IL-4 appears to be a distinctive feature of patients with common atopy.

Additional cellular and/or molecular signals seem to be involved in the induction of IgE synthesis, but their precise role still needs to be determined. It is clear that alterations of one or more of these regulatory mechanisms are responsible of the hyperproduction of IgE, which characterizes the atopic patient.

However, besides the great effort reserved to the study of the basic mechanisms involved in the IgE regulation, a great body of work has been carried out from the biochemical point of view, in order to investigate if the allergen (and/or its presentation by accessory cells to T cells) is able to lead in certain individuals, with an appropriate genetic background, to the hyperproduction of IgE whereas in others it drives antibody response toward other isotypes.

Characterization of allergenic preparations

Many substances have been recognised to have allergenic properties; for example mites, bee and wasp venom, from animal origin, and pollens, from plant origin; allergens have been also found in foods [18].

From the analysis of the characteristics of the allergens some common properties have been defined. Generally they are glycoproteins, with a molecular weight ranging from a few thousands up to 70,000 Da and an isoelectric point generally acidic. They are easily absorbed through the mucous membranes and easily released from the allergenic sources, together with other non allergenic molecules [19]. However, these features are shared by many other antigenic substances, thus indicating that they do not represent a peculiarity of allergenic molecules.

Allergens are generally obtained by extractive procedure from a raw material, and the crude extract represents the starting material for the purification procedures. Moreover, the raw material has been obtained in the past by collecting the sample of interest from the environment, but now it is commonly accepted that a standardised source (cultured plants for pollens, cultured mites, clearly identified strains for molds, etc.) is preferable.

The crude extract is characterized by using several biochemical and immunochemical techniques, some of them specially developed for this purpose. Table 1 reports a list of the most important techniques used. In order to facilitate the description of these methods, they have been conventionally divided into three groups, the *in vivo* methods, the *in vitro* methods and the biochemical methods.

The *in vivo* methods are essentially two, the skin-prick test (SPT) [20] and the provocation tests (PT) [21]. The former method is based on the interaction between the soluble allergen and the sensitised cell in the skin of the patients. The SPT is easily performed by adding a drop of allergens preparations over the skin of the volar region of the arm, this addition followed by a gentle scarification in correspondence with the drop. This procedure allows the contact of the active molecules with the IgE-bearing cells resident in the skin, eliciting a wheal reaction in positive sensitised subjects. The reaction can be quantitated by

Table 1. - *Methods applied to pollen allergen characterization*

<i>In vivo</i> methods
Skin prick test (SPT)
Provocation test
<i>In vitro</i> methods
Radio allerge sorbent test (RAST)
RAST inhibition
Gel precipitation techniques
Crossed immuno electrophoresis (CIE)
Crossed radio immuno electrophoresis (CRIE)
Immunoblotting
Histamine release from Basophils
Biochemical methods
Determination of protein content
Determination of protein nitrogen content
Isoelectric focussing
Polyacrylamide gel electrophoresis (SDS-PAGE)
High performance liquid chromatography (HPLC)

measuring the wheal area, either in relation or not with the wheal produced by a standard histamine solution. This method has been also used for the standardisation of allergenic extracts and for monitoring the efficacy of the immunotherapy.

The provocation tests are essentially based on the same principle than the SPT, although in this case the allergen solution is applied either on the conjunctival mucosa or, as an aerosol, on the mucosa of the respiratory tract. In both instances, the effect of the exposure can be recorded in a semiquantitative way by scoring the degree of the hyperaemia of the conjunctive or the degree of impairment of several respiratory parameters. Of course, these two techniques, especially the latter, have some disadvantages, due to the high degree of risk accompanying the tests, and therefore are generally carried out on a non-routine basis.

The *in vitro* methods have been more recently introduced in order to simplify and improve the *in vivo* methods for the diagnosis of allergic diseases and for allergen characterization. These methods are generally based on immunochemical techniques and have been of great importance in the characterization of allergenic extracts. They are generally based on the interaction of the different epitopes present in the various components of the extract with antibodies present in the patient serum or raised in experimental animals.

