

## Review article

# Analytic aspects of the standardization of allergenic extracts

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## IgE-based standardization compared with allergen measurements

Should allergen standardization always take IgE-based techniques (e.g., skin testing, RAST inhibition, *in vitro* histamine release) as its starting point, or can we rely on allergen-specific antibodies derived from laboratory animals (monospecific polyclonal and monoclonal antibodies [mAbs])? The early 1980s saw the production of the first allergen-specific mAbs (1–9). Since then, allergists have debated whether mAbs are only supplementary to IgE-based standardization, or whether they can replace it (9–16). In these debates, the necessity of IgE-based techniques has been argued; mainly, by the crucial role that IgE antibodies play in type I allergy. Only IgE-based standardization would enable measurement of all relevant allergenic structures, major as well as minor, provided, of course, that enough patients are skin tested, or that serum pools for *in vitro* tests are composed of large enough numbers of sera. Do IgE-based techniques really guarantee coverage of the complete spectrum of allergens?

Let us take as an example cat epithelial extract, containing essentially one major and one minor allergen. Of all cat-allergic patients, more than 90% recognize Fel d 1, and between 15% and 20% cat albumin. This was recently confirmed in our

laboratory in a study of 539 sera with IgE antibodies against cat dander extract: 507 had a positive RAST for Fel d 1 (94%) and 95 for albumin (18%). Let us now assume that we want to select patients from this group for *in vivo* standardization (skin testing) of cat dander extracts. Essentially, we have the choice between two systems: the (B)AU-system of the US Food and Drug Administration (FDA) and the BU-system, formulated in the Nordic Guidelines (14). In the former case, a minimum of 15 highly sensitized patients is required; in the latter, 20 patients with clinical sensitivity to the allergen in question, but not necessarily highly sensitized. Clinical data on the group of 539 patients were not available, but we can safely assume that the vast majority of patients with high anticat IgE titers (4+/5+) are clinically sensitive. In this way, 136 of 539 fulfilled the requirements of both systems. Of these 136 sera, 47 demonstrated positive RAST to albumin. All had positive RAST to Fel d 1. For only 10 patients, ratios of serum IgE antialbumin/anti-Fel d 1 were  $>0.5$ , ranging from 0.51 to 136.6 (mean  $\pm$  SE:  $31.6 \pm 15.0$ ). For the remaining 37 albumin-positive sera, the mean ratio ( $\pm$ SE) was  $0.12 \pm 0.02$ . In other words, if we select 15–20 patients from the group of 136, we will end up with approximately one patient whose anti-cat-dander IgE response contains a significant antialbumin fraction. Although

methods differ, both systems for biologic standardization, essentially, calculate the potency of an extract from the reactivity of the median-sensitive patient. The inclusion of one patient with clear IgE reactivity to a minor allergen will not significantly influence the outcome of such a calculation.

Similarly, potency measurements by RAST inhibition with serum pools will not be influenced by the presence of minor allergens: IgE antibodies against minor allergens are diluted in a "sea" of IgE against major allergens. Therefore, a large pool serum is in fact nothing more than a polyclonal antiserum against major allergens. In other words, the fullest possible coverage of potential allergens by IgE-based techniques for standardization is merely fictive: major allergens determine the outcome of potency measurements. What about the alternative, measurement of major allergens by means of monospecific polyclonal or monoclonal antibodies? The number of available mAbs has increased enormously since the beginning of the 1980s (Table 1). For the measurement of major allergens from most of the important inhalant allergen sources, reagents are now available (1,

4–8, 17–116). For food allergens, the situation is not yet as favorable (2, 3, 117–146). What argues against the use of major-allergen-specific mAbs for standardization, if IgE-based techniques in fact measure the same molecules? An argument advanced is that mAbs are not always directed to IgE epitopes (12). There is, however, no reason to assume that IgE epitopes have different inherent characteristics from those recognized by mAbs. Both can be used to monitor the presence of major allergens in allergen extracts. The advantages of mAbs are clear: well-defined reagents of constant quality with unlimited availability. The variability of pool sera is illustrated in Fig. 1, from an experiment in which 10 grass pollen extracts were compared in RAST inhibition with two different pool sera, each composed of more than 20 sera. Potencies were consistently lower (average: 2.5 times) with pool serum A. The two extracts with the lowest potency, calculated with pool serum A, were the strongest for pool serum B.

This review aimed to evaluate analytic aspects of the application of monospecific antibodies against major allergens in allergen standardization.

