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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1995 purified and partially characterized both Hev b 2 [4] and Hev b 13 (H. Alenius, N. Kalkkinen, T. Palosuo *et al.*, unpublished observations on early nodulin-like latex protein, 1995).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

22 amino acid residues in the C-terminus, is 35 072 Da. The differences in the obtained masses can be interpreted by the presence of one or more glycans in the proteins, indicating that the two forms of Hev b 2 protein are post-translationally modified.

In the case of Hev b 13, in agreement with Dr Yeang's statement, the differences we noted in the molecular mass of our purified protein and the mass deduced from the known sequence suggested post-translational modifications. We had also analysed the glycosylation sites and the nature of the glycans in the purified protein in considerable detail. Our interpretation is that our final purified Hev b 13 protein represented the allergenic protein with putative post-translational modifications at several glycosylation sites. Although the putative isoforms were not significantly separated by their protein chemical properties, it seems highly likely that they were not removed from the final product.

Altogether, for the time being, the clinical relevance of the different isoforms and the impact of glycosylation on IgE-binding properties are not yet fully established. Because the nature of the epitopes on Hev b 2 (or Hev b 13) molecules, responsible for IgE binding, is not known or even tentatively characterized, the discussion on their relevance remains largely speculative.

Dr Yeang then questions the specificity of our monoclonal Hev b 6.02 antibody and suggests that it could perhaps combine with a cross-reactive epitope in Hev b 13. As we discuss in detail in the article, this explanation is entirely speculative. The monoclonal antibodies were produced by immunization using purified Hev b 6.02 that does not contain glycans. As we have pointed out, there is no apparent homology between the primary sequences of Hev b 6.02 (or Hev b 6.01) and Hev b 13. It is thus very difficult to envision how the developed monoclonal antibodies, while recognizing protein epitopes of Hev b 6.02, could have simultaneous specificity to moieties in Hev b 13. Moreover, not only did our monoclonal anti-hevein antibodies bind to the final, ostensibly pure Hev b 13 preparation, but also human IgE to Hev b 6.02 from a large number of sera (without antibodies to Hev b 13) bound to the same Hev b 13. As reported in our article, this binding activity to Hev b 13 could often be inhibited by added Hev b 6.02.

Although this Hev b 6.02-like immunological reactivity could not be totally abolished in the course of the purification process, it was stepwise reduced to a very low level. We also acknowledge that the exact molecular nature of the copurifying Hev b 6.02-like component(s) still remains to be identified, but, as we point out, a minimal contamination of Hev b 6.02 would have been difficult to detect with the use of classical protein chemistry methods. In line with this contention we have had the chance to analyse some other seemingly pure preparations of native Hev b 13 (and Hev b 2) made by us and also by

other investigators and found in some preparations significant contamination with other allergens, in particular Hev b 6, even in concentrations high enough to be demonstrated also by standard protein chemistry methods.

Finally, Dr Yeang takes up the possibility that the selection of subjects used in the present prevalence study may not have been optimal to diagnose latex allergy. He suggests that the concentration of e.g., Hev b 13 in the skin prick test reagents may have been below the threshold required to elicit a positive result in patients sensitized to Hev b 13 but not to Hev b 6 [5]. The low concentration of Hev b 13 protein in the source material and in some test reagents may indeed be true but the absolute and relative concentration of Hev b 13 in manufactured rubber products, although not yet unambiguously established, may also be generally low [6] and indicative of low sensitizing ability. In any case, latex allergy needs to be diagnosed in a reliable manner using generally accepted guidelines such as those used in the present study. We note that three different skin prick test reagents were used in the three centres (Stallergènes, Greer and ALK-Abelló reagents) along with two different immunoassays for the measurement of latex-specific IgE (Alastat and ImmunoCAP). In addition, the majority of Finnish patients had been subjected to a glove use (provocation) test. Despite some differences in the diagnostic algorithms, the observed prevalence rates were mostly of the same order of magnitude across the three countries, indicating a roughly similar reactivity to the tests reagents and, eventually, a low or relatively low prevalence of IgE to Hev b 2 and Hev b 13.

In summary, our experience shows that unless scrupulous attention is paid to the purification methods, adequate purity of two 'new' alleged major latex allergens, Hev b 2 and Hev b 13, may be difficult to achieve. Impure allergens in turn can result in false-positive skin prick test results and erroneous IgE prevalence rates in *in vitro* tests. We see no reason to change our view in concluding that IgE prevalence to properly purified Hev b 2 and Hev b 13, when assessed by serological tests, is low and classifies these two proteins to the category of minor latex allergens.

References

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