

# Affinity purification of latex antigens

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*Latex extracts are complex mixtures of antigenic peptides. We attempted to raise monoclonal antibodies to latex and to use these antibodies to purify latex antigens. A monoclonal antibody, CRI-C, was raised by standard techniques. Peptides of nonammoniated latex (NAL) and ammoniated latex were electrophoretically separated and transferred for immunoblots. CRI-C was covalently attached to an agarose column. NAL was passed over the column, and purified antigen was then eluted. The eluate was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and RAST inhibition with sera from health care workers and children with spina bifida. CRI-C recognized a single band in ammoniated latex immunoblots and several distinct bands in NAL. The affinity-purified antigen of CRI-C (C-Ag) had multiple bands of less than 20 kd and was 3.9 times more potent in RAST inhibition than NAL when sera from patients with spina bifida were used. However, when health care workers' sera were used, there was no significant difference in the inhibitory potency of NAL and C-Ag. CRI-C appears to recognize a distinct and important epitope in the IgE immune response to latex of patients with spina bifida. (J ALLERGY CLIN IMMUNOL 1994;93:644-9.)*

**Key words:** Latex, rubber, immediate hypersensitivity, IgE, monoclonal antibodies, RAST, RAST inhibition, immunoblots, affinity purification

IgE-mediated reactions to natural rubber latex are associated with protein antigens present in finished rubber products. These antigens have been detected by a variety of methods in gloves,<sup>1</sup> condoms,<sup>2</sup> ammoniated and nonammoniated latex,<sup>3</sup> as well as in leaf extracts from the natural rubber tree *Hevea brasiliensis*.<sup>4, 5</sup>

Latex is an antigenically complex material, and molecular weight estimates of antigenic peptides have varied widely.<sup>6</sup> Many technical subtleties may explain these discrepancies, such as different antigen sources and preparation, separation or filtration techniques, and antigen detection methods. In addition, patient sera were used to detect

## Abbreviations used

AL:	Ammoniated latex extract
C-Ag:	C antigen (the affinity purification product of NAL passed over a CRI-C column)
CRI-C:	A Balb/C hybridoma that produces a monoclonal antibody that recognizes AL and NAL
NAL:	Nonammoniated latex extract
PBS:	Phosphate-buffered saline
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

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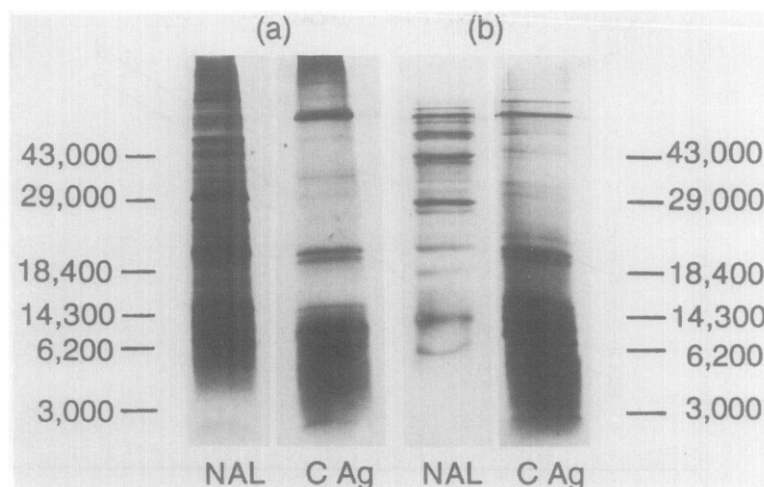
antigen in virtually all of the investigations, and the possibility exists that different at-risk patient populations may produce IgE that recognizes distinct latex peptides.

In this study we have developed a mouse hybridoma that produces antibody specific for latex antigens. This antibody binds to specific latex peptides and can be used to affinity purify latex antigens. The antigen so purified appears to be significant in binding latex-specific IgE from children with spina bifida.

## METHODS

### Latex sources and extract preparation

Nonammoniated latex was obtained from Dr. Katrina Cornish, U.S. Department of Agriculture. It had



**FIG. 1.** Fifteen percent SDS-PAGE of (lane a) nonreduced NAL and C-Ag, (lane b) reduced NAL and C-Ag. Molecular weight markers are indicated on the right and left.

been collected from *H. brasiliensis* in Costa Rica in Tris buffer (0.1 mol/L, pH 7.5) and sodium azide (0.01%) and shipped frozen on dry ice.<sup>7</sup> Ammoniated latex concentrate was obtained from Guthrie Latex (Baltimore, Md.). Nonammoniated latex extract (NAL) and ammoniated latex extract (AL) were prepared as previously described.<sup>3</sup>

