ALLERGEN STANDARDIZATION

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The article summarises current knowledge about the preparation and standardisation of treatment and diagnosis of allergies. It summarises the basic conceptions, units, methods and conditions for preparation, conservation and the storage of allergens. The article is especially orientated towards grass pollen.

INTRODUCTION

Allergens

Allergens are defined as antigens being capable to initiate an allergic reaction[1]. Traditionally, this indication has been used in connection with a reaction of the 1st immunological type (IgE mediated reaction). Allergens used for diagnostics in the human medicine as well as allergens being specific for immunotherapy of allergic states are prepared through extraction of allergen substances from materials of biological origin most of the time. Extraction procedures are various, nevertheless they all respect physiological conditions enabling an isolation of protein structures without denaturation or a change of molecular conformation[2]. They are formed by a mixture of various substances largely of glycoprotein character. The relations between structure and immunogenic activity of allergen proteins have not been quite cleared up. Only a small part of extracted proteins shows an allergen activity, the remaining substances are the ballast ones from the allergist's point of view or they form adjuvant background. Namely as to pollen allergens, a number of short amino acid sequences was described which present specific determinants[3] for idiotypes of IgE antibodies. Such sequences are determined as allergen motives. They are comparatively conserved in the plant kingdom and show a high level of sequence homology in various taxa. This fact is a frequent cause of a cross – reactivity[4, 5, 6]. There are necessary methods of determination of their composition as well as characterization of allergen active components for study of immune response in allergic patients as well as study of allergen in general. The problem of standardization and characterization of preparations is largely a question of a choice of the suitable units and techniques. World Health Organization (WHO) has endeavoured to reach an agreement on an approach to testing and standardization of preparations used for allergy treatment for many years. However, the effort of WHO has not been successful up to now. A great number of methods, terms and units[7, 8, 9, 10] still exists.

Allergen activity

A study of allergen activity is of importance not only from the point of view of theoretical immunology, but it is of use in the clinical practice, too. From the point of view of a choice of suitable method for quantification of allergen activity it is necessary to choose between exact procedures of analytical chemistry on the one hand and evaluating of biological activity on the other hand. A biological activity is determined by a reactivity of the subject. Its quantification is then relative and has got an information value rather on the statistic level. On the other hand the chemical procedures are insufficient for reproducible information on allergen potential of a preparation, because a pure biological characteristic is concerned. The development of the methods used for standardization of the allergen preparations is a search of a compromise between the both above – mentioned approaches. The goal of the standardization is to prepare reference allergen(s) with a known and reproducible composition and with a biological potential which is suitable for calibration of industrial production, or of experimentally prepared extracts of particular allergens used for diagnostic and therapeutic purposes[11, 12, 13] .

In origin the standardization of allergen activity of preparations was based only on spectrum of molecular weight of proteins (MW), later volume units were introduced. The first unit taking into consideration protein quantification in an extract was PNU (protein nitrogen unit). Using the unit can be traced back to the 1944, when American Academy of Allergy recommended to give the protein content in allergen preparations just in PNU[12] . However, a protein concentration given in PNU does not correlate regulary with biological activity of allergen preparations, because only amino-acid sequences of various lengths – so-called allergen epitopes show allergen activity in particular proteins. They are represented in accordance with their kinds. A real picture of biological activity are in vivo techniques (skin – prick testing). They are frequently used for diagnostic purposes, thus in treatment of the concrete patient. However, from the ethical point of view these techniques are

not to be used for a real testing of commercially produced preparations. Likely the best solution seems to be in vitro tests (RAST or ELISA techniques) in connection with molecular-biological approaches. The entrance of molecular biology in these problems was expressed by an increase of information about so-called main allergens of the given pollen, i.e. pollen proteins inducing anaphylactic reaction in more than one half of patients being allergic to this pollen and by preparing some allergens by means of recombinant technology[13]. In some main allergens the amino-acid sequence or the nucleotide one of their genes[13, 14, 15, 16] has been already known.

TERMS AND UNITS

In connection with a standardization of allergens some terms and special units are used[17].

Allergen extract is defined as an extract containing allergen – active components of animal or plant origin in a suitable solvent.

Allergen preparation is a biological product containing allergen extract in a suitable solvent, or bound to a suitable pharmaceutical carrier, affecting immunological properties of the preparation (e.g. aluminium hydroxide) which is designated for diagnostic, prevention and treatment of allergies[8].

Since 1980 the following units for allergen extracts have been used:

NOON units	(world-wide)
Wt/vol	(world-wide)
PNU	protein nitrogen units (world-wide)[18]
IU	international unit (WHO)[8]
BU	biological unit (Europe)[19]
AU	allergy unit (U.S.FDA)[12]
BAU	bioequivalent allergy unit[8]

A biological standardization of allergens also includes taxonomy and a nomenclature[7] being connected with it. The name of allergen is derived from the source, which allergen was obtained from. The procedure is as follows:

1. the first three letters of gender name, space
2. the first letter of specific epithet, space
3. arabic numeral which designates an identification order of the given allergen.

