

Bacterial Protease Treatment of Natural Rubber Latex Alters Its Primary Immunogenicity in a Mouse Model of Sensitization

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The purpose of the present investigation was to determine whether enzyme-treated (ET)-NRL is less immunogenic than untreated NRL in a BALB/c mouse model of primary *in vivo* sensitization following repeated subcutaneous injections with the aqueous phase of ammoniated NRL or ET-NRL. Mice immunized with NRL produced IgE against NRL and ET-NRL, indicating that protease treatment did not completely destroy IgE antibody epitopes. In contrast, ET-NRL-immunized mice did not produce IgE against either NRL or ET-NRL, suggesting that enzyme treatment reduced the number of antigenic polypeptides associated with NRL below the threshold for sensitization. T_{helper}-lymphocytes from NRL-immunized mice proliferated and produced IL-4 when stimulated *in vitro* with polypeptides from NRL, but not ET-NRL. In contrast, T_{helper}-lymphocytes from ET-NRL-immunized mice were nonresponsive to ET-NRL or NRL. We conclude that lack of IgE production by ET-NRL-immunized mice is likely related to a lack of T-cell help in the form of IL-4, rather than enzyme digestion of IgE antibody epitopes. These data indicate that there is an immunologic rationale for production of enzyme-treated NRL-containing medical devices. © 2002 Elsevier Science (USA)

Key Words: natural rubber latex; immunogenicity, IgE, bacterial protease; Th2 lymphocytes.

INTRODUCTION

Type I hypersensitivity reactions to natural rubber latex (NRL)² are an important clinical problem because these allergies can result in potentially life-threatening reactions in patients frequently exposed to NRL-

containing medical devices and occupational disability in health care workers (1–6). IgE-mediated allergic reactions to NRL-containing medical devices are thought to result from sensitization to the polypeptides originating from NRL of the *Hevea brasiliensis* tree. These hypersensitivity reactions can occur because of repeated exposures through cutaneous, mucosal, or surgical body cavity surfaces.

While fresh NRL has been estimated to contain as many as 240 different polypeptides that can be identified by two-dimensional immunoblotting, IgE from the serum of NRL-allergic patients can recognize a smaller number of these polypeptides (7–11). These allergenic polypeptides range in size from 5 to 110 kDa and are probably derived from a limited number of proteins in different aggregate states or degraded to various degrees (12, 13).

Extractable proteins associated with NRL films are likely to be involved in the sensitizing process. Processing of NRL in the field or during industrial manufacturing procedures will affect the extractable proteins associated with raw or processed NRL in the form of films such as those used in glove manufacturing. Addition of ammonia, sulfur, accelerators, antioxidants, and other chemicals to NRL during its processing may alter the polypeptides associated with NRL.

Since total extractable antigenic proteins associated with NRL and reactivity with IgE from allergic patients can be related (14), there is interest in developing novel methods to decrease the total protein content and potential immunogenicity of NRL by digesting the polypeptides associated with NRL with proteases. There have been several technical reports describing the use of exogenous proteases to degrade polypeptides associated with NRL (15–20). Although this approach successfully reduces the total protein content associated with NRL, there is no information about whether this treatment affects the immunogenicity of NRL.

Herein we report that enzyme treatment of NRL alters the biochemical properties of the associated polypeptides and affects its immunogenicity in a mouse

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² Abbreviations used: NRL, natural rubber latex; sNRL, “serum” or aqueous phase of NRL; ET-NRL, enzyme-treated natural rubber latex; sET-NRL, “serum” or aqueous phase of enzyme-treated NRL.

model of sensitization to such polypeptides. Enzyme treatment of NRL reduces antigen-specific IgG1 and eliminates IgE production after repeated subcutaneous exposures. Similarly, T-lymphocytes from mice immunized with enzyme-treated (ET)-NRL do not proliferate *in vitro* to ET-NRL or untreated NRL. This is in contrast to T-lymphocytes from NRL-sensitized mice, which react to NRL (but not ET-NRL) *in vitro* by proliferating and producing IL-4. We conclude that enzyme treatment of NRL renders this material less immunogenic in this *in vivo* mouse model of sensitization induced by repeated subcutaneous exposures.

MATERIALS AND METHODS

Experimental mice. Female BALB/c mice (6–8 weeks old) were used as the experimental species for all immunizations. These animals were purchased from Jackson Laboratories (Bar Harbor, ME). All work with experimental animals was reviewed and approved by the University Committee for Animal Resources (UCAR).