### Production of mouse hybridomas

Balb/C mice (6 to 8 weeks old) were injected intraperitoneally with 200  $\mu$ g AL in complete Freund's adjuvant and were reinjected 2 weeks later with 200  $\mu$ g AL in incomplete Freund's adjuvant. After 3 more weeks, the mouse with the highest anti-AL antibody titer was injected with 60  $\mu$ g AL in sterile saline solution. Four days later, the mouse was killed; splenocytes were fused, by using polyethylene glycol 1500, with the myeloma cell line P3/NS1/1-Ag4-1 (ATCC, Rockville, Md.). Hybridoma cells were selected with medium containing hypoxanthine, aminopterin, and thymidine. Cell lines were tested for production of anti-AL and anti-NAL antibodies; these lines were selected and cloned at least four times by limiting dilution techniques.

Animal care and protocols were reviewed by the Animal Research Committee of the Children's Research Institute and followed the guidelines set out in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication no. 86-23, revised 1985).

### ELISA to detect rubber-specific antibody

Polyvinyl chloride microtiter plates (Falcon, Oxnard, Calif.) were coated with lyophilized AL or NAL (1  $\mu$ g/well) dissolved in a sodium carbonate-bicarbonate buffer (0.1 mol/L, pH 9.6). Wells were washed with 0.9% NaCl containing 0.1% Tween 20 and blocked with phosphate-buffered saline (PBS) containing 3% bovine

serum albumin, 0.05% Tween 20 and 0.01% sodium azide ("blocking buffer"). Then 100  $\mu$ L of tissue culture supernatant or dilutions of mouse serum or ascites were added and incubated at room temperature for 2 hours. Wells were washed, and anti-mouse IgG conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) in blocking buffer was added. After 2 hours, the wells were washed, and p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml in 0.1 mol/L glycine buffer, pH 10.4, containing 5 mmol/L  $MgCl_2$ ) was added. Absorbance was measured at 414 nm with a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, Calif.).

