

Measurement of natural rubber proteins in latex glove extracts: comparison of the methods

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Background: Healthcare workers and individuals with frequent contact with latex are at risk for latex protein allergy.

Objective: The purpose of this study was to compare several established methods for measuring protein in extracts from latex-containing medical devices.

Methods: Extracts from latex gloves were analyzed for natural rubber proteins using a modified Lowry assay and two different immunochemical assays. The immunochemical methods were competitive inhibition assays that employed either immune rabbit serum or human serum with antibodies directed against natural rubber proteins.

Results: Seventy extracts representing five different brands of gloves from four manufacturers were analyzed. A good linear correlation ($R = 0.88$) was found between the immunoassay methods. Correlation to the modified Lowry method was not possible because many of the samples were below the limit of detection for the Lowry assay. Reference extracts and antisera were further characterized by Western blot analysis. The data demonstrate that the proteins recognized by rabbit antisera and the proteins recognized by human IgE are similar. The greatest difference in the immunochemical assays appears to be the relative binding of the antibody sources to high and low molecular weight natural rubber proteins in the reference extracts.

Conclusions: The immunochemical assays are specific for latex proteins and provide a more sensitive and biologically relevant method for determining protein levels in latex medical products.

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INTRODUCTION

Latex allergy currently affects thousands of individuals, primarily in healthcare professions.¹ While the prevalence of the allergy in the general population is thought to be less than 1%, latex-specific IgE has been observed in up to 6% of the general population.² Certain occupations and medical conditions result in an increased risk for latex allergy. The prevalence of latex allergy in health care workers is between 7% and 10%,^{3,4} in dentists between 8.8% and 13.7%,⁵ and in children with spina bifida between 28% and 67%.⁶ Immediate hypersensitivity reactions to latex proteins develop after repeated exposure to these proteins

by contact or inhalation.^{7–10} Latex protein allergens have been measured in: gloves^{11,12}; air samples from laboratories and personal breathing zones¹³; dental offices and surgical suites^{13,14}; and in one report, on city streets.¹⁵ Because natural rubber latex proteins are capable of inducing immediate type latex allergies, it is necessary to establish methods to quantify these sensitizing agents accurately.

Several methods to measure natural rubber proteins have been developed and tested.^{11,12,16–19} Standard protein assays such as the Bradford, Lowry, or the bicinchoninic acid protein assays have been used to estimate protein concentrations in glove samples. These assays are subject to error because a variety of chemicals in latex products interfere in these tests.²⁰ The Lowry method can be improved by the use of a precipitation step to remove these interfering substances.²¹ Biologically, a more relevant way to quantify latex

proteins is to use immunochemical methods. Several types of immunochemical assays are now routinely employed for natural rubber protein determinations; however, a comparison of these methods has not been reported.

This study focuses on the comparison of two competitive inhibition immunochemical methods using rabbit IgG or human IgE antibodies specific for natural rubber proteins and a modified Lowry assay for estimation of total protein. Western blot analysis of laboratory reference standards and several glove extracts demonstrated similar antigenic and allergenic protein patterns of the preparations. These data demonstrate greater sensitivity using the immunochemical assays than the Lowry method and also show a good correlation between these immunoassays.

MATERIALS AND METHODS

Sampling and Extraction of Gloves

We tested 70 different lots of medical gloves by randomly sampling one glove from each lot. The gloves represented four different manufacturers and all gloves were purchased in 1994. For immunochemical testing, gloves were extracted in 0.1 M phosphate buffer (1:5 wt/vol extraction ratio) containing 0.2% bovine serum albumin and 0.01% sodium azide for 18 hours at 20 °C. For the modified Lowry assay, the gloves were weighed, cut into small strips (2 to 5 cm²) and extracted at a 1:4 (wt/vol) extraction ratio with phosphate buffered saline pH 7.2 for two hours at 37 °C.