The most important *in vitro* techniques are radio-allergo-sorbent-test (RAST) [22], RAST-inhibition [23], basophil degranulation [24], gel precipitation techniques (CIE, CRIE) [25], immunoblotting [26] and immunoassays with monoclonal antibodies (MoAbs) [27].

The RAST procedure is able to directly detect specific IgE in the serum of the patients. The allergens preparations are covalently coupled to a solid phase (originally paper disks activated by cyanogen bromide) that is incubated with the human sera. After washings, the solid phase is incubated with an ^{125}I -labelled rabbit anti-human IgE and, after further washings, the radioactivity, proportional to the amount of human IgE bound to the solid phase, is counted in a γ -counter. This system has been recently standardised by several companies and results are now expressed as units per ml. Unfortunately, the different unitage systems do not allow a direct comparisons of the specific IgE values obtained by using the kits of different companies.

This technique, opportunely modified (RAST-inhibition), has been easily applied to the characterization of allergenic extracts; in this case, a reference solid phase-allergen/human IgE antibody system (RAST) is inhibited by serial dilutions of standard allergenic extract or unknown extract: the results are plotted, analysed by mathematical interpolation and the amounts of the extracts producing 50% of inhibition of the system are compared.

Another *in vitro* technique is the degranulation test, based on the measurement of the histamine released from IgE-sensitized basophils upon allergenic challenge. This test has been called degranulation test since granules

present in the basophils and containing several mediators, including histamine, are no longer detectable after the reaction has occurred.

The test can be carried out either on basophils-enriched peripheral blood lymphocytes (B-PBL) from allergic subjects, already sensitized with IgE, or with B-PBL from normal subjects, that can be passively sensitized upon incubation with serum from allergic subjects. In both cases, the reaction can be elicited by incubating the sensitized cells with the allergen preparation, and the released histamine can be quantitated by a chemical procedure. Alternatively, the number of cells with cytoplasmic granules can be counted before and after the allergenic challenge; this system, although very simple and not expensive, is only semiquantitative. A great advantage of the degranulation test is that it resembles *in vitro* what happens *in vivo*, avoiding any direct exposure of the patient with the allergenic extract. However, the complexity of the equipments required for quantitative histamine determination renders this method more suitable for research purposes than for the diagnosis of allergic diseases.

Other than RAST and RAST-inhibition, other techniques (such as crossed-immuno-electrophoresis, CIE, and crossed-radio-immuno-electrophoresis, CRIE) have been applied in order to elucidate the complexity of the raw allergenic preparations commonly employed and of the purified allergens. Differently from the other techniques, CIE requires a step in which the various components of the extract are precipitated in agar gel by using a polyclonal rabbit antiserum; the precipitin arcs can be clearly evidenced by staining the agarose gel in different ways, thus producing a pattern which allows the extract to be characterized. Furthermore, in order to recognise which precipitin arc/s are produced by allergenic molecules, the gel is incubated with the patient serum (CRIE). The IgE contained in the serum sample will bind to the epitopes available on the precipitated components and will be evidenced by incubation with radiolabelled anti-human IgE and autoradiography. The results will indicate which precipitin arcs obtained in the CIE are allergens and how many individual patients sera are able to recognise a given precipitated component, allowing an estimation of which one is more frequently recognised by a given panel of allergic sera, and leading to the definition, firstly introduced by Lowenstein [28], of major (more frequent) and minor (less frequent) allergens.

In the last decade, a new powerful technique, the immunoblotting, has been applied to the characterization of allergenic extracts. The test is based on the properties that proteins and glycoproteins, and allergenic extracts as well, have to react with specific ligands (antibodies, lectins, etc.) once bound in a strong way to nitrocellulose paper (blotting), even after they have been separated by either sodium-dodecyl-sulphate-polyacrylamide-gel-electrophoresis (SDS-PAGE) or isoelectric focussing (IEF). The blotted allergenic extract is incubated with the patients sera, then with a radiolabelled anti-human IgE and finally developed by X-rays exposure. The results allow to indi-

viduate the relevant components able to interact with the human IgE (allergens), and to estimate which one is more frequently recognised by a given panel of sera from allergic patients, again allowing the definition, by a different technique, of major and minor allergens.

