

REVIEW

Microarrayed recombinant allergens for diagnosis of allergy

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Summary

We suggest that the coapplication of recombinant allergens and microarray technology can lead to the development of new forms of multi-allergen tests which allow the determining and monitoring of complex sensitization profiles of allergic patients in single assays. The allergen extracts which have so far been used for diagnosis only allowed the determining of whether an allergic patient is sensitized against a particular allergen source, but the disease-eliciting allergens could not be identified. Through the application of recombinant DNA technology a rapidly growing panel of recombinant allergen molecules has become available which meanwhile comprises the epitope spectrum of most of the important allergen sources. We demonstrate that microarray technology can be used to establish multi-allergen tests consisting of microarrayed recombinant allergen molecules. Microarrayed recombinant allergens can be used to determine and monitor the profile of disease-eliciting allergens using single tests that require minute amounts of serum from allergic patients. The wealth of diagnostic information gained through microarray-based allergy testing will likely improve diagnosis, prevention and treatment of allergy.

Introduction

Atopy is commonly described as the increased, genetically predisposed tendency to mount IgE antibodies against *per se* harmless antigens (i.e. allergens) [1]. In the industrialized countries, more than 20% of the population suffer from type I allergy, which therefore represents a major health problem of the western world [2]. Recognition of allergens by IgE, which constitutes the least abundant class of immunoglobulins [3, 4] causes effector cell activation (e.g. mast cell, basophil). The subsequent release of inflammatory mediators such as histamine and leukotrienes [5] causes the immediate symptoms of allergy (e.g. hayfever, allergic asthma, anaphylactic shock). Upon repeated contact with allergens, chronic manifestations involving the activation of eosinophils [6] and allergen-specific T cells occur [7]. Moreover, allergen contact induces increased levels of serum IgE as well as a higher sensitivity to allergens [8].

In vitro diagnosis of type I allergy is based on the detection of allergen-specific IgE antibodies in the serum of atopic patients. Currently available forms of diagnosis rely on allergen extracts prepared from various allergen-containing biological materials (e.g. pollen, fruits, animal hair, etc.). These extracts contain a variety of allergenic and non-allergenic components and are often cumbersome to standardize regarding their allergen content. For example, expression of allergens can be restricted to certain developmental stages, as was shown for certain pollen

allergens who are only expressed in mature pollen [9]. Furthermore, allergen extracts may be contaminated (e.g. mite allergens in animal hair dander) [10], or the molecules which actually elicit the disease may be under-represented or become degraded due to instability [11]. Although allergen-specific antibodies can be used to detect and quantify certain allergens in a given allergen extract, the presence of many other allergens cannot be measured, the ratio of the individual allergens as well as the composition of different extract batches may greatly vary and degradation of certain allergens is possible [12]. Disadvantages of allergen extract-based diagnosis are therefore: 1) allergen extracts cannot identify the disease-eliciting allergens and; 2) false negative as well as false positive results are possible. In this review we develop a future scenario for improved allergy diagnosis which is based on a combination of recombinant allergens and chip technology.

History of allergy diagnosis

The timeline in Fig. 1 displays milestones in the evolution of allergy diagnosis. More than 130 years ago, Charles Blackley, a pioneer of allergy research who himself suffered from severe seasonal allergic rhinitis (hayfever) prepared allergen extracts. In a study lasting for over 15 years and with experiments mostly carried out on himself, he delivered the first scientific evidence for pollen to be the causative agent of hayfever, which had been proposed already in 1831 by Elliotson [cited in 13]. Blackley administered fresh or dried pollen directly to his eyes, nose, skin and tongue or inhaled it, which resulted in the typical symptoms of rhinitis. He also found a correlation between the number of pollen he collected on microscopy slides covered with adhesives