Human IgE Inhibition

The human IgE inhibition assay was performed as previously described.^{11,17} A laboratory reference standard extract was prepared from powdered latex examination gloves (Bodyguards™, TK Glove Products Co., Ltd., Huntington

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Beach, CA). The extract was prepared as a 1:5 wt/vol extract in Dulbecco's phosphate-buffered saline pH 7.5 (PBS). The extract was centrifuged 15,000 *g* for 20 minutes and the supernatant ultrafiltered and concentrated 20-fold with a YM2 (1000 MW cut off) Amicon Diaflo membrane (Amicon Division of W R Grace & Co, Danvers, MA). This concentrate was then assayed for protein content by the bicinchoninic acid method and assigned a protein concentration of 7 mg/mL.

Latex allergens were quantified by an inhibition immunoassay using latex-specific IgE. Extracts of gloves or the reference standard glove extract compete with the immobilized reference latex allergens for specific IgE antibodies in pooled sera obtained from five latex-sensitized healthcare workers. Immulon IV microtiter plates (Dynatech Inc, Chantilly, VA) were adsorbed with 1 μ g of the reference standard glove extract. Latex glove reference standards or various glove extracts were delivered (50 μ L) in the well along with 50 μ L human anti-latex IgE. Radioiodinated affinity-purified rabbit anti-IgE was used for detection. The results were calculated with logistic regression and expressed as mass protein per gram of rubber. The assay has an analytical range between 5 and 3500 ng.

Rabbit IgG Inhibition

The Latex ELISA for Antigenic Protein¹⁶ was modified to be performed as an indirect competitive inhibition assay. The competitive inhibition step was carried out in microfuge tubes by making four 2-fold dilutions of each glove extract (150 μ L/tube) and seven 2-fold dilutions of the latex reference protein (ammoniated latex proteins) beginning at 2 μ g/mL. Rabbit anti-latex sera (1/500 dilution) was added to each dilution before incubating the tubes overnight at room temperature. Solid phase antigen was prepared by coating the wells of a 96-well polystyrene ELISA plate (Corning) with 0.1 μ g/well of ammoniated latex protein for four hours at 37 °C. Solid phase

antigen was washed once with phosphate buffered saline containing 0.1% Tween 20 (TPBS), after which the unreacted sites were blocked for one hour with 5% bovine serum albumin in PBS and washed two times with TPBS.

After overnight incubation, samples (100 μ L/well) were added to 96-well plates containing the solid phase antigen and incubated for four hours at 37 °C. The plates were washed three times and reacted with peroxidase-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO) for one hour at 37 °C. After washing the plates, a colored reaction was produced by using o-phenylenediamine (OPD, 1 mg/mL) in H₂O containing 0.1% H₂O₂. The reaction was stopped by the addition of 50 μ L/well of 4 N sulfuric acid. The reaction product was quantified by reading the optical density at 490 nm using a MR 5000 ELISA plate reader (Dynatech, Chantilly, VA). The concentration of latex protein in glove extracts was determined by comparing the optical density of unknowns to the laboratory reference standard at three consecutive dilutions using a customized computer spreadsheet and expressed as mass protein per gram of latex. The assay has a linear working range between 30 and 2000 ng/mL.

Lowry with Precipitation

Glove extracts and ovalbumin (reference standard) were assayed by the American Society for Testing and Materials standard method D5712-95 that incorporates a precipitation step to reduce the interfering substances in latex extracts prior to analysis by the Lowry assay. In brief, the precipitation step was performed on 0.5 mL of each sample by first adding 50 μ L of 0.15% (wt/vol) sodium deoxycholate (DOC). The samples were incubated at room temperature for ten minutes. Next, 50 μ L of 72% (wt/vol) trichloroacetic acid (TCA) and 50 μ L of 72% (wt/vol) phosphotungstic acid (PTA) were added, and the samples incubated at room temperature for 20 minutes. The samples were centrifuged for 15 minutes at 6,000 *g*, the supernatant discarded, and the pellet dissolved in 125

μ L of 0.1 M NaOH (yielding a four-fold concentration of each sample). Protein was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Each sample was tested in duplicate using four 2-fold serial dilutions. The optical density was read at 700 nm and the concentration of protein determined by comparing the optical density of unknowns to the ovalbumin protein standard.