A great improvement has been achieved by applying the MoAbs technology to the characterization of allergenic extract. MoAbs have been produced against a large number of major and minor allergens (Table 2), and after they have been characterized in terms of specificity, affinity constant, epitope recognised, they have been employed both in quantitative binding assays for analytical purposes or in analytical and preparative affinity chromatography. Several radioimmunoassays have been developed against many allergens [30]: they are generally two-sites immunoassays and require two monoclonal antibodies specific for different epitopes of the same allergen. A few allergenic preparations, containing a relevant component quantitated by using in this assay as a standard the corresponding highly purified material, are now available on the market.

In the third group of techniques are included all the biochemical techniques, which have been applied in the past to the characterization of many biological substances and also to several relevant allergens. Basically, these classical biochemical techniques are SDS-PAGE [31], low pressure gel chromatography [32] and high performance liquid chromatography (HPLC) [33], and isoelectric focussing (IEF) [34]. However, in some circumstances the high number of components contained in the extracts has often required the combined use of these techniques with immunochemical techniques (*i.e.* RAST, RAST-inhibition, CRIE, immunoblotting, etc.) in order to get the various fractions characterized in terms of allergenic activity. However, even with the combined use of several methods, the complexity of the extract under investigation did not allowed, in some cases, a good characterization of the component(s) of interest.

As a result of these studies, many allergens have been extensively investigated, the relevant molecules identified, allowing a better approach to the standardization of the products still used for the diagnosis and the therapy of allergic diseases.

Purification of allergens

Once the allergenic preparations have been characterized by using the techniques already described, a great effort has been put in the purification of the relevant allergens, in order to make pure reference materials available for the standardization of the allergenic extracts used, and also for the preparation of balanced mixtures of well-known allergens to be used in the diagnosis and immunotherapy. For this reason, allergens preparations from several relevant sources were deeply characterized and major and minor allergens purified.

Several procedure have been used in order to get relevant allergens purified. They are represented by common biochemical procedures, such as general chromatographic

Table 2. - Pollen allergen specific monoclonal antibodies

Source	Specificity	Sequence	Epitopes
Grass			
<i>Lolium perenne</i>	Lol p I	(a)	> 6
<i>Dactylis glomerata</i>	Dac g I		4
<i>Poa pratensis</i>	Ag27		1
Weeds			
<i>Ambrosia</i>			
<i>artemisiifolia</i>	Amb a I	(a)	3
	Amb a II		2
<i>Parietaria judaica</i>	Par j I		2 (b)
Trees			
<i>Betula verrucosa</i>	Bet v I		
<i>Olea europaea</i>	Ole e I		
House dust mites			
<i>D. pteronyssinus</i>	Der p I	(a)	5 (b)
	Der p II	(a)	1 (b)
<i>D. farinae</i>	Der f I	(a)	2 (b)
	Der f II	(a)	3 (b)
Cat			
<i>Felis domesticus</i>	Fel d I		4
	Fel d I		2 (b)
Insects			
<i>Chironomus tummi</i>	Chi t I	(a)	5
<i>Apis mellifera</i>	Mellitin	(a)	1

(a) Partial or N-terminal sequence available.

(b) Two-site monoclonal antibody immunoassay developed.

(Modified from Chapman [29]).

procedures [35], salt fractionation [36], and preparative isoelectric focussing [37], by immunochemical methods - affinity chromatography on lectins [38] and monoclonal antibodies [39] - and, more recently, by genetic engineering [40].

Table 3 reports a partial list of the most important allergens investigated, purified and characterized.

