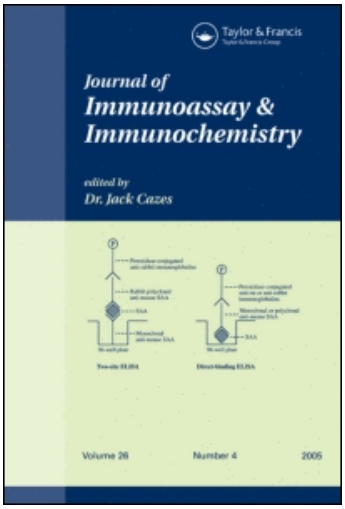
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

ELISA INHIBITION ASSAY FOR THE QUANTITATION OF ANTIGENIC PROTEIN IN NATURAL RUBBER LATEX

Vesna J. Tomazic-Jezic[a]; Michael R. Woolhiser[b]; Donald H. Beezhold[c]
[a] Center for Devices and Radiological Health, USFDA, Rockville, MD, U.S.A. [b] National Institute for Occupational Safety and Health, Morgantown, WV, U.S.A. [c] Guthrie Research Institute, Sayre, PA, U.S.A.

Online publication date: 08 July 2002

To cite this Article Tomazic-Jezic, Vesna J. , Woolhiser, Michael R. and Beezhold, Donald H.(2002) 'ELISA INHIBITION ASSAY FOR THE QUANTITATION OF ANTIGENIC PROTEIN IN NATURAL RUBBER LATEX', Journal of Immunoassay and Immunochemistry, 23: 3, 261 — 278
To link to this Article: DOI: 10.1081/IAS-120013026
URL: http://dx.doi.org/10.1081/IAS-120013026

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

JOURNAL OF IMMUNOASSAY & IMMUNOCHEMISTRY
Vol. 23, No. 3, pp. 261–278, 2002

ELISA INHIBITION ASSAY FOR THE QUANTITATION OF ANTIGENIC PROTEIN IN NATURAL RUBBER LATEX

Vesna J. Tomazic-Jezic,[1,*] Michael R. Woolhiser,[2] and Donald H. Beezhold[3]

[1]USFDA, Center for Devices and Radiological Health, 12709 Twinbrook Parkway, Rockville, MD 20852, USA
[2]National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA
[3]Guthrie Research Institute, Sayre, PA 18840, USA

Downloaded At: 10:32 16 January 2011

ABSTRACT

Evaluating allergenicity of natural rubber latex (NRL) products is essential for the successful reduction of the consumer's exposure to potentially allergenic NRL proteins. We have developed an ELISA Inhibition method for the quantitation of extractable proteins from NRL products which has good sensitivity and specificity. The method utilizes rabbit anti-NRL protein serum as a detection mechanism. The source of NRL proteins for immunization and as a reference protein in the assay is ammoniated raw latex (AL). By comparison with the Western blot analysis of the rabbit sera, it appears that the ELISA detects most of the latex proteins present in extracts. To investigate, further, this assumption,

*Corresponding author. Fax: (301) 596-6775.

DOI: 10.1081/IAS-120013026
1532-1819 (Print); 1532-4230 (Online)
Copyright © 2002 by Marcel Dekker, Inc.
www.dekker.com

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

we compared the ELISA Inhibition test with two other methods for total protein measurement. We also compared the values generated by the ELISA Inhibition test with other immunological methods for quantitation of antigenic and allergenic proteins. Comparisons were performed with 15 extracts from randomly selected surgical and examination gloves. The samples were coded separately for each test to insure the objectivity of evaluation. The antigenic protein values obtained by the ELISA Inhibition test correlated best with the HPLC amino acid analysis (correlation coefficient (cc) = 0.79) and with the LEAP assay (cc = 0.97). The antigenic protein levels obtained by the ELISA test were 3–10 times lower than those obtained by the HPLC analysis. A lesser correlation was observed with the Modified Lowry assay (cc = 0.45), which is likely due to chemical interference bias in the Lowry method. Our findings suggest that the antigenic proteins measured by the ELISA Inhibition test described here more closely represent the measure of the total protein content in the extracts. It is important to note that the relative values obtained by this method are lower than the values obtained by other total protein methods, possibly due to a large number of small peptides present in NRL products, that would only be measured by the chemical method.

Downloaded At: 10:32 16 January 2011

INTRODUCTION

A major factor in the development of an allergy is the degree of exposure to the allergen. A number of studies have revealed the correlation of sensitization to natural rubber latex (NRL) proteins with duration and frequency of the exposure to NRL products.[1,2] Similarly, in patients with spina bifida, the level of sensitization correlated with the number of surgical procedures performed on the individual child.[3,4] While avoidance of NRL is the most effective way to prevent sensitization or latex allergic reactions, it cannot be easily accomplished considering the ubiquitous nature of natural rubber. Logically, reducing the levels of allergens on the finished products should reduce the sensitization. It has been observed that the use of low allergen gloves appears to reduce the rate of sensitization among NRL product users.[1]

Reliable quantitation of NRL allergens is crucial for the adequate prediction of the potential allergenicity of finished NRL products. Several

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

methods for the quantitation of total NRL proteins,[5–7] antigenic NRL proteins,[8,9] or allergenic NRL proteins[10–13] have been developed in the past several years. The reproducibility and uniformity of the measurements and their relevance as an indicator of allergenicity has not been, however, fully established. The American Society for Testing Materials (ASTM) standard method, the Modified Lowry (D5712), that is presently used, is a chemical assay measuring total protein content.[7] After several years of experience with this method, it was established that interference of chemical additives in NRL products represent a significant impediment to accurate measurement of protein levels in NRL manufactured products. The method also lacks the sufficient sensitivity needed to quantitate the reduced protein levels found on more recently manufactured products.