Table 1. Allergen-specific monoclonal antibodies (mAbs). Attempt was made to generate complete overview of all available allergen-specific mAbs. Certainly, we cannot guarantee completeness. Whenever possible, we have listed references in which mAb was reported for first time. References on additional mAbs are welcome

| Pollen                | Mites                 | Insects               | Molds/yeasts          | Foods                   |
|-----------------------|-----------------------|-----------------------|-----------------------|-------------------------|
| <i>Grasses</i>        | <i>House-dust</i>     | <i>Cockroach</i>      | <i>Aspergillus</i>    | <i>Peanut</i>           |
| Dac g 1 17            | Der p 1 8, 68–72      | Bla g 1 93, 94        | Asp f 1 103           | Ara h 1 117             |
| Lol p 1 4, 18–21      | Der f 1 68, 70, 72–75 | Bla g 2 93, 94        | Asp f HSP88 104       | Ara h 2 118             |
| Phl p 1 22, 23        | Der p 2 71, 76        | Per a 72 kDa 95       |                       | Ara h "misc" 119        |
| Poa p 1 24            | Der f 2 77, 78        | Per a 28–32 kDa 96    | <i>Alternaria</i>     |                         |
| Cyn d 1 25–27         | Der f 3 77            | Per a 12–18 kDa 96    | Alt a 1 7             | <i>Apple</i>            |
| Group 1 28, 29        | Der p 7 79            |                       | Alt a 70 kDa 105, 106 | Mal d 1 120             |
| Lol p 4 5, 30         | Der p 10 80           | <i>Vespid venoms</i>  |                       |                         |
| Phl p 4 31            | Der p ?? 81           | Dol m 5 97            | <i>Penicillium</i>    | <i>Mustard</i>          |
| Cyn d 4 25            |                       | Ves g 5 98            | Pen n 68 kDa 107, 108 | Sin a 1 121             |
| Lol p 5 4             | <i>Storage</i>        |                       |                       |                         |
| Dac g 5 32, 33        | Lep d 2 82            | <i>Bee venom</i>      | <i>Trichophyton</i>   | <i>Soybean</i>          |
| Phl p 5 22, 31, 34–38 | Lep d 39 kDa 83       | Api m 1 99            | Tri t 1 109           | Gly m Bd 30 kDa 122     |
| Poa p 5 1, 39         | Lep d 79–93 kDa 84    | Api m 3 99            | Tri t protein IV 110  |                         |
| Lol p 11 40           |                       |                       |                       | <i>Egg</i>              |
|                       | <b>Epithelia</b>      | <i>Fire-ant venom</i> | <i>Candida</i>        | Gal d 1 2, 3, 123–128   |
| <i>Trees</i>          |                       | Sol i 2 100           | Can a 40 kDa 111      | Gal d 3 126, 129, 130   |
| Bet v 1 41–45         | <i>Cat</i>            | Sol i 3 100, 101      |                       |                         |
| Car b 1 46            | Fel d 1 85–89         | Sol i 4 100           | <i>Pityrosporum</i>   | <i>Milk</i>             |
| Bet v 2 47            | Fel d 2 85            |                       | Pit o 67 kDa 112      | β-Lactoglobulin 131–135 |
| Ole e 1 48–50         | Albumin 85, 90        | <i>Midge</i>          | Pit o 37 kDa 112      | α-Lactalbumin 136–138   |
| Cry j 1 51–57         |                       | Chi t 1 102           |                       | Casein 139–143          |
| Cry j 2 52, 53, 58    | <i>Dog</i>            |                       | <b>Latex</b>          |                         |
|                       | Can f 1 91            |                       | Hev b 55–65 kDa 113   | <i>Shrimp</i>           |
| <i>Weeds</i>          | Can f 2 91            |                       | Hev b C-Ag 114        | Pen a 1 144             |
| Par j 1 59–63         | Albumin 85            |                       | Hev b 23 kDa 115, 116 | Par f 1 145             |
| Par o 1 64            |                       |                       |                       | <i>Fish</i>             |
| Amb a 1 6, 65–67      | <i>Cow</i>            |                       |                       | Gad c 1 146             |
|                       | Bos d 1 92            |                       |                       |                         |