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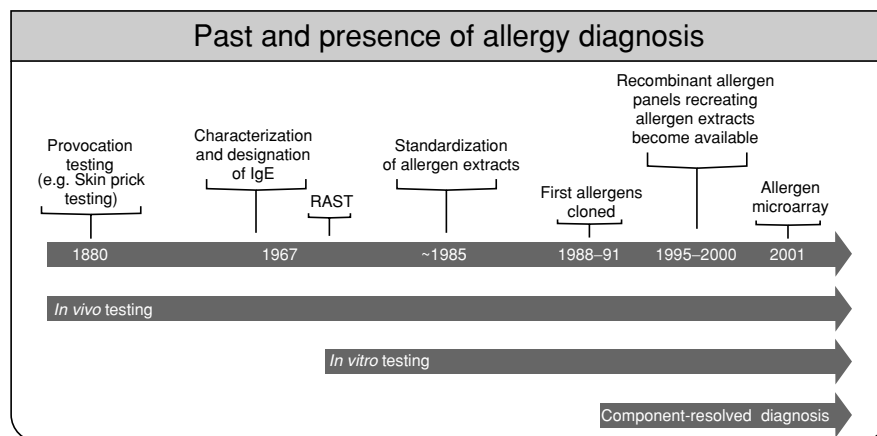


Fig. 1. Timeline: milestones in allergy diagnosis.

and the severity of his disease. Of importance for the diagnosis was Blackley's finding that when pollen was applied to abraided skin, a typical erythema and weal reaction could be induced. This principle is – in a modified form – today one of the most widely applied methods for allergy diagnosis, namely the skin prick test [14].

The possibility to identify sensitized patients by provocation testing provided the basis for the first attempts of specific immunotherapy. Although based on the erroneous belief that hayfever was caused by a pollen 'toxin' Leonard Noon and John Freeman administered injections containing pollen extracts to sensitized patients and were able to demonstrate successful outcomes with acquired immunity after treatment was discontinued.

Experimental evidence for the involvement of antibodies in the pathogenesis of type I allergy was provided by experiments carried out by Prausnitz and Küstner in 1920. By injecting serum from Küstner, a fish-sensitive individual, into Prausnitz's skin, they were able to transfer the sensitivity to fish extract [15]. The designation of the sensitizing agent as 'reagin' results from this classic experiment. Nevertheless, it took almost another 50 years until the molecular entity of this reaginic antibody was elucidated.

In 1967, the work of two research groups finally led to the characterization and designation of IgE as the reaginic antibody. In a laborious series of experiments Ishizaka and co-workers had purified IgE and characterized an antiserum against the new immunoglobulin which did not belong to the subclasses known at that time (IgG, IgA, IgM and IgD) and therefore proposed the presence of an additional Ig class [16]. The latter finding was confirmed by Johansson and Bennich, who had characterized a myeloma protein which blocked the skin-sensitizing activity of atopic serum [4]. The fact that an antiserum produced against the myeloma protein had the same specificity as the anti-IgE globulin prepared by Ishizaka yielded the definitive proof that IgE represented the reaginic antibody. In the same year, the first radioallergosorbent assay (RAST) was introduced by Wide, Johansson and Bennich, which for the first time allowed doctors to perform allergy diagnosis *in vitro* on the basis of specific IgE measurements [17].

Attempts to standardize allergen extracts regarding their major allergen contents with specific antibody probes have resulted in the improvement of the quality of allergen extracts [18–20]. However, only since the application of recombinant

DNA technology to the field of allergen characterization has it become possible to reveal the molecular nature of allergens. The first reports on the cloning of important allergens appeared between 1988 and 1989 [reviewed in 21]. Since then, a continuously increasing number of allergen sequences has become available and panels of recombinant allergens could be produced which assemble the epitope complexity of natural allergen sources [22].

