

CORRESPONDENCE

Clinical and Experimental Allergy

Assessing the prevalence of sensitization to specific latex allergens

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I read with interest the paper entitled 'Latex allergy: low prevalence of immunoglobulin E to highly purified proteins Hev b 2 and Hev b 13 by Palosuo et al. [1]. The authors dispute that prevalence of sensitivity to Hev b 2 and Hev b 13 is sufficiently high for them to be classified as major latex allergens (>50% binding to IgE of latex-allergic individuals), contrary to the results from recent research [2, 3] and earlier reports [4, 5]. They contend that only Hev b 5 and Hev b 6 are the major allergens among latex-allergic adults.

In their paper, the authors isolated and purified Hev b 2 and Hev b 13 from natural rubber latex, and then went on to refine further what they describe as already highly purified proteins. Might these allergens have ended up being over purified?

In the first instance, is it even possible to 'over-purify' a substance? If the subject of purification is homogeneous, then the answer is generally 'No'. But many allergens are heterogeneous by nature and occur as multiple molecular variants (isoforms). The International Union of Immunological Societies has provision for naming such isoallergens and their variants, although many more isoforms have been reported than officially classified. Except where the isoallergen is specified, a named allergen should refer to a balanced representation of its isoforms that are commonly encountered.

Purifying something from a mixture is a matter of isolating the target substance and rejecting the remainder through a sequence of refining steps. There is no reason to doubt what the authors isolated at the end of their elaborate purification regime as indeed pure extracts of Hev b 2 and Hev b 13. But of no lesser importance here are the fractions that were thrown out in the course of purification. Were key isoallergens discarded *en route*?

It is not always easy to distinguish between different isoallergens because changes in the DNA are often difficult to detect in the translated proteins. In the case of Hev b 2, however, the existence of isoforms is evident even from routine SDS-PAGE where it commonly appears as a

doublet of about 35 kDa [6–8], with both protein bands similar enough to be recognized by the same monoclonal antibody [9]. Yagami et al. [8] resolved the Hev b 2 doublet into three distinct isoforms (from at least five molecular variants that exist [9]). The slower migrating (slightly larger) isoform was glycosylated, whereas the fast-migrating protein band yielded one glycosylated and another unglycosylated isoform. All three isoforms were shown by skin prick tests to be allergenic, but individual patients exhibited varying sensitization to the three variants. More recent work on Hev b 2 isoallergens indicate that glycans in the glycosylated isoforms play an active role in allergy, rather than being passive cross-reactive carbohydrate determinants [3, 10].

The purification procedure described for Hev b 2 is closely similar to an earlier protocol (similar gel filtration technique, separation column, buffer and elution rate) from the same research group [11]. But rather than a doublet, Hev b 2 was purified to a single SDS-PAGE protein band on that occasion. It is not clear if this were also the case in the present study and the question hence arises whether all common isoforms of Hev b 2 were captured. In describing their purified Hev b 2 as 'homogeneous' in the current paper, the authors might have lost more than just isoallergens; it appears they have lost the plot. For their purpose, there is little merit in setting the goal of homogeneous Hev b 2 when, in its natural state, the allergen is clearly not.

Concern over a balanced representation of isoallergens in ultra-purified proteins extends to Hev b 13, although dissimilarities in its isoforms are not as readily discernible to the naked eye as for Hev b 2. That notwithstanding, the discrepancy in the Hev b 13 molecular mass of 43 375 Da determined by mass spectrometry in the present study with the value of 42 975 Da from an earlier report [12] already hints of molecular variation.

The authors attribute the previously reported [2–5] high allergenicity of Hev b 13 to contamination by other latex allergens, especially Hev b 6. They cite as evidence the persistent positive reaction of their highly purified Hev b 13 to an antibody against Hev b 6. But when

exhaustive purification still fails to rid an antigen of interaction with an unrelated antibody, the likelihood increases that the problem lies not with antigen purity but with antibody specificity. Nevertheless, the authors discount cross-reactive epitopes because binding of Hev b 13 with anti-Hev b 6 diminishes with successive stages of purification. That would certainly be indicative of a contaminant being removed stepwise, but only provided that the target of purification were homogeneous to begin with. The very same observation would be expected if specific isoforms of Hev b 13 had been actively selected against over the course of purification.

When further refinement of the already highly purified Hev b 13 yields fractions with no obvious distinguishing chemical characteristics among them, how do the authors select which fractions to keep and which to discard? If binding with anti-Hev b 6 were adopted as a criterion for rejection, would not ultra-purification be systematically stripping away molecular variants of Hev b 13 that happen to cross-react with anti-Hev b 6?

In any case, ultra-pure Hev b 13 from the final cut has not spared the alleged presence of contaminating Hev b 6, even though evidence for this was looked for but not found in a battery of tests, including reversed phase chromatography, N-terminal sequencing, electro spray/MALDI-TOF mass spectrometry and mass spectrometric peptide mass fingerprinting. The authors point to an interaction of Hev b 13 with IgE specific for Hev b 6. (It is, of course, equally true to view this as Hev b 6 that is cross-reacting with IgE specific for Hev b 13.) Even if (an isoform of) Hev b 13 were recognized by IgE that also recognized Hev b 6, there is no reason why such cross-reaction *per se* should render Hev b 13 irrelevant as an allergen. (Should Hev b 6 be deemed irrelevant because it cross-reacts with Hev b 13?)

Estimating the prevalence of sensitization to specific latex allergens requires more than just samples of the individual proteins. Latex-allergic patients (or their blood sera), too, must be available. In this connection, the selection of subjects for the study group has an important bearing on the outcome of the prevalence calculation.