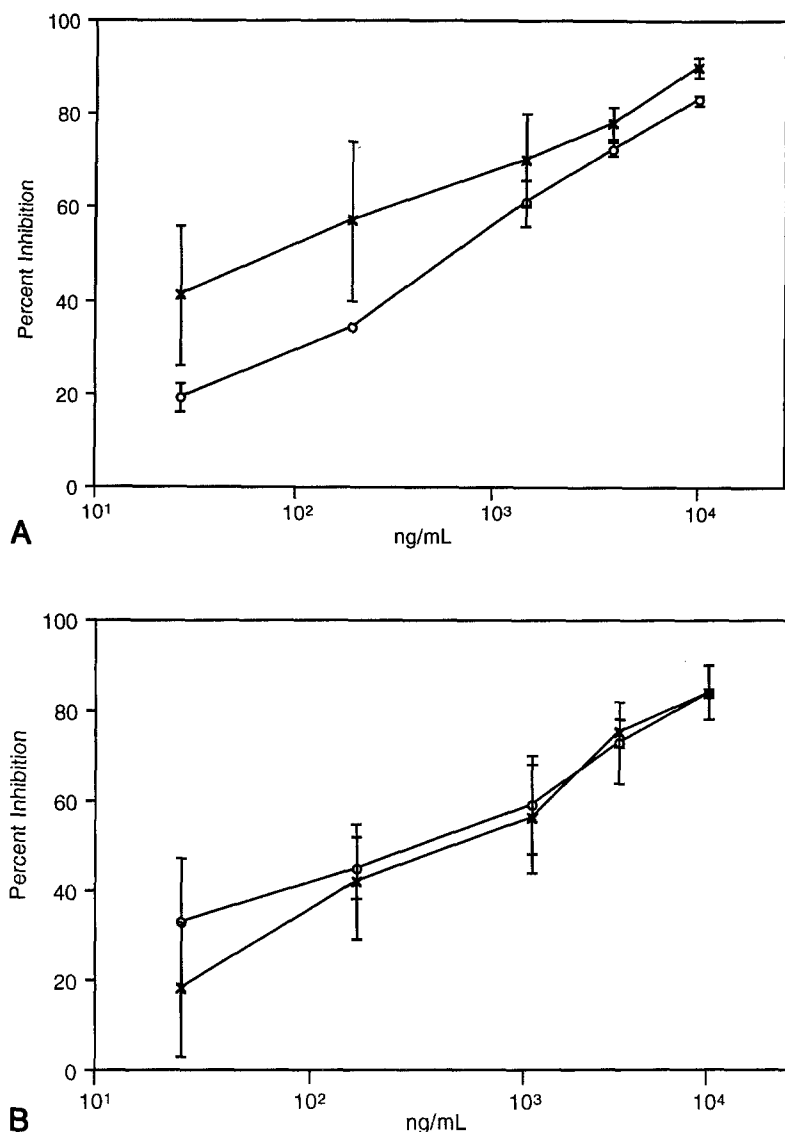
### Antibody purification

Monoclonal antibodies were purified from mouse ascites fluid. Ascites was raised in pristane-primed mice by the injection of rapidly growing cells suspended in sterile PBS. Antibody was purified by ammonium sulfate precipitation followed by ion-exchange chromatography and was at least 90% pure as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibody was isotyped with a kit purchased from Sigma.

### Electrophoresis

SDS-PAGE was performed with the Mini-Protein II system (Bio-Rad, Richmond, Calif.) in Laemmli's buffer. For immunoblotting, approximately 30  $\mu$ g of latex, with or without  $\beta$ -mercaptoethanol (5% vol/vol), was applied to each lane, and 15% gels were run at 200 V for 45 minutes. For silver staining of gels, about 10  $\mu$ g of antigen was applied to each lane.

For determination of mean molecular weights, stained gels were scanned with a Shimadzu CS9000 densitometer (Kyoto, Japan). Mean molecular weights



**FIG. 2.** AL-RAST inhibition, with pooled sera from (A) children with spina bifida and latex allergy or (B) health care workers with latex allergy. O, NAL; X, C-Ag. Each point is the average of three experiments  $\pm$  standard deviation.

were determined by the measurement of areas under the scanning peaks, with reference to molecular weight standards.

### Transfer and immunostaining

The separated latex peptides were transferred to a polyvinylene difluoride membrane (Millipore, Bedford, Mass.) at 240 A for 2 hours in Towbin's buffer.<sup>8</sup> The membrane was then stained with Coomassie Brilliant Blue (Sigma) (0.1% in 50% methanol) and cut into strips for immunostaining. These strips were blocked in blocking buffer for 2 hours. The strips were then incubated in sequence with purified antibody (50  $\mu$ g/ml) in blocking buffer and alkaline phosphatase

conjugated to anti-mouse IgG (Kirkegaard and Perry Laboratories). They were developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Bethesda Research Laboratories, Gaithersburg, Md.).

### Affinity purification protocol

Ten milligrams of pure antibody was bound to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden); nonreacted sites were blocked by alkaline hydrolysis. After extensive washing, 10 mg of NAL in PBS was added; the column was then washed with 10 column volumes of PBS, and bound antigen was eluted with glycine buffer (0.1 mol/L, pH 2.8). Eluted material was neutralized with 1/10 volume Tris buffer

(1 mol/L, pH 8) and analyzed on a 15% SDS-polyacrylamide gel, which was stained with silver nitrate (Silver Stain Plus, Bio-Rad).

### Protein assays

The protein contents of AL, NAL, and the purified antigen were estimated by absorption of unmodified protein at 280 nm and by absorption of Coomassie Brilliant Blue bound to protein at 570 nm (Bradford protein assay; Pierce Chemical Co., Rockford, Ill.). Absorbance was compared with a known quantity of bovine serum albumin for all assays. When the two assays were compared, the absorption coefficient of the latex antigens was found to be  $0.38 \pm 0.05$ . The Bradford assay was used as the standard for all subsequent calculations.

### RAST inhibition

Serum for RAST inhibition studies was pooled from two sources. One pool was from six children with spina bifida who had high titers of anti-latex IgE. Serum samples were collected after informed written consent was obtained as part of a study approved by the Institutional Review Board of Children's National Medical Center. Serum samples were also obtained for study from five health care workers with clinical latex allergy and a positive AL RAST result.

AL RAST was performed as previously described.<sup>9</sup> Briefly, 1  $\mu$ g of AL was bound to each well in a polyvinyl chloride microtiter plate, which was then blocked with blocking buffer and incubated sequentially with patient serum and iodine 125-labeled rabbit anti-human IgE (Pharmacia Diagnostics, Uppsala, Sweden). RAST inhibition studies were performed by adding NAL or affinity-purified antigens in blocking buffer to the pooled RAST-positive serum and preincubating overnight at 4° C before addition to AL-coated plates.

