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**IDENTIFICATION OF ANTIGENIC AND
ALLERGENIC NATURAL RUBBER LATEX
PROTEINS BY IMMUNOBLOTTING***

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ABSTRACT

Quantitation of proteins in finished natural rubber latex (NRL) products is essential in predicting their allergenic potential. The ASTM standard Modified Lowry method for measuring total protein content has been used for several years. Most recently, ASTM published a standard for more sensitive and more specific enzyme immunoassay for quantitation of antigenic NRL proteins. It is an ELISA inhibition assay, using rabbit anti NRL sera. Since the measurement of proteins in this method depends on recognition capacity of rabbit antibodies, the selection of an appropriate protein source for rabbit immunization is crucial for the accuracy

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of such test. In this study, we evaluated the composition of NRL proteins from ammoniated (AL) and nonammoniated (NAL) raw latex and from finished NRL products, and compared the effectiveness of sera from rabbits immunized with NRL proteins, to react with those extracts. Immune rabbit sera were analyzed by immunoblotting against extracts of several samples of AL, NAL, and glove proteins. In the NAL extracts, we identified 26–28 protein bands by SDS-PAGE. AL samples had between 6 and 9 bands with a great variation in the band positions among the samples. The Western blot analysis showed that anti-AL rabbit serum reacted with 4–9 protein bands in various AL extracts. The highest intensity of reaction was observed with the extract used to immunize the rabbits. Similar reaction was observed with anti-NAL serum. However, when the antisera were blotted against NAL extracts, anti-NAL serum reacted more strongly and with a larger number of proteins than anti-AL serum. In summary, anti-NAL serum recognized an equal number of proteins in AL extract as anti-AL serum. However, anti-AL serum recognized fewer protein molecules in NAL extract than anti-NAL serum. Our findings suggest that NAL extract contains more individual proteins than other extracts, and sera from rabbits immunized with this antigen have a greater capacity to react with a wide spectrum of NRL proteins. This finding may be helpful in selecting the representative reference antigen and antiserum for further efforts in NRL protein quantitation.

INTRODUCTION

The principal approach to prevent or minimize further sensitization of natural rubber latex (NRL) product users is the reduction of allergen levels on the finished latex products and the development of reliable methods for measuring the amounts of remaining allergens. A large number of studies are describing attempts to characterize NRL proteins and to identify those responsible for allergy induction. Presently, several different NRL proteins are identified as allergenic^[1–4] and ten of them are recognized by the International Union of Immunological Sciences (IUIS) as “major allergens.” As the research continues, more proteins in NRL protein mixture may be found to have an allergenic potential.



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Although some proteins are intrinsically more allergenic than others, there are other factors that may affect the potential allergenicity of individual proteins. The genetic potentialities of the host, the environmental factors concomitant to the exposure, and protein resistance to biodegradability are important in allergy development. However, the amount of protein and the route of exposure are the two most significant contributors to overall allergenicity. Some of these factors cannot be controlled, but the quantitation of proteins on finished NRL products may indirectly predict a potential allergenicity. As identification of the individual allergens is still an ongoing effort, to insure the most accurate prediction of allergenic potential, it is necessary to include in the measurement process all potentially allergenic proteins on finished NRL products.

It has been observed among NRL products that, not only the total protein levels can vary significantly, but also the relative ratios of individual proteins.^[5] A wide diversity of responses to particular NRL allergens among sensitized individuals clearly reflects the significance of the above factors in an overall allergic reaction.^[5,6] Until all allergens are identified with certainty, the safest approach is to quantitate all proteins in NRL as an indirect indication of the potential allergenicity level. This approach has been applied in the past to the development of test methodology for evaluation of the allergenic potential of latex products.^[7] Recently, an ELISA inhibition assay has been developed to measure antigenic NRL proteins using rabbit anti-NRL antiserum.^[8] A selection of the most suitable and representative source of NRL proteins as a standard reference antigen is critical to minimize the possibility of missing some potential allergens. The available sources include proteins from raw ammoniated latex (AL), raw nonammoniated latex (NAL) and proteins extracted from finished latex products. Both AL and NAL extracts have been used for skin testing and for *in vitro* assays.^[9-13] However, the published literature is still not conclusive regarding which of these sources contains the most of potential allergens in NRL and would be the most suitable antigen to produce the complete repertoire of rabbit antibodies.