A number of research laboratories applied various formats of RAST or ELISA tests to quantitate either allergenic proteins, using sera from NRL allergic patients,[11–13] or antigenic proteins using rabbit anti-NRL serum.[8–10,14] In recent years, great progress has been achieved in identifying allergenic proteins, but an accurate and reproducible test for allergen quantitation has not been developed. The uncertainty of yet unidentified NRL allergens, differences in the relative proportions of individual allergens in various NRL products and the heterogeneity of responses in sensitized patients, represent a hindrance to developing a relevant standardized allergen test.

Using an ELISA Inhibition test format, we have developed a sensitive and reproducible method for quantitation of antigenic NRL proteins. The protocol has been validated by round robin testing in collaboration with the ASTM and developed into a new standard, ASTM D6499-00.[15] Furthermore, we compared the antigenic protein values of several NRL glove extracts obtained using the ELISA Inhibition Assay with the values obtained by the other chemical and immunological methods.

Downloaded At: 10:32 16 January 2011

EXPERIMENTAL

Antigen Preparation

For the extraction of latex proteins, we have used raw latex from *Hevea brasiliensis*, which was either treated with ammonia immediately after the harvest or preserved with glycerol in its natural form. A pool of several samples of ammoniated latex (AL) was used to prepare AL antigen. For the preparation of nonammoniated latex antigen (NAL), the raw latex was obtained from *H. brasiliensis* clone 600 in Malaysia.

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

AL was sampled immediately upon arrival to the US (five to ten weeks from harvest). One lot of full ammonia (0.9% ammonia) and one lot of low ammonia latex (0.4% ammonia) were collected from three different latex suppliers. The latex was centrifuged at $76\,000 \times g$ for 2 h. The aqueous protein extract was recovered by puncturing the side of the centrifuge tube below the rubber layer to remove the aqueous fraction (C serum). Additional rounds of centrifugation were used if the C serum remained cloudy. The clarified aqueous extract was passed through a 0.45 μm filter and dialyzed against a 0.1 M carbonate buffer, pH. 9.6 using dialysis tubing of MWCO 1000 (>20 volumes each over 4 changes of carbonate buffer) for 3 days at 4°C to remove the ammonia and other chemical compounds. The latex protein concentration was determined using ASTM D5712-95 Modified Lowry assay and the protein was freeze-dried (lyophilized) under nitrogen into small vials by Greer Laboratories (Lenoir, NC).

NAL was diluted with phosphate buffered saline (PBS) pH—7.2–7.4 containing 0.1% SDS and centrifuged at $10\,400 \times g$. The aqueous fraction underneath the rubber layer was collected using a canula and syringe. The aqueous fraction was additionally centrifuged at $40\,000 \times g$ for 60 min to separate the remaining latex particles. The extract was passed through a 0.45 μm filter and dialyzed against a 0.1 M carbonate buffer pH. 9.6 using dialysis tubing of MWCO 1000 (>20 volumes each over 4 changes of carbonate buffer) for 3 days at 4°C. The latex protein concentration was determined using ASTM D5712-95 and the protein solution was lyophilized under nitrogen into small vials by Greer Laboratories.

Downloaded At: 10:32 16 January 2011

Antisera Preparation

New Zealand white female rabbits (5–6 lb) were immunized with either AL or NAL antigen, using Complete Freund's Adjuvant (CFA, Sigma, St. Louis, MO) or TiterMax adjuvant (CytRx, Norcross GA). For rabbits immunized with CFA, an equal volume of antigen was mixed with CFA to form a homogenous emulsion. Rabbits were injected subcutaneously at 2–6 sites on the dorsum with 150 μL aliquots, containing total of 750 μg of protein. After a rest period, 2 booster injections were given using incomplete Freund's adjuvant (IFA) on week 4 and 5. The total amount of antigen administered per rabbit was 1.5–3.0 mg of AL and 1–1.5 mg of NAL. Starting at week 7, rabbits were bleed weekly and the anti-NRL antibody titers were checked by an indirect ELISA. After 5 to 6 bleedings, the rabbits were exsanguinated.

For rabbits immunized with TiterMax adjuvant, a 50/50 mix of antigens and adjuvant was injected subcutaneously at four injection sites

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

(0.4 mL per rabbit). Four injections of 100 μL each were given, one site over each shoulder and one site over each hind quadriceps. Antibody titer was checked by indirect ELISA. Two subsequent boost injections were given at 2 and 10 weeks. Three weeks following the last injection the rabbits were bled and exsanguinated.

Extraction of Proteins from NRL Products

Water-soluble proteins were extracted from surgical and examination gloves according to the standard ASTM procedure described in D5712 and D6499 protocols. Briefly, extraction was done with 100 mM PBS, pH 7.4, in a 5 : 1 v/w ratio at 37°C for 2 h with shaking every 10–15 min. Extracts were centrifuged to remove glove powder and other particulates, and the cleared supernatants were used in the assays.

