

Protein and allergen assays for natural rubber latex products

Vesna J. Tomazic-Jezic, PhD, and Anne D. Lucas, PhD *Rockville, Md*

The major issues in the efforts to alleviate allergy to natural rubber latex (NRL) proteins are prevention of allergic reactions and decreasing future sensitization. An accurate estimate of the potential allergenicity of NRL products is essential as guidance to manufacturers and users. Development of a reliable *in vitro* method for the quantitation of NRL allergens has been impeded by (1) limited knowledge of the individual allergens among NRL proteins, (2) variations in the protein profiles among NRL products, and (3) heterogeneity of responses to NRL allergens among sensitized individuals. The current methods include measurement of either the total or the antigenic NRL proteins. Although these methods provide valuable information, all except one have not been validated or standardized. The lack of sensitivity and specificity of this standardized method and poor correlation with the allergenic protein levels have mandated the need for a better assay. In the past year, an immunologic method for the quantitation of all antigenic proteins has been developed as a national standard. The method provides a significant improvement in sensitivity and specificity of NRL protein measurement, but the question of relevance to allergen content remains open. In the last several years, the intensive research aimed at purifying and characterizing individual allergens among NRL proteins resulted in the identification of more than 10 major allergens. On the basis of this knowledge, present efforts are focused on the development of a method for quantitation of relevant allergenic NRL proteins. (*J Allergy Clin Immunol* 2002;110:S40-6.)

Key words: *Natural rubber latex, protein measurement, glove powder, standard test, reference protein*

After more than 10 years of intensive research and other efforts, the problem of natural rubber latex (NRL) allergy is still not resolved. In spite of significant advances during that time, many questions and issues remain. Significant progress has been made in the education and prevention of NRL allergy. The protein levels on finished NRL products across the market are lower than they were 5 to 10 years ago. Our knowledge about specific NRL allergens responsible for sensitization has markedly increased, and there has been significant

Abbreviations used

AL: Ammoniated raw latex
ASTM: American Society for Testing Materials
LEAP: Latex ELISA for antigenic proteins
NAL: Nonammoniated raw latex
NRL: Natural rubber latex

progress in diagnostic and risk evaluation methodology. However, allergens are still present on the NRL products, and users are still being sensitized.

NRL allergy results from extended and repeated exposure to NRL proteins. The dose, the duration, and the route of exposure are the determining factors.¹⁻³ From the Food and Drug Administration's perspective, the safety and effectiveness of regulated medical devices are the main concerns. The available data clearly support the fact that NRL medical gloves are effective products, providing an excellent infection barrier. The problem of allergic reaction to NRL proteins has raised concerns regarding the safety of NRL gloves. Reducing the overall protein levels of NRL in medical devices was an initial approach, but to ensure safety, it is essential to identify and protect sensitized individuals and to prevent or minimize further sensitization by controlling exposure to allergenic proteins. This cannot be accomplished without adequate diagnostic tests and reliable methods for allergen quantitation. Although ongoing research is directed toward quantitation of biologically relevant proteins in NRL, no standard allergen test has been developed.

This article attempts to summarize the past and present efforts in the development of methods for quantification of NRL proteins, antigens, and allergens, including a discussion regarding future steps toward the accomplishment of this goal.

METHODS FOR MEASUREMENT OF TOTAL NRL PROTEIN

In a discussion of methodology for the quantitation of allergens on NRL products, the critical point is that the test reagents recognize all relevant proteins that may be responsible for allergy induction. Efforts to develop a reliable and accurate *in vitro* method for the quantitation of NRL allergens have been hampered by 2 major obstacles. One of them is a limited knowledge of the identity of individual allergenic proteins. However, in the last several years, there has been significant progress in this area, and more than 10 allergens have been identified.⁴⁻⁷ Because NRL contains other proteins that have not been characterized, this number is probably not final. Two-

From the Food and Drug Administration, Center for Devices and Radiological Health, Rockville, Md.