The purpose of this study was:

- a) to determine source to source variations in molecular weight distribution of individual proteins in three sources of latex proteins,
- b) to evaluate antibodies from rabbits immunized with proteins from these three sources for their specificity and their capacity to recognize NRL proteins, and
- c) to compare rabbit sera recognition capacity with the recognition pattern of human sera.



EXPERIMENTAL

Preparation of Antigen and Antibodies

Several protein extracts from raw nonammoniated latex (NAL), raw ammoniated latex (AL), and from finished NRL products were used in this study. Raw material was obtained from Malaysia and extracted as described earlier.^[5] The NRL products used for protein extraction in this study were commercially available brands of surgical and examination gloves and a dental dam. All extracts were concentrated with an Amicon filtration unit using a YM1 membrane, MW cutoff = 1000 Da. The samples were stored at 4°C for up to 1 week or at -20°C and -70°C for the extended periods. The preliminary evaluation confirmed the stability of protein preparations under these conditions.

Rabbit sera used in this study were obtained from animals immunized with latex proteins extracted from various sources. The sources included raw NAL collected from *Hevea brasiliensis* in Malaysia and in India, two raw AL samples collected from *H. brasiliensis* in Malaysia and extract from medical gloves. A total of three NAL and two AL protein antisera were used in this study to evaluate their capacity to recognize individual proteins in various latex protein extracts. A pool of adult human sera was prepared from more than 30 samples of sera from NRL-sensitized individuals pre-screened by immunoblotting to include a wide range of antibody specificities for different NRL allergens. A pool of pediatric sera was obtained from the spina bifida children, which frequently present a reaction pattern markedly different from the adult population.

Protein Electrophoresis and Immunoblotting

The molecular weight distribution of latex protein extracts was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All protein samples were diluted in 10 mM Tris-HCl, pH 7.4, and sample buffer (62.5 mM Tris-HCl, pH 8.0, 2% mercaptoethanol, 0.01% bromophenol blue, and 1 mM EDTA) to a concentration of 4 mg/mL. Gel electrophoresis was performed with commercial 8-25% gradient minigels (Phast System, Pharmacia/LKB Biotechnology, Piscataway, NJ), applying 1 µL of each sample per run. Gels not used for western blots, were subsequently stained with Coomassie R-350 or double stained with Coomassie and silver stain. For Western blots, proteins were transferred from gels to a transfer membrane (PolyScreen, NEN—Research Products, Boston, MA) using 40 mA per gel for 45 min. A pre-stained molecular weight marker



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verified the successful transfer of proteins. The blots were then blocked with a 3% solution of bovine serum albumin (BSA) in Tris buffer solution (TBS) for 3–6 h at room temperature. After washing three times with blocking buffer containing 0.05% Tween-20 (TTBS), the blots were incubated with the rabbit anti-NRL sera for 12–15 h. The blots were then washed three times with TTBS and incubated with alkaline phosphatase-conjugated (AP) polyclonal goat anti-rabbit IgG, before the addition of the substrate.

The same procedure was applied to determine allergenic proteins, using human sera from latex allergic individuals as a source of IgE antibodies. AP-labeled goat anti-human IgE antibodies (Cat. # A-3525, Sigma Immunochemicals, St. Louis, MO) were used as secondary antibodies. The specificity for human IgE was confirmed by the manufacturer using an ELISA test. An AP substrate kit (Bio-Rad Laboratories, Richmond, CA) was used for the visualization of antibody binding.

RESULTS

The comparison of extracts from NRL products with extracts from raw NRL in our earlier study demonstrated significant variations in both the numbers of protein bands and their molecular weight distribution.^[5] In this study, the NRL protein extracts were evaluated by SDS-PAGE to compare the molecular weight distribution of proteins with the recognition patterns of different rabbit sera. Figure 1 shows SDS-PAGE analysis of three types of extracts used in this study. The protein profiles in NAL extracts are usually quite uniform when a standard extraction procedure is used. There are usually about 22–28 protein bands observed on the gel. The protein distribution patterns of AL extracts can vary significantly from one another, depending on how much the native proteins were affected by ammonia. The extracts from finished NRL products were found to vary even more, as manufacturing processes and chemical additives may change or eliminate some proteins. The samples used in this study have a number of common proteins but, in some cases, marked differences were observed in the relative amounts of individual proteins. The total amount of protein in each sample was equalized before the analysis.