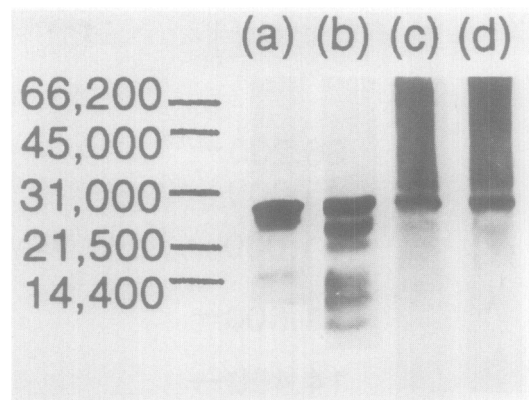
### IgE immunoblot inhibition

Aliquots of pooled sera were preincubated with NAL or C-Ag in 50-fold excess of the inhibitory concentration of 50% of these antigens, as determined in the RAST inhibition assays. Strips of polyvinylene difluoride with separated peptides of NAL were blocked and incubated overnight at 4° C with these pooled sera (1:10, vol/vol, in blocking buffer). They were then washed and incubated for 6 hours in blocking buffer containing <sup>125</sup>I-labeled rabbit anti-human IgE (Pharmacia Diagnostics). The strips were then washed, air-dried, and exposed to X-omat film (Kodak, Rochester, N.Y.) at -70° C.

## RESULTS

### Monoclonal antibodies

Several hybridoma cell lines that produce antibodies that recognize AL and NAL were devel-



**FIG. 3.** Immunoblot with antibody CRI-C of (lane a) non-reduced NAL, (lane b) reduced NAL, (lane c) nonreduced AL, and (lane d) reduced AL. Molecular weight markers are indicated on the left.

oped. CRI-C was an IgG<sub>1</sub> with the highest affinity and was used in all subsequent experiments.

### SDS-PAGE of purified antigens

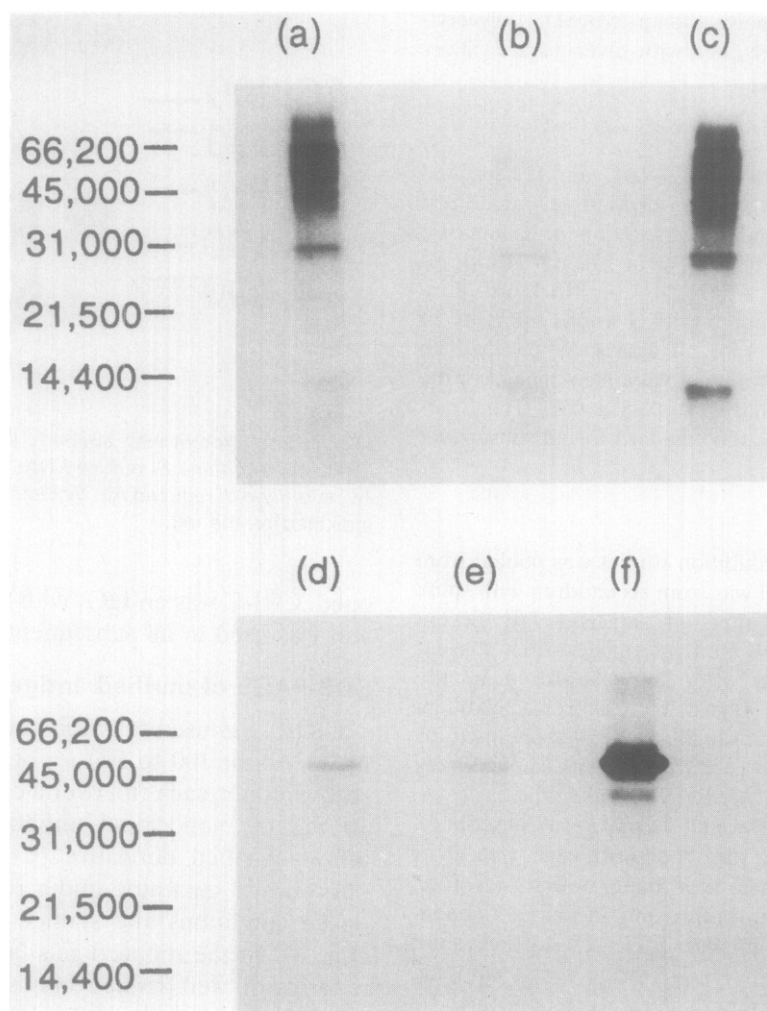
CRI-C was used to affinity purify material from NAL. About 100 to 300  $\mu$ g of material could be recovered for each run over a column bearing 8 to 10 mg of monoclonal antibody. NAL and its affinity-purified derivative (C-Ag) were electrophoretically separated under reduced and nonreduced conditions and stained with silver nitrate (Fig. 1). In the reduced gels NAL separated into a series of well-defined bands, the most prominent of which were at 6.2, 14.3, 21.3, 27.5, and 43 kd, with a few bands migrating more slowly than the 43 kd standard. C-Ag had a prominent broad band from 3 to 6.2 kd, a series of bands from 8.8 to 15.5 kd, a doublet at 20 kd, minor bands above 20 kd, and several bands greater than 43 kd.