The transition from extract-based to component-resolved diagnosis

By the application of recombinant DNA technology to the field of allergen identification it was possible to characterize many important allergens on a molecular level [reviewed in 21–24]. Until recently, the major allergens from the most prevalent allergy causing sources were identified and produced in recombinant form (e.g. pollens, mites, animal dander, food, moulds, venoms). This effort resulted in the assembly of panels of recombinant allergens comprising the epitope complexity of the corresponding allergen extracts [25–33]. For example, it has been demonstrated for birch and timothy grass pollen allergies, by using only a few recombinant proteins for diagnosis, that more than 95% of all patients suffering from birch pollen and grass pollen allergy could be diagnosed correctly [25, 29].

The use of recombinant allergens instead of allergen extracts has several major advantages. Extract-based diagnosis can only identify the allergen source, but cannot reveal the molecular entity of the disease-eliciting molecules (Fig. 2). Therefore, extract-based diagnosis does not discriminate between patients who are sensitized to unrelated allergen sources, and those who are cross-sensitized towards a certain allergen which cross-reacts with allergens from different allergen sources [34]. The occurrence of cross-reactivity between different allergen sources was already demonstrated in the mid-1970s by RAST inhibition experiments [35]. Nevertheless, the molecules involved could not be identified until purified allergens (e.g. recombinant allergens) became available (Fig. 2). The following example shall illustrate how recombinant allergen-based diagnosis can aid the improvement of the selection of currently available treatment forms.