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Reprint requests: Vesna J. Tomazic-Jezic, PhD, FDA, Center for Devices and Radiological Health, Office of Science and Technology, Division of Life Sciences, HFZ-112, 12709 Twinbrook Pkwy, Rockville, MD 20852.

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TABLE I. Methods for measurement of natural rubber latex proteins, antigens, and allergens

Method	Type	Advantages	Disadvantages
Modified Lowry method (D5712)	Chemical method, measures total proteins	Standardized, available as a kit	Insufficient sensitivity, chemical interference
Amino acid analysis (HPLC)	Chemical method, measures amino acids	Sensitive, reproducible	Expensive equipment, technical expertise
LEAP	Immunologic method, measures antigenic proteins	Sensitive, available as a kit	Assay format, not standardized
RAST inhibition assay	Immunologic method, measures antigenic or allergenic proteins	Good assay format, very sensitive	Radioactive isotope, not standardized
ELISA inhibition test	Immunologic method, measures allergenic proteins	Good assay format, colorimetric, good sensitivity	Uses human serum, cannot be standardized
ELISA inhibition test (D6499)	Immunologic method, measures antigenic proteins	Sensitive, standardized	New as a standard, unknown reproducibility of standard reagents

HPLC, High performance liquid chromatography; *LEAP*, latex ELISA for antigenic proteins.

dimensional electrophoresis revealed about 60 proteins that can react with human IgE antibodies. Although some of them may be isomers or cleaved fragments of larger molecules, they may be important contributors to overall allergenicity of NRL proteins.⁸ More recently, 30 significant allergens in NRL were described.⁹ Another important factor to be considered in methodology development is the heterogeneity of the IgE responses among sensitized individuals.¹⁰⁻¹² This is, at least in part, due to the variations in the protein composition from product to product. The most pronounced differences in the response patterns were observed between sensitized health care workers and sensitized children with spina bifida.¹³

When these findings were considered, the initial approach in method development was to quantitate all NRL proteins. The assumption was that the total level of protein is proportional to the level of potential allergens. The Modified Lowry method, a chemical assay that measures all proteins, became the American Society for Testing Materials (ASTM) standard (D5712) in 1995.¹⁴ It was the first standardized and validated method with great utility for screening NRL products (Table I). As a result of the availability of this standard, the Food and Drug Administration could issue a recommendation that manufacturers may state a total protein level on their products as a guide for users in product selection.¹⁵ With the frequent use of this method, several disadvantages became evident. As manufacturers successfully decreased the protein levels in NRL products, the assay was not sufficiently sensitive to accurately quantitate the remaining proteins. However, NRL proteins even below the detection level could induce a positive skin reaction.¹⁶ Furthermore, the Lowry assay is prone to the interference of chemical additives in NRL products and also includes in the measurement other proteins, which may have been added during the manufacturing process. These factors are probably responsible for the generally poor correlation of the Lowry method with the biologically active protein content of the products. At present, there is an initiative to standardize amino acid analysis as another chemical method for the quantitation of NRL proteins. The analysis is based on a complete protein

hydrolysis and therefore includes in the measurement all small peptides and single amino acids that may not have any biologic relevance. However, it is a sensitive analytical method, and it may serve as a good reference method for total protein evaluation. Because of the need for expensive high performance liquid chromatography equipment and specific technical expertise, this would be an impractical method for routine testing.

QUANTIFICATION OF NRL ANTIGENIC PROTEINS

In the pursuit of a method for allergen quantitation, the next step after the chemical analyses of total protein content was an immunologic method for the measurement of antigenic proteins. The latex ELISA for antigenic proteins (LEAP) test, designed by Guthrie Research Institute,¹⁷ is an ELISA that uses rabbit anti-NRL protein antibodies (Table I). This test has a greater sensitivity than the Modified Lowry standard test and is available as a kit. However, the test format requires binding of unknown protein samples to the assay plate, to which rabbit anti-NRL serum is then applied. The protein binding to the plate depends on the overall concentration and the molecular weight of individual proteins. Because the test samples vary in both concentration and size distribution, uniform binding of the sample proteins can be neither ensured nor validated.

