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The Importance of Minor Allergens in Allergen Standardization

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Introduction

The aim of allergen standardization has been to overcome the allergen extract heterogeneity and variability. Allergenic extracts for therapeutic use are crude mixtures of proteins and non-protein components isolated from natural sources. Following the discovery in 1966 of the IgE molecule, scientific methods were introduced to standardize allergenic extracts in the seventies and eighties [1]. These methods were based on the comparison of new production batches to a reference extract, the potency of which had been previously measured by skin testing in selected patients. The methods mostly used for *in vitro* assessment of relative allergenic potency are RAST inhibition or related methods [2]. The essential reagent in these methods is a serum pool from a panel of allergic patients. The potency is measured by comparing the inhibition caused by the test extract on the binding of specific IgE from the serum pool to a reference extract, coupled to a solid phase, with the inhibition reached by the reference extract. In this way, the potency of an allergen extract is the sum of the contribution to the allergenic activity of any individual IgE molecule specific for any epitope on any molecule in the allergen extract. Therefore, the potency values will always depend on the serum pool or patient panel selected, as well as on the reference extract. Moreover, these methods do not provide any information on the quantitative composition of the extracts, that is, the relative concentration of individual allergens.

Once a better understanding of the nature of allergen components was obtained, the concept of major allergens emerged. These are individual protein molecules recognized by the majority of an allergic population. When a single major allergen is present, there is a good correlation between biological activity and major allergen content. A standardization method measuring the amount of major allergens in an extract was developed [3]. This significant advance has been greatly acknowledged and major allergen dose is considered as a key parameter in the forecast of the efficacy of immunotherapy [4].

An international project has been running with the aim of establishing methods and references for the most relevant major allergens [5]. The most advanced standardization methodology consists of a combination of biological activity measurement, major allergen quantification and qualitative antigenic/allergenic profile. However, it should be noted that the term “major allergen” is based on a statistical estimation of the prevalence of sensitization to a particular allergen within a general population of patients allergic to a certain allergen extract. Thus, only those allergens that cause sensitization in >50% of patients are called “major allergens”. However, a clinically relevant factor is the amount and affinities of IgE antibodies directed against a particular allergen, either major or minor, that an individual patient can produce. This is not taken into consideration in the current standardization methods.

The role of minor allergens: Olive as a model

In some particular geographical areas where olive pollen exposure was rather high, an unexpected rate of adverse events seemed to accumulate in the course of olive pollen immunotherapy. Interestingly, a significant number of patients from these areas, with clear olive pollinosis, were not diagnosed by skin prick testing. There was no clear explanation for this and we thought that variations of minor allergens could possibly account for these phenomena.

The allergenic composition of olive has been widely studied [6]. Olive allergens belonging to the group of profilins (Ole e 2) and polcalcins (Ole e 3 and 8) have been described. The fact that adverse events were almost exclusively limited to two particular areas (Jaén and Córdoba) led us to postulate that allergens different from pollen panallergens (profilins and polcalcins) might be involved. Of the other allergens described, Ole e 9 [7] and Ole e 7 (olive pollen LTP) [8] were investigated.