Five rabbit anti-NRL sera, generated by the immunization with NRL proteins from different sources, were evaluated for their capacity to react with NRL proteins in three NRL extracts. Two sera were from rabbits immunized with NAL, one from Malaysia (#1) and other from India (#2). Sera #3 and #4 were from rabbits immunized with two different extracts of AL proteins, and serum #5 was from rabbit immunized with a glove extract.

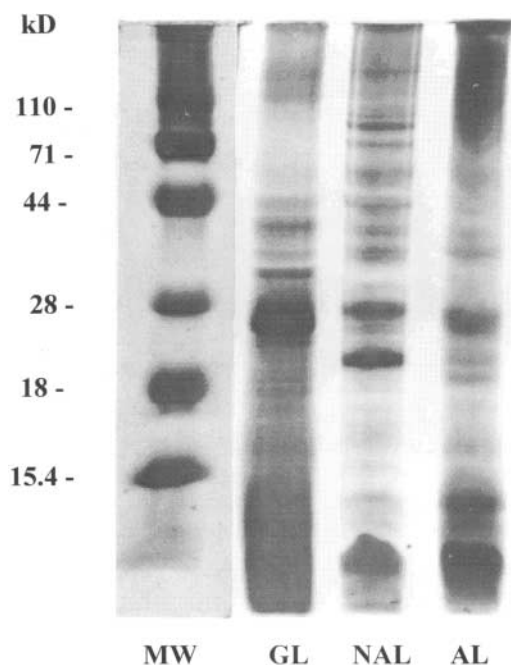


Figure 1. Molecular weight distribution of proteins in extracts from natural rubber latex gloves (GL), nonammoniated (NAL), and ammoniated (AL) raw natural rubber latex (SDS-PAGE, silver stained).

When exposed to NAL extract (Fig. 2-A), two anti- NAL sera reacted with the majority of proteins. Serum 2 from rabbit immunized with NAL from India reacted with fewer proteins than the serum from rabbit immunized with a Malaysian NAL. Anti-AL sera (Sera 3 and 4) reacted with even fewer proteins, most likely only with proteins that were present in the immunizing antigen. Anti-GL serum from rabbit immunized with glove extract showed a markedly different pattern, reacting strongly with the lower molecular weight proteins, 20 kDa and lower, while there was a very weak or no reaction at all with the higher molecular weight proteins. The same rabbit sera recognized only 9–15 proteins in the AL extract (Fig. 2-B). The largest number of proteins was again recognized by both anti-NAL sera and with a very similar pattern. Anti-GL serum reacted with the AL extract in a similar pattern as with the NAL extract, reacting strongly with lower molecular weight proteins. When evaluated against glove extracts (Fig. 2-C), anti-NAL sera reacted strongly with the proteins in MW range of 28–45 kDa,



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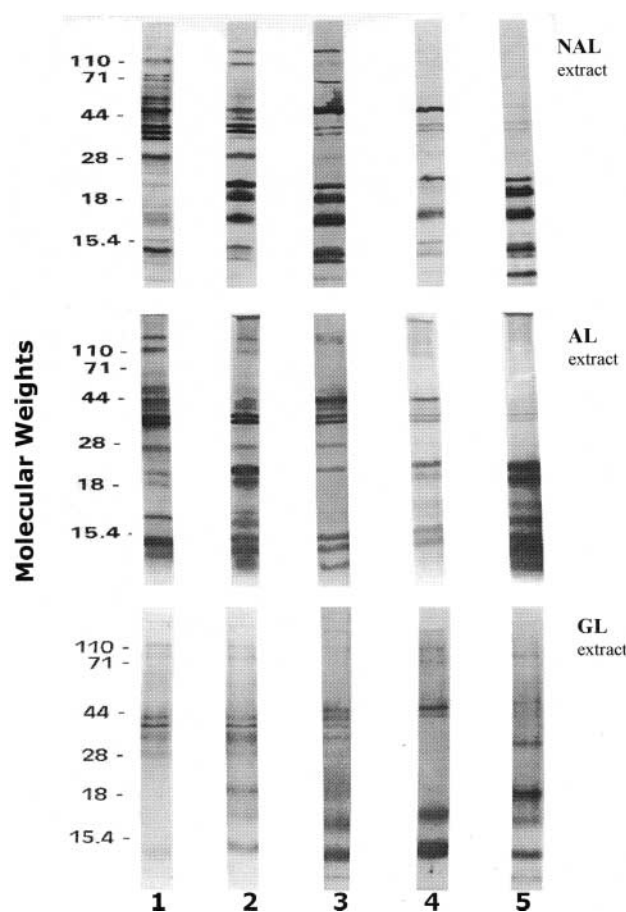


Figure 2. Recognition of the NRL proteins by various rabbit anti-NRL sera. Rabbit sera are from animals immunized with various sources of NRL proteins; 1—NAL extract (Malaysia), 2—NAL extract (India), 3—AL extract (Malaysia), 4—AL extract (Malaysia) and 5—Glove extract (Malaysia). Each of the sera was evaluated against (A) NAL protein extract, (B) AL protein extract and (C) glove extract proteins.

while anti-AL and anti-GL sera reacted stronger with the low molecular weight proteins, less than 20 kDa.