Two other tests used in research laboratories, the RAST inhibition and the ELISA inhibition test, have avoided this problem by applying a different format. The measurement of antigenic protein levels in both tests is based on the capacity of rabbit anti-NRL serum to react with the NRL protein sample. A test protein is exposed to anti-NRL serum in a separate inhibition step. After the antigen-antibody reaction is completed, the unbound antibodies are exposed to the reference antigen, and the percent of serum inhibition represents the amount of protein in the test sample. The reference antigen is a defined source of NRL protein, which is either passively attached to the assay plates or covalently bound to a solid phase. The inhibition format is both sensitive and reproducible. In addition, this format can be easily used for the quanti-

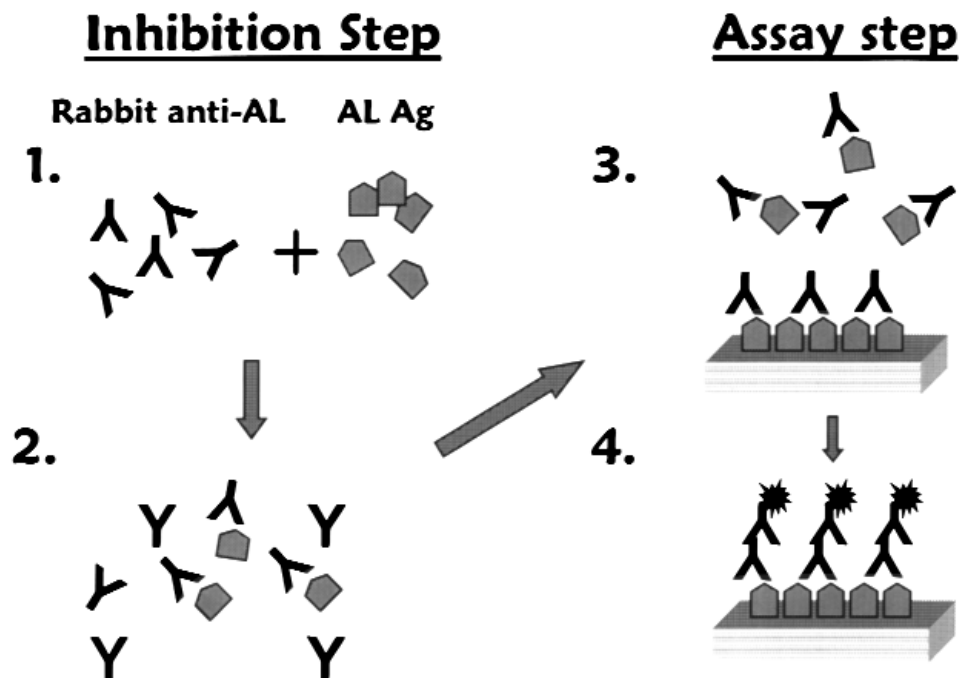


FIG 1. ELISA inhibition test (D6499) standard format. AL antigen is pool of 6 samples of high and low ammonia raw NRL. Rabbit antisera generated by immunization with standard antigen in complete (CFA) and incomplete (IFA) Freund's adjuvant. Inhibition step performed in separate plates, sera transferred in the antigen-coated assay plates. Horse radish peroxidase-conjugated secondary antibodies used to visualize reaction.

tation of antigenic proteins with rabbit anti-NRL serum and the quantitation of specific NRL allergenic proteins with human anti-NRL serum.^{18,19} However, neither of these methods can be developed into a standard allergen assay because the potency and specificity of human anti-NRL IgE antibodies cannot be standardized as a reference antiserum.