Downloaded At: 10:32 16 January 2011

ELISA Inhibition Test for Latex Protein

Inhibition plates were prepared using low protein binding microtiter plates, blocked with 300 μL/well of 3% nonfat dry milk/PBS overnight at room temperature. After washing plate 3× with saline containing 0.05% Tween 20 (T-PBS), seven two-fold dilutions of each test extract (100 μL/well) and seven two-fold dilutions of AL or NAL reference protein beginning at 2 μg/mL were made in duplicate wells. Rabbit anti-NRL serum (1/15 000 dilution) was added to each sample dilution (100 μL/well). The plates were incubated for 2 h at 37°C.

Microtiter plates containing the solid phase antigen were prepared by coating the polystyrene ELISA plates (Maxisorb, Nunc) with 0.3 μg/well of latex protein in a carbonate buffer pH 9.6. After incubation for 2 h at 37°C, solid phase antigen was washed 3× with T-PBS. The non-reacted sites were blocked for 1 h with 3% nonfat dry milk/PBS and washed two times with T-PBS.

After the 2 h incubation of the inhibition plates to allow antibody reaction with the test samples, the inhibited antiserum was transferred to the 96-well assay plates containing the solid phase antigen. The assay plates were then incubated for 2 h at 37°C to allow remaining unbound antibody to bind to the solid phase antigen. After washing the plates three times with T-PBS, a 1/5000 dilution of peroxidase-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO) was added and incubated for 1 h at 37°C. The plates were again washed three times, and a colored reaction was developed by the addition of *o*-phenylenediamine (1 mg/mL) in H_2O containing 0.1% H_2O_2.

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

The color development was stopped by the addition of 50 μL/well of 4N sulfuric acid. The reaction product was quantified by reading the optical density at 490 nm using a microtiter plate reader. The concentration of latex protein in the test samples was determined by comparing the optical density of the samples to the optical density of the latex reference standard at seven consecutive dilutions of the original extract. The inhibition assay has a linear working range between 30 and 2000 ng/mL.

RESULTS

Pilot Studies

The inhibition format of the ELISA test has been successfully used in the past for the quantitation of various individual allergens. Considering that natural latex proteins contain a large number of individual proteins, ranging in molecular weights from very small peptides to over 200 kD proteins, the basic test format had to be adapted to measure such a heterogeneous group of proteins. Several pilot studies were conducted to determine the optimal test parameters specific for these antigens. We also investigated the option to perform competitive instead of noncompetitive inhibition. As an alternative to the two step assay, described in the method section, the test samples together with rabbit antiserum were added directly to the assay plates. These studies showed a higher level of inhibition with a noncompetitive format, than with competitive inhibition in the assay plates (Table 1). Also, the carbonate buffer pH—9.6 resulted in a greater level of inhibition than when PBS pH—7.4 was used as a coating buffer. The noncompetitive inhibition format and carbonated buffer were selected for further study.

A crucial parameter for this test was the selection of a standard antigen. The protein composition on finished NRL products differ from one another, as well as from ammoniated and nonammoniated raw latex, which further complicates the selection of an appropriate standard protein. As glove proteins could not be standardized, we evaluated two protein sources, nonammoniated and ammoniated latex protein extracts, as potential standard antigens. Earlier data with immunoblots indicated that both AL and NAL contain native proteins that may be potential allergens in humans.[16–18] Proteins in AL extracts are partially hydrolyzed, and have quite a different appearance in immunoblots. We choose to study both sources of antigens to determine which of them would be more appropriate and provide more accurate measurements of glove protein levels.

Downloaded At: 10:32 16 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Table 1. Percent of Inhibition in Competitive and Noncompetitive Inhibition Assay

Antigen Conc. (μg/mL)	Competitive Inhibition		Non-Competitive Inhibition	
	pH = 9.6	pH = 7.4	pH = 9.6	pH = 7.4
4	66	58	84	72
2	56	44	72	62
1	46	36	71	53
0.5	33	26	60	45
0.25	19	19	44	33
0.12	12	12	27	23
0.06	5	9	19	15
0.03	−1	5	11	9
0.015	−5	−3	2	2
0	0	0	0	0

Values represent average of values from two independent laboratories Guthrie Research Institute and the FDA Research Laboratory. The data include evaluation of two coating buffers, carbonate buffer at pH = 9.6 and PBS at pH = 7.4.

Downloaded At: 10:32 16 January 2011

Figure 1 shows an evaluation of protein levels in 8 glove extracts using either a combination of a) NAL antigen and anti-NAL antiserum on NAL coated plates (NAL assay, Fig. 1A), or b) AL antigen and anti-AL serum on AL-coated plates (AL assay, Fig. 1B). With the NAL assay, 2 of the 8 extracts showed clear dose-response inhibition of the serum, while the other extracts only mildly inhibited anti NAL serum, suggesting a very low level of NRL proteins in those extracts. However, with the AL assay, 7 of 8 glove extracts showed various levels of dose-response inhibition. We also observed that AL was a poor inhibitor in the NAL assay and that NAL was a poor inhibitor in the AL assay. This finding was unexpected, as the allergenic similarity of these two protein sources has been documented in vivo and in vitro.[13,19]

We anticipated that a possible reason for the lack of antigen recognition by rabbit antibodies might be the selection of adjuvant for rabbit immunization. The sera used in the above pilot studies were generated using TiterMax adjuvant. To address this question we evaluated the binding capacity and specificity of our rabbit antisera in comparison with rabbit anti-NRL sera from other laboratories, where complete Freund's adjuvant was used. We evaluated two rabbit sera generated by immunization with NAL proteins, using either TiterMax (NAL-T) or CFA (NAL-F) as the adjuvant and two sera from rabbits immunized with AL proteins and the same combination of adjuvants (AL-T and AL-F). The assay of a direct