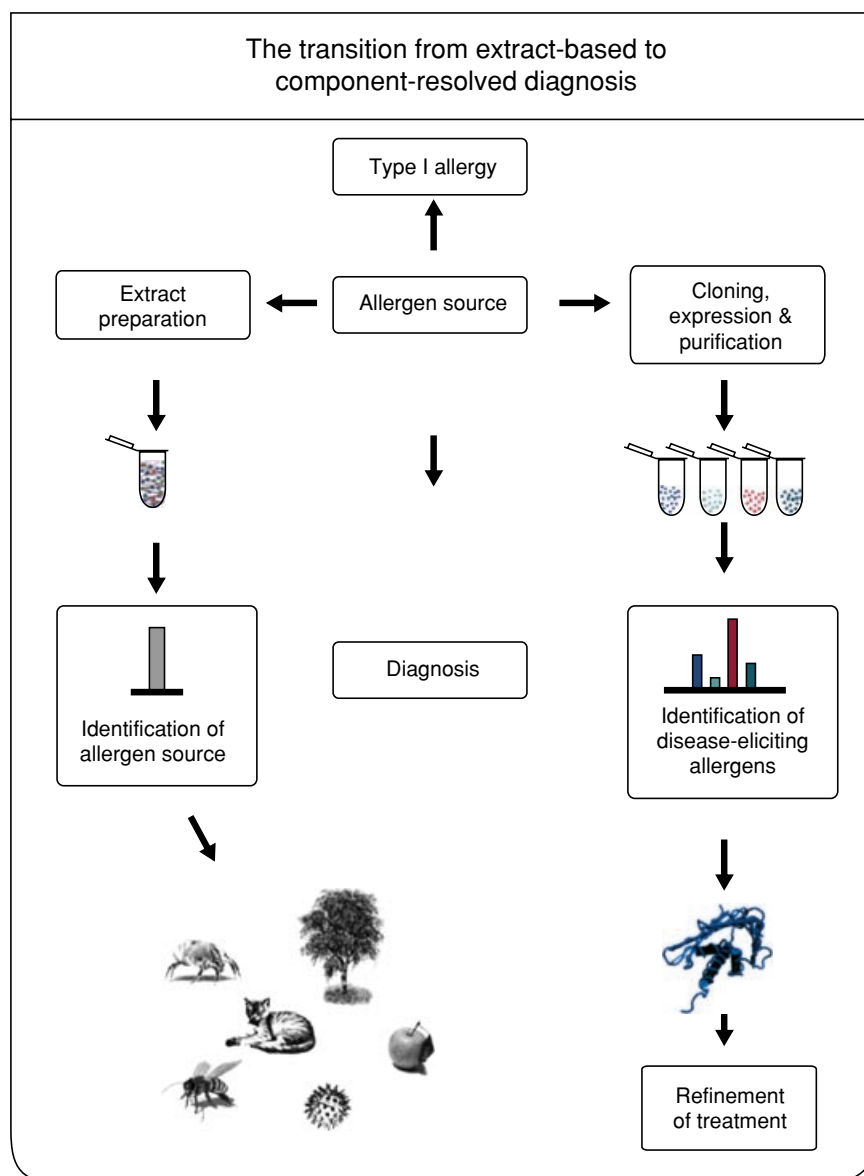


Fig. 2. Diagnosis of type I allergy with allergen extracts can identify allergen sources, but only component-resolved diagnosis identifies disease-eliciting molecules and provides a rational basis for specific immunotherapy and prediction of cross-reactivity towards other allergen sources containing structurally-related allergens.

Bet v 1, a 17-kDa protein, is the major allergen of birch (*Betula verucosa*). More than 95% of all patients sensitized towards birch develop IgE against Bet v 1 [26]. It has been demonstrated, that trees belonging to the order of *Fagales* (alder: Aln g 1; hazel: Cor a 1; hornbeam: Car b 1) as well as some plant-derived food (e.g. carrot: Dau c 1; celery: Api g 1; apple: Mal d 1) contain Bet v 1-related allergens that cross-react with Bet v 1-specific IgE [36–41]. Accordingly, sensitization to birch can result in a cross-sensitization towards many other tree pollen allergens or plant-derived food [42, 43]. Today, most birch pollen extracts are standardized mainly regarding their Bet v 1 content. Therefore, it is very likely that a patient who is sensitized towards Bet v 1 will benefit from specific immunotherapy with birch pollen extract and that allergy to related allergen sources (fagales, plant-derived food) will also improve. On the other hand, if a patient reacts with birch pollen extract but does not actually produce Bet v 1-specific IgE, it is likely that the individual was originally sensitized by another allergen source. In that case, treatment with Bet v 1-standardized

extracts is not recommended [44, 45]. Further examples for cross-reactive allergens are reviewed in detail elsewhere [34, 45].

As described for birch pollen allergy it will be possible to identify molecules for most allergen sources which are either major allergens or represent allergens which are specifically expressed in these sources. These molecules may be considered as diagnostic gatekeepers for choosing specific immunotherapy or symptomatic pharmacotherapy. For example, immunotherapy with the corresponding extract may be preferred for those patients who are sensitized against the major allergens in the given extract [45]. Patients lacking reactivity to the major allergens of a particular allergen extract or who are polysensitized may be preferably treated by symptomatic pharmacotherapy.

It is very likely that traditional allergen extracts used for current forms of immunotherapy will be soon replaced by recombinant allergen-based forms of specific immunotherapy. Using recombinant allergens, it will become possible to tailor the treatment according to the individual patient's sensitization profile. Furthermore, recombinant DNA technology and

synthetic peptide chemistry have been successfully applied to generate hypoallergenic allergen derivatives which hold promise to reduce the risk of side-effects during specific immunotherapy [46–53].

Applications for microarrayed allergens: an overview

Since a variety of recombinant allergens are available today, a comprehensive monitoring of the patient's IgE reactivity profile to a great number of different allergen molecules requires a new type of test. Therefore, a multi-allergen assay for IgE diagnosis employing purified recombinant allergens in a novel microarray-based test system was developed. The feasibility to use 'allergen chips' for the determination of complex IgE reactivity profiles of atopic patients was demonstrated recently [54]. Results obtained from allergen chip experiments were in good agreement with the clinical sensitization pattern of these patients as well as the state-of-the-art diagnostic methods (e.g. skin prick test). Some general considerations regarding the use of protein microarrays for diagnostic applications are now discussed.

The microarray technology was originally developed to monitor the expression pattern of cellular transcripts in parallel [55]. In the last decade, DNA microarrays have proven to be a useful tool for basic and applied biological research.

