Hev b 5 and Hev b 13 as allergen markers to estimate the allergenic potency of latex gloves

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Background: Sensitization to natural rubber latex has been linked to proteins from medical latex gloves. Various assays to estimate the amount of residual allergenic proteins extractable from latex gloves to assess their potential exposure hazard have inherent weaknesses.
Objective: This investigation was aimed at developing 2-site immunoenzymetric assays and identifying appropriate protein markers to assess the allergenic potential of latex gloves.
Methods: The presence of 6 latex allergens—Hev b 1, 2, 3, 5, 6, and 13—was measured in a cross-section of commercial latex medical gloves by using monoclonal and polyclonal antibody-based 2-site immunoenzymetric assays. The overall allergenic potential of these gloves was assessed by IgE-inhibition assay. Stepwise multiple regression analyses were performed to identify marker allergens that best explained the variation in latex glove allergenicity.
Results: All 6 latex allergens were detected in at least some of the glove samples. Hev b 5 and Hev b 13 were identified as the marker allergens that combined best to explain the variation in the glove allergenicity. The significant multiple correlation ($R = 0.855$) between these 2 markers and glove allergenic potency forms the basis of an assay to gauge latex glove allergenicity.
Conclusion: The overall allergenic potential of latex gloves can be estimated by using Hev b 5 and Hev b 13 as indicator allergens. The correlation between glove allergenicity and the level of these allergens was maintained for low-protein gloves (< 200 µg/g). This estimation of glove allergenicity was superior to that obtained by using total protein readings. (J Allergy Clin Immunol 2004;114:593-8.)

Key words: *Latex allergy, latex glove, allergen, Hev b 5, Hev b 13, step-wise multiple regression, immunoassay, protein, IEMA*

Latex sensitization, especially among healthcare workers, has been linked to proteins that are extractable from natural rubber latex gloves.[1] The latex used in glove manufacturing is the laticifer cytoplasm of the commercial rubber tree, *Hevea brasiliensis*. Natural rubber latex contains several hundred proteins, of which 13—Hev b 1 to Hev b 13—have been recognized by the International Union of Immunological Societies (IUIS) as latex allergens.

Several assays have been developed to estimate the amount of residual allergenic latex proteins to assess the potential hazard posed by exposure to latex gloves from different manufacturers. The micro Lowry assay[2-6] is the most commonly used assay for quantifying the total protein level in extracts from latex gloves, which serves as a coarse gauge of latex glove allergenicity. Although the total protein content of an extract provides an indication of latex glove allergenicity,[7-9] the assay is time-consuming, and its analytical sensitivity is limited. Moreover, it cannot discriminate between allergenic and nonallergenic proteins extracted from latex gloves. Alternative assays that quantify latex antigens in an indirect ELISA format[10] or an inhibition ELISA format (American Society for Testing and Materials D6499)[11-14] have the aim of narrowing the range of latex proteins detected but still fall short of distinguishing the allergens from the nonallergenic antigens. Immunoenzymetric assay (IEMA)-based quantitation for a limited number of specific allergenic proteins (Hev b 1, Hev b 3, Hev b 5, and Hev b 6) is available commercially, with separate assays providing individual results for each allergen.[15] Hence, a single assay that can determine the overall allergenicity of a rubber product is still needed. The laboratory method that probably provides the best overall measure of allergenicity of latex gloves is the human IgE antibody–based inhibition immunoassay[7,9,16,17] because it actually measures the amount of inhibition of IgE antilatex binding to solid phase latex

TABLE I. Antibodies and calibration proteins used in 2-site IEMAs

Antigen	Capture antibody		Signal antibody		Calibration reference protein
	Type	Source	Type	Source	
Hev b 1	Monoclonal	JHU/RRIM	Polyclonal, biotinylated	JHU/RRIM	Native latex protein
Hev b 2	Monoclonal	USM/RRIM	Polyclonal, biotinylated	JHU/RRIM	Native latex protein
Hev b 3	Monoclonal	USM/RRIM	Polyclonal, biotinylated	JHU/RRIM	Native latex protein
Hev b 5	Monoclonal	FIT-Biotech	Monoclonal, enzyme-conjugated	FIT-Biotech	Recombinant MBP fusion protein
Hev b 6.01/6.02	Monoclonal	BGFA	Polyclonal	RRIM	Native latex protein (Hev b 6.01)
Hev b 13	Monoclonal	JHU/RRIM	Polyclonal, biotinylated	JHU/RRIM	Native latex protein

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allergens. Although this immunoassay has been useful in research, it has been difficult to standardize or to extend its use to monitoring the allergenicity of latex gloves in manufacturing facilities because of the requirement for large amounts of blood from patients allergic to latex.