One out of the most important problems related to allergen purification is represented by the fact that in the crude extract the relevant allergen(s) are often contained in very minute amounts, thus rendering the biochemical approach quite complicate. Moreover, the yield of the biochemical purification is generally low, while on the other hand the purified allergen(s) should be available in large quantities, suitable for further biological studies. The availability of purified allergenic components does not seem to be useful at the diagnostic level since some false negative reactions could occur in those patients reactive only with other allergenic components different from those tested; on the other hand, single purified allergenic components could be used for a better immunotherapy, providing the identification of the relevant components for a given patient is available. In this case, in fact, it would be advisable to use in the SIT the relevant allergenic component only, in order to prevent the subject to get in contact with, and eventually sensitised to, other components for which he was negative before the treatment. From this, the

Table 3. - List of some highly purified allergens

Allergen	Molecular weight	pI	Allergen	Molecular weight	pI
1. POLLEN ALLERGENS: GRASSES, WEEDS AND TREES					
Grass					
Timothy grass - <i>Phleum pratense</i>			Dog		
Phl p VIII	10,000	3.9	Albumin (a)	65,000	4.7
Phl p VI	15,000	4.9	globulin fraction (a)	-	-
Phl p V	15,000	4.5	Ag2 (a)	65,000	4.5
Phl p VII	34,000	9.4	Ag13 (a)	-	-
			AgD4 (a)	16,000-20,000	4.5-5.5
Cocksfoot/Orchard grass - <i>Dactylis glomerata</i>			Farm animals		
Ag26 (a)	75,000	-	Horse		
Ag32 (a)	23,000	-	Fraction b2 (a)	34,000	4.1
			Component I (a)	22,000	4.2
Rye grass - <i>Lolium perenne</i>			Component III (a)	38,000	-
Lol p I	27,000-34,000	5.1-5.25	Equ c I	19,000	4.1
Lol p II	11,000	5.3	Equ c II	51,000	3.8
Lol p III	11,000	9.4	Equ c III	31,000	3.9
Lol p IV	50,000	-	Ag15 (a)	36,000	3.75
			Cow		
Ag1 (a)	-	5.4	Bos d I	24,000	3.6
Ag6 (a)	<10,000	3.9	Bos d II	20,000	4.2
Weed			Bos d III	22,000	4.6
Short ragweed - <i>Ambrosia elatior</i>			Ag14 (a)	>200,000	-
Amb a I	37,800	5.0	Invertebrates		
Amb a II	38,200	5.9	Mites		
Amb a III	12,100	8.6	<i>Dermatophagoides p.</i>		
Amb a V	5,000	9.5	Agp1 (a)	24,000	4.7-5.4
Amb a VI	11,000	basic	Dpt12 (a)	27,000	5.8-7.0
Tree			Der p I	30,000	4.6-6.9
Birch - <i>Betula verrucosa</i>			Der p II	12,000	6.4
Major BP allergen (a)	20,000	-	<i>Dermatophagoides f.</i>		
Ag7-8 (a)	17,000-10,000	5.6	Der f I	28,000	7.0
Ag4 (a)	29,000	5.2	Cockroaches		
Bet v I	17,000	5.5	<i>Periplaneta americana</i>		
Ag19 (a)	27,000-30,000	6.7	Cr-I (a)	25,000	<prealbumin
Alder - <i>Alnus incana</i>			Cr-II (a)	63,000-65,000	β -globulin
Aln i I	22,500	4.8	4. FOOD ALLERGENS		
Plane - <i>Platanus acerifolia</i>			Milk		
DE IV (a)	22,000	3.8	β -lactoglobulin (a)	36,000	-
Hazel - <i>Corylus avellana</i>			Egg		
Cor a I	13,500	5.4	VE-54B (a)	31,500	4.1-4.5
2. FUNGAL ALLERGENS			Ag6 (a)	36,000	-
Fungus			Ag12 (a)	27,000	-
<i>Alternaria alternata</i> (= <i>Alternaria tenuis</i>)			Cod		
Alt a I	25,000-50,000	4.0	Gad c I	16,200	4.75
Ag1 (a)	60,000	4.0	5. STINGING INSECTS		
AgB (a)	40,000	4.3	<i>Apis mellifera</i>		
Ag8 (a)	30,000	4.3	Api m I	15,800	10.5
<i>Cladosporium herbarum</i>			Api m II	50,000	basic
Cla h I	13,000	3.4	Api m III	2,840	basic
Cla h II	25,000	5.0	Allergen B (a)	49,000	acidic
<i>Aspergillus fumigatus</i>			<i>Vespula spp.</i>		
Ag3 (a)	24,000	4.5	Ves g V	25,000	basic
Ag10 (a)	-	-	Ves g I	35,000	basic
Ag40 (a)	-	-	Ves g II	45,000	basic
3. ANIMAL ALLERGENS			Acid phosphatase (a)	-	acidic
Domestic pets			Ag24-25 (a)	6,000	basic
Cat					
Main epithelia (a)	32,000	<albumin			
Allergen 1 (a)	30,000-60,000	3.8			
Fel d I	24,000	3.5			
Ag3 (a)	70,000	4.5			