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

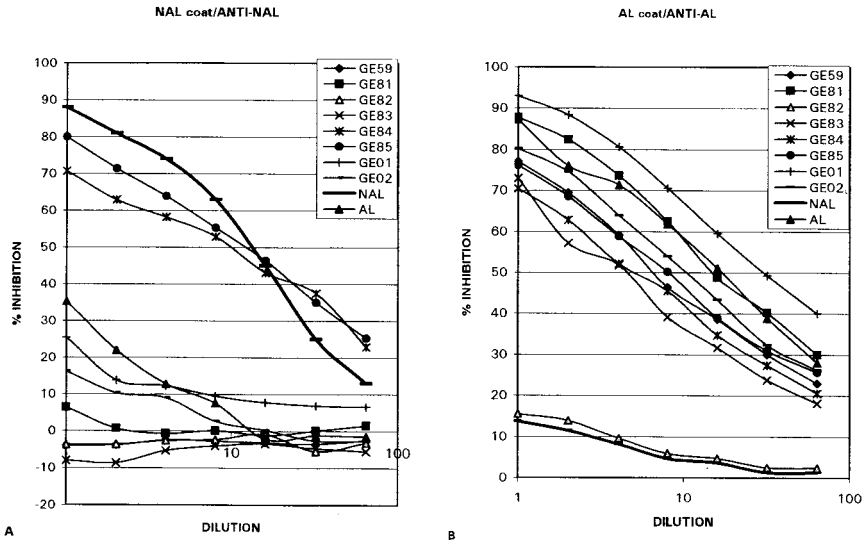


Figure 1. Evaluation of antigenic protein levels in glove extracts, comparing two sources of reference antigen. The assay plate coating antigen and the inhibition antigen was either NAL (A) or AL (B) protein. The inhibition curves of glove extracts were compared to inhibition curves of both NAL and AL antigens.

Downloaded At: 10:32 16 January 2011

binding and antigen recognition capacity was performed in plates coated with preparations of NAL or AL protein extracts (Fig. 2). The data shows variability in the capacity of these sera to react with different protein preparations. While anti-NAL sera demonstrated a higher titer for both NAL and AL proteins, the most critical factor for binding with protein from various sources appeared to be the adjuvant used for immunization. CFA was clearly a stronger adjuvant than TiterMax for the generation of anti-NRL antibodies. Both, the adjuvant used and the form of the protein antigen for the rabbit immunization appeared critical for the capacity of immune sera to react with NRL proteins.

Preparation and Validation of Assay Reagents

Our pilot data indicated that the AL reagents react better with glove extracts, therefore we selected AL as a preferred source of antigen for the further development of the assay protocol. To minimize possible effect of the variations in the protein composition among different samples of AL,

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

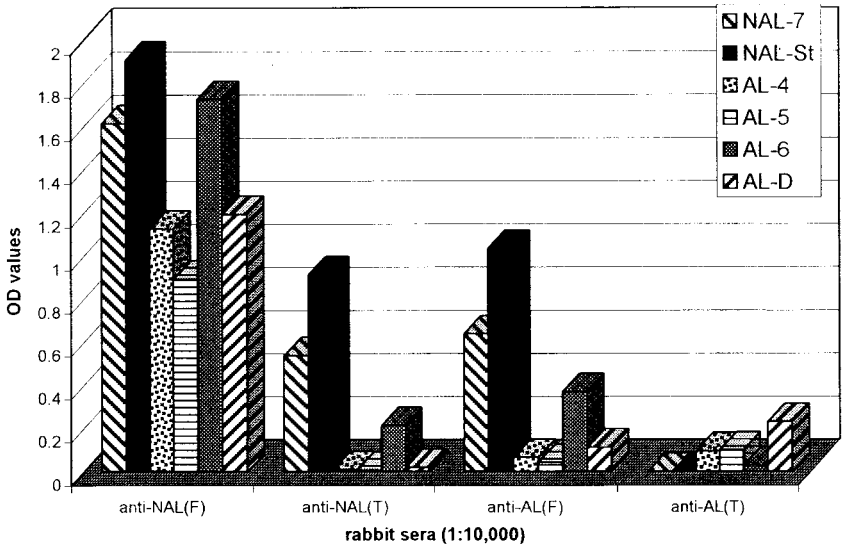


Figure 2. Evaluation of rabbit anti-NRL sera reactivity with various sources of NRL protein. Plates were coated with (2 μg/well amount) various preparations of AL and NAL antigens. Sera (1/10 000 dilution) from rabbits immunized with either NAL or AL antigen using Titermax adjuvant (NAL-T, AL-T) or complete Freund's adjuvant (NAL-F, AL-F).

a new AL protein antigen was prepared as a mixture of extracts from three high-ammonia and three low-ammonia NRL samples. Figure 3 shows SDS-PAGE analysis of the individual AL samples (top). The Western blots of the individual samples and the mixture of all six samples (bottom) were performed with rabbit anti-AL and anti-NAL sera.