In the current study, our objective was to develop a panel of 2-site IEMAs for multiple latex allergens and to identify suitable protein indicators of rubber product allergenicity. By using these assays, we identified Hev b 5 and Hev b 13 as the best indicator allergens, and we showed the limitations of the total protein assay as a monitoring device for assessing glove allergenicity.

METHODS

Glove extraction and analysis

Thirty-two brands of powdered examination gloves manufactured in Malaysia were initially extracted in phosphate buffer-saline (NaCl adjusted to 0.2 mol/L) for 2 hours and tested for total protein according to the modified Lowry American Society for Testing and Materials D 5712-99 method.[6] Six gloves of each brand were tested, and brands that showed an intralot coefficient of variation in extractable protein >12% were removed from the study. Gloves from 1 box each of the 24 remaining glove brands were systematically allotted to the 3 participating laboratories at the Rubber Research Institute of Malaysia (RRIM), the Johns Hopkins University School of Medicine (JHU), and the Research Institute for Occupational Medicine (BGFA). The means of total protein readings from the 3 laboratories were used for the statistical analyses in this study. Besides the 24 gloves, 3 additional low-protein, nonpowdered latex gloves (2 of them chlorinated and 1 polymer-coated) were added to the analyses in the validation experiments.

Immunoenzymetric assays of 6 latex allergens

The quantity of 6 latex allergens (Hev b 1, Hev b 2, Hev b 3, Hev b 5, Hev b 6, and Hev b 13) was measured in extracts of the 24 glove samples by using a panel of 2-site immunoenzymetric assays. The assay design as previously described[18] was used to measure Hev b 1, 2, 3, 6, and 13. Carbonate-bicarbonate coating buffer containing the capture antibodies was pipetted into wells of the microtiter plates and incubated at room temperature for 3 hours, followed by incubation at 4°C overnight. Blocking was performed the next day at room temperature for 1 hour by using 1% bovine serum albumin in PBS. The plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). Test samples were then pipetted into the wells of their respective microtiter plates in triplicate and incubated at room

temperature for 3 hours and overnight at 4°C. The next day, the plates were rewashed 3 times with PBS-T, and the bound latex protein was detected with the addition of its respective biotinylated polyclonal detection antibody, which was diluted in PBS containing 1% BSA. Only the detection antibody specific for Hev b 6 was not directly biotinylated; rather, it was detected with alkaline phosphatase–conjugated antirabbit IgG secondary antibody. After a 2-hour incubation at room temperature, the plates were washed once with PBS-T and then twice with TRIS-buffered saline (TBS) containing 0.05% Tween 20, pH 8.0. After this, streptavidin-conjugated alkaline phosphatase in TBS containing 0.2-mmol/L magnesium chloride was added (except for the Hev b 6 assay). After a 1-hour incubation, the plates were washed twice with TBS containing 0.05% Tween 20, pH 8.0, and once with TBS, pH 9.5, containing 50-mmol/L magnesium chloride. Color development was initiated by adding p-nitrophenyl phosphate in 10% diethanolamine buffer and monitoring the absorbance at 405 nm in a microtiter plate reader. Absorbance readings of calibration standards for the purified native proteins Hev b 1, 2, 3, 6, and 13 were obtained in a similar manner. (Although native proteins were used for calibration, they could be replaced by their respective recombinant proteins that were similarly reactive with the monoclonal and polyclonal antibodies.) The highest concentrations for the calibration standards ranged from 1 µg/mL to 10 µg/mL. They were serially diluted 4-fold or 5-fold to obtain 7 concentrations of each allergen. Absorbance readings were obtained at intervals ranging from 20 minutes to 4 hours to optimize precision of the readings. Readings for the same calibration concentration taken at different intervals showed good agreement (results not presented). Readings that differed from the buffer blank readings by <2 times the SD of the latter were considered below detection. Absorbance readings were interpolated from the dose-response curves in nanograms of allergen per gram glove. The IEMA for Hev b 5 was performed by using a commercial kit (FITkit Hev b 5; FIT Biotech Oyj Plc, Tampere, Finland) according to the manufacturer's instructions. Details of the antibodies and calibration proteins used in these assays are presented in Table I. The monoclonal and polyclonal antibodies against Hev b 6 recognized epitopes on Hev b 6.02. Because the Hev b 6.01 molecule encompasses the entire Hev b 6.02 domain,[19] the antibodies detected both the proteins.