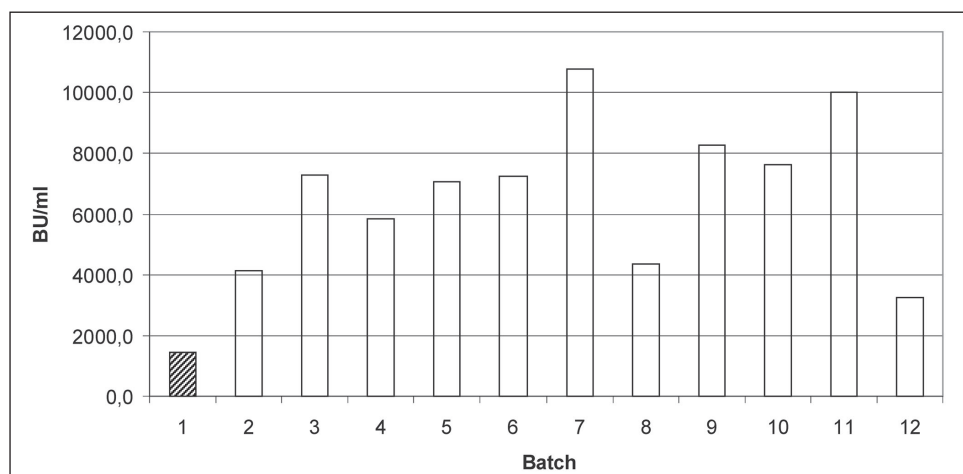


Fig. 1. Allergenic activity, expressed in BU/ml, of twelve different batches of olive pollen. Shaded bars indicate batches that should be rejected because of not fulfilling specifications of allergenic activity.

Minor allergen variability

A specific monoclonal antibody-based ELISA for Ole e 9 quantification was developed [9]. Twelve olive pollen batches from different national and international suppliers were tested for biological activity, Ole e 1 and Ole e 9 content. A high variability was observed in the allergenic activity. The activity of the most potent extract was almost ten times higher than that of the extract with the lowest activity. The latter (batch sample 1) should be rejected for production of an olive allergy vaccine, according to the specifications of our Production Department, because of its low activity (Fig. 1).