NRL reagents. The following materials were utilized for the *in vivo* immunization studies, *in vitro* ELISA, and *in vitro* stimulation of T-lymphocytes: The serum phase of centrifuged, ammoniated NRL (designated sNRL) was provided by Tillotson Healthcare Corporation. Commercial grade, centrifuged, ammoniated NRL was either left untreated (control) NRL or treated with a 0.01% (100 μ g/ml) aqueous solution of a bacterial alkaline protease (source: *Bacillus subtilisin* serine-type alkaline protease) for 24 h at 80°F with constant agitation (Allotex NRL, United States Patent, US 6,380,283 B1, 2002) (21). Subtilisin proteases are known for their broad range of substrate, temperature, and pH activity.

The protease was not removed from the NRL solution after its addition, but it constituted a small fraction of the total protein. The protein content of the supernatant of NRL using the Lowry test was 2.9 mg/ml for NRL and 3.0 mg/ml for ET-NRL. Therefore, approximately 98% of the total protein consisted of latex proteins.

The serum phase of untreated NRL or enzyme-treated s(ET)-NRL was prepared by freezing and thawing the NRL, followed by centrifugation of the thawed NRL for 2 h at 15,000g. The surface rubber material was removed from the tube, and the supernatant was recentrifuged for 1 h at 27,000g. The resulting supernatant of NRL or ET-NRL was then filtered through a 0.22- μ m filter. The pH of the sNRL or sET-NRL was highly basic (pH $\sim$ 11.0); the pH of these solutions was adjusted to a more physiologic level (pH 7.4) by diluting in phosphate-buffered saline and then bubbling CO₂ gas through the sNRL or sET-NRL to remove ammonia and lower the pH. These sterile, pH-adjusted

solutions were utilized for *in vivo* immunizations, *in vitro* ELISA, and T-lymphocyte proliferation assays.

The polypeptide content of sNRL or sET-NRL for the *in vivo* immunizations and the *in vitro* ELISA or T-lymphocyte studies was quantified using a modified Lowry assay (using the American Society for Testing and Materials standard method D5712-95) (22).

Guthrie-ammoniated NRL polypeptides (3.3 mg total protein) and rabbit-ammoniated NRL-specific antiserum (high-titer antiserum, estimated ELISA inhibition titer 1:15,000) were generously provided by Donald Beezhold (Guthrie Research Foundation, Sayre, PA.) The Guthrie reagents were utilized to validate the NRL-specific ELISA (data not shown) (23).

Immunizations. BALB/c mice ($N = 6$ /group) were immunized by treatment with subcutaneous injections of saline solution, sNRL, sET-NRL, or keyhole limpet hemocyanin (Calbiochem, San Diego, CA) at the base of the tail with 50 μ g of antigen in a 50- μ l volume. For the antibody responses, the mice were immunized on days 0, +14, +28, and +42. The animals were bled via the tail vein on days -1 , +21, and +56. Alum adjuvant (Alhydrogel, Accurate Chemical, Westbury, NY) was administered during the immunizations, and it was in a 1:1 ratio with the NRL solutions in the indicated volume.

For the *in vitro* T-lymphocyte proliferation assays (see below), mice (pooled lymphocytes from three animals/group) were used; this experiment was repeated three times) were treated with intradermal injections on days 0 and +7 with 50 μ g of sNRL, sET-NRL, or saline solution in a 1:1 ratio without alum adjuvant in a 140- μ l volume. Popliteal lymph node cells were harvested and disaggregated for the *in vitro* T-lymphocyte proliferation assay (see below) on day +14. Different immunization protocols were used to study antibody and T-cell responses because of a difference in the kinetics of the two types of immune responses (see data).

NRL-specific ELISA. To test sera from immunized mice for antigen-specific IgG1, IgG2a, and IgE, a specific ELISA was developed using standard techniques (24). Briefly, ELISA plates (Corning Glass Works) were coated with 10 μ g of sNRL or sET-NRL and the plates were blocked with 0.25% BSA in Tween/PBS. A 1:25 dilution of the sera was then plated in triplicate to allow for antibody binding. Unbound antibody was washed off and biotinylated antimouse IgG1, IgG2a, or IgE (BD-Pharmingen, San Diego, CA) was added at 2 μ g/ml. The level of NRL- or ET-NRL-specific immunoglobulin was then analyzed by adding streptavidin-horseradish peroxidase at a dilution of 1:2,000. o-phenylenediamine dihydrochloride (Sigma Chemical Co.) was added according to Sigma instructions for color

development. Plates were read on a Bio-Rad benchmark microplate reader, and optical density was read at 570 nm.