Estimation of allergenicity by IgE inhibition

JHU analyses. The JHU IgE inhibition assay to evaluate allergenicity of the latex gloves used a nonammoniated latex allergosorbent (Pharmacia CAP System-K82, not spiked with rHev b 5; Uppsala, Sweden) and a human serum pool (n = 100 subjects) blended to contain IgE antibodies to known latex allergens by enzyme immunoassay (data not shown). Each subject providing serum to the pool had a positive history of latex allergy, a positive puncture skin test with an investigational latex allergen preparation

TABLE II. Allergen and protein content in 24 brands of latex gloves

	Hev b 1 (ng/g)	Hev b 2 (ng/g)	Hev b 3 (ng/g)	Hev b 5 (ng/g)	Hev b 6 (ng/g)	Hev b 13 (ng/g)	Protein (μg/g)
Mean	254	9908	17.96	332	2525	154	429
Median	211	4620	2.20	317	2223	8.6	272
Minimum	<5.8	<80	<1.0	36	1195	<2.4	105
Maximum	750	74,924	111	680	5695	1502	1164
Coefficient of variation (%)	83	174	195	56	41	227	77

from Greer Laboratories (Lenoir, NC), and positive IgE antilatex serology. The reference latex used to construct the dose response curve was a nonammoniated latex (E8) from the US Food and Drug Administration Center for Biologics Evaluation and Research that was assigned 100,000 allergen units/mL and had a total protein of 3.89 mg/mL. The assay was performed by incubating 100 μL reference or test extract with 100 μL human IgE antilatex overnight. The extract-antibody mixtures were then pipetted (50 μL in duplicate) into separate latex ImmunoCAPs (Pharmacia). After an overnight incubation at 23°C, the amount of bound IgE antilatex was assessed in the CAP System. Test extract response data were interpolated from the E8 calibration curve.

BFGA analyses. A second IgE inhibition analysis was performed at BGFA to assess the latex allergen content of the 24 test gloves. In this analysis, a serum pool of 12 German healthcare workers allergic to latex was used as the latex-specific IgE antibody source. Subjects providing these sera had a positive clinical history of workplace-related latex allergy symptoms (urticaria, rhinitis, conjunctivitis, and/or bronchial asthma), a positive skin prick test to latex, and a positive latex-specific IgE test in the Pharmacia CAP System (average of 16.6 kU/L latex-specific IgE). The combined serum was tested for IgE binding to individual recombinant latex allergens prepared as fusion proteins linked to the maltose binding protein (MBP) and coupled to ImmunoCAPs. The serum pool showed significant reactivity (0.82-13 kU/L) with each of the recombinant Hev b 1, 3, 5, 6.01, 6.02, and 6.03. MBP alone showed reactivity equivalent to <0.35 kU/L. Nonammoniated natural rubber latex from Thailand was used as the reference allergen. An inhibition CAP calibration curve was constructed by incubating 20 μL of the different concentrations of the reference allergen with 40 μL of the IgE antilatex pool and adding the mixtures to their own solid-phase ImmunoCAP latex allergosorbents (k82, not spiked with rHev b 5). The content of latex allergen in the test glove extracts was analyzed in the same manner, and absorbance results were interpolated from the inhibition calibration curve.

Statistical analyses

Associations between the total protein and allergen measurements were assessed by Pearson correlation or the multiple correlation statistics by using the SAS/STAT Statistical Package (SAS Institute, Cary, NC). IgE inhibition data were untransformed or log-transformed. Analyses using log-transformed allergen data were also performed, but they are not presented because they do not represent any enhancement of the untransformed results appearing in this report.

RESULTS

Protein, individual allergen content, and overall allergenicity of the latex gloves

The results of the modified Lowry total protein assays performed at RRIM, JHU, and BGFA showed good agreement, with correlation coefficients exceeding 0.96 ($P < .001$) between the mean measurements obtained in

the 3 laboratories and the measurements from each individual laboratory. The IgE inhibition results obtained at JHU and BGFA were also significantly correlated ($r = 0.830$; $P < .001$). Of the 24 brands of gloves tested, Hev b 1 was detected in 22, Hev b 2 in 23, Hev b 3 in 15, Hev b 5 in 24, Hev b 6 in 24, and Hev b 13 in 13. The contents of these allergens in the 24 gloves are presented in Table II. Among the allergens, Hev b 2 and Hev b 6 were quantitatively the most abundant on the basis of their median levels in the gloves. Hev b 3 and Hev b 13 had the lowest median levels, but they also exhibited the highest variation. Hev b 6 and Hev b 5 had the least variation in their contents among the latex gloves.