ELISA INHIBITION TEST: THE ASTM D6499 STANDARD

Although all previously described methods provide valuable approaches and information, none except the Modified Lowry method have been validated or standardized. The lack of sensitivity and specificity of the Modified Lowry method mandated the development of a better standard test. Because the knowledge about the identity of specific NRL allergens was still insufficient for development of an allergen assay, a test for quantitation of antigenic NRL proteins was the next logical step. In collaboration with Guthrie Research Institute and the National Institute for Occupational Safety and Health, we developed a protocol for the ELISA inhibition test,²⁰ which was recently accepted as a standard by the ASTM.²¹ The 2-step protocol includes inhibition of rabbit anti-NRL serum with a test protein sample as a separate initial step (Fig 1). The inhibited serum is transferred

from the inhibition plates to the antigen-coated assay plates. The binding of remaining antibody to the plates is visualized by the enzyme-conjugated second antibodies and the appropriate substrate. This method measures all proteins capable of inducing an antibody response in rabbits, and it is therefore defined as an antigenic protein assay. The inter- and intra-laboratory reproducibility and sensitivity of the test are good.²¹ Considering variations in the amount and relative ratio of individual proteins in different NRL product samples, the accuracy and specificity of the measurement will be established with further use and validation.

Furthermore, an issue that still requires clarification and further discussion is the measurement of powder-bound protein. The NRL protein binding to glove powder has been well documented,²²⁻²⁴ but the proportion of powder-bound protein to the glove-bound protein and to the total protein content has not been determined. Because of the role of powder-bound protein in sensitization and allergic reactions to NRL, this information is important for proper risk assessment. It has been suggested that the amount of protein on the glove powder might not be proportional to the protein level on the glove, and it may depend on the specific steps in the manufacturing procedures.²⁵

Current ASTM methods (D6499²¹ and D5712) quantitate soluble proteins in glove extracts after centrifugation

TABLE II. Measurement of powder-bound and extractable protein in medical gloves

Protein level (μg/mL)	Low protein gloves*	High protein gloves†
Whole extract (with powder)	0.45	14.48
Clear extract (powder removed)	0.37	12.14
Powder pellet	0.15	2.08
Calculated total (clear + powder pellet)	0.52	14.22
% Difference		
Whole extract vs clear extract	21.6	19.3
Whole extract vs calculated total	-14.5	1.8

*Average of 11 glove samples, SD in the range of 15% to 25%.

†Average of 9 glove samples, SD in the range of 10% to 15%.

and elimination of all particulate material. The protein in the powder pellet excluded from the measurement may have a significant impact on the total allergenicity of the NRL product. To clarify this issue, we evaluated the centrifuged extracts in comparison with the whole, uncentrifuged extracts from a randomly selected group of high-protein gloves (9 samples, <2 μg/mL) and a group of low-protein gloves (11 samples, >1 μg/mL). The data indicated that the whole extracts, those that include glove powder, contain an average of 19% to 21% more protein than the clear centrifuged extracts (Table II).

Using the same procedure, we also measured protein values of the powder pellets and added them to protein values of the respective clear centrifuged extracts. The whole extract values were compared with calculated total protein values (clear extract + powder protein). For the high protein gloves, the values were very close, with about 2% difference. For the low protein gloves, the difference was up to 15%, which is probably due to a higher experimental error in the low-dose range, close to the detection limits of the method. Correlation coefficients for whole and centrifuged extracts were 0.82 for the low protein gloves and 0.84 for the high protein gloves (Table III). Correlation coefficients for the measured total protein (whole extract) and the calculated total protein levels (clear extract + pellet) were 0.73 and 0.91 in the low- and the high-protein gloves, respectively. The protein levels on the powder pellet showed a poor correlation with either whole or clear extracts in both groups of gloves. This finding suggests that the level of powder-bound protein may not be proportional to the protein level of the finished product and that it may be affected by specific manufacturing processes. Powder may need to be included in the measurement to ensure an accurate evaluation of the potential risk for sensitization.