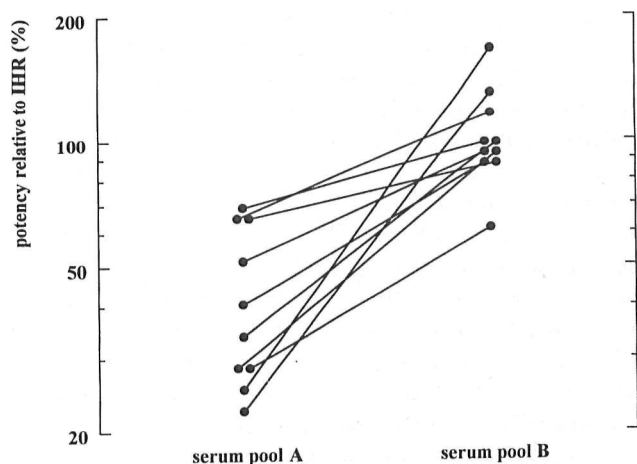


Fig. 1. Inhibitory potencies of *D. glomerata* pollen extracts were calculated relative to in-house-reference (IHR=100%) preparation. Two different serum pools (A and B) were compared in RAST inhibition.

### Contamination of allergen extracts with allergens: an underexamined problem

In the draft text of the monograph on allergen products of the European Pharmacopoeia Commission, there are several references to contamination of source materials for allergen products (147). Attention is mainly focused on chemically or biologically active contaminants or possible transmissible agents of disease. Twice, potential contamination with foreign allergens is mentioned: "Pollens contain not more than 1 per cent of foreign pollens as determined by microscopic examination" and "Care must be taken to minimise any allergenic constituents of the media used for the cultivation of mites and moulds as source materials". These demands are either too vague ("care must be taken to minimise") or not strict enough ("not more than 1 per cent"). A contamination of 1% grass pollen in, for example, weed pollen will result in a false-positive RAST for weed pollen when weed-pollen negative/grass-pollen positive sera are tested. This is illustrated in Fig. 2. Sera with high IgE titers against grass pollen (4+/5+) were tested on dilutions of a routinely used Sepharose-coupled extract of *Dactylis glomerata*. Even at a dilution of 1:256, positive RASTs were found. If we bear in mind that skin tests are more sensitive than RAST (148, 149), a contamination of 1% of foreign pollen will result in frequent false-positive diagnoses. Therefore, more stringent limits for contamination are needed. They should be based on the demonstration of specificity of a diagnostic product: in individuals (or their serum), highly sensitized to the potential contaminant, no reactivity should be found. More convenient methods than microscopy would facilitate monitoring of contamination at levels below 1%. Species-specific

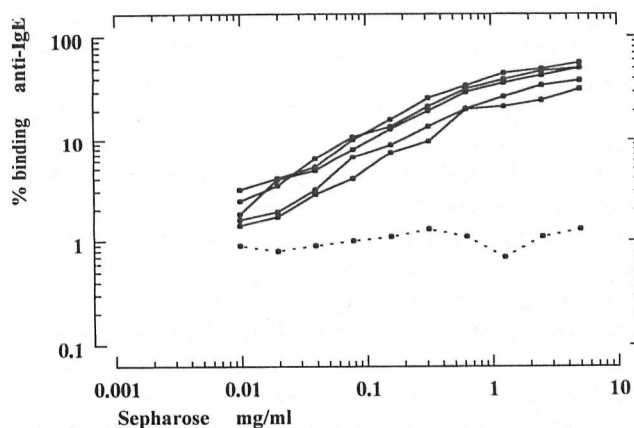


Fig. 2. Five sera, highly sensitized to grass pollen, were tested in RAST with Sepharose-coupled *D. glomerata* pollen. Sepharose was tested in several dilutions. Even at dilutions up to 1/256, percentages of binding were still significantly above background (without serum: dotted line).

mAbs are good candidates to detect contaminants. Contamination of weed pollen with grass pollen can, for example, be detected in a competitive radioimmunoassay (RIA) with mAb 4B1 against the grass-pollen-specific group 5 allergen (32). By this method, extracts from four different weed species were analyzed. Levels of contamination ranged from 0.2% to 1.0%.