Among the individual allergens, a comparison of correlation coefficients showed that the allergen potential of gloves, as represented by JHU IgE inhibition, was best explained by Hev b 13 ($r = 0.831$; $P < .001$) and Hev b 5 ($r = 0.637$; $P < .01$). When the JHU CAP data were log-transformed, the same 2 allergens were prominent, with Hev b 5 having the best correlation ($r = 0.771$; $P < .001$) and Hev b 13 the next best correlation ($r = 0.650$; $P < .001$). Hev b 13 and Hev b 5 thus appear to play important roles in determining the allergenicity of latex gloves.

Identifying marker allergens and estimating the allergenic potential of gloves

Although all 6 of the allergens can contribute to the allergenicity of the latex gloves, we attempted to identify 2 or 3 allergenic proteins that would serve as useful indicator allergens to predict glove allergenicity. The markers were to be combined in a manner that reflected their relative importance (weight) in explaining the variation in glove allergenicity. The stepwise multiple regression analysis achieved these objectives. In this analysis, the computer selected, as the first step, the *x* variable (allergen) that best explained the variance in *y* (IgE inhibition potency measurement). In a second iteration, the next best *x* variable that explained the variance in *y* was identified, independent of the variance explained in the first step. This evaluation was repeated until further addition of an *x* variable no longer increased the amount of variation in *y* significantly.

The stepwise multiple regression performed with data from the JHU CAP inhibition assay as the dependent variable and the levels of each of the 6 allergens as independent variables selected Hev b 13 and Hev b 5 as the significant variables that accounted independently for the variance in glove allergenicity. Incorporation of additional
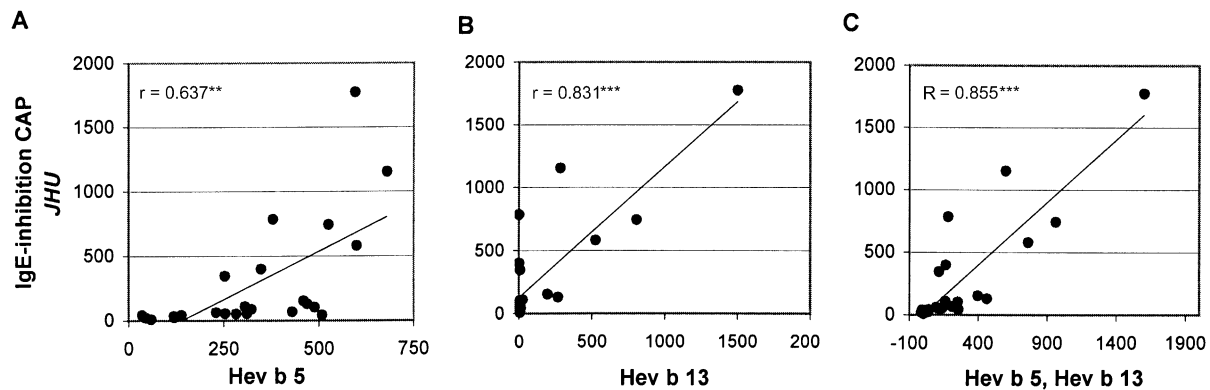
FIG 1. Relationships between JHU IgE inhibition CAP (μg/g) and latex allergen contents (ng/g) in 24 glove eluates. The abscissa values are **(A)** Hev b 5 content, **(B)** Hev b 13 content, and **(C)** 0.576 (Hev b 5 content) + 0.864 (Hev b 13 content) − 39.05. *******P* < .01. ********P* < .001.