The appeal to use microarrays for basic research as well as for diagnostic applications results mainly from the highly parallel nature of measurement, the minute amounts of biological material necessary for the investigation and the sensitive detection by applying fluorescence-labelled probes (e.g. DNA, RNA, protein). Therefore, the motivation to develop protein microarrays for the quantification of cellular protein levels was obvious. Nevertheless, it was not before the year 2000 when the development of the first protein microarray, comparable with

the state-of-the-art methodology known for DNA microarrays, was reported [56]. Since then, protein arrays have been designed for a number of different scientific applications. For example, protein arrays covering almost the complete yeast proteome have been constructed and successfully used to study a wide range of protein–ligand interaction [57]. The use of protein arrays for quantitative monitoring of interaction partners in complex samples (e.g. protein expression profiling) has been widely discussed and shown to be feasible to some extent [58–63]. The molecular interactions between proteins are more complex than on the level of nucleic acids. The same is true for gene expression regulation on protein level compared to RNA level. Therefore, the monitoring of protein expression on a genome/proteome-wide scale as it is available for RNA (Affymetrix Human Genome U133 probe set; for technical information, see www.affymetrix.com) is not foreseeable yet. Nevertheless, protein microarrays will have a strong impact on diagnostic applications in the future. As recently shown, protein or peptide arrays can be successfully applied for the diagnosis of allergy or autoimmune disease by using purified antigens in a simple and rapid assay [54, 64]. With more sophisticated signal amplification techniques, even allergenic extracts were applied to microarray-based allergy diagnosis [65], despite the disadvantages of extracts.

The basic steps employed in the production of allergen microarrays are briefly outlined in Fig. 3. Purified or recombinant allergen components are printed to a chemically preactivated glass slide in the form of circular shaped spots by an automated process, termed spotting. Delivery of nanolitre volumes of the protein solution proceeds in a contact (e.g. pin-and-ring or split pin technology) or non-contact manner (e.g. piezo or capillary technology). If the coating chemistry of the glass slide and the spotting conditions are optimized for the immobilization of proteins, a sufficient portion of the protein retains its biologically active form, allowing to be recognized by affinity interaction partners, e.g. antibodies. In total, not more than a few