(a) For these allergens it was not possible to unequivocally introduce the new nomenclature recently adopted for purified allergens [46]. (Modified from Dreborg *et al.* [18]).

idea of the balanced mixture of purified allergenic components for the diagnosis and of an *ad hoc* preparation for therapy. All these concepts have been clearly discussed by Aas [41], who proposed a model for grading of allergen extract quality. According to this Author at the bottom of the list are placed those preparations that have been until recently used, that are merely crude extracts submitted to any purification procedure. At the top of the list is placed a theoretical product defined as a mixture of purified, chemically defined characterized and quantitated material representing all major and minor allergens found in the original crude extract. Of course, non-allergenic material and irritants are removed. At present, the use in diagnosis and immunotherapy of the theoretical mixtures proposed by Aas is not possible because highly purified allergens are not yet available in large amount.

Practically, between the former theoretical and the latter dismissed preparations, a number of intermediate products are available on the market and are commonly employed for diagnosis and immunotherapy: they are still crude extracts which underwent some steps of purification in order to remove irritants and low molecular weight components. In some circumstances, when reagents are available, the major allergen(s) is/are quantitated by immunoassays by using MoAbs and by other techniques by using polyclonal antibodies: this procedure allows the final product to be characterized and the active ingredients at least in part quantitated, thus partially ensuring a batch to batch consistency.

However, in order to get as close as possible to the highest level of quality of the extracts proposed by Aas, two different approaches, based on methods alternative to the classical purification procedures, have been attempted with the aim of recovering pure allergens in large amount: affinity chromatography on lectins or monoclonal antibodies and genetic engineering. The usefulness of affinity chromatography with the lectins has been evaluated in several instances, and the results have clearly indicated that this technique can be used in addition to the others with the aim of improving the purity of the final product. However, since almost all the components of the crude extract are glycoproteins, the use of lectin-chromatography has been found without utility when performed on the early stages of the purification procedure. On the contrary, affinity chromatography with monoclonal antibodies has been revealed to be of some utility, since several papers have been published concerning either single-step or multi-steps purification of a relevant allergen with good purity and good yield of the product [42, 43]. However, problems have been encountered in the scaling up of the purification procedure: up to now, although monoclonal antibodies against a number of allergens have been produced, only a few have been used for purification purposes and none has been routinely introduced by any Company in the manufacturing procedures of allergenic extracts. At the same time great effort was put in the application to allergen preparations of the possibilities recently offered by genetic engineering. By using this technique, a few major aller-

gens have been already cloned, and produced in large amounts [44, 45]. Interestingly, the presence on the natural allergens of sugar residues absent in the cloned product, a problem which has been often encountered for other products, does not seem to represent a problem in this case since the IgE of the patients are generally directed against proteic structures. However, to fully evaluate the possibilities offered by this new approach it will be advisable to wait until several of these products will become available for the clinical evaluation.