For the ELISA inhibition assay, serum from sNRL immunized mice was preincubated with dilution buffer or buffer containing 2–200 $\mu\text{g/ml}$ of soluble sNRL or sET-NRL for 60 min at 37°C prior to use in an ELISA using sNRL-coated plates (see above). Binding of IgG1 or IgE antibodies to the coated plates was detected as described above. ELISA inhibition was calculated using the formula $\text{ELISA inhibition} = [1.00 - (\text{OD serum}_{\text{unblocked}} - \text{OD serum}_{\text{blocked}}) / \text{OD serum}_{\text{unblocked}}] \times 100$.

T-lymphocyte proliferation assays. Unfractionated lymph node cell suspensions from NRL-, ET-NRL-, or sham-immunized mice were assayed for their ability to proliferate in response to medium alone, NRL, ET-NRL, or mitogens such as concanavalin A (Calbiochem, La Jolla, CA). Cell suspensions were prepared by disaggregation of dissected lymph nodes between the frosted ends of sterile glass slides. A total of 1×10^5 lymph node cells were plated with the indicated stimuli in 96-well round bottom microtiter plates (Corning Glassworks) using standard T-lymphocyte medium (20). The 96-well plates were incubated at 37°C in a 5% CO_2 atmosphere for 96 h. One microcurie of [^3H]TdR (Amersham Life Science, Arlington Heights, IL) was added to each well for the last 18 h of the incubation to measure proliferation. DNA from the labeled cells was harvested with a PhD Model 200A cell harvester (Cambridge Technology, Inc., Watertown, MA), and the level of [^3H]TdR was counted on a Beckman LS 6500 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Lymphokine ELISA. Supernatants from culture wells of control or antigen- or mitogen-stimulated lymph node T-lymphocytes were harvested on day 3 of culture and subjected to sandwich ELISA assay for the presence of IL-4 using standard techniques (24). Briefly, 25 μl of supernatant from each well was applied to wells of a 96-well ELISA plate (Corning Costar, Cambridge, MA) that had been treated with 1 $\mu\text{g/ml}$ of antimouse IL-4 capture antibody (BVD4-1D11, Pharmingen), according to the supplier's recommendations (Pharmingen OptEIA Set). Bound IL-4 was measured using 1 $\mu\text{g/ml}$ of biotinylated antimouse IL-4 antibody (BVD6-24G2, Pharmingen) followed by avidin peroxidase (Sigma Chemical Company). Plates were developed for approximately 30 min in the presence of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) and H_2O_2 (Sigma Chemical Company). Plates were read at 405 nm using a Bio-Rad benchmark microplate reader. The OD readings were compared to those of recombinant mouse IL-4 (Pharmingen) concentration standards, which ranged from 15 pg/ml to 4 ng/ml . The

limit of detection of this assay is 7.8 pg of IL-4 per milliliter of culture supernatant (Becton-Dickinson/Pharmingen Catalogue, 2000).

SDS-PAGE of NRL polypeptides. Using standard methods (25), 10–30 μg of sNRL or sET-NRL polypeptides was electrophoretically separated using a precast 4–20% gradient Tris-HCl gel (Bio-Rad, Hercules, CA) and stained with Coomassie brilliant blue. The stained gels were photographed using a Nikon FG single reflex camera equipped with a ring flash, using Kodachrome ASA 64 film.

Statistical analysis. Data were analyzed for statistical significance using the two-tailed Student's *t* test (Statview Student, Abacus Concepts, Berkeley, CA). Probability values of <0.05 were considered statistically significant.

RESULTS

The effects of protease digestion on the biochemical properties of ammoniated sNRL peptides was studied using SDS-PAGE (Fig. 1). Untreated, ammoniated sNRL peptides migrated as a smear ranging in relative size from 10 to 25 kDa on a Coomassie blue-stained gel, which is similar to previous observations (12). An equal amount of ammoniated sET-NRL migrated at <10 kDa, suggesting that protease treatment degraded the polypeptides into smaller fragments, altering the apparent molecular weight as estimated by gel migration.