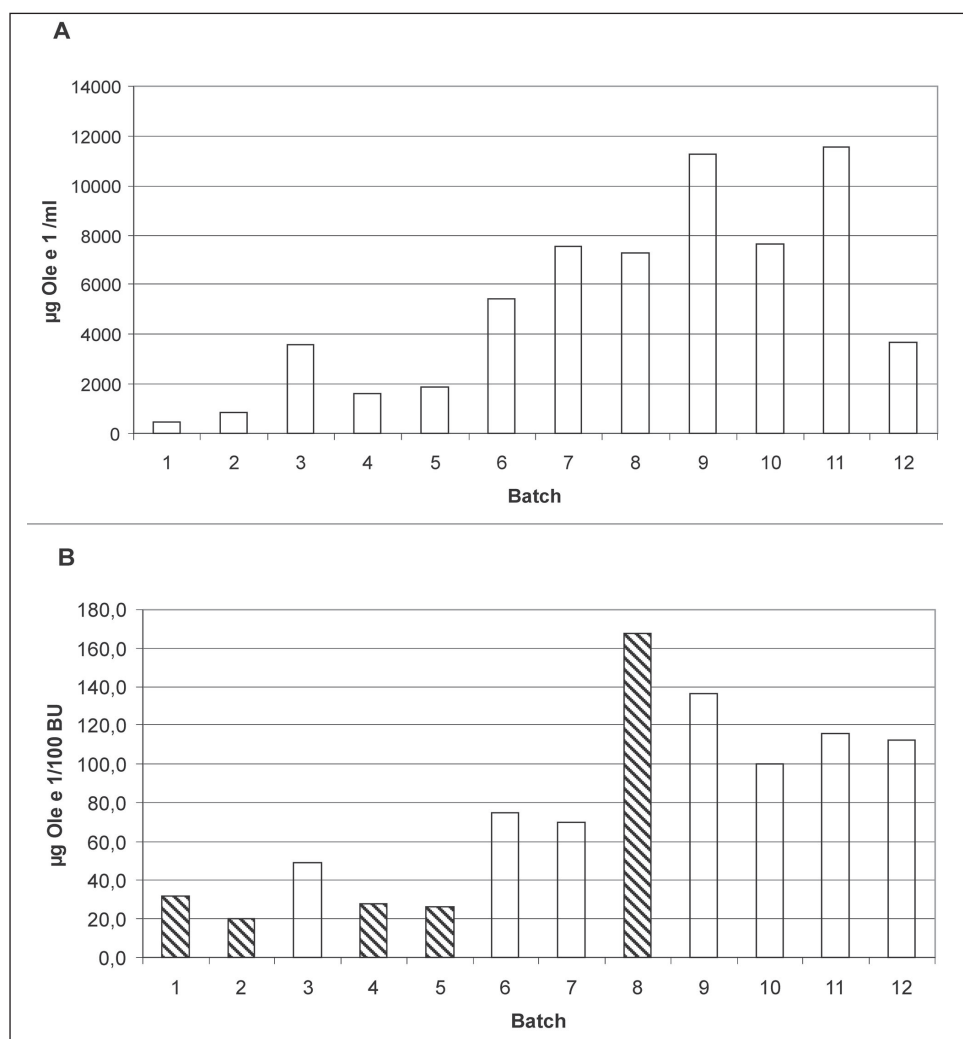
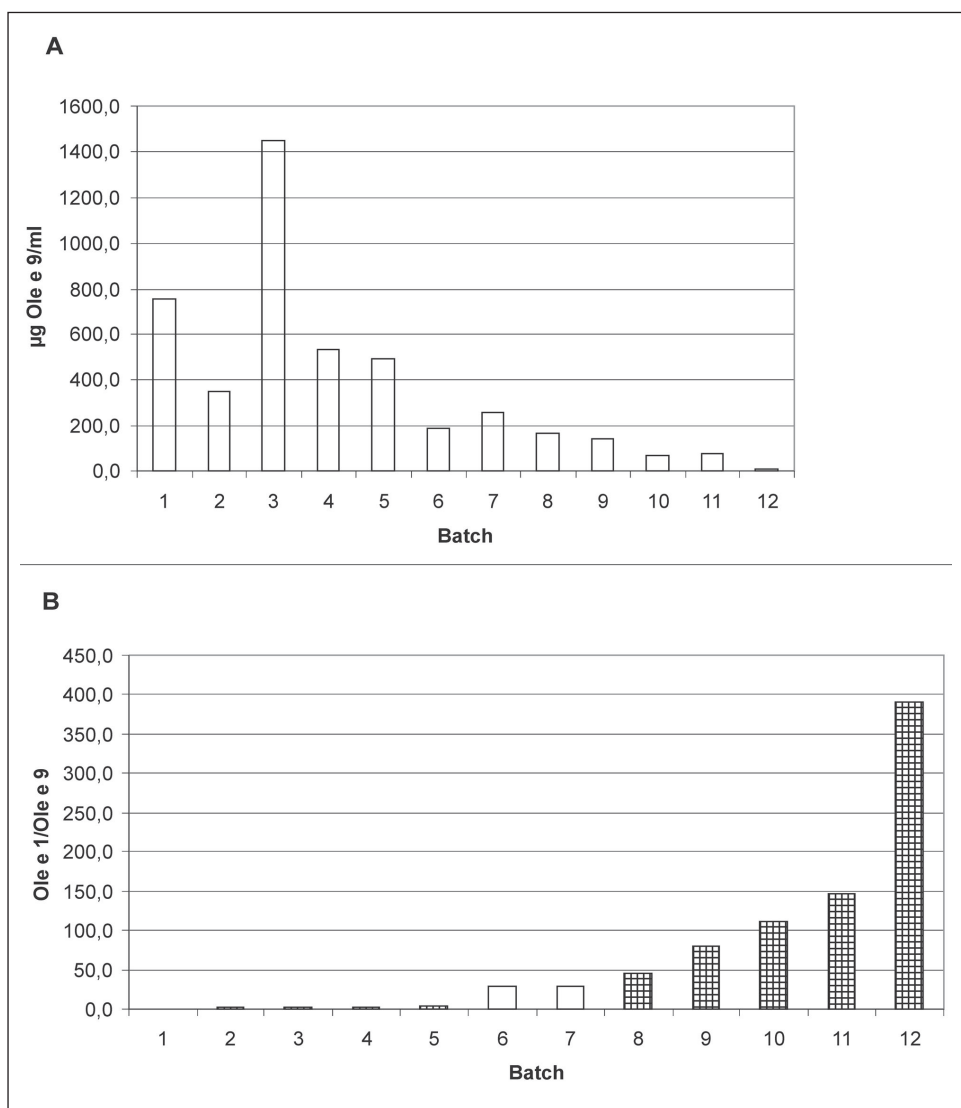


Fig. 2. (A) Content of major olive pollen allergen Ole e 1 in twelve batches of olive pollen. Values, in µg/ml, were determined by an ELISA method. (B) Ratio between concentration of Ole e 1 and activity, expressed as µg of Ole e 1/100 BU. Shaded bars indicate batches that should be rejected for not fulfilling this criterion.