Standardization of allergenic extracts

The heterogeneity of the components present in the crude extract has dramatically risen the issue of standardization and quality control of allergen preparations. A few points have to be considered. As stated above, theoretical preparations, as those indicated by Aas, are not available, and will not be available for many years. Therefore, in order to have a satisfactory product, the extracts should be at least qualitatively and quantitatively standardised. For this standardisation process, international standard preparations should be prepared and used, and clearly described and evaluated methods should be available. A few years ago, manufacturers were used to produce and sale their

Table 4. - *Units commonly employed in the evaluation of allergenic extracts potency*

<i>In vivo</i> units
Histamine equivalent units, HEP
Biological units, BU (1 HEP = 1000 BU)
Allergic units, AU
<i>In vitro</i> units
RAST inhibition units, PRU
Others
Weight/volume units
Noon units
Protein nitrogen units, PNU

Table 5. - *International organizations and regulatory agencies involved in standardization of allergens*

World Health Organization
International Union of Immunological Societies - Allergen Standardization Subcommittee
Nordic Council on Medicines
Food & Drug Administration
European Economic Community - Committee for Proprietary Medicinal Products
Council of Europe - European Pharmacopoeia Commission

allergenic preparations without any in process or final quality control. This led to the presence on the market of preparations displaying a broad degree of allergenic activity evaluated at the diagnostic level in terms of sensitivity and specificity and at the therapeutic level in terms of efficacy and safety. With a few exceptions, this trend has been in recent years replaced by the idea that at least batch to batch consistency has to be obtained by the manufacturers. This means that a given batch of a given allergenic preparation obtained by one manufacturer has to be comparable within definite limits to a previous batch and to a future batch. This internal standardization has been essentially carried out by quantitative SPT, RAST-inhibition, immunoblotting, always comparing by statistical analysis the batch under investigation with an internal standard, different from company to company. Moreover, this standardization process led to the presence on the market of an increasing number of units (Table 4), essentially defined on the basis of the techniques employed by a manufacturer

in the standardization process and different from those used by another manufacturer. Therefore, several international organizations (Table 5) have been involved with the aim of defining international reference preparations each containing an assigned amount of international units. At present, three standards for three common allergens only (*Dermatophagoides pteronyssinus*, *Ambrosia artemisiifolia* and *Phleum pratense*) have been defined by the International Union of Immunological Societies (IUIS) Subcommittee and are available for standardization purposes, while others are under evaluation. However, in order to achieve an international harmonization of the unitage system, standard methods to be used with these reference preparations must be established, and well defined pools of sera from allergic patients must be settled by the International Committee.

Received on November 5, 1990.

Accepted on November 22, 1990.

REFERENCES

1. ISHIZAKA, T. & ISHIZAKA, K. 1975. Biology of immunoglobulin E. *Prog. Allergy* **19**: 60-121.
2. BOUSQUET, J. & MICHEL, F.B. 1988. Specific immunotherapy. *Allergy* **43**(suppl.8): 16-22.
3. DJURUP, R. 1985. The subclass nature and clinical significance of the IgG antibody response in patients undergoing allergen-specific immunotherapy. *Allergy* **40**: 469-486.
4. TAMIR, R., CASTRACANE, J.M. & ROCKLIN, R.E. 1987. Generation of suppressor cells in atopic patients during immunotherapy that modulate IgE synthesis. *J. Allergy Clin. Immunol.* **79**: 591-598.
5. OKUMURA, K. & TADA, T. 1971. Regulation of homocytotropic antibody formation in the rat. III. Effect of thymectomy and splenectomy. *J. Immunol.* **106**: 1019-1025.
6. TADA, T., TANIGUCHI, M. & OKUMURA, K. 1971. Regulation of homocytotropic antibody formation in the rat. II. Effect of X irradiation. *J. Immunol.* **106**: 1012-1018.
7. TANIGUCHI, M. & TADA, T. 1971. Regulation of homocytotropic antibody formation in the rat. IV. Effects of various immunosuppressive drugs. *J. Immunol.* **107**: 579-585.
8. BUCKLEY, R.H. & FISCUS, S.A. 1975. Serum IgD and IgE concentration in immunodeficiency diseases. *J. Clin. Invest.* **55**: 157-165.
9. ISHIZAKA, K. 1988. IgE-binding factors and regulation of the IgE antibody response. *Annu. Rev. Immunol.* **6**: 513-534.
10. CAPRON, A., DESSAINT, J.P., CAPRON, M., JOSEPH, M., AMEISEN, J.C. & TONNEL, A.B. 1986. From parasite to allergy: a second receptor for IgE. *Immunol. Today* **7**: 15-18.
11. YUKAWA, K., KIKUTANI, H., OWAKI, H., YAMASAKY, K., YOKOTA, A., NAKAMURA, H., BARSUMIAN, E.L., HARDY, R.R., SUEMURA, M. & KISHIMOTO, T. 1987. A B cell specific differentiation antigen, CD23, is a receptor for IgE (Fc_εR) on lymphocytes. *J. Immunol.* **138**: 2576-2580.
12. BONNEFOY, J.Y., AUBRY, J.P., PERONNE, C., WIJENES, J. & BANCHEREAU, J. 1987. Production and characterization of a monoclonal antibody specific for the human lymphocyte low affinity receptor for IgE: CD23 is a low affinity receptor for IgE. *J. Immunol.* **138**: 2970-2978.
13. SARFATI, M., RECTOR, E., WONG, K., RUBIO-TRUJILLO, SEHON, A.H. & DELESPESE, G. 1984. *In vitro* synthesis of IgE by human lymphocytes. II. Enhancement of the spontaneous IgE synthesis by IgE-binding factors secreted by RPMI 8866 lymphoblastoid B cells. *Immunology* **53**: 197-205.
14. SCHERR, E., MACY, E., KIMATA, H., GILLY, M. & SAXON, A. 1989. Binding of the low affinity Fc_εR on B cells suppresses ongoing human IgE synthesis. *J. Immunol.* **142**: 481-489.
15. VERCELLI, D. & GEHA, R.S. 1989. Regulation of IgE synthesis in humans. *J. Clin. Immunol.* **9**: 75-83.
16. COFFMAN, R.L., OHARA, J., BOND, M.W., CARTY, J., ZLOTNIK, A. & PAUL, W.E. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* **136**: 4538-4541.