In the next group of experiments, we evaluated the capacity of anti-AL and anti-NAL sera to recognize antigenic proteins in three NRL protein extracts. The SDS-PAGE analysis indicated that NAL extract preparations



appear very uniform under the same extraction conditions. The patterns with two NAL extracts from Malaysia samples were almost identical, while the extract from Indian NRL (N-3) showed more bands, most likely due to the different clone of *H. Brasiliensis* tree (Fig. 3). The immunoblots of three NAL extracts with anti-NAL serum (#2) and anti-AL serum (#3) showed that anti-AL serum recognized fewer proteins than anti-NAL serum in all three samples.

The same two antisera were evaluated against three AL extracts (Fig. 4), which in SDS-PAGE showed a significant variation in their protein distribution, with very few proteins detected in the larger molecular weight range. The immunoblots analysis indicated that the anti-NAL serum reacted with a larger number of proteins than the anti-AL serum, some of which were not detectable in SDS-PAGE. The reaction pattern was similar to two of three AL extracts. On the other hand, the anti-AL serum reacted with a very few proteins in two AL extracts, but showed a strong reaction with the homologous AL-D extract, used for the particular antiserum production.

Figure 5 shows the reactions of the same two antisera with three NRL product extracts, two from medical gloves and one from a dental dam. The anti-NAL serum recognized a large number of proteins, some of which were not in sufficient concentration to be detected by the Coomassie staining of the gels. The anti-AL serum demonstrated only a partial recognition pattern in comparison with the anti-NAL serum. The most significant difference was

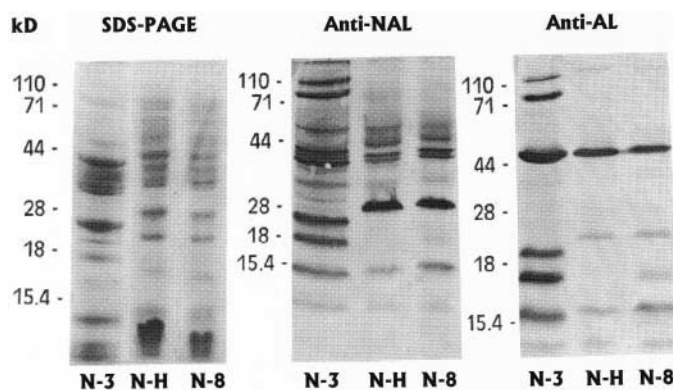


Figure 3. Comparison of the anti-NAL serum (#2) and anti-AL serum (#4) reaction with the NAL extracts prepared by the same procedure from three individual batches of the NAL. N-3, extracted from NAL sample from India, N-H and N-8 from Malaysia.



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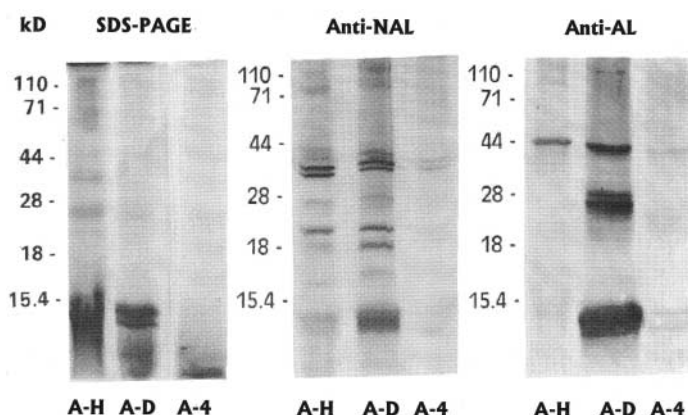


Figure 4. Comparison of the anti-NAL serum (#2) and anti-AL serum (#4) reaction with AL extracts from three different samples of AL from Malaysia. A-D extract was used as antigen for the generation of serum #4.