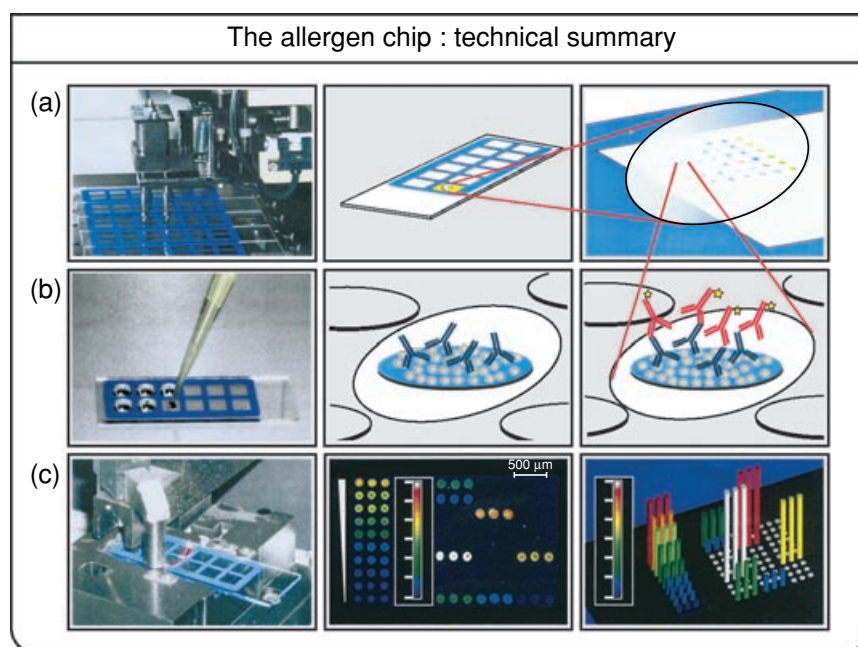


Fig. 3. (a) (left) Microarraying of purified or recombinant allergen molecules onto a preactivated glass slide coated with a Teflon mask by an automated spotting robot. (middle, right) Schematic representation of arrayed allergen molecules inside the Teflon mask wells. Each well can carry several hundred spots of different allergen molecules. (b) (left) Up to 12 different sera can be simultaneously assayed on one allergen chip with $\sim 15 \mu\text{L}$ of diluted serum. In the second step, allergen-specific IgE antibodies (middle) are detected with a fluorescently labelled anti IgE antibody (right). Considering that each well could contain up to 800 different allergens, this corresponds to 9600 ELISA or CAP equivalents. (c) (left) Scanning of allergen microarray with conventional confocal microarray scanner. (middle) Typical image of an allergen microarray containing an IgE standard calibration curve for semiquantitative analysis of the results. (right) Quantification of assay results with customized microarray analysis software resulting in the reactivity profile of the atopic patient.

hundred picograms of protein become covalently attached to the surface. As a consequence, with only a few micrograms of allergenic protein, a vast number of allergen microarrays can be produced.

Basically, about 10 000 features or protein spots can be deposited on a standard microscopy glass slide (75×25 mm) which by far exceeds the number of recombinant or purified allergens available. Therefore, a preferable test format should allow the performing of several independent assays on a single slide. A possible design is proposed in Fig. 3. The glass slides contain a Teflon mask that renders part of the surface hydrophobic. The Teflon mask generates separate reaction wells and up to 12 samples can be applied simultaneously (see Fig. 3c). These samples can be different serum dilutions or different detection reagents (e.g. against various Ig classes). A further advantage of this spatial array design is the reduced amount of liquid necessary to cover each array. Using the allergen chips shown in Fig. 3, up to 800 distinct allergens can be accommodated in a single well, requiring only 15 μ L of diluted serum or secondary antibody to perform an assay.

During the first assay step, all immobilized allergens are exposed to the patient's serum. Consequently, allergen-specific IgE binds to the individual allergen spots on the array. In a second step, bound IgE is detected with a fluorescence-labelled secondary antibody (Fig. 3). Allergen arrays can be scanned with a conventional microarray laser scanner (Fig. 3) and the resulting 16 bit TIFF images can be quantified with any available microarray analysis software. Using simple software automation tools, the evaluation of the complex IgE reactivity profile including the formulation of a comprehensive report requires only a few seconds, once the images are acquired. This simple and robust assay procedure with an overall assay time of less than two hours, allows a single investigator to perform the equivalent of several thousand ELISA assays in one day.

For the quantification of the allergen chip results strategies are currently being developed to determine the allergen specific IgE concentration on a semi-quantitative scale. Standardization of allergen-specific IgE levels may be achieved by different concentrations of spotted purified IgE antibodies to calibrate the signal obtained with the labelled anti-IgE antibodies. By comparing allergen-specific signals to those of a standard IgE calibration curve (see Fig. 3) an accurate correlation between the assay results and the severity of the allergic symptoms the patient suffers from will become possible.

Regarding the sensitivity of microarray-based immunoassays, it has to be taken into account that in contrast to established immunological assay methods (e.g. ELISA, RAST), the available spotting technology does not allow allergen material to be immobilized in excess to IgE analytes. As a consequence of the small feature size (radial spots $< 200 \mu$ m in diameter) and the low amount of immobilized allergen, microarray assays fall into the category of so called 'ambient analyte assays', [reviewed in 66]. These assays do not measure the total concentration of analyte present in the sample, but only the analyte concentration in close proximity to the immobilized target molecules. Nevertheless, if the fluorescence read-out device is sensitive enough and the surface coating chemistry provides an optimal signal-to-noise ratio, detection limits and assay linearity as for conventional immunoassays can be achieved with microarray-based assays [66]. Since commercially

available microarray scanners have been particularly optimized towards sensitivity, the detection of IgE antibodies, which are only present in trace amounts in patient serum, is possible even when using only a single fluorescence labelled anti-IgE antibody [54]. The sensitivity of an unamplified assay may be even increased by the use of a mix of several detection antibodies or signal amplification by enzymatic reactions (e.g. rolling circle amplification [67]). The latter manipulations will provide means for the further enhancement of sensitivity and make allergen chips a highly sensitive platform for allergy diagnosis.