Contamination of cat dander extract with mite allergens was demonstrated by the application of mite-specific mAbs (16). A recent report by van der Veen et al. illustrates that the presence of mite allergens in epithelial extracts of pets is a problem that cannot be neglected (150). In this study, skin prick test (SPT) materials for dog allergy from five different European companies were analyzed for their Can f 1 and Der p 1 and 2 content by mAb-based immunoassays. It was found that all batches tested ( $n=10$ ) contained demonstrable amounts of Der p 1 and 2, ranging from 1 to 35 ng/ml. Dilutions of mite extract with Der p 1 levels of 3–6 ng/ml still elicit positive skin tests in mite-allergic subjects (van der Veen, personal communication). This implies that the contaminated dog-SPT materials can result in false-positive diagnosis for dog allergy. This was indeed confirmed by SPT analysis in >1000 subjects suspected of inhalant allergy: approximately 50% of positive dog SPT results were not supported by a positive clinical history or RAST. Most of these patients turned out to be mite allergic. When dog SPT material, coupled to Sepharose at supraoptimal concentrations, was used in RAST, positive dog RAST was found. This reactivity was inhibited by mite extract, confirming that contamination of dog extract with mite allergens is the cause of the observed false-positive diagnoses. Titration of mite

SPT material showed that the mite contaminations are, indeed, high enough to elicit significant skin reactivity.

The simplest and most reliable way to determine the degree of contamination of allergen extracts with foreign allergens is the application of mAb-based allergen measurements. In order to guarantee specificity of diagnostic products, these measurements should be introduced to allergen standardization. Decisions on maximum acceptable levels of contamination should not be made by what can be reasonably distinguished under a microscope, or by the assurance that "care was taken to minimise contamination", but by demonstrating when specificity of tests can no longer be guaranteed.

### **Different ratios of individual allergens but similar biologic potency**

One of the main goals of biologic standardization is to ensure safety. As already pointed out, the outcome of SPT (and RAST inhibition) is largely determined by the major allergen content of the extract tested. In allergen sources where there is only one major allergen, biologic potency and major allergen content will closely correlate. This is the case, for example, for cat dander, birch pollen, and olive pollen. Lombardero et al. analyzed different batches of source materials for cat dander extract on their Fel d 1 content by mAb-based RIA (151). Relative to total protein content, Fel d 1 levels varied about 10-fold. Chapman et al. measured differences in Fel d 1 content up to 300-fold, although these were not corrected for total protein levels (87). For olive pollen extracts, Carreira et al. reported that the Ole e 1 content also varied considerably: 1–20% of the total protein (152). As a consequence, the amount of extract needed to reach a certain biologic potency will also differ. The concentration of minor allergens will probably be relatively high in allergen products based on source materials with a low major allergen content. For patients with a significant IgE response to minor allergens, biologic standardization does not really ensure safety. mAb-based monitoring of major allergens does not achieve that goal either, but it facilitates selection of source materials with sufficiently high major allergen contents.

Several allergen sources contain at least two major allergens (153). This is, for example, true of house-dust mite and grass pollen. The most important mite allergens are Der p 1 and Der p 2. Standardization by skin test or RAST inhibition will not detect different Der p 1/Der p 2 ratios. Application of Der p 1- and Der p 2-specific mAbs revealed that house-dust mite extracts with similar biologic potency can differ extremely in their Der

p 1/Der p 2 ratio: up to a factor of 1000 (152). Der p 1 and 2 levels are highly dependent on the nature of the source material used: whole-body versus total-culture extract. In the latter, Der p 1/Der p 2 ratios are much higher, because Der p 1 is primarily excreted in fecal particles. The choice of source material does not only influence the composition of mite extracts. Another clear example is cat allergen extract: albumin levels vary greatly between dander- and pelt-based extracts. Perhaps the allergen sources most notorious for variable composition are mold extracts: strain-differences, culture supernatants or mycelia, and culturing conditions all contribute to variability.

Of course, not only the ratio of recognized major allergens is dependent on the nature of the source material. The level of other less well-known allergens can be influenced as well. A good example is the group 10 allergen of mite, the muscle protein tropomyosin (153). This allergen is almost absent in spent-medium-based extracts (154). Skin test preparations from European manufacturers are usually based on total-culture extracts. By means of mAb 1A6 (80), no tropomyosin was detected in preparations of three of these manufacturers. In contrast, a whole-body mite extract contained significant amounts: relative to the Der p 1 content, at least a factor of 1000 more than that found in the skin test preparations. As a consequence, tropomyosin-specific IgE will not be picked up in skin tests. A recent report from Japan indicates that tropomyosin is recognized by approximately 80% of all mite-allergic patients (155), emphasizing the importance of its presence in preparations for diagnostic purposes. mAbs are ideal reagents to measure the presence of an allergen such as tropomyosin.