Western Blot Analysis

Reference standards and glove extracts were analyzed by Western blotting. In order to concentrate the protein in the glove samples, extracts were precipitated using DOC/TCA/PCA as described above for the Lowry assay (ASTM D5712). The precipitate from 2 mL of extract was dissolved in 30 μ L of reducing sample buffer and 10 μ L of 5 N NaOH was added to neutralize the residual TCA and PTA in the sample. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% polyacrylamide. The proteins were visualized using silver staining (Bio-Rad).

Western blot analysis was performed as previously described.¹⁰ The SDS-PAGE separated latex proteins were transferred for two hours at 150 mA to 0.1 μ m nitrocellulose (Schleicher and Schuell, Keene, NH). The membranes were stained using 0.5% Ponceau S (wt/vol) to visualize molecular weight markers and assess transfer efficiency. The membranes were blocked for one hour at room temperature with 5% bovine serum albumin and then incubated overnight with a 1/2000 dilution of rabbit anti-latex antisera or with a 1/5 dilution of human anti-latex sera pooled from five sensitized individuals. The blots were washed with TPBS and reacted with an alkaline-phosphatase labeled anti-rabbit IgG or a mouse monoclonal anti-human IgE followed by an alkaline-phosphatase anti-mouse IgG. After thoroughly washing the blots, immunoreactive bands were visualized using nitro-blue tetrazolium and 5-bromo

4-chloro 3-indolyl phosphate (Promega, Madison, WI).

Statistical Analysis

Correlations (R value) between test methods were determined using linear curve fits of log transformed data. Log transformation of the data was necessary to overcome skewness when comparing protein values obtained from different glove brands. Data from a single glove brand was not skewed.

RESULTS

Protein Levels in Glove Extracts

Protein concentrations in extracts of 70 different gloves were compared using three assay methods. The gloves were sampled from multiple lots of five different brands from four manufacturers. Data from the modified Lowry assay were compared to the immunoassays, and the immunoassays were compared with each other (Fig 1). The modified Lowry assay was less sensitive (higher detection limit) than the immunoassays (Table 1). The detection limit for the Lowry and rabbit IgG inhibition assays was defined as 3 times the standard deviation at zero and the reporting limit as 10 times the standard deviation at zero.²² The standard deviation at zero for the Lowry assay was determined using water as the zero point. The reporting limit for the modified Lowry assay was calculated to be 5 $\mu\text{g/mL}$ extract. Since a 1:4 wt/vol extraction ratio was used, the reporting limit of the Lowry method was 20 $\mu\text{g/g}$. The detection limits for the immunochemical assays were determined by extrapolating the standard deviations from known protein determinations to zero and determining the least amount of protein that was significantly different from zero.²² The assay reporting limits were calculated to be 0.06 $\mu\text{g/mL}$ for rabbit IgG inhibition and 0.04 $\mu\text{g/mL}$ for human IgE inhibition corresponding to method reporting limits of 0.3 $\mu\text{g/g}$ and 0.2 $\mu\text{g/g}$ respectively.