To study the effects of enzyme treatment on the antigenicity of ammoniated sNRL, we coated ELISA plates with enzyme-treated ammoniated sNRL and studied the reactivity of serum from mice immunized with untreated, ammoniated sNRL (Figs. 2A and 2B). Immunization with 100 μg of ammoniated sNRL induced a vigorous, antigen-specific, IgG1 antibody response (Fig. 2A) and specific IgE production against sNRL (Fig. 2B). This serum from sNRL-immunized mice contained high-titer, NRL-specific IgG1 that titrated out to a >1:1000 dilution; NRL-specific IgE reactivity titrated out only to a 1:100 dilution (data not shown). Serum from sNRL-immune mice exhibited weak IgG1 reactivity against ammoniated sET-NRL (Fig. 2A), suggesting that enzyme treatment of ammoniated sNRL significantly decreased the immunogenic epitopes recognized by IgG1 in NRL-immune mice. Surprisingly, serum from sNRL-immune mice exhibited IgE seroreactivity with ammoniated sET-NRL that was identical to that of untreated, ammoniated sNRL (Fig. 2B).

The primary immunogenicity of ammoniated sET-NRL in naïve mice was studied. Immunization of naïve mice with 100 μg of ammoniated sET-NRL (in the presence of adjuvant) did not result in a detectable IgG1 or IgE response on ELISA against the primary

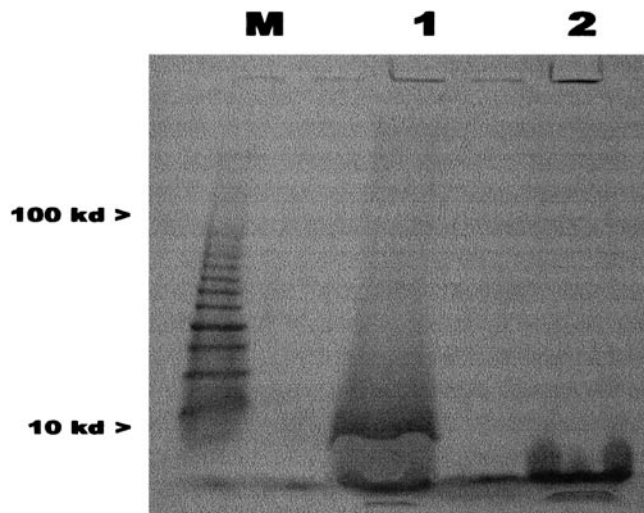


FIG. 1. Biochemical characterization of ammoniated sNRL and sET-NRL. SDS-PAGE analysis of the serum phase of ammoniated sNRL (lane 1) and sET-NRL (lane 2); 10 μ g of ammoniated sNRL or sET-NRL was loaded in the indicated lanes and separated on a 4–20% gradient Tris-HCl gel.

immunogen (ammoniated sET-NRL) or against untreated, ammoniated sNRL (Figs. 3A and 3B).

To further confirm these ELISA data, an ELISA inhibition assay was used to inhibit NRL-specific binding of IgG1 and IgE from ammoniated sNRL-sensitized mice, by using either soluble sNRL or sET-NRL (Fig. 4). Whereas preincubation of immune mouse serum with soluble sNRL inhibited ELISA IgG1 reactivity, soluble ET-NRL did not inhibit ELISA IgG1 reactivity. In contrast, both soluble sNRL and sET-NRL inhibited IgE to an equal degree. These data suggest that protease treatment of sNRL has differential effects on IgG1 immunogenic epitopes (protease-sensitive) and IgE immunogenic (protease-resistant) epitopes.

T-lymphocyte proliferative responses and cytokine production to immunization with untreated sNRL and enzyme-treated sNRL were further studied using an *in vitro* model. Lymph node T-lymphocytes from mice immunized with untreated sNRL proliferated in response to untreated sNRL, but not enzyme-treated NRL (Fig. 5A) and exhibited vigorous proliferation in response to concanavalin A (data not shown). Lymph node T-lymphocytes from mice immunized with enzyme-treated NRL were unresponsive to either untreated NRL or enzyme-treated NRL (Fig. 5A), but were fully responsive to concanavalin A (data not shown). As expected, mice immunized with saline (sham control) were unresponsive to either untreated NRL or enzyme-treated NRL (Fig. 5A) but were fully responsive to concanavalin A (data not shown).