The new AL protein preparations were evaluated by inhibition assay using either a mix of (a) three low ammonia preparations, (b) three high ammonia preparations, or (c) a pool of all six preparations as a coating antigen (Fig. 4). The rabbit anti-AL serum was inhibited by both high and low ammonia preparations in a similar pattern and the ability of the inhibited serum binding to the coating antigen was comparable. With the pool of both high and low ammonia preparations used as a coating antigen, the inhibition curves showed the best dose-response pattern. The mixture of all six preparations was used for further studies and to generate antisera. Rabbits were immunized with the pool of low and high ammonia AL antigen in complete Freund's adjuvant. The assay produced consistent data when based on the standard curve obtained with these reagents.

Downloaded At: 10:32 16 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

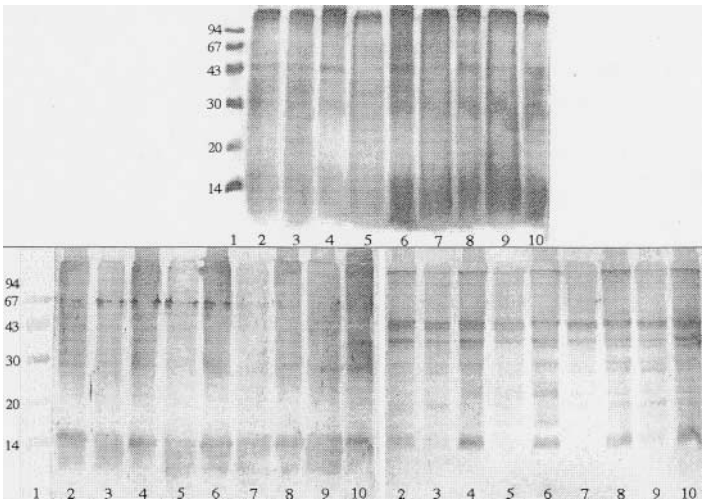


Figure 3. SDS-PAGE and Western blots of three low-ammonia and three high-ammonia NRL extracts and a pool of all six preparations in comparison with previously used AL preparation.

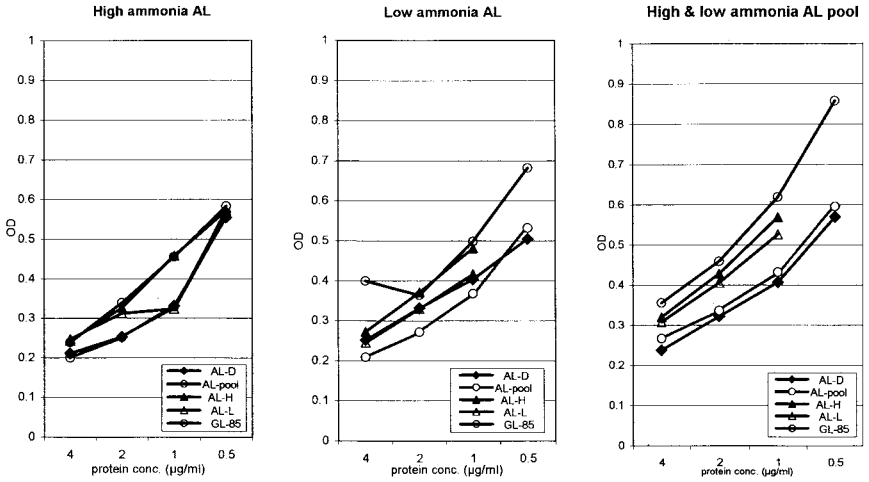


Figure 4. Comparison of new AL antigen preparations in the ELISA inhibition assay. Anti-AL serum was inhibited with several AL antigen preparations (AL-D = original AL antigen, AL-H = pool of three high-ammonia preparations, AL-L = pool of three low ammonia preparations, AL-POOL = pool of AL-H and AL-L preparations, GL-85 = glove extract). All preparations were evaluated using either AL-L, AL-H or AL-POOL as a coating antigen.

Downloaded At: 10:32 16 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

The inhibition curves generated in the three individual laboratories had an intra-laboratory relative standard deviation (RSD) of 4.3–5.3% within the assay. When combined, the inter-laboratory RSD averaged 9.4% across the standard curve. In order to evaluate the specificity of the antigen pool and the capacity of the new antiserum to react with glove proteins, we tested a number of extracts from NRL medical gloves (Fig. 5). The intra-laboratory variability of the inhibition curves was again very low, while an increased variability in analysis of the glove extracts, with an RSD of 27%, was found among laboratories.

Comparison of the ELISA Inhibition Test with Other NRL Protein Assays

To evaluate relationship of the ELISA Inhibition test with other methods, the protein levels obtained by this test were compared to the levels obtained by other methods routinely used to quantitate NRL proteins. The antigenic protein levels obtained by the ELISA inhibition assay were compared with the total protein values measured by either the Modified Lowry assay (ASTM D5712), or by amino acid analysis. The ELISA inhibition assay was also compared with another antigenic protein

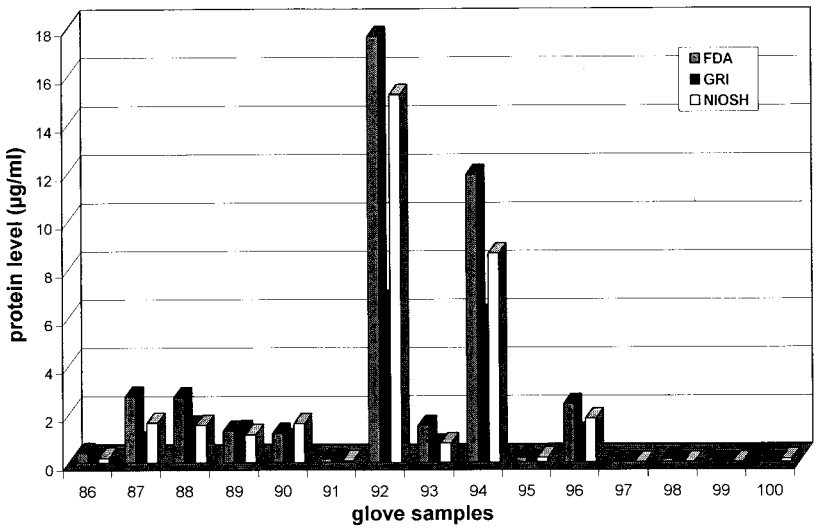


Figure 5. Antigenic protein values in 15 glove extracts determined by the ELISA Inhibition.