Many of the glove extracts had protein levels below the reporting limit of the Lowry method (Fig 1, panel A and B) and regression analysis comparing

the Lowry method with the immunochemical assays could not be determined. When the samples below the reporting limit were deleted, regres-

sion analysis demonstrated a lack of correlation with the rabbit IgG inhibition ($R = 0.32$) and the human IgE inhibition ($R = 0.19$) assays. How-

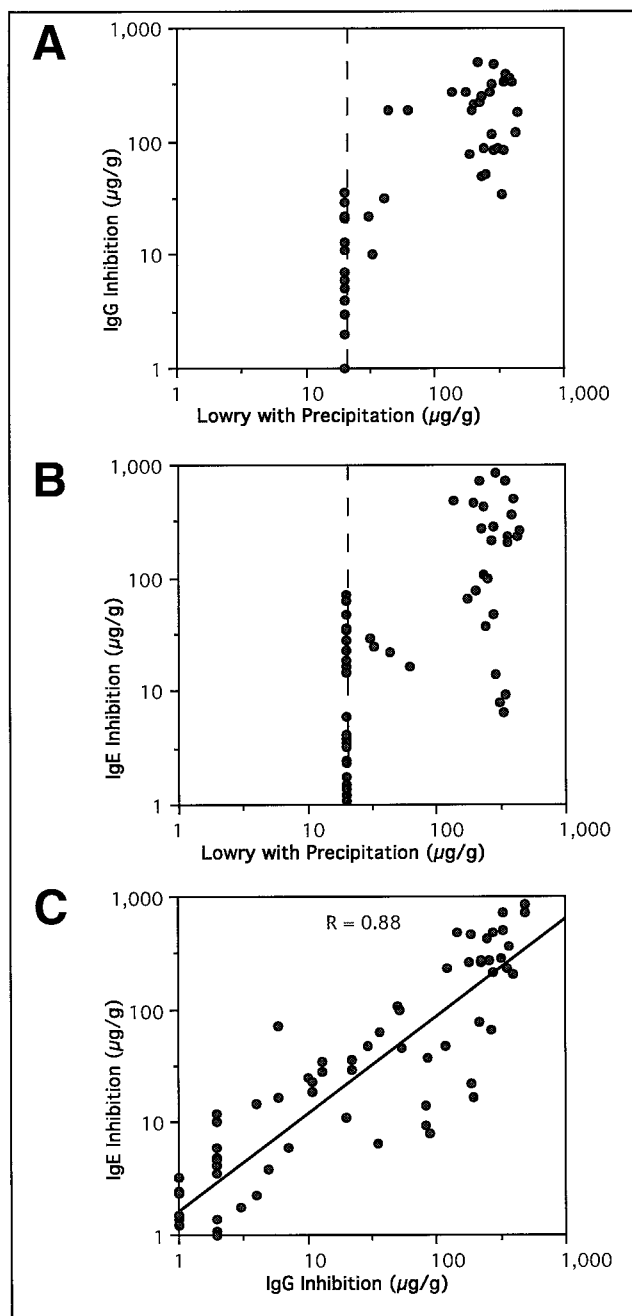


Figure 1. Correlation between latex protein test methods. Each point corresponds to a separate glove extract. A total of five separate brands are represented. Graph A represents rabbit IgG inhibition vs. Lowry, Graph B represents human IgE inhibition vs. Lowry, and Graph C shows human IgE inhibition vs. rabbit IgG inhibition. The correlation value (R) for a linear fit on log transformed data is given in graph C.

Table 1. Comparison of Protein Assay Detection and Reporting Limits

Method	Assay Detection limit ($\mu\text{g/mL}$)	Assay Reporting limit ($\mu\text{g/mL}$)	Method* Reporting limit ($\mu\text{g/g}$)
Lowry with precipitation	2.5	5	20
Rabbit IgG inhibition	0.03	0.06	0.30
Human IgE inhibition	0.04	0.04	0.20

* Method reporting limit is based on a 1:4 wt/vol extraction ratio for the Lowry method and a 1:5 wt/vol extraction ratio for the immunochemical assays.

ever, these data represent only three brands of gloves (brands B,C,D) and are likely to be skewed. A good correlation ($R = .88$) was observed when comparing the rabbit IgG and human IgE inhibition assays (panel C). Table 2 summarizes the statistical evaluation of the data obtained by the three different methods.