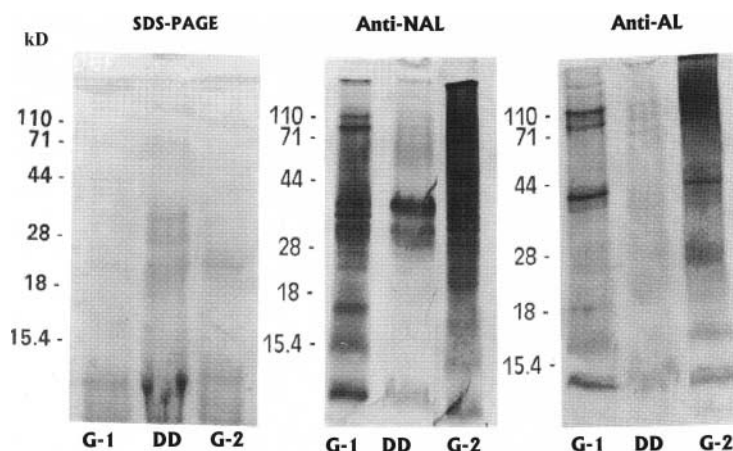


Figure 5. Comparison of the anti-NAL serum (#2) and anti-AL serum (#4) reaction with two medical gloves (GL-1 and GL-2) and a dental dam (DD) extract.

observed with the dental dam extract, where the anti-NAL serum reacted strongly with 2–3 proteins in the MW range of 30–40 kDa, while the anti-AL serum did not recognize those proteins at all. The data presented in Figs. 4 and 5 indicate that the specificity of rabbit anti-NRL sera is restricted to proteins that were present in the immunizing extract. With

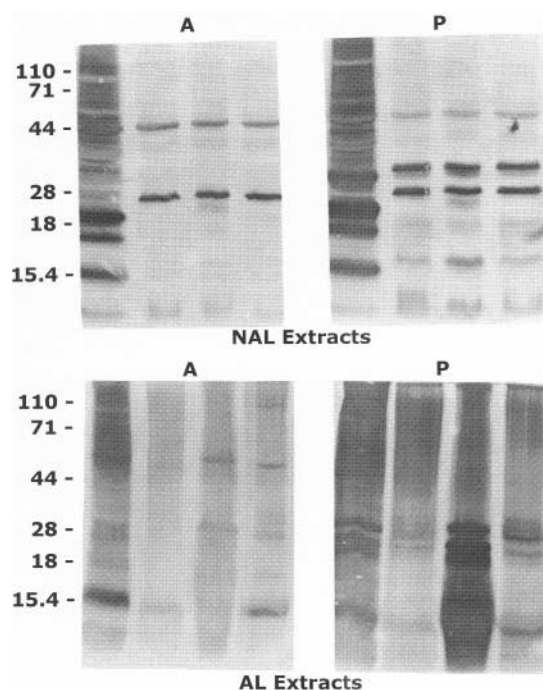


Figure 6. Identification of allergenic proteins in NAL (top) and AL extracts (bottom) using pool of immune sera from the adult population (A) and the pediatric population (P). Protein extracts in columns 2, 3, and 4 on the top are N-3, N-H and N-8 respectively. The lower part columns 2, 3, and 4 are extracts A-H, A-D and A-4 respectively. The first column in each picture shows reaction of rabbit antiserum for comparison.

both anti-AL sera and anti-GL sera, we observed only partial recognition of proteins present in extracts from other sources.

Since rabbits produce antibodies to all or to the majority of proteins used for immunization, their serum antibodies actually detect all antigenic proteins in NRL extracts. To investigate which of those antigenic proteins are actual allergens in humans, we exposed our NAL and AL extracts to a pool of sera from adult (A) NRL allergic individuals and to a pool of sensitized pediatric (P) population (Fig. 6). It has been suggested that the sensitized adult population and the pediatric population have markedly different patterns of response to individual allergens, more pronounced than among the adult population itself.^[4,14,15] It is assumed that these variations are a result of a difference in the type of sensitizing NRL products.



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Our data presented in Fig. 6 support this finding. Both human pools reacted uniformly with all three NAL extracts, but the pediatric pool reacted with more proteins than the adult sera pool (top). The difference between these two pools of human antisera was even more pronounced in the reaction with the AL protein preparations (bottom). The children sera pool demonstrated strong reaction with proteins in 25–30 kDa range and lower, but the intensity varied among different AL extracts. In addition, comparing the reaction of the human sera with protein extracts from two sources of raw latex indicated that some of the allergens present in the NAL extracts were not detected in the AL extracts. Therefore, the allergenic response may, in a large part, be dependent on the type and the level of proteins present in the sensitizing product.