E.g. Lol p 1 means, that the first discovered allergen from *Lolium perenne* is concerned. Also allergens of on species can contain several allergen molecules. These molecules are indicated as isoallergens – they have the same MW (molecular weight), identical biological functions, enzymatic activity and also the same sequence of amino acids of 67%. The partial or complete sequences of amino acids are known in the whole number of allergens at present. A good source of information about the known allergens is Allergen Database[20] (ALBE) which contains beside sequence data also biochemical and immunological data.

COLLECTION OF MATERIALS

As to the source of materials for preparing of allergen preparations, these source are very strongly controlled, a great stress is laid on preserving a taxonomy identity which is very difficult with mites and moulds. A collection of materials concerning the usual allergens has been fully described in the literature[21, 22].

EXTRACTION, AGITATION, INFLUENCE OF INGREDIENTS AND PRESERVING

Extraction of pollen (without contaminants and adulterants) is carried out most of the time as follows: 0.0125 M $NaHCO_3$ or 0.05 M $NaHCO_3$ + 0.4 % phenol in the ratio of 1 : 10 (pollen weight : extraction buffer) at the temperatures of 2–8 °C for the time of 24 hours at continual agitation, the extract is then filtrated through Whatman No. 4, the pellet is usually reextracted by means of a half – quantity of extraction buffer under the same conditions, supernatants from the both filtrations are connected and filtrated in a sterile way through a membrane of 0.2 μm[23, 24].

If the patient is allergic to more substances (and it is usual), it is suitable to administer a vaccine containing all substances to which he is allergic. The situation is e.g. with pollens complicated by a cross – reactivity of patients to various pollens. The nature consists in molecular mechanism of induction of anaphylactic reaction, on which only a small part of proteins takes part, a so-called allergen epitope which is characterized by a primary amino-acid sequence. Allergen epitopes are not proteins contained in various kinds of pollen grains and no functional or other connection has been ever found between these proteins. Furthermore, it can be assumed that also pollen grains of one species have got a spectrum of proteins depending on regional and actual climatic conditions of a culture. There should be an optimal doses for such a patient selected so, that the particular substances do not affect each other. E.g. the pollens can be degradated in the mixture with dust or moulds[8].

It is known that the effectiveness of allergen preparations drops in dependence on time. It was found that 50% glycerol exerts positive influence on a stability of the preparations, an influence of human serum albumin (HSA) and Tween 80 has been observed, too; silicates from glass exerts positive influence, too and on the other hand phenol reduces the effectiveness[25, 26]. A direct RAST test, eventually inhibition was used for observing of activity decrease[27].

An activity decrease is usually quicker with diluted extracts. This phenomen is explained by an adsorption of protein on the side of the phial. Hence it was recommended to maintain the preparations at 4 °C in a as high as possible concentration. A decrease of activity with mites and fungi is caused by proteases. Pollen extracts are relatively resistant against proteases[28]. An influence

of ingredients in an extract diluting of 1:100 up to 1:100000 w/v has been observed to. As ingredients 50% and 10% glycerol and 0.03% HSA were used. All these substances has got a significant preservation effect. It is better for diagnostic administration in the form of prick testing to add 10% glycerol + 0.03 % HSA (the same effect as 50% glycerol, however its administration is not so painful and does not cause an inflammation).

A one year study has been carried out with allergen extracts. The study has observed an influence of the storage temperature on the activity of the preparations. The samples were preserved at the temperatures of 4 °C, 20 °C and a simulation of tempering before administration to a patient (extract was removed from a temperature of 4 °C for approx. 13 hours/week) was carried out. The study brought the following conclusion: with preserving within 3 months no significant difference was observed in activity at temperatures of 4° and 20 °C; with preserving within 12 months a loss in activity was observed with a preparation which has not been preserved at the temperature of 4 °C all the time[27]. A stability of the preparations can be enhanced by a lyophilization or by preserving in 50% glycerol. Lyophilization is preferred with the most of allergens, however, e.g. mites lose a great part of their activity during a lyophilization process[12].

TESTING OF PREPARATIONS AND STANDARDIZATION

Standardization is based on in vitro and in vivo determinations of IgE antibodies against allergens[29]. Skin tests enable to define allergen vaccines by means of biological units. Several methods are used, all of them depending on a selection of suitable patients. The sera of selected patients are usually tested by means of RAST technique 3 or 4 types of systems exist, by means of which a biological activity of allergen preparations is given:

1. IU – international unit	in WHO
2. BU – biological unit	Europe
3. AU – allergy unit	USA
4. BAU – bioequivalent allergy unit	USA

Evaluation of activity in IU is based on electrophoreses (SDS – PAGE, IEF (isoelectric focusation), CRIE (cross immunoelectrophoresis), immunoblots and skin tests. In 1989 the international standards for *D. pteronyssinus,* short ragweed, timothy grass, dog hair and birch[30] were available.

Methodology of European system is somewhat different. Standardization is based on skin tests[31], CRIE, IEF and SDS-PAGE[32]; the essential difference from the methodologies of WHO is introducing of RAST inhibitions for evaluating total activity. The RAST test[31] (radioallergosorbent test) is a radioimmunomethod on the solid phase using designation by radioactive I^{125}. Prick – tests are carried out with 20 allergic patients. A size of wheel is measured (geometric mean) and the results are given in BU.