Western Blot Analysis of Reference Standards

The repertoire of antigenic determinants in the reference standards is critical for performance of the immunochemical assays. The rabbit IgG inhibition assay uses protein extracted from a dried film of ammoniated latex (AL) as the reference standard, while the human IgE inhibition assay uses a glove extract (glove extract). The standard extracts were analyzed following SDS-PAGE by silver staining for total protein and by Western blot for antigenic and allergenic determinants (Fig

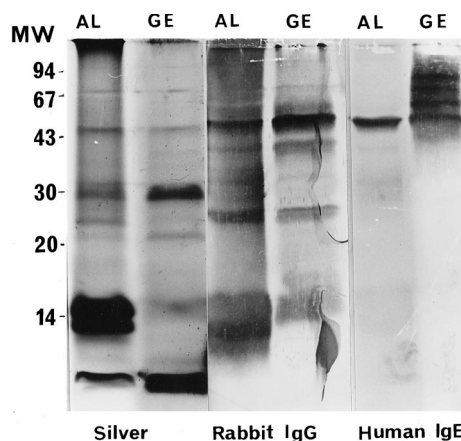


Figure 2. SDS-PAGE and Western blot analysis of reference standards. Ammoniated latex (AL) reference standard and the glove extract (GE) reference standard were compared by SDS-PAGE and silver staining, and by Western blotting with rabbit anti-latex and human latex specific IgE. In each lane, 100 μg of extract was separated by SDS-PAGE on a 15% polyacrylamide gel. Molecular weights are indicated in the left margin.

2). Both extracts have a similar protein distribution pattern as indicated by silver staining (Fig 2A). The ammoniated latex protein preparation has a more

diffuse polypeptide staining and a greater proportion of a 14-kD doublet protein. While the glove extract had a similar banding pattern, there was a

Table 2. Comparison of Protein Measurements Determined by 3 Different Latex Protein Test Methods

Brand	Method	Lots Tested	Mean Protein, $\mu\text{g/g}$	Standard Deviation	Median	Range
A	Lowry with precipitation	6	<20	n.d.	<20	n.d.
	Rabbit IgG inhibition	13	1.6	1.1	1	4
	Human IgE inhibition	12	3	1.5	2.8	4.7
B	Lowry with precipitation	6	259	149	212	403
	Rabbit IgG inhibition	27	229	132	225	466
	Human IgE inhibition	28	299	236	262	846
C	Lowry with precipitation	6	439	96	451	259
	Rabbit IgG inhibition	6	80	32	84	97
	Human IgE inhibition	6	21	17	12	40
D	Lowry with precipitation	6	60	13	60	18
	Rabbit IgG inhibition	14	28	47	13	186
	Human IgE inhibition	14	33	17	27	56
E	Lowry with precipitation	6	<20	n.d.	<20	n.d.
	Rabbit IgG inhibition	10	2.6	1.6	2	5
	Human IgE inhibition	9	1.0	0.4	1	1.5

n.d. = not determined.

greater amount of small molecular weight material running near the dye front.

Using the rabbit anti-latex sera the protein profile of the two reference extracts were found to be similar (Fig 2B); the major difference was proportionally less immunoreactive material in the 14-kD doublet. The most obvious difference noted between the two standard extracts was observed using the pooled human IgE. In the ammoniated latex protein extract, human IgE strongly recognize the band at 46 kD, with some diffuse staining of lower molecular weight polypeptides. The 14-kD doublet could be faintly detected. In the glove extract preparation, human IgE strongly recognized proteins greater than 40 kD but some faint reactivity could be seen at lower molecular weights. Difference between the rabbit and human antibody reactivity may be attributed to differences in the titers of the antisera. IgE is present at low levels in human sera making it more difficult to detect by the Western blot technique.