17. LEBMAN, D.A. & COFFMAN, R.L. 1988. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J. Exp. Med.* **168**: 853-862.
18. DREBORG, S., EINARSSON, R. & LONGBOTTOM, J.L. 1986. The chemistry and standardization of allergens. In: *Handbook of immunochemistry*. D.M. Weir (Ed.). Blackwell Scientific Publication. Chapter 10.
19. MARSII, D.G. 1975. Allergens and the genetics of allergy. In: *The antigens*. O. Sela (Eds). Academic Press, New York. Vol. III. pp. 271-359.
20. DREBORG, S. 1989. Skin tests used in type I allergy testing. *Allergy* **44** (suppl. 10): 1-59.
21. CHOI, H. 1988. Bronchial challenges. *Clin. Rev. Allergy* **6**: 231-257.
22. WIDE, L., BENNICH, H. & JOHANSSON, S.G.O. 1967. Diagnosis of allergy by an *in vitro* test for allergen antibodies. *Lancet* **ii**: 1105-1107.
23. YMAN, L., PONTERIUS, G. & BRANDT, R. 1975. RAST-based allergen assay methods. *Dev. Biol. Stand.* **29**: 151-165.
24. HAYDIK, I.B., MA W.S. 1988. Basophil histamine release: assays and interpretation. *Clin. Rev. Allergy* **6**: 141-165.
25. AXELSEN, N.H. 1983. Handbook of immunoprecipitation-in-Gel techniques. *Scand. J. Immunol.* **17**(suppl. 10).
26. TOWBIN, H., STAHELIN, T. & GORDON, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 4350-4354.
27. CHAPMAN, M.D., HEYMANN, P.W., WILKINS, S.R., BROWN, M.J. & PLATTS-MILLS, T.A.E. 1987. Monoclonal immunoassay for major dust mite (*Dermatophagoides*) allergens, *Der p 1* and *Der f 1*, and quantitative analysis of the allergen content of mite and house dust extracts. *J. Allergy Clin. Immunol.* **80**: 184-194.
28. LOWENSTEIN, H. 1980. Allergy and characterisation of allergens. *Allergy* **35**: 210-211.
29. CHAPMAN, M.D. 1989. Purification of allergens. *Curr. Opin. Immunol.* **1**: 647-653.
30. CHAPMAN, M.D. 1988. Allergen specific monoclonal antibodies: new tools for the management of allergic disease. *Allergy* **43** (Suppl. 5): 7-14.
31. LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**: 680-685.
32. KING, T.P. 1976. Chemical and biological properties of some atopic allergens. *Adv. Immunol.* **23**: 77-105.
33. BASSOLI, A., CHIOCCARA, F., DIGREGORIO, G., RINDONE, B., TOLLARI, S., FALAGIANI, P., RIVA, G. & BOLZACCHINI, E. 1988. Analysis of allergenic components of *Parietaria judaica* pollen extract by chromatographic methods for the evaluation of purification procedures. *J. Chromatogr.* **444**: 209-218.
34. BRIGHTON, W.D. 1975. Profiles of allergen extract components by isoelectric focusing and radioimmunoassay. *Dev. Biol. Stand.* **29**: 362-369.