latex allergen data into the regression equation did not further explain the variation observed among glove allergenicity levels. As stated, both Hev b 13 and Hev b 5 were individually correlated with glove allergenicity as determined by IgE inhibition (JHU CAP inhibition). As shown in Fig 1, *A,* Hev b 5 alone generally lacked selectivity, with a wide range of values lying along the x-axis. Moreover, Hev b 13 alone lacked sensitivity (Fig 1, *B*). Some gloves with very low Hev b 13 (points lying on the y-axis) showed moderately high JHU CAP inhibition results that could not be attributed to Hev b 13. These deficiencies in Hev b 5 and Hev b 13 were resolved substantially when the 2 variables were incorporated into a multiple regression. The linear regression equation

$$\text{IgE inhibition CAP} =$$
$$0.576 \,(\text{Hev b 5}) + 0.864 \,(\text{Hev b 13}) - 39.05$$

had a multiple correlation coefficient, *R,* of 0.855 (*P* < .001; Fig 1, *C*). This showed that just 2 allergens in combination, Hev b 13 and Hev b 5, could account for the variability in glove allergenicity (IgE binding capacity) to a large extent. Log transformation of the JHU CAP inhibition data did not change the allergen variables that were selected by multiple regression analysis. Using the log-transformed data, the multiple correlation coefficient was *R* = 0.814 (*P* < .001). The JHU IgE-CAP inhibition results also correlated with total protein levels (*r* = 0.869; *P* < .001).

Result validation with low-protein and nonpowdered gloves

The 24 glove samples selected for this study contained total protein levels ranging from 105 to 1164 μg/g (Table II). With latex glove manufacturers actively taking steps to reduce extractable proteins from their products, current gloves tend to have lower total protein levels. With this trend expected to continue into the future, and to subject the multiple regression formula to an even more stringent test for robustness when extractable proteins were very low, data from the 6 gloves with extractable proteins

below 200 μg/g were grouped together with additional data from 3 nonpowdered gloves. Two of the nonpowdered gloves were chlorinated and had extractable proteins of 15 μg/g and 57 μg/g, whereas the polymer-coated glove had 105 μg/g proteins. The results of this analysis showed that total proteins explained 68% of the variation in allergenicity as determined by the JHU IgE inhibition CAP (*r* = 0.824; *P* < .01; *r²* = 0.680). When a regression analysis was performed with protein content as the variable replaced by the combination of Hev b 5 and Hev b 13 contents, the variation in allergenicity that was accounted for rose to 80% (*r* = 0.897; *P* < .01; *r²* = 0.805). These results showed that the regression equation that was derived from analysis of the original 24 latex gloves was applicable to gloves with very low (< 200 μg/g) extractable proteins, including nonpowdered gloves that were chlorinated or polymer-coated. Moreover, the use of the allergen markers Hev b 5 and Hev b 13 gave an estimate of glove allergenicity superior to that obtained by using total extractable protein content.

Result validation with independent IgE inhibition measurements

The relationship between glove allergenicity as determined by the JHU IgE inhibition assay and the levels of the markers Hev b 5 and Hev b 13 was validated by using a separate set of allergenicity readings. These measurements were the IgE inhibition CAP readings for the same gloves that were made independently at BGFA. In a regression analysis, the glove allergenicity values earlier estimated from the allergen markers (0.576 [Hev b 5 content] + 0.864 [Hev b 13 content] − 39.05) served as the independent variables, whereas the BGFA IgE inhibition readings were the dependent variables. By using all 27 latex gloves (including the 3 nonpowdered samples), a correlation coefficient of *r* = 0.894 (*P* < .001) was obtained. This compared with *r* = 0.634 (*P* < .001) when BGFA IgE inhibition was correlated with total protein.

When similar regressions were performed for the 9 low-protein gloves with extractable proteins below 200 μg/g (inclusive of the 3 nonpowdered gloves), total protein
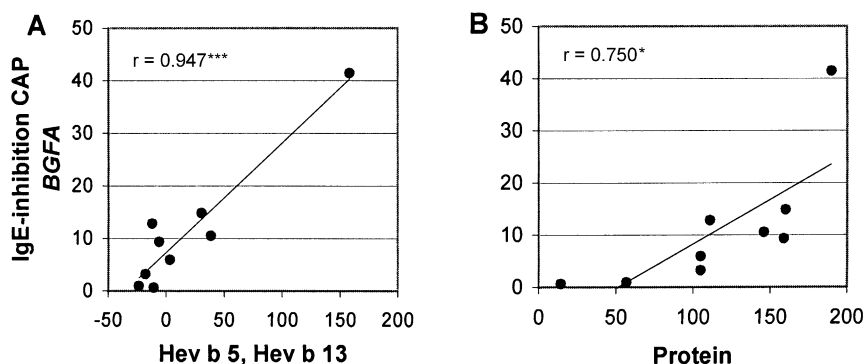
FIG 2. Validation of the JHU allergen marker regression model using data from low-protein gloves and a different reference serum in the IgE inhibition measurements. Relationships between BGFA IgE inhibition CAP (µg/g) and latex allergen contents (ng/g) or total protein (µg/g) in eluates from 9 gloves with less than 200 µg/g protein are shown. The abscissa values are **(A)** 0.576 (Hev b 5 content) + 0.864 (Hev b 13 content) − 39.05, and **(B)** total protein. *$P < .05$. ***$P < .001$.