Western Blot Analysis of Glove Extracts

The antisera were also used to compare the profile of immunoreactive proteins in glove extracts. Extracts of the five glove brands described in Table 1 were analyzed by SDS-PAGE and immunoblot analysis using the anti-latex sera. Because glove extracts are dilute, proteins were first concentrated by precipitation with DOC/TCA/PTA to obtain sufficient protein for detection. Both Ponceau S staining of blotted membranes and Coomassie staining of SDS-PAGE demonstrated detectable protein in extracts from brands B & C gels (data not shown). As shown in Figure 3, the rabbit and human anti-latex sera had similar profiles of antibody reactivity against the glove proteins as well as the ammoniated latex reference proteins used as a positive control.

DISCUSSION

Measuring protein concentrations in latex products is important for the

study of latex allergy and for health-care institutions wishing to make informed decisions about the latex products they purchase. In this paper, we have compared the three most common methods for determining protein concentrations in latex devices, the modified Lowry and two immunoassays. Each assay measures protein utilizing different parameters and has its own advantages and disadvantages.

The Lowry assay is a widely used chemical method to estimate protein levels. The method is based on the binding of a chromogenic dye to tyrosine and tryptophan residues, and to a lesser extent cystine, cysteine, and histidine residues, and to peptide bonds.²³ The assay is easy to perform and is reproducible but lacks specificity and sensitivity.²⁴ Many substances, particularly ones used in latex production, are known to interfere in this method.²⁰

The immunochemical assays are based on the binding of antibodies to specific antigens. The two most common immunochemical assays for latex proteins are the Latex ELISA for Antigenic Protein (LEAP) assay¹⁶ and several RAST inhibition type assays that employ human IgE to quantify

latex allergens.^{11,17,19} The LEAP assay is an indirect ELISA that utilizes antisera (IgG) from rabbits immunized with the reference standard protein extract from ammoniated latex.¹⁶ Rabbits immunized with ammoniated latex were shown to recognize the entire repertoire of potential protein allergens in latex.¹⁰ In this study, the LEAP assay was modified as a competitive inhibition assay to allow a more direct comparison to the human IgE inhibition assays. The various IgE inhibition assays utilize pooled human sera from latex allergic patients and a source of latex allergens as a reference standard. The allergen reference standard and the pooled human sera differ depending on the laboratory performing the assay; however, reference standards from non-ammoniated latex, ammoniated latex, and glove extracts have been used in the different IgE inhibition assays and produce remarkably consistent results.¹⁸

We report here that the D5712 Lowry method consistently lacks the sensitivity to detect proteins in glove extracts. We modified the method to include a 1:4 (wt/vol) extraction ratio rather than the 1:10 recommended in the D5712 protocol and a 4-fold con-

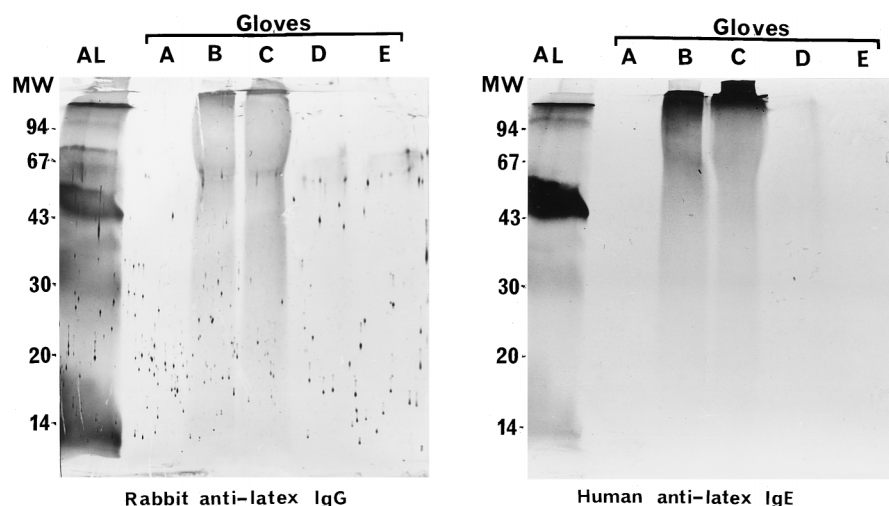


Figure 3. Western blot analysis of glove extracts. Extracts (six lots) from five glove brands were each pooled separately, and the proteins precipitated using DOC/TCA/PTA. The concentrated extracts were separated by SDS-PAGE and immunoblotted with rabbit anti-latex (A), or human anti-latex (B) sera. Gloves A–E are the same brands represented in Table 1. Ammoniated latex protein (AL) was included in the first lane as a positive control. Molecular weights are indicated in the left margin.