DISCUSSION

This study demonstrates a significant variation in the number and the relative amount of NRL proteins among extracts from different sources. The protein profiles of NAL preparations appear uniform if the origin of the sample and the extraction procedure are the same. A marked heterogeneity was observed among extracts of various AL samples, as the amount of ammonia added in the field and the time elapsed between the harvest and the extraction can markedly change protein composition. The protein profiles in extracts from finished NRL products, as expected, varied the most. The differences in the manufacturing procedures and chemical additives contribute further to changes in the protein profiles of finished products. Therefore, it is not surprising that the specificity of the rabbit anti NRL antibodies also differs greatly, depending on the antigen used for immunization. The NAL preparations contain the highest number of proteins, as the entire repertoire of protein molecules present in the *H. brasiliensis* is preserved through the preparation. The subsequent ammoniation and processing of the liquid NRL may lead to the loss or change of some proteins.

The observed differences in the response of latex allergic individuals may be influenced by the individual genetic characteristics, but the differences in the protein content of NRL products have the most significant impact.^[5,6] We have observed restrictions to homologous antigen with rabbit anti-AL sera (Fig. 4). The same explanation should apply to a difference in the reaction patterns between pools of sera from adult and pediatric population. A number of studies already showed that the “major allergens” in allergic health care workers and allergic spina bifida children are different proteins.^[4,14–18] The major allergens have been defined on the basis of frequency of response in sensitized individuals. It is not known if the



allergenicity in this case is a result of the intrinsic properties of specific proteins or is it simply a dose-response relationship. If the latter is correct, one has to assume that other NRL proteins may also become allergenic under different circumstances. Therefore, to insure the proper diagnosis of latex sensitivity, it is important that all major allergens are included in the test reagents. Present tests for specific anti-NRL IgE antibodies showed approximately 75% specificity,^[19] but the positive diagnosis of latex allergy is usually determined in combination with other allergy tests.

The efforts to develop a methodology for the accurate measurements of the potential allergenicity of NRL products is a much harder task, because it is a single test which requires a high level of quantitative accuracy. Therefore, it is critical for such measurement to include all allergenic proteins; those already known and those that may become allergens if presented in a specific route and sufficient dose. The source of NRL proteins that would be the most reflective of the entire repertoire of allergenic and potentially allergenic proteins remains an open question. It is generally accepted that the NRL product extracts are not suitable because of a great variation in protein composition among them. Some investigators consider the AL proteins a better source than the NAL proteins, as AL represents an actual source material for the finished NRL products to which the users are exposed.^[11,20] It is theorized that the hydrolysis of proteins by ammonia may expose some new epitopes that were not exposed in the native protein and, consequently, present a "neo-allergen." While this may be a realistic possibility, our studies with polyclonal rabbit antibodies did not reveal any antigens in the AL preparations that were not recognized by the anti-NAL sera. This finding, and the fact that we observed a marked preference in specificity of reaction with a homologous combination of AL proteins and rabbit anti-AL antisera, raises a question of the appropriateness of AL as a reference protein. The protein hydrolysis is a continuous process which depends on the level of ammonia and the duration of exposure, which would make a standardizing and reproducing an AL protein source quite difficult to achieve.

On the other hand, the NAL may be easier to standardize. With a defined clone of *Hevea brasiliensis* tree, defined harvesting and standard extraction procedure, it can be easily reproduced. All native proteins are present in the NAL extracts and all allergenic proteins identified so far have been purified from the NAL preparations. Our study confirms that NAL extract contains the largest number of proteins, and that anti-NAL serum has the capability of recognizing and reacting with the largest number of proteins in all of the extracts we have evaluated. Clinical studies have also demonstrated that the NAL extract is a relevant skin testing reagent for the diagnosis of latex allergy.^[9,12,21] Comparison of NAL, AL, and glove



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extracts as skin testing reagents have demonstrated that the NRL sensitized individuals responded equivocally to all three reagents at the same total protein level.^[9]

In conclusion, our study shows that the NAL extract appears to have the most complete repertoire of NRL proteins and that serum from the NAL immunized rabbits has the highest capacity for recognizing and reacting with NRL proteins, regardless of the source. This information may be useful in the process of development and standardizing the methodology for evaluation of NRL product allergenicity.

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