accounted for 56% of variation in BGFA IgE inhibition ($r = 0.750$; $P < .01$; $r^2 = 0.562$; Fig 2, *B*). This compared with the 90% variation explained when the allergen markers Hev b 5 and Hev b 13 were used in the regression analysis ($r = 0.947$; $P < .001$; $r^2 = 0.897$; Fig 2, *A*). The results show that the regression equation derived from the relationship between the allergen markers and glove allergenicity determined by using American reference sera were equally applicable to allergenicity measurements on the same gloves when German reference sera were used.

DISCUSSION

Of the 13 latex allergens recognized by the IUIS, more than half of adult subjects allergic to latex are known from prick skin tests and serologic tests to be sensitive to Hev b 2, recombinant Hev b 5, Hev b 6.01, and Hev b 13.[20] These may therefore be considered major latex allergens,[21] which is why we selected them for our investigation. Rubber particle associated proteins (Hev b 1 and Hev b 3) were also included because they are particularly relevant to patients with spina bifida.[22-24] Because much latex protein is removed during glove production, the profile of residual proteins is not expected to be similar to that of whole natural rubber latex. One objective of this investigation was therefore to determine which latex allergens remained in the finished gloves.

The results of the current study showed that all 6 allergens were found in at least some of the gloves tested, with Hev b 5 and Hev b 6 present in all 24 brands of gloves. It is noteworthy that some gloves contained very high levels of Hev b 13, whereas in other gloves, Hev b 13 was undetectable. In this respect, it was surprising that the brands with the second and third highest protein levels among in the 24 samples did not contain detectable Hev b 13. These observations help explain why total protein levels can fail to predict accurately the overall allergenicity of a glove on occasion.

Although it is possible to devise an assay that determines the content of all 13 of the known latex allergens, such an immunoassay would be complicated to standardize and perform. Even an assay to quantify 6 major allergens would be expensive and cumbersome. Thus, it would be more practical to use 2 or 3 allergens that could serve as indicators to predict the allergenicity of latex gloves. Of the 6 proteins evaluated, the stepwise multiple regression analysis identified Hev b 5 and Hev b 13 as the 2 marker allergens that, in combination, best reflected the overall allergenicity of the gloves. The computer algorithm did not select allergen indicators solely on the basis of their abundance in latex gloves or how well each allergen correlated with the overall allergenicity of gloves. Rather, it selected the allergens as the best combination of independent variables that explained maximal variation of the dependent variable (allergenic potency) with minimal redundancy. The linear multiple regression of JHU IgE inhibition CAP with Hev b 5 and Hev b 13 gave a multiple correlation coefficient, R, of 0.855 ($P < .001$). This regression model showed that just 2 allergens in combination, Hev b 5 and Hev b 13, could account for 73% of the variability in glove allergenicity ($R^2 = 0.731$).

The primary statistical analyses involving data from the IgE inhibition assays were performed by using the American (JHU) serum pool that was combined from a large number of active sera and was therefore deemed broadly representative of subjects allergic to latex. The JHU allergen marker model was found compatible when tested against IgE inhibition assays conducted independently in Germany (BGFA) by using sera from a different and smaller patient population. In these analyses, total protein readings for gloves with extractable protein below 200 µg/g explained only 56% of the glove allergenicity variation. On the other hand, the variation that was explained by Hev b 5 and Hev b 13 in combination rose to approximately 90%. These results demonstrate the robustness of the JHU allergen marker regression model even in situations in which (1) the protein concentrations

were low, (2) the reference serum was changed, and (3) data from nonpowdered gloves were included. Although the multiple regression incorporating Hev b 5 and Hev b 13 leaves a proportion of glove allergenicity still unaccounted for, this extent of imprecision should not be critical if the primary objective of the immunoassay is to identify and eliminate gloves that are unacceptably high in allergens. The patient sera used in this study were biased toward adult subjects allergic to latex. Because children with spina bifida—another important group prone to latex allergy—tend to be particularly reactive with Hev b 1 and 3,[22-24] the regression model could well have been different if reference sera in the IgE inhibition assays had been derived specifically from patients with spina bifida.

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