It is also necessary to determine whether the binding of proteins to powder is a selective or a random process. The relative allergenic potential of individual allergens depends on their amounts, which may not be uniform in all NRL products.²⁶ Allergenic potential also depends on the route of exposure. Because more proteins are absorbed through mucous membranes than through the skin, powder-bound proteins may have a relatively high-

TABLE III. Correlation coefficients among protein levels in glove extracts with and without powder*

Extracts compared	Low protein gloves	High protein gloves
Whole extract with clear extract	0.82	0.84
Whole extract with powder pellet	0.40	0.49
Whole extract with calculated total	0.73	0.91
Clear extract with powder pellet	0.55	0.08

*Numbers represent correlation coefficients for groups of 11 samples in low protein gloves and 9 samples in high protein gloves.

er allergenic potential than those exposed through the skin only.²⁷ This issue will become even more important in the future, as specific allergen tests are developed.

THE SELECTION OF THE NRL REFERENCE PROTEIN

The commonly used sources of NRL proteins for research purposes and test development include extracts from nonammoniated raw NRL, ammoniated raw NRL, and finished NRL products. The selection of the most appropriate and the most relevant source of NRL proteins to be used as a standard antigen has been repeatedly discussed in the process of development of both diagnostic tests and methods for antigen and allergen measurement. The same question has been extensively discussed during the development of the ELISA inhibition test.

Nonammoniated latex (NAL) proteins are native, unchanged *Hevea brasiliensis* proteins, preserved in glycerol or frozen immediately after harvesting. When NRL is collected from a particular clone of the *H. brasiliensis* tree, by means of a standardized extraction procedure, the protein content and specificity are uniform and reproducible. NRL was the main source for studies of protein size distribution and identification of the individual proteins. It has been widely used as a reference protein for in vitro assays, including 3 assay kits for human IgE quantitation. NAL extracts are used as skin testing reagents and as antigens for generation of anti-NRL antisera in rabbits. Studies show that NAL extracts contain the largest number of proteins in comparison with other sources. Rabbit anti-NAL sera demonstrate the widest repertoire of antibody specificities in reactions with various sources of NRL protein.²⁸

Another source of NRL proteins is ammoniated raw latex (AL). Ammonia, added to NRL immediately after harvesting, hydrolyzes the native proteins, resulting in a relative reduction of large molecular weight proteins and clustering of proteins in a lower molecular weight range. It has been suggested that AL may be a better source of reference protein than NAL, because it is actually the source material for NRL products, and therefore has a better representation of proteins present on the finished

TABLE IV. Affinity of rabbit anti-NRL protein antisera to react with AL and NAL proteins*

Antigen tested	Anti-NAL (CFA)	Anti-NAL (Titermax)	Anti-AL (CFA)	Anti-AL (Titermax)
NAL-7	+++	++	++	—
NAL-St	+++	++†	++	—
AL-4	+++	—	—	—
AL-5	+++	—	+/-	+/-
AL-6	+++	+	+	—
AL-D	+++	—	+	+†

*Index of binding intensity determined on the optical density (OD) level under the same experimental conditions (+++, OD 2-4; ++, OD 1-2; +, OD 0.3-1; +/-, OD <0.3).

†Antigen tested is the same as used for rabbit immunization.

TABLE V. Correlation coefficients of ELISA inhibition test and other methods for NRL protein measurement

Test	ELISA inhibition test	
	AL antigen*	NAL antigen*
ELISA inhibition (D6499)	1	0.17
Modified Lowry (D5712)	0.55	0.5
Amino acid analysis HPLC	0.77	0.21
LEAP assay	0.73	0.14
RAST-1†	0.43	0.89
RAST-2†	0.45	0.92
SPT	0.52	0.53

Analyses were performed on a group of 15 glove samples.

AL, Ammoniated latex; NAL, nonammoniated latex; HPLC, high-performance liquid chromatography; LEAP, latex ELISA for antigenic proteins; SPT, skin prick test.

*The same antigen was used for immunization, for coating the assay plates, and as a reference antigen.