Because Th-lymphocyte responses to polypeptides from experimental animals (26–28) or humans (29, 30)

that are allergic to NRL tend to have polar Th2 lymphokine patterns (an IL-4 producing subset of T-helper cells) after antigen stimulation, the IL-4 content of supernatants from antigen-stimulated T-lymphocytes was studied (Fig. 5B). There was a dose-dependent increase in IL-4 content in culture supernatants from sNRL (Fig. 5B)- or mitogen (data not shown)-stimulated Th-lymphocytes from sNRL-immunized mice. These same lymphocytes did not produce IL-4 upon stimulation with sET-NRL. In contrast, Th-lymphocytes from sET-NRL-immunized mice did not produce detectable IL-4 after sET-NRL or sNRL stimulation *in*

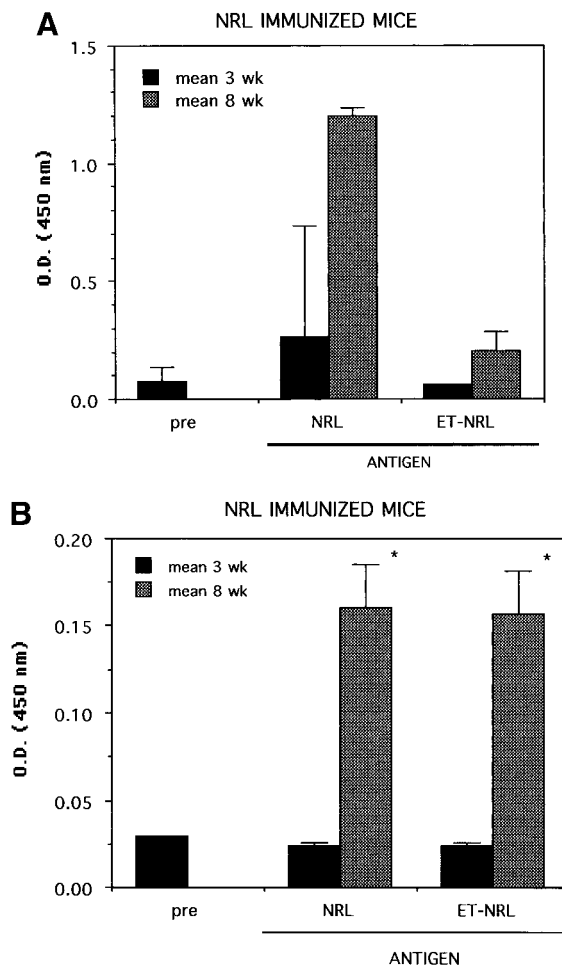


FIG. 2. sNRL immunized mice develop IgG1 and IgE antibodies against NRL-associated polypeptides, but only IgE against sET-NRL-associated polypeptides. (A) Antigen-specific (sNRL) IgG1 and (B) IgE were assayed using an ELISA after immunizations with sNRL. Sera were obtained from experimental mice ($N = 5$ /group) at baseline at 3 and 8 weeks after a series of immunizations with 100 μ g of sNRL (days 0, +14, +28, +42). These sera were studied for reactivity against sNRL- and sET-NRL-coated plates (A and B). Data represent means $\pm$ standard deviations of data from five animals, with each condition assayed in duplicate. Asterisks indicate statistically significant increases above prebleed level (* $P < 0.05$; ** $P < 0.01$).