Downloaded At: 10:32 16 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

method, the LEAP assay, and with the RAST inhibition assay that measures allergenic proteins using patient IgE. The same glove extract preparations were used in all tests.

The antigenic protein levels obtained by the ELISA inhibition assay were markedly lower than the total protein values for the same extracts (Table 2) This was not surprising, considering the different nature of protein detection for the various methods. While the ELISA is an immunological method, and appears to measures all proteins capable of inducing antibody response in rabbits, the Modified Lowry assay D5712 is a chemical method that measures all protein in the NRL extracts.[7] The protein measurement in the Lowry method can be biased by the interference of chemical additives in NRL products.[6] The amino acid analysis also measures total protein content including all small peptides and single amino acids that may have no antigenic properties and probably are not be recognized by the ELISA test. Correlation coefficients among values obtained by these methods are presented in Table 3. The highest correlation of the ELISA

Downloaded At: 10:32 16 January 2011

Table 2. Comparative Evaluation of Protein/Antigen/Allergen Levels in Various Glove Extracts (in μg/mL)

Sample #	ELISA[a]	LOWRY[b]	LEAP[c]	RAST[d]	AA[e]
86	0.33	69	0.31	0.27	6.2
87	2.90	133	15.95	1.46	8.9
88	3.30	118	9.98	0.02	59.2
89	2.70	21	3.40	0.01	9.6
90	2.06	165	13.00	0.32	6.2
91	0.13	10	0.25	0.01	11.4
92	17.46	128	114.00	0.49	120.5
93	1.76	110	5.90	0.08	90.9
94	12.37	106	20.90	2.80	64.1
95	0.24	41	0.28	0.00	2.5
96	2.80	37	6.50	0.08	9.6
97	0.02	10	0.02	0.00	2.8
98	0.10	24	0.02	0.00	8.0
99	0.06	16	0.02	0.00	2.0
100	0.14	10	0.02	0.00	3.8

[a]ELISA values—average of data from three different laboratories.
[b]LOWRY values—average of data from four different laboratories.
[c]LEAP data was determined at GRI.
[d]RAST values—average of data from two different laboratories.
[e]AA data was determined at Baxter Corp.

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Table 3. Correlation Coefficients of Glove Extracts Values of Total/Antigenic/Allergenic Protein Levels*

	Testing Methods				
	ELISA	LOWRY	LEAP	RAST	AA
ELISA	**1.00**	0.51	0.89	0.58	0.78
LOWRY	0.51	**1.00**	0.48	0.46	0.55
LEAP	0.89	0.48	**1.00**	0.23	0.75
RAST	0.58	0.46	0.23	**1.00**	0.28
AA	0.78	0.55	0.75	0.28	**1.00**

*Data from Table 3 used for CC calculations.

Downloaded At: 10:32 16 January 2011

Inhibition assay was with the LEAP assay and with the amino acid analysis. When the ELISA inhibition values were compared to the levels of allergenic proteins obtained by the RAST inhibition assay, a lower level of correlation was observed. Limited skin testing performed with the same NRL protein extracts indicated a similar correlation level as with the RAST inhibition assay data (not shown).

DISCUSSION

The past experience in quantitation of NRL proteins and/or allergens has indicated a significant degree of complexity. Potential factors contributing to the complexity are: (a) the multitude and heterogeneity of NRL proteins; (b) variations in the composition and relative proportion of individual proteins on finished NRL products; (c) heterogeneity of human IgE responses and (d) incomplete identity of allergenic proteins. In view of a need for standardized quantitative evaluation of the allergenicity of NRL products, the most appropriate approach was to develop methods that measure most NRL proteins. The basic assumption was that the total amount of protein is proportional to the amount of antigenic proteins, and thus would indirectly reflect the potential allergenicity of NRL products. The Modified Lowry assay was developed as an ASTM standard (D5712) and has been used to quantitate total proteins on NRL products for several years. With continuing problems of chemical interferences and a relatively high limit of detection, the need for a better assay was apparent. In the process of developing new assays, efforts have been aimed at methodologies that would quantitate only biologically relevant proteins, i.e., those proteins that have antigenic and/or allergenic properties.

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Several methods have been already used in research and clinical laboratories, but the accuracy or relevance of the measurement has not been fully established, and none of the methods have been standardized outside of the individual laboratories.

The main purpose of this study was to develop an assay that: (a) could be suitable for the quantitation of biologically pertinent proteins, and (b) could be validated and standardized. The comparison of the inter- and intra-laboratory data obtained by the ELISA Inhibition showed good reproducibility and the sensitivity to 0.5 μg/mL level. With the common well-defined NRL protein source used as a reference protein and as the antigen for rabbit antisera generation, the test could be easily standardized. The ELISA Inhibition test was recently accepted as a new national ASTM D6499 standard. A critical factor for the measurement accuracy was found to be the selection of proper reagents. The choice between NAL extract as a source for NRL proteins in the native form, and AL extract, which represents the actual source material for NRL products, has been a subject of many discussions. We evaluated the performance of both sources of NRL proteins as a potential reference antigen in the ELISA Inhibition assay.