Fig. 3. (A) Content of olive pollen allergen Ole e 9 in twelve batches of olive pollen. Values, in $\mu\text{g/ml}$, were determined by an ELISA methods. (B) Ratio between concentration of Ole e 1 and Ole e 9. Shaded bars indicate batches that should be rejected if a range of 50-200% of the median value found (30.0) was defined as an acceptance criterion.



A higher variability was observed in Ole e 1 content (Fig. 2A). Thus, a ratio of 25 was estimated between the sample with the highest Ole e 1 content (batch sample 11) and the one with the lowest content (batch sample 1). Since a concentration of 60 μg of Ole e 1/ml has been established for an activity of 100 BU/ml, with an acceptance range between 30 and 120 $\mu\text{g/ml}$, four additional batches (samples 2, 4, 5, and 8) were found to be out of specifications (Fig. 2B).

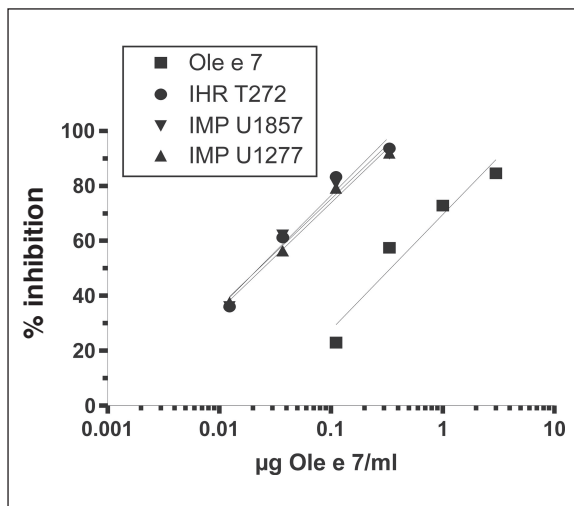


Fig. 4. Quantitation of Ole e 7 by specific inhibition assay in three batches manufactured with major (Ole e 1)/minor (Ole e 9) allergen ratio adjustment. Ole e 7 content per treatment dose was determined to be 0.50, 0.54 and 0.84 micrograms.

Moreover, an unexpected and extremely high variability was found when the samples were analyzed for Ole e 9 content. The concentration of Ole e 9 in sample 3 was 161-fold higher than that of batch sample 12 (Fig. 3A). It should be noted that batches with differences in Ole e 9 content as high as 161-times could be released according to current standardization methods, since both batches were within specifications according to the former criteria.

When the ratio between the concentrations of both allergens was estimated, an even higher variability was observed (Fig. 3B). Furthermore, a significant inverse correlation between the Ole e 1 and Ole e 9 contents in olive pollen batches was found ($r_s = -0.7203$, $p = 0.0082$). The Ole e 1/Ole e 9 ratio ranged from 0.6 to 390.4, with a median value of 30. If acceptance criteria were established based on this value, with a range between 15 and 60, five additional batches would be rejected (samples 3, 9, 10, 11, and 12). Therefore, only two batches (samples 6 and 7) would fulfil the acceptance criteria.

In conclusion, the new method developed enabled us to detect an unknown source of variability in batches of olive pollen. This high variability in Ole e 9 content, previously unnoticed, might have been the origin of severe adverse reactions to olive immunotherapy.