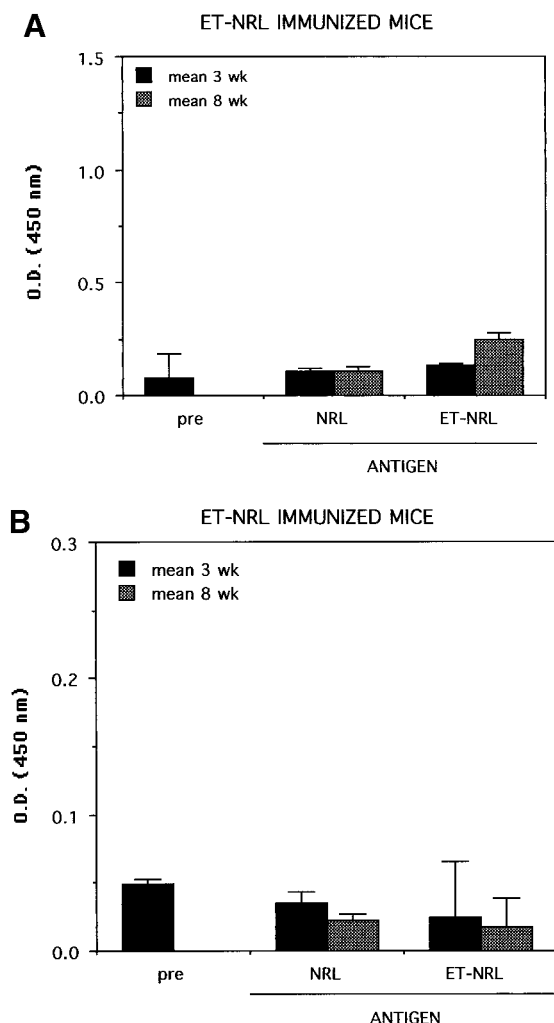


FIG. 3. sET-NRL-immunized mice do not develop IgG1 or IgE antibodies against sET-NRL- or sNRL- associated polypeptides. Antigen-specific (sET-NRL) (A) IgG1 or (B) IgE were assayed using an ELISA after immunizations with sET-NRL. Sera were obtained from experimental mice ($N = 5$ /group) at baseline and 3 and 8 weeks after a series of immunizations with 100 μ g of sET-NRL (days 0, +14, +28, +42). These sera were studied for reactivity against sNRL- and sET-NRL-coated plates (A and B). Data represent means $\pm$ standard deviations of data from five animals, with each condition assayed in duplicate.

vitro (Fig. 5B). However, mitogen stimulation induced IL-4 in these lymphocytes (data not shown). As expected, Th-lymphocytes from sham-immunized mice did not produce detectable IL-4 after stimulation with either sNRL or sET-NRL, but produced significant IL-4 only after mitogen stimulation (data not shown).

DISCUSSION

The purpose of these studies was to evaluate whether protease treatment of NRL altered its primary

immunogenicity in an *in vivo* model of sensitization of naïve mice. Protease digestion of polypeptides associated with NRL could potentially alter the conformational epitopes recognized by NRL-specific antibodies and thereby reduce their immunogenicity (31, 32). The amino acid sequences of some of the immunodominant antigens, such as rubber elongation factor associated with NRL, have been determined, and there are multiple protease digestion sites associated with these polypeptides (28). It is therefore possible that protease digestion may affect the primary and secondary structures of NRL-associated polypeptides, quantitatively and qualitatively altering IgE and IgG antibody epitopes, potentially rendering the polypeptides less immunogenic.

Enzyme treatment of ammoniated sNRL rendered it less immunogenic in this mouse model of primary immunization, because T-cell cell epitopes were completely destroyed by enzyme treatment of sNRL. This is surprising, because dogma suggests that T-lymphocytes exclusively recognize proteolytically processed, linear oligopeptides presented in the context of self-class I or II major histocompatibility antigens (33, 34). Therefore, proteolytic digestion of sNRL would be expected to have minimal impact on T-cell epitope recognition.

The ablation of T-cell epitopes by enzyme treatment of ammoniated sNRL is the likely reason that mice

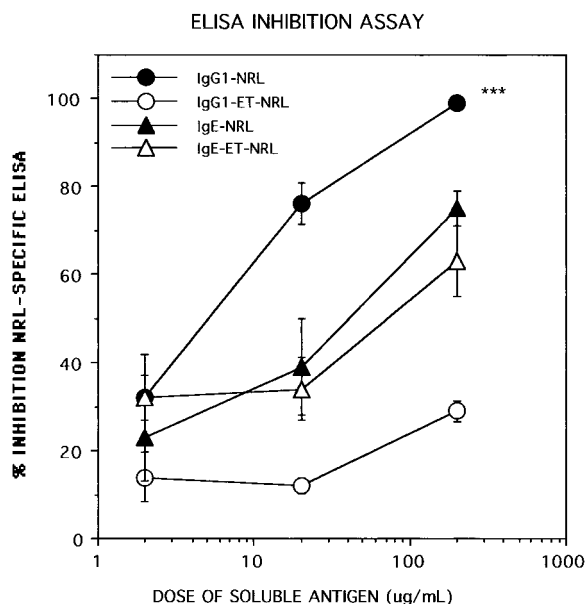


FIG. 4. Enzyme treatment of sNRL destroys ELISA inhibitory activity for IgG1 but not IgE for reactivity with sNRL. Mouse sera ($N = 5$) from sNRL-sensitized mice were preincubated with either buffer or buffer containing the indicated doses of sNRL or sET-NRL. Binding of IgG1 or IgE was assayed as described under Materials and Methods. Data represent means $\pm$ standard deviations of data from five animals, with each condition assayed in duplicate.