35. BERRENS, L. 1971. *The chemistry of atopic allergens*. Karger, Basel, New York.
36. GIULIANI, A., PINI, C., BONINI, S., MUCCI, N., FERRONI, L. & VICARI, G. 1987. Isolation and purification of a major allergen from *Parietaria officinalis* pollen. *Allergy* **42**: 434-440.
37. BLANCA, M., BOULTON, P., BROSTOFF, J. & GONZALEZ-REGUERA, I. 1983. Studies of the allergens of *Olea europea* pollen. *Clin. Allergy* **13**: 473-478.
38. LAUZURICA, P., MARURI, N., GALOCHA, B., GONZALEZ, J., DIAZ, R., PALOMINO, P., HERNANDEZ, D. GARCIA, R. & LAHOZ, C. 1988. Olive (*Olea europea*) pollen allergens. II. Isolation and characterization of two major antigens. *Mol. Immunol.* **25**: 337-344.
39. DEGROOT, H., VAN SWIETEN, P., VAN LEEUWEN, J., LIND, P. & AALBERSE, R.C. 1988. Monoclonal antibodies to the major feline allergen *Fel d 1*. I. Serologic and biologic activity of affinity purified *Fel d 1* and of *Fel d 1* depleted extract. *J. Allergy Clin. Immunol.* **82**: 778-786.
40. BALDO, B.A. & DONOVAN, G.R. 1988. The structural basis of allergenicity: recombinant DNA-based strategies for the study of allergens. *Allergy* **43**: 81-97.
41. AAS, K. 1975. Clinical and experimental aspects of standardization and purification of allergen. *Int. Arch. Allergy Appl. Immunol.* **49**: 44-54.
42. COCCHIARA, R., LOCOROTONDO, G., PARLATO, A., GUARNOTTA, G., RONCHI, S., ALBEGGIANI, G., AMOROSO, S., FALAGIANI, P. & GERACI, D. 1989. Purification of *Par j 1*, a major allergen from *Parietaria judaica* pollen. *Int. Arch. Allergy Appl. Immunol.* **90**: 84-90.
43. CHAPMAN, M.D., AALBERSE, R.C., BROWN, M.J. & PLATTS-MILLS, T.A.E. 1988. Monoclonal antibodies to the major feline allergen *Fel d 1*. II. Single step affinity purification of *Fel d 1*, N-terminal sequence analysis, and development of a sensitive twosite immunoassay to assess *Fel d 1* exposure. *J. Immunol.* **140**: 812-818.

44. CHUA, K.Y., STEWART, G.A., THOMAS, W.R., SIMPSON, R.J., DILWORTH, R.J., PLOZZA, T.M. & TURNER, K.J. 1988. Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p I*. Homology with cysteine proteases. *J. Exp. Med.* **167**: 175-182.
45. FANG, K.S.Y., VITALE, M., FEHLNER, P. & KING, T.P. 1988. cDNA cloning and primary structure of a white-face hornet venom allergen, antigen 5. *Proc. Natl. Acad. Sci. USA* **85**: 895-899.
46. MARSH, D.G., GOODFRIEND, L., KING, T.P., LOWENSTEIN, H. & PLATTS-MILLS, T.A.E. 1988. Allergen nomenclature. *Int. Arch. Allergy Appl. Immunol.* **85**: 194-200.