Although the major advantage of the chip-based assay is the simultaneous detection of allergen-specific IgE to a great number of different allergens, it should in principle be possible to determine also total serum IgE levels. For this purpose monoclonal anti-human IgE antibodies binding to different epitopes on human IgE can be utilized, of which one antibody will be spotted to catch IgE from serum samples and the second, with different epitope specificity, as a tracer. On this basis it will be possible to use the International Reference preparation for IgE to establish a calibration curve for total serum IgE levels.

Chip-based allergy diagnosis: a future scenario

Accurate diagnosis of type I allergy requires careful clinical history as well as serological and provocation testing to identify precisely the disease-eliciting allergens. The use of multi-allergen tests based on microarrayed recombinant allergens will greatly improve and facilitate the serological part of diagnosis. While the so far used allergen extracts only describe sensitivity to unspecified allergens present in a given allergen source, microarrayed recombinant allergens reveal the patient's sensitization profile to the individual allergen molecules. One advantage of the allergen microarray is that only small amounts of serum will be sufficient to determine the reactivity profile against a great variety of different allergen molecules in a single test. Using microarray technology it should be possible to assay for reactivity towards several thousand different antigens or epitopes. However, due to extensive cross-reactivities among certain allergens, it will not be necessary to test for such a large number of different allergens. It is rather likely that several specific epitopes for each allergen can additionally be included to allow a more refined diagnostic analysis. Using detection reagents for the different antibody classes and subclasses which are labelled with different fluorescent dyes it will be possible to analyse IgE, IgG, IgM and IgA reactivity profiles in parallel. Allergen microarrays may therefore be extremely useful tools to analyse the development of allergy and to monitor the disease process during its natural course as well as under treatment.

A possible future scenario for chip-based allergy diagnosis is depicted in Fig. 4. Due to the small format of the chip it should be possible to perform the first incubation step without sophisticated laboratory facilities. The subsequent detection steps and the read-out of the signals will require additional laboratory and analysis equipment. Information about the reactivity profile together with background knowledge regarding the individual allergen molecules or epitopes and their predictive value may then be delivered online from an expert database to the clinician. A continuous influx of clinical information that can be compared with the *in vitro* reactivity profiles may allow to train

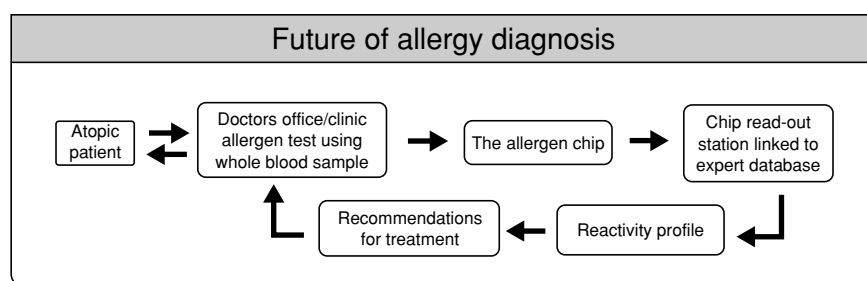


Fig. 4. Future scenario for diagnosis of type I allergies.

the expert database so that certain reactivity patterns may be linked to specific clinical disease phenotypes or predictive parameters. According to the test results, recommendations may be given for further diagnostic testing (e.g. additional provocation tests), to chose preventive measures and to select the most appropriate form of treatment (e.g. pharmacotherapy, specific immunotherapy) for the individual patient.

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