Participants selected to assess prevalence of sensitization to latex allergens would be those who exhibit clinical symptoms of latex allergy. In the present investigation, as has been commonly the case in other similar studies, the participants were also required to be skin prick-positive against natural rubber latex. Whereas this might appear to be an additional safeguard to ensure the patients were truly allergic to latex, note should be taken of the fact that the concentrations of individual allergens vary widely in natural rubber latex. These discrepancies are carried over to commercial diagnostic skin test reagents that are commonly prepared by diluting whole natural rubber latex without adjusting for individual allergen concentra-

tions [13], other than – in some cases – supplementation with recombinant Hev b 5 [14].

Hev b 6 is by far the most abundant protein in natural rubber latex, its molecular concentration being, for example, some 25 times that of Hev b 2 and some 270 times that of Hev b 13 [13]. The disproportionately high concentration of Hev b 6 means that skin prick diagnostics with whole latex are likely to be biased towards detection of allergy to Hev b 6 in particular. That may be a factor explaining the high prevalence of sensitivity to Hev b 6 observed in this and other similar studies. (A positive bias for Hev b 5 would similarly apply where its recombinant protein has been added to the test reagent.) On the other hand, patients who exhibit clinical symptoms of latex allergy, but who are nevertheless *not* sensitized to Hev b 6, may be skin-test false negative because other latex allergens (e.g. Hev b 13) in the test reagent are frequently below the threshold concentrations required to elicit a positive result [13].

An assessment of prevalence rates for allergen sensitization is only as good as the allergen test reagent used. Not only should the protein be substantially pure, but the full complement of its common isoallergens and molecular variants should be present. There must, of course, also be an unbiased study population on which to make the assessment.

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**Response by T. Palosuo, N. Kalkkinen, H. Alenius,
R. G. Hamilton and C. Blanco**

We would like to thank Dr Yeang for his interest and careful review of our article. He asks whether it is possible that our proteins were 'over-purified' and suggests that due to extensive purification we could have removed from the final preparations some important isoforms with IgE-binding properties. At a general level concerning purification of proteins, our methods represent well-established protein chemistry techniques that are aimed at purifying proteins in their native conformation. A major focus of our work has been to eliminate other possible interfering allergens.

Recent reports, in which a high prevalence of IgE antibody specific for native Hev b 2 and Hev b 13 proteins, both produced in one laboratory [1-3], gave us the stimulus for the present study. Our group in Finland had in 1995 purified and partially characterized both Hev b 2 [4] and Hev b 13 (H. Alenius, N. Kalkkinen, T. Palosuo *et al.*, unpublished observations on early nodulin-like latex protein, 1995). Because of the relatively rare occurrence of IgE to Hev b 2 and Hev b 13 in our unselected latex-allergic patients (10-20%) we discontinued our studies on these proteins for several years. Our interest was renewed when we saw the paper of Bernstein *et al.* [1] showing an astonishingly similar (63%) prevalence of IgE in skin prick testing to three latex allergens, Hev b 6.01, Hev b 2 and Hev b 13. We were earlier concerned about the problem that Hev b 6.02 may copurify as a contaminant with other latex proteins. Even a slight contamination (a few per cent, not readily discerned by standard protein chemistry techniques) could totally distort the IgE antibody results in prevalence studies.

Dr Yeang underlines the generally acknowledged situation that many allergens are heterogeneous by nature and occur as multiple molecular variants (isoforms). We fully agree on this and note that possible isoforms of Hev b 2 and Hev b 13 allergens are due either to alternative mRNA splicing (generation of splice variants) or post-translational modifications, such as differences in glycosylation, proteolytic processing or deamidation. In the context of the present study the question may be asked whether the possible isoforms would express the so-marked protein chemical differences that they could become unnoticed and lost during the sequential purification steps. Considering this possibility, our purification methods for Hev b 2 and Hev b 13 in protein chemical terms can be considered as very broad and in our judgment efficiently enabled the capture of the relevant protein components.

In addition, Dr Yeang suspects that we had selected fractions of interest based on their IgE-binding properties, which could have led to a bias. This was not the case because during the purification process, the components or fractions representing the proteins or polypeptides of interest were picked up using protein chemistry properties, such as absorbance at different wavelengths in the chromatographic purification steps and bands in SDS-PAGE. Assessment of the IgE-binding properties of all protein peaks using monoclonal antibodies and pooled human sera with specific IgE to the complete spectrum of allergens was of course performed; however, it was used as an independent indicator of the purification process. Because the specific antibodies did not bind to other protein components, it is very difficult to perceive that there were IgE-binding isoforms with substantially deviant physical-chemical properties. We thus conclude that the putative isoforms of Hev b 2 and Hev b 13 allergens shared reasonably comparable structural properties and, in all likelihood, these isoforms were present as a representative mixture in the final purified preparations.

Specifically, with regard to Hev b 2, Dr Yeang points out that isoforms with different glycosylation and IgE-binding properties have been described for Hev b 2 and doubts that we could have 'discarded key isoforms *en route*'. According to our data, not shown in the article, the Hev b 2 preparation used in the prevalence studies gave two closely-spaced bands in SDS-PAGE. Further analysis of this preparation by reversed phase chromatography gave two closely eluting protein peaks, of which the first (33.54 min) gave in electrospray Q-TOF mass spectrometric analysis a mass of 37 628 Da and the second (34.10 min) a mass of 36 432 Da. Both of these masses are larger than the calculated mass of the non-glycosylated form of Hev b 2. The theoretical calculated mass of the non-glycosylated form of Hev b 2 (P52407) with the signal sequence removed, containing a cyclic pyroglutamic acid at its N-terminus and lacking the potential propeptide of