Based on these results, a new specification for Ole e 9 has been set. An adequate extract can be manufactured by mixing different batches of olive pollen to ensure adequate allergen levels and ratios. Variability of Ole e 7 is currently being investigated. A method for quantification of Ole e 7 using an inhibition assay of biotinylated Ole e 7 has been developed (Lombardero M et al.; unpublished). Interestingly, the first batches manufactured using the above-mentioned procedure displayed a low variability of Ole e 7 content (Fig. 4), thus supporting the utility of the new standardization strategy to control minor allergen variability in a better way.

Pattern of sensitization to Ole e 1/ Ole e 9 in regions with different exposure levels to olive pollen

In collaboration between the Spanish Health Ministry public-funded *Vegetalia* Net and ALK-Abello, around 1000 pollen-allergic patients from different geographical areas in Spain have been screened for specific IgE with a wide panel of single-allergen components. To our knowledge, this is the first trial to compare significant populations of different areas with similar inclusion criteria and validated analytical methodologies. The results have not yet been published. Ole e 1, Ole e 9 a 2EF hand polcalcin (Che a 3) and a mixture of four profilins (Mal d 4A and B, Che a 2, Cuc m 4) were included in the screening panel.

The results obtained for the prevalence of sensitization and the median values of specific IgE levels found in patients from each area clearly evidence that Ole e 9 is not a prevalent allergen (<5%) in low-exposure areas (up to 1000 pollen grains/m³), whereas it is far more prevalent in Jaén (35%) (up to 14,000 pollen grains/m³). Therefore, it can be concluded that patients tend to produce IgE antibodies to Ole e 9 when the intensity of exposure increases. Moreover, the specific IgE levels to this allergen in sensitized patients from both Jaén and low-exposure areas are similar to those found for Ole e 1, showing that Ole e 9 has a great clinical relevance for these patients. In addition, we did not find any patient sensitized to Ole e 9 without concomitant sensitization to Ole e 1. This suggests that Ole e 9 may be considered as a marker for clinical severity of allergy to olive pollen. Obviously, patients with this pattern of allergen recognition are at a high risk of suffering from adverse reactions if an allergy vaccine with a high content of Ole e 9 is administered to them. Because of the extreme variability in the Ole e 9 content of olive extracts previously reported and the high specific IgE levels found in some patients, this allergen has probably been the agent responsible for clustered adverse reactions occasionally reported in Jaén. Profilin and polcalcin prevalence in Jaén was not significantly different from that found in other areas, the range being 10-25% in all areas. These data support the idea that the side effects observed are linked to allergens other than profilin/polcalcins in these populations.

In conclusion, we have demonstrated that allergens considered as minor may have a very relevant clinical significance for certain patient populations. To minimize the risk of eliciting adverse reactions when these patients are subjected to immunotherapy, we propose the implementation of methods to measure the concentration of minor allergens. This, combined with methods for major allergen quantitation and total allergenic activity, will allow a proper selection of starting materials and the production of extracts with allergen ratios adequate to the patient sensitization profile. These extracts with controlled concentration of minor allergens will increase the safety of allergen vaccines and, probably, also their efficacy.

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Discussion

Pauli: I think we have the same problem with the ash pollen allergen because we could also show that polcalcin and profilin can be involved. And sometimes we also had high reactions. Do you think that we also have something homologous like Ole e 9 in *Fraxinus*?

Barber: We might state that probably all pollen systems are having similar kinds of antigens. So it does not matter if you read 14,000 pollen grains per cubic metre or not: In the Jaén area we have found a minor olive allergen that is equivalent to a major *Salsola kali* allergen. That means that you have a sensitization mainly independent from olive allergens. β -Glucanases are described in many other pollen systems as well. So, of course when you have patients who are heavily exposed, it seems that they establish sensitization. It seems that you have a "switch on-off" level of sensitization because you do not see a clear progress from 0 - 40%, it seems that you either have quite a low sensitization and suddenly it goes up steep. But I think that we have to start to understand what is going on here. We need to establish the adequate dose, that is the adequate allergen concentration, and measure efficacies and so on. We still have a lot of work to do in that particular area.