In the earlier studies, the Western blot analysis showed that NAL contains the largest number of discreet protein bands that are recognized by either human or rabbit sera.[16] On the other hand, human anti NRL serum showed a more complete inhibition of AL than NAL protein preparation.[18,19] Based on the linearity of standard curves and the recognition of proteins in glove extracts obtained in our study, we have selected a pool of six extracts of AL proteins as the preferred antigen. We further showed that not only the type of protein affects the assay, but also that the adjuvant used for immunization of rabbits is affecting specificity of rabbit antibodies. The Freund's complete adjuvant appeared to produce antisera with higher titer and less dependent on the conformation of immunizing antigen than the TiterMax adjuvant. The AL protein and Freund's complete adjuvant was determined to be more appropriate for the assay, based on our testing of glove extracts and the fact that AL is the source material for NRL gloves.

When glove extracts were evaluated using the AL reagents, the resulting values of antigenic protein were comparable to the LEAP assay, which is very similar to the ELISA Inhibition assay in regards to both, the mechanism of detection and the source of reagents.[8] The chemical methods that measure total protein content, however, showed higher values than the two ELISA tests. This was especially evident with the Modified Lowry method, where all proteins and peptides are detected and in addition a chemical interference can cause false positive reactivity and produce markedly higher values. The higher values obtained by the amino acid

Downloaded At: 10:32 16 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

analysis, which does not have the same bias as the Modified Lowry, can be attributed to the detection of small peptides and single amino acids. Another cause for the observed higher values obtained by chemical methods in comparison with immunological methods is that chemical assays measure casein, a non-latex protein component to some latex formulations that would not be detected by the ELISA tests.[20] Small peptides, single amino acids and casein are not detected by the immunological methods and are irrelevant as potential allergens.

The values of the protein levels of 15 glove extracts obtained by the ELISA Inhibition test correlated well with the LEAP test and with the amino acid analysis. A lesser correlation was found with the Modified Lowry assay. The interference of chemical additives to NRL gloves is probably the main reason for the lack of good correlation between the Modified Lowry and the ELISA Inhibition assay. A good correlation with the amino acid analysis indicate that the ELISA Inhibition assay indeed measures most proteins, except small peptides and single amino acids.

The ELISA Inhibition assay values demonstrated a less good correlation with the allergen measurement by the RAST inhibition assay. A likely reason for the observed discrepancy is a variation in relative proportions of individual allergenic proteins on NRL products and a variation in relative levels of the IgE antibodies specific for individual allergens in human sera. This will be also an important issue in the development of a general allergen test, as these variations may significantly affect the accuracy of the test. The similar argument can be used in the comparison of the ELISA Inhibition test with the skin test values. The preliminary evaluation of glove extracts studied herein on a small sample of only 10 NRL allergic individuals provided an equivocal information. A recent skin testing study with individual allergens showed that some patients, who had positive skin test with the crude AL protein preparation, reacted to only one or none of the seven major allergens tested.[21] As in the case of the RAST test, this data suggest that sensitized individuals have been exposed to different allergenic proteins and different amounts of particular allergens. These data also suggest that other proteins, in addition to known major allergens may be allergenic.

In general, a direct comparison of absolute protein values obtained by the ELISA Inhibition test with the values obtained by other methods for the quantitation of NRL proteins, antigens or allergens may be difficult due to significant differences in their detection mechanisms, differences in the reference proteins and the specific biases of each method. The ELISA Inhibition assay appears to accurately reflect the protein content of NRL products and indirectly estimates their relative potential allergenicity. The quantitation of antigenic proteins, includes in measurements all proteins

Downloaded At: 10:32 16 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

that are known allergens or could be potential allergens, This approach overcomes the significance of the variability in protein composition among NRL products that may be an issue in development of a specific allergen test.

ACKNOWLEDGMENTS

The authors wish to acknowledge: Drs. R. Hamilton, Johns Hopkins University and M. Swanson, Mayo Clinic, for performing RAST tests; P. Turner, Baxter Corporation, for amino acid analysis, G. Sussman, University of Toronto, for the skin testing. We also acknowledge the Round Robin participants Dave Albery, Ansell-Perry Corp., Judy Genzale, Tillotson Health Care Corp. and Wunan Huang, Maxxim Medical.