†The same test was performed in different laboratories.

products. Furthermore, there is the possibility that protein hydrolysis may expose or create new epitopes, which are not present in the native NAL. Although this is a valid point, the presence of "neoantigens" in AL extracts has not been well documented. When considering AL as a potential standard antigen, one must account for the variability of AL as a source material. A different concentration of ammonia added to the native material and time differences between harvest and product manufacturing result in large variations in the protein composition among NRL products.

The third option, use of extracts from the finished NRL products (eg, gloves), has been shown to be even more variable in protein composition. In addition to variations in the source material, the difference in manufacturing procedures introduces additional factors that change protein levels and their composition. The use of glove extracts as a reference protein for testing was generally abandoned, especially in the light of the observed heterogeneity in responses of sensitized individuals.

However, the choice of AL or NAL as a reference protein in the various tests is still the subject of ongoing discussions. AL has been used as a source antigen in the LEAP assay and is a standard antigen in the new ELISA

inhibition test (Fig 1). NAL has been used in other formats of the ELISA and in RASTs and is a basis for several in vitro kits for human IgE detection. Both AL and NAL proteins have been used as skin test reagents,^{16,29,30} but NAL is used more widely. A direct comparison of 2 reagents in the same individuals showed equivocal responses at the same protein level.²⁹ Some investigators consider NAL superior to AL because of the constant availability of the source and better reproducibility. NAL has been used as a source for purification of all allergenic proteins identified so far, which supports the hypothesis that all major allergenic proteins of NRL products are present in the NAL preparation.

The reference antigen in the present standard ELISA inhibition test is a pool of 6 AL extracts,²⁰ each with a specifically defined origin and known time and dose of ammonia exposure. One high ammonia and one low ammonia sample were obtained from Firestone Inc. (Akron, Ohio), Guthrie Latex Inc. (Tucson, Ariz), and Ore & Chemical Corp. (Virginia Beach, Va). The standard antiserum in this test is a pool of sera from 3 to 4 rabbits immunized with the standard antigen in 2 different laboratories. With the well-defined source of NRL proteins and immunization protocol, a reasonable reproducibility of standard reagents is assumed. For the continuity of the standard test performance, the reproducibility of the reagents remains to be validated.

Our experience indicates that the specificity of the rabbit anti-NRL antibodies can vary significantly, depending on both the differences in the source of immunizing antigen and the immunization protocol. Table IV presents the binding affinity of several rabbit anti-NRL antisera to protein preparations from various sources. Sera from rabbits immunized with Titer Max adjuvant (Cyt Rx Corp., Norcross, Ga) demonstrated a clear preference binding to autologous antigen. Sera from rabbits immunized with complete Freund's adjuvant did not show such preference. Also, antisera from rabbits immunized with NAL antigen presented a wider spectrum of specificities than antisera from rabbits immunized with AL antigen. These data show effects of the immunizing protein source and of the immunization protocol on the capability of the rabbit sera to react with NRL proteins and its impact on the test end points. The choice of adjuvant played a significant role in antibody affinity.

We further investigated the performance of the AL and NAL antigens in the ELISA inhibition test (Table V), comparing the protein values measured with 2 sets of reagents. The protein levels obtained by using AL antigen and anti-AL serum correlated well with the values obtained by the similar LEAP test and by amino acid analysis. The protein levels in the same samples obtained with NAL reagents correlated well with results of the RAST inhibition test. Skin testing data indicated equivocal correlation in both cases. However, only 10 individuals were tested, and the correlation was based on a positive or negative response. More extensive skin testing is needed for a more definitive conclusion. Our data demonstrate that the selection of one or the other antigen source may generate markedly different protein values with a very low correlation between them. Therefore, for accurate risk assessment, it is essential to determine which of the test reagents generates measurements that reliably reflect the allergenic potential of NRL.