In conclusion, mAbs facilitate the selection of the source material with a sufficiently high major allergen content. In contrast to IgE-based techniques, mAbs can distinguish between extracts of similar biologic potency but with totally different major allergen ratios. Safety can only gain from this.

### **mAbs can be too specific: be aware of isoforms!**

Isoforms have been described for a number of allergens from grass pollen, tree pollen, weed pollen, house-dust mites, and cat. Most of this knowledge comes from cDNA cloning of allergens. In most cases, not much is known about the natural occurrence of isoforms and their immune-reactivity. For some mAbs, reactivity with a selection of isoforms was described, as in the case of Bet v 1 and its homologs in other tree pollen. Breiteneder

et al. studied the reactivity of mAb BIP1, raised against Bet v 1, with four recombinant isoforms of the homologous protein from alder pollen, Cor a 1 (156). The mAb selectively recognized a single isoform of Cor a 1. Akkerdaas et al. performed IEF/immunoblotting studies with natural Bet v 1, and showed that mAb 7F7 failed to recognize one isoform of Bet v 1 that was recognized by mAb 5H8 (45). In two-dimensional immunoblotting, Løwenstein et al. identified over 20 charge variants of Bet v 1, and analyzed their reactivity with nine different mAbs (157). A broad variability in reactivity was observed.

For the mite allergen Der p 2, sequence polymorphisms were reported at different sites of the molecule (158). One of the exchanges observed, in comparison to the originally published sequence, was asparagine at position 114 instead of aspartic acid. Recently, in our laboratory, recombinant versions of both variants were produced in yeast (Hakkaart et al., paper submitted). Several mAbs raised with natural Der p 2 were analyzed for their reactivity. For one mAb, the presence of aspartic acid at position 114 completely eliminated binding, whereas the binding of most of the mAbs was not affected. For IgE, no significant differences in reactivity were observed.

For standardization, one should select mAbs that recognize the whole spectrum of isoforms. Alternatively, a mixture of two or more different mAbs could be used to ensure complete coverage of isoforms.

### **“Monospecific” for a highly cross-reactive epitope: *N*-glycans**

Immunization of laboratory animals with plant- or invertebrate-derived glycoproteins frequently results in highly cross-reactive antibodies (159–164). This is a result of the presence of highly immunogenic *N*-linked carbohydrate determinants, *N*-glycans. They are so immunogenic because they contain several monosaccharides linked to the core structure of the sugar group in a fashion not observed in mammals: an  $\alpha(1,3)$ -linked fucose on the proximal *N*-acetylglucosamine and a  $\beta(1,2)$ -linked xylose on the core-mannose (159, 163). These linkages are ubiquitous in plants (pollen and vegetable foods) and invertebrates (insects, animals used as seafood, snails). Therefore, antibodies against these structures demonstrate a broad spectrum of cross-reactivity (159, 165). It is important to realize that this is not only true of polyclonal antisera raised after immunization with, for example, a total grass pollen extract. Monospecific polyclonal antibodies (32, 159–162) and even mAbs (164) raised with highly purified major allergens can be cross-reactive for the same

reason. What does this mean for allergen standardization?

An example of a major allergen carrying an *N*-glycan is the group 1 grass pollen allergen (166). We performed a competitive RIA with radiolabeled purified Dac g 1, using four different antibody reagents: three polyclonal rabbit antisera against *D. glomerata* pollen extract, purified Lol p 1 (32), and a synthetic C-terminal peptide of Lol p 1 (167), respectively, and a group 1-specific mAb. Purified group 1 allergen was used as a reference to measure group 1 allergen in pollen extract. As shown in Fig. 3, measurement of Dac g 1 with the polyclonal rabbit antiserum against *D. glomerata* resulted in a Dac g 1 content that was about three times higher than the total protein content. It is obvious that this is an overestimation: other glycoproteins in *Dactylis* extract also inhibit the binding of radiolabeled Dac g 1 to the fraction of antibodies that are carbohydrate-specific. With anti-Lol p 1, the calculated Dac g 1 level decreased to 25%. Calculations based on measurements with the rabbit antipeptide and the mAb gave similar results: about 2% of the total protein was Dac g 1, indicating that the measurement with the “monospecific” antiserum against Lol p 1 was also an overestimation. On immunoblot, it was confirmed that anti-Lol p 1 binds to a wide variety of bands, predominantly of high molecular weight, besides the Lol p 1 band (167). The antipeptide antiserum exclusively recognized the Lol p 1 band. When anti-Lol p 1 was preincubated with potato extract before the Dac g 1 measurement, the calculated Dac g 1 content dropped from 25% to 3%. This agrees with the results obtained with the mAb and the antipeptide antiserum. Carbohydrate determinants in potato extract prevented interference in the Dac g 1 measurement of antibodies directed to *N*-glycans.