Downloaded At: 10:32 16 January 2011

REFERENCES

1. Tarlo, S.M.; Sussman, G.L.; Holness, D.L. Latex Sensitivity in Dental Students and Staff: A Cross-Sectional Study. J. Allergy Clin. Immunol. **1997**, *99*, 396–401.
2. Gautrin, D.; Infante-Revard, C.; Dao, T.V.; Magnan-Larose, M.; Desjardins, D.; Malo, J.L. Specific IgE-dependent Sensitization, Atopy and Bronchial Hyperresponsiveness in Apprentices Starting Exposure to a Protein-Derived Agents. Am. J. Respir. Crit. Care Med. **1997**, *155*, 1841–1847.
3. Kelly, K.J.; Pearson, M.L.; Kurup, V.P.; Havens, P.L.; Byrd, R.S.; Setlock, M.A. A Cluster of Anaphylactic Reactions in Children with Spina Bifida During General Anesthesia: Epidemiologic Features, Risk Factors and Latex Hypersensitivity. J. Allergy Clin. Immunol. **1994**, *94*, 53–61.
4. Bernardini, R.; Novembre, E.; Lombardi, E.; Mezzeti, P.; Cianferoni, A.; Danti, D.A. Risk Factors for Latex Allergy in Patients with Spina Bifida and Latex Sensitization. Clin. Exp. Allergy **1999**, *29*, 681–686.
5. Yeang, H.Y.; Yusof, F.; Abdullah, L. Precipitation of *Hevea brasiliensis* Latex Proteins with Trichloroacetic Acid and Phosphotungstic Acid in Preparation for the Lowry Protein Assay. Anal. Biochem. **1995**, *226*, 35–43.
6. Tomazic-Jezic, V.J.; Lucas, A.D.; Lamanna, A.; Stratmeyer, M.E. Quantitation of Natural Rubber Latex Proteins: Evaluation of Various Protein Measurement Methods. Toxicol. Meth. **1999**, *9*, 153–164.

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

7. Standard Test Method for Analysis of Protein in Natural Rubber and Its Products, ASTM D5712. In *Annual Book of ASTM Standards*; ASTM: Philadelphia, June, 1995; Vol. 14.
8. Beezhold, D.H. LEAP: Latex ELISA for Antigenic Proteins. Guthrie J. **1992**, *61*, 77–81.
9. Chabane, H.; Barbara, J.; Hrabina, M.; Leynadier, F. Competitive Immunoassay for Antigenic Latex Protein Measurement: Rabbit Antiserum-based Assay Compared to Modified Lowry and Human IgE-inhibition Methods. Invest. Allergol. Clin. Immunol. **1999**, *9*(6), 372–379.
10. Rauf-Heimsoth, M.; Sander, I.; Chen, Z.; Borowitzki, G.; Diewald, K.; vanKampen, V.; Baur, X. Development of a Monoclonal Antibody-Based Sandwich ELISA for Detection of the Latex Allergen Hev b1. Int. Arch. Allergy Immunol. **2000**, *123*, 236–241.
11. Palosuo, T.; Makinen-Kiljunen, S.; Alenius, H.; Reunala, T.; Yip, E.; Turjanmaa, K. Measurement of Natural Rubber Latex Allergen Levels in Medical Gloves by Allergen-Specific IgE ELISA Inhibition, RAST Inhibition and Skin Prick Test. Allergy **1998**, *53*, 59–67.
12. Yuninger, J.W.; Jones, R.T.; Fransway, A.F.; Kelso, J.M.; Warner, M.A.; Hunt, L.W. Extractable Latex Allergens and Proteins in Disposable Medical Gloves and Other Rubber Products. J. Allergy Clin. Immunol. **1994**, *93*, 836–842.
13. Hamilton, R.G.; Charous, B.L.; Adkinson, N.F.; Yuninger, J.W. Serologic Methods in the Laboratory Diagnosis of Latex Rubber Allergy: Study of Nonammoniated Ammoniated Latex and Glove (End-Product) Extracts as Allergen Reagent Sources. J. Lab. Clin. Med. **1994**, *123*, 594–604.
14. Lu, L.-J.; Kurup, V.P.; Hoffman, D.R. et al. Characterization of a Major Latex Allergen Associated with Hypersensitivity in Spina Bifida Patients. J. Immunol. **1995**, *155*, 2721–2728.
15. Standard Test Method for the Immunological Measurement of Antigenic Protein in Natural Rubber and Its Products. *Annual Book of ASTM Standards*; ASTM: Philadelphia, January, 2000; Vol. 9.02.
16. Tomazic-Jezic, V.J.; Truscott, W. Identification of Antigenic and Allergenic Natural Rubber Latex Protein by Immunobloting. J. Immunoassay Immunochem. In press.
17. Beezhold, D.H., Chang, N.-S.; Kostyal, D.A.; Sussman, G. Identification of a 46 Kilodalton Latex Protein Allergen in Healthcare Workers. Clin. Exp. Immunol. **1994**, *98*, 408–413.
18. Beezhold, D.H.; Swanson, M.; Zehr, B.D.; Kostyal, D. Measurement of Natural Rubber Proteins in Latex Glove Extracts: Comparison of the Methods. Ann. Allergy **1996**, *76*, 520–526.

Downloaded At: 10:32 16 January 2011

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

19. Akasawa, A.; Hsieh, L.-S.; Lin, Y. Comparison of Latex Specific IgE Binding Among Nonammoniated Latex, Ammoniated Latex and Latex Glove Allergenic Extracts by ELISA and Immunoblot Inhibition. J. Allergy Clin. Immunol. **1996**, *97*, 1116–1120.
20. Makinen-Kiljunen, S.; Reunala, T.; Turjanmaa, K.; Cacioli, P. Is Cows' Milk Casein an Allergen in Latex-Rubber Gloves? Lancet **1993**, *342*, 863–864.
21. Yip, L.; Hickey, V.; Wagner, B.; Liss, G.; Slater, J.E.; Breiteneder, H. Skin Prick Test Reactivity to Recombinant Latex Allergens. Int. Arch. Allergy Immunol. **2000**, *121*, 292–299.

Received December 1, 2001
Accepted January 8, 2002
Manuscript 3061

Downloaded At: 10:32 16 January 2011