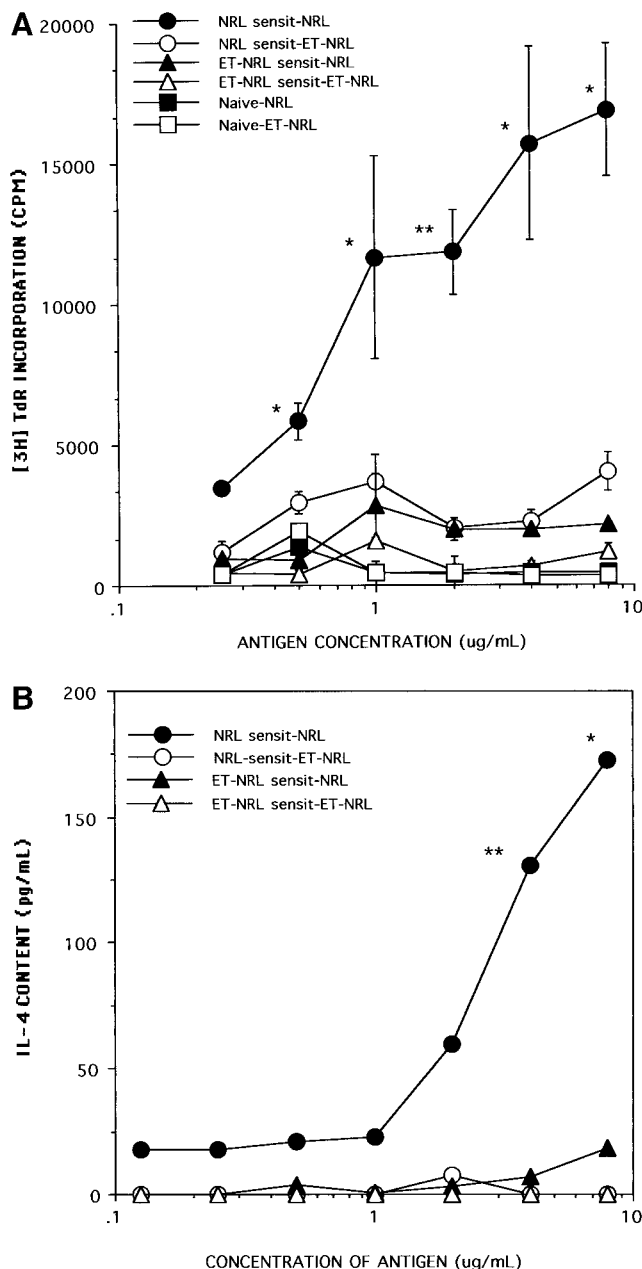


FIG. 5. *In vitro* T-lymphocyte proliferation and IL-4 production in response to ammoniated sNRL or ET-NRL. Experimental mice were immunized with sNRL, sET-NRL, or saline solution on days 0 and +7 (intradermal footpad injections with 50 μ g of sNRL or sET-NRL or sham solution). Seven days later, dissociated popliteal lymph node cells were cultured with the indicated doses of (A) sNRL or sET-NRL (* P < 0.05; ** P < 0.001 when comparing proliferation of T-cells from NRL-sensitized or ET-NRL-sensitized mice, Student's *t* test). Proliferation was measured after a 72-h culture, using [^{3} H]TdR incorporation. Data represent means of triplicate specimens $\pm$ SD from a single representative experiment of pooled lymphocytes from three animals, which was repeated three times and found to be reproducible. Lymphocytes (from NRL- or ET-NRL-immunized or naive mice) cultured in medium alone incorporated little [^{3} H] TdR (cpm < 1000), whereas concanavalin-A-stimulated lymphocytes from these experimental groups proliferated vigorously (cpm > 75,000). In

immunized with sET-NRL did not develop specific IgE antibodies. Alternatively, changes in peptide structure resulting from protease digestion could lead to inefficient antigen uptake by antigen presenting cells.