These observations urge us to be very cautious with monospecific antibodies, polyclonal as well as monoclonal. Immunization with highly purified glycosylated allergens does not guarantee monospecificity. It is not enough to show that anti-Lol p 1 binds to purified Lol p 1, and not to, for example, purified Lol p 5 and profilin, to demonstrate its monospecificity. When antisera or monoclonals clearly detect more bands on immunoblot than the band corresponding to the allergen used for immunization, this observation cannot be ignored. The possible involvement of *N*-glycans has to be investigated. The immunogenicity of plant-type *N*-glycans hampers the production of truly monospecific polyclonal antisera against major allergens. A (variable) fraction of the antibodies is usually carbohydrate-specific. This activity can, however, be absorbed with plant *N*-glycan-containing extracts. When available, mAbs are preferred, provided of

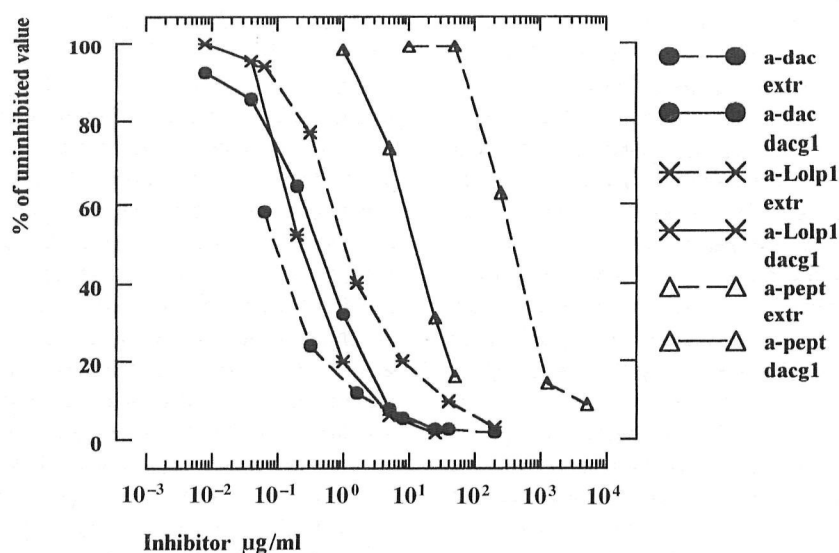


Fig. 3. Application in measurement of Dac g 1 of three different polyclonal rabbit antisera, reactive with grass pollen group 1 allergen, was compared. Binding of radiolabeled Dac g 1 to each antiserum was inhibited with *D. glomerata* extract and purified Dac g 1. Results are expressed as percentage of uninhibited value (without inhibitor). A-dac = anti-*D. glomerata*, a-Lolp1 = anti-Lol p 1, a-pept = anti-Lol p 1 C-terminal peptide, extr = *D. glomerata* extract, dacg1 = purified Dac g 1.

course that they are not directed to the carbohydrate epitope.

## Concluding remarks

The claim that IgE-based techniques for allergen standardization facilitate complete coverage of major and minor allergens does not hold. This takes away one of the main reasons to promote these techniques for allergen standardization. An important disadvantage of IgE-based techniques is the variability of the IgE source. Batches of pool sera have finite volume, and are different all over the world. IgE characteristics of candidates for biologic standardization are subject to the same type of variability. mAbs are well-defined reagents of constant quality with an unlimited availability. They allow the use of identical reagents all over the world. In addition, they are ideal tools to trace allergen contaminations which have been shown to cause false-positive diagnoses. Biologic potency measurements aim to guarantee safety. The potency of an extract, however, is determined by the sum of the biologic activities of its major allergens. Ratios of these allergens were shown to be highly variable. Therefore, for the individual patient, biologic standardization does not necessarily guarantee safety. Detailed knowledge of the composition of allergen extracts, regarding major and, where possible, minor allergens, is the best guarantee. mAbs offer opportunities to achieve this goal, provided that they are selected with care. They should be neither too specific nor too cross-reactive.

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