AU used in the USA are based on using biological standardization by means of chemical analysis and intradermal tests. Recently AU have been replaced by BAU[8]. There has been used a complex of 15 "highly allergic patients" for quantification of the preparations. An erythema is produced by intradermal tests and its size (arithmetic mean) is measured again. The result is given in BAU – Bioequivalent Allergy Unit.

It is coincident for BU and AU that they are based on allergic reactions of the patients. It is in place to mention a selection of suitable patients. The selection is based on personal history, results of skin tests and as to patients being allergic to pollen also on the season. By means of BU and AU the preparations of the same as well as various species can be compared, e.g. birch and cat fur. Nevertheless, RAST – inhibition test is better for activity evaluating. Also here it is necessary to select suitable patients as donors of the serum which is needful for tests. The used pooled sera can be characterized only with difficulty. A certain disadvantage of RAST tests is besides a work with radioactive material also the fact that activity of the preparations of various types, e.g. epithelium versus grass pollens cannot be compared in this way.

Lately ELISA techniques have been introduced into a laboratory practice[23]. A modification of RAST tests is concerned; radioactive labelling is replaced by an enzymatic label, e.g. peroxidase. For the purposes of evaluating allergen activity it is suitable to use ELISA – inhibitions. The principle of determination consists in incubation of the series of dilution of tested allergen with a serum of allergic patients being sensitive to the given allergen and in the following determination of free allergen – specific IgE antibodies. It is determinated such a concentration (or dilution, as the case may be) of tested allergen which produces a 50% inhibition of bond of serum IgE antibodies to a linked allergen. Thus, an inhibition rate is a measure of allergen activity of the given preparation. By using ELISA - inhibition it is possible to compare allergen activity with allergens of the same kind comparatively.

INTERNATIONAL STANDARDS

In 1981 Allergen Standardization Subcommittee carried out a world-wide study leading to a preparation of international standard[33] for the group "short ragweed pollen" (SRW). Then the preparation achieved a certificate IRP (International Reference Preparation).

Pollen of Ambrosia elatior – from four crops (1962, 1964, 1978, 1979) was used for the study. Pollen was dried, put into capped phials, embedded with bee-wax and preserved at 2–10 °C. Pollen was checked by means of electron microscope, in order to find a presence of foreign particles as well as to carry out an identification.

For testing preparations the following techniques were used: RAST test, electrophoreses, cross immunoelectrophoresis (CIE); gel was coloured by Brilliant Coomassie Blue. The content of protein nitrogen according to Kjeldahl was determinated and a series of tests for sterility, ELISA tests and HPLC was carried out.

A temperature stability was investigated at –20 °C, +5 °C, +23 °C, +37 °C and +55 °C. The result was as follows: by preserving at the temperatures of –20 °C the activity does not drop, at 5 °C the activity increases slightly within 12 months of preserving and otherwise it drops. The activity is undetectable with samples being preserved at 55 °C after 6 months.

The data achieved in this way served for preparing of national and local reference standards for SRW. By using several physical-chemical and immunochemical methods a relative potency of SWR extracts has been determinated. After carrying out the study SWR extract and concentration 100000 IU/phial has been recognized as IRS[33].

A similar study was carried out for Phleum pratense, too[18]. A task of the study was to collect the data and to investigate the following items:

1. Relative potency
2. Relative allergen activity
3. Thermal stability
4. Method of preparing preparations
5. Achieving a concentration of 100 000 IU

There were used RAST inhibition sets by Pharmacie, IEF (scale pH 3.5–9.5), SDS-PAGE, HPLC, ELISA inhibition, quantitative electrophoresis for testing. The proteins have been determinated by the method of Kjeldahl and Lowry.

The tests for stability have been carried out for the temperatures of –80, –20, 5, 22, 33 and 58 °C and a period of 2 and 6 months of preserving, evaluating has been carried out by means of RAST inhibition test. After 2 months the samples preserved at <33 °C showed no loss in activity, after 6 months signs of degradation occurred at >33 °C. It has followed from the study that preserving at the temperature range of 4–20 °C exerts no influence on activity.

CONCLUSION

WHO has endeavoured to unify the approach to allergen standardization for many then fifty years. Although the interest in standardization problems has increased rapidly during last ten years, WHO has not succeeded despite every effort.

There are available international standards for the most of frequently spread allergens which can be used for calibration of domestic standards, too. However, some producers give “activity” of allergen preparations in concentration units (PNU most frequently), only leading world producers of allergens also give a so-called biological activity, i.e. a capability of an allergen to produce a reaction of skin hypersensitivity with a group of monospecific patients.

By developing of in vitro techniques RAST inhibition as well as its modification in ELISA performance is in the limelight. The both techniques complete each other and they replace an unethical testing of preparations on man. A further way to standardization is a molecular-biological approach, consisting in preparing of recombinant protein being characterized as the main allergen. The recombinant main allergen as well as an antibody being prepared for it enable to use the basic immunological methods for standardization procedures.

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