There was a lack of T-lymphocyte-derived help in the form of cytokines, such as IL-4, which is known to be a critical cytokine in driving IgE production (35, 36). Mice immunized and challenged with sET-NRL in the footpad did not produce detectable IL-4 transcripts in the local lymph node above that of a sham immunization, whereas sNRL immunization induced IL-4 transcripts in local lymph nodes (data not shown). Similarly, lymph node T-lymphocytes from sET-NRL-immunized mice did not proliferate or produce IL-4 when restimulated *in vitro* with either sET-NRL or sNRL. Lymph node T-lymphocytes from sNRL-immunized mice proliferated vigorously and produced abundant IL-4 in response to sNRL polypeptides but not in response to sET-NRL. These data suggest that protease treatment of NRL-associated polypeptides destroys T-cell epitopes required for a strong Th2-lymphocyte response. Alternatively, enzyme treatment of sNRL polypeptides may generate T-cell receptor antagonistic peptides, also known as altered peptide ligands, potentially resulting in clonal anergy to sNRL (37). This possibility warrants further investigation.

The route of exposure during immunization with nonammoniated sNRL in naïve mice has important effects on antigen recognition by IgE, as well as the response to respiratory challenge (38). Topical, intranasal, intratracheal, and subcutaneous immunizations all increase total serum IgE that immunoblot different NRL-associated antigens, which has parallels with the pattern of NRL-specific IgE isolated from healthcare workers and spina bifida patients, whose IgE recognize different NRL-associated polypeptides (27, 38, 39). Sensitization of BALB/c mice with raw, nonammoniated NRL after intranasal administration results in a significant increase in total serum IgE and NRL-specific IgG1 and increased airway resistance after intravenous challenge (39). Sensitization of naïve mice with nonammoniated latex by a variety of different routes results in increases in total serum IgE that immunoblots NRL antigens (38, 39) and NRL-reactive IgG of all subclasses (27). Other perturbations associated with NRL immunizations included peripheral eosinophilia, increases in serum IL-4 and IL-5, along with

selected experiments, IL-4 content was assayed from supernatants derived from (B) sNRL- or sET-NRL-stimulated T-cells (* P < .002; ** P < 0.001 when comparing IL-4 production by T-cells derived from NRL-sensitized to those of ET-NRL-sensitized mice, Student's *t* test). A commercially available ELISA was used to assay IL-4 content (in the ELISA, the standard deviations were <10% of the mean and are omitted for clarity).

mRNA encoding these cytokines in splenic lymphocytes (27).

Our study is the first to demonstrate that repeated exposures to ammoniated NRL by the subcutaneous route of exposure result in strong NRL-specific IgG1 antibody responses and detectable NRL-specific IgE responses. We utilized the subcutaneous route of immunization, which is highly relevant to the sensitizing exposures that are thought to occur in patients with spina bifida who undergo multiple surgical procedures and develop NRL allergy as a result of repeated exposures to NRL-containing medical devices (1–5). As well, this route of exposure to ammoniated NRL resulted in vigorous T-lymphocyte proliferation and IL-4 production *in vitro* upon restimulation with NRL.

Experimental mice sensitized to NRL (with NRL-specific IgE) by various routes of exposure developed increased airway resistance (26, 38, 39) or eosinophil-rich inflammatory infiltrates (27) after airway challenges. Future studies of mice immunized with sET-NRL will examine immediate hypersensitivity reactions to intradermal or intratracheal challenges with sET-NRL or sNRL. Based the lack of IgE responses in the sET-NRL-sensitized mice, it is unlikely that these mice will develop allergic symptoms after ET-NRL or NRL challenges.

Our studies provide evidence that protease treatment of NRL does, in fact, decrease its immunogenicity. This conclusion is based on the following observations: First, mice immunized with sET-NRL did not develop detectable IgG1 or IgE antibodies specific for either sET-NRL or sNRL. Second, T-lymphocytes from sET-NRL-immunized mice did not proliferate when restimulated *in vitro* with either sET-NRL or sNRL. Third, T-lymphocytes from sNRL-immunized mice did not proliferate *in vitro* in response to sET-NRL, whereas they proliferated vigorously and produced IL-4 in response to sNRL. Fourth, sera from mice immunized with sNRL reacted with sNRL-specific IgG1 and did not react with sET-NRL.

Last, this study provides evidence that there is an immunologic rationale for reducing the content of antigenic polypeptides associated with NRL using approaches such as bacterial protease to digest these polypeptides.

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