centration step to increase the level of detection. The precipitation step has been used to remove the interfering chemicals from the extracts.²¹ In spite of these steps, the modified Lowry is approximately 150 times less sensitive than the immunochemical methods. Furthermore, the precipitation step does not appear to completely remove all interfering substances. Synthetic gloves manufactured without the addition of exogenous protein often give readings by this assay, even when no protein can be detected by SDS-PAGE with Coomassie staining.²⁵

Although differences exist in the methodology employed for the immunochemical assays, good agreement ($R = 0.88$) between the rabbit IgG and human IgE methods was observed. Potential sources for variation between these two methods might include differences between the antigens (rabbit IgG) and allergens (human IgE) detected by the antisera employed and differences in the reference preparations. Differences in the antigens or allergens detected depends on the pool of sera chosen for use in the individual assays. In this study, the human IgE represented a pool of sera from five healthcare workers. These sera were chosen for their high anti-latex titer and their broad spectrum of reactivity to latex proteins.¹⁷ It is noted that this pool did not contain sera from patients with spina bifida, thus it is possible that certain allergens uniquely recognized by patients with spina bifida may be underrepresented by the IgE inhibition assay. Seventy-six percent of latex allergic patients with spina bifida have IgE to a 27-kD protein while only 20% of healthcare workers recognize this allergen.²⁶ This protein, however, is only one of many that are allergens^{8-10,19,26,27} and likely represents a qualitative rather than quantitative difference.

The potential differences between the antisera and the reference preparations used in these assays were further analyzed. Proteins in the reference extracts were compared using SDS-PAGE with silver staining or Western blotting and appear to represent similar

protein profiles (silver staining) and similar antigen profiles (rabbit antisera). The relative proportions of certain protein bands were different (ie, 14 kD). The major difference observed between the antisera was in the reactivity of the pooled human sera with the glove extract. Human IgE reacted with ammoniated latex proteins with a profile similar to that of rabbit, but less intensely. This difference may be due to the lower levels of IgE found in human serum. The reactivity of the pooled human serum to the glove extract was considerably different, with much of the reactivity directed against polypeptides greater than 40 kD. Except for the 46-kD protein found in both ammoniated latex proteins and the glove extract, the IgE reactive proteins in glove extract were not evident by silver staining or by reactivity with the rabbit antisera. Since the protein antigens in gloves must be derived from the same *Hevea brasiliensis* proteins found in ammoniated latex proteins, the data suggest these antigens may be altered during manufacturing. It is not known whether these antigenic determinants are different or if they just appear in altered form such as multimers or aggregates with similar antigenic determinants. It is likely that these IgE reactive proteins represent similar antigenic determinants and not neoantigens. This is supported by the fact that the immunochemical assays which employ an ammoniated latex protein extract and a glove extract yield similar results in this study and one previously reported.¹⁸ Comparisons of the immunochemical assays would be simplified if a common reference standard were used by all laboratories.

Finally, we compared the antigens and allergens in several glove extracts. The Western blot analysis demonstrated that the immunoreactive profiles of the proteins in these glove extracts were nearly identical. The data demonstrate that the immunochemical methods recognize similar antigens. In spite of the differences in the methodology of the immunochemical assays, these assays correlate well and are spe-

cific for latex allergens/antigens. Furthermore, these assays are more sensitive than the modified Lowry assay.

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