EMERGING APPROACHES TO ALLERGEN EVALUATION

With the great progress in the identification and evaluation of the biologically relevant allergenic proteins, future testing for NRL allergens may now be focused in a different direction. Attempts have been to replace preparations containing all NRL proteins with combinations of several major allergenic proteins as a standard reference.³¹

Several of the known allergens have been screened for their capacity to react with human anti-NRL IgE.³²⁻³⁵ In a recently published article by Kurup et al,³⁵ allergens Hev b 1 to Hev b 7 showed IgE antibody binding in an ELISA with 53% to 100% of the sera. The percentage of positive responses varied for the individual allergens and was different between sera from health care workers and sera from children with spina bifida. These data indicate that a combination of 2 to 3 allergens may be sufficient to identify all IgE-positive individuals. However, skin testing of sensitized individuals with 6 major allergenic recombinant proteins³² showed that 7% did not respond to any of the allergens, and an additional 15% responded weakly to only 1 of the 6 allergens. Although these limited data are not yet conclusive, it appears that a combination of a few allergens might be sufficient and appropriate for detection of serum-positive individuals. A relative ratio of individual allergens in such a combination is an additional issue in the light of a recent finding that the amount of individual allergens, in proportion to one another and to a total protein level, varies from product to product.²⁶ Another approach that may be promising is detection of individual allergens with monoclonal antibodies. A sandwich ELISA for one of the allergens (Hev b 1) has been reported recently.³⁶ Quantitation of allergens with a panel of specific monoclonal antibodies would eliminate concern regarding different ratios of individual allergens in test samples.

When discussing the development of future methods, one should consider the relative importance of *in vitro* tests for serum IgE antibody detection and tests for NRL

allergen quantitation. The positive diagnosis of NRL allergy is based on patients' medical history, clinical symptoms, positive skin test responses, and the presence of serum IgE antibodies. Serum anti-NRL IgE measurement is therefore only one of the methods, which actually represents more qualitative than quantitative support to other methods for diagnosis of allergy. On the other hand, the measurement of allergen level on the NRL product would be a stand-alone test for the assessment of the risk of allergic reaction and sensitization. Therefore, the quantitative accuracy and specificity of such a test is of immense importance. A reference protein for evaluation of allergen levels must include not only the proteins to which sensitive individuals respond most frequently but also all other proteins in NRL that, under special circumstances, have an allergenic potential.

With a good number of allergens already identified, the ultimate goal of measuring only relevant allergenic proteins on finished NRL products appears closer to realization. The attempt to use a combination of individual allergenic proteins as a reference antigen is a move in the right direction. However, several lingering issues need to be addressed before a practical allergen test can be developed: (1) selection of appropriate allergens, (2) the optimal relative ratio of individual allergens, (3) whether recombinant allergenic proteins that will be used in test development are identical to native allergens, and (4) the need for careful evaluation of test format and test parameters. It has been suggested that only few proteins may be used as "indicator allergens."³¹ Future studies should reveal adequacy of only a few representative allergens versus all allergenic proteins to be included in a reference antigen. The significance of the variations among the products and possible differences among powder-bound and water-soluble proteins need to be determined. The limited published data indicate that there may be a difference in the allergenicity between recombinant and native NRL proteins.^{32,35} The tertiary protein structure may be responsible for the change in allergenic epitopes. At present, mouse monoclonal antibodies and rabbit polyclonal antibodies against individual allergens have been considered for use in the test. Depending on the test format selection, one or both antibodies may be used. Regarding the specific test format, the ELISA inhibition test and the sandwich ELISA are being evaluated for suitability as NRL allergen test formats.

In conclusion, the accurate assessment of the potential allergenicity of NRL products is of paramount importance for alleviation of the NRL allergy problem. The primary factors to be considered in this process are the accuracy, sensitivity, and reproducibility of the test. It is also important that the test be practical, that it can be performed in a large spectrum of laboratories, and that the reagents can be standardized. The process of developing better methods for the quantitation of NRL proteins is evolving in several directions. There are ongoing efforts to improve the present standard test for antigenic proteins and validate the standard reagents. On the other hand, as sufficient data become available, the efforts will be focused on the development of a specific NRL allergen assay.

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