Mean molecular weights, estimated by scanning the nonreduced gels, were 26 kd for NAL and 21 kd for C-Ag.

### RAST inhibition

RAST inhibition data with pooled sera from children with spina bifida were used to assess the relative potency of NAL and C-Ag. On the basis of the 50% inhibition points, C-Ag was 3.9 times more potent than NAL (54 ng/ml compared with 210 ng/ml; Fig. 2, A).

When RAST inhibition was performed with pooled sera from health care workers allergic to latex, the inhibitory concentration of 50% of C-Ag and NAL were virtually identical (150 ng/ml for NAL and 200 ng/ml for C-Ag; Fig. 2, B).



**FIG. 4.** IgE immunoblot inhibition of separated reduced NAL. *Lanes a to c*, Pooled spina bifida sera; *lanes d to f*, pooled health care worker sera. Antigen added: *lanes c and f*, none; *lanes b and e*, NAL; and *lanes a and d*, C-Ag.

### Immunostain

Immunostains with CRI-C detected a clear band in AL extracts; this band had an apparent molecular weight of 26 kd. In contrast, CRI-C detected several distinct peptides in the NAL extract. For reduced NAL, the darkest bands detected by CRI-C were at 26 and 22.5 kd, but four bands from less than 14.4 kd to 20.6 kd were also detected. In nonreduced NAL, CRI-C detected a prominent band at 26.8 kd (Fig. 3).

### IgE immunoblot inhibition

Western blot of reduced NAL with pooled sera from health care workers showed IgE binding predominantly to the 43 kd band; other bands were bound to a lesser degree. Preincubation of the sera with excess NAL and C-Ag led to a

dramatic inhibition of IgE binding to the strips, and the intensity of the bands decreased proportionately.

In contrast, when sera from children with spina bifida were used, IgE binding was mainly found at the 14.3 and 27.5 kd bands, as well as a series of peptides from 43 kd to more than 66.2 kd. Although preincubation with excess NAL led to a significant decrease in the binding of IgE to all bands, preincubation with C-Ag produced a selective decrease in IgE binding to the 14.3 kd band (Fig. 4).

### DISCUSSION

Latex antigens were used to raise an antigen-specific mouse monoclonal antibody, and this antibody was used to affinity purify antigenic pep-

tides from NAL. Silver-stained polyacrylamide gels of affinity-purified peptides suggest that CRI-C recognizes an epitope found predominantly on peptides with molecular weights of less than 20 kd. In the immunostain studies, CRI-C bound to different peptides than during affinity purification, probably because of different binding affinities of the antibody to soluble and membrane-bound antigens.

RAST inhibition studies with pooled sera from patients with spina bifida showed C-Ag to be 3.9 times as potent as NAL; pilot studies with individual sera had produced similar results.<sup>10</sup> Although this may in part be due to the lower mean molecular weight of C-Ag, it cannot fully account for this observation. First, C-Ag was estimated to have a mean molecular weight only 19% less than NAL as determined by scanning densitometry. Second, C-Ag was no more potent than NAL in blocking latex-specific IgE in sera from health care workers with latex allergy. Thus the lower 50% inhibition concentration appears to reflect the importance of C-Ag in the IgE immune response to latex in patients with spina bifida.

At first glance, the Western blot inhibition studies appear to contradict the RAST inhibition data: C-Ag appears to be more effective at binding to latex-specific IgE in health care workers' sera than in sera from patients with spina bifida. However, because these experiments (unlike the RAST inhibition experiments) were performed with large excesses of antigen, it is likely that the nonspecific inhibitory effect of C-Ag in health care workers' sera was due to small amounts of contaminating NAL. In contrast, in the sera of the patients with spina bifida, C-Ag blocks only the 14.3 kd-specific IgE, and the contaminating NAL has insufficient affinity for other latex-specific IgE in the sera to inhibit IgE binding to higher molecular weight peptides on the immunoblot strips.

We believe that CRI-C recognizes a major antigen of *H. brasiliensis* for children with spina bifida. It binds to a 26 to 27 kd peptide band present in both AL and NAL and can be used to purify a material from NAL (C-Ag) with greatly enhanced ability to block the binding of latex-specific IgE to solid-phase antigen. IgE immuno-

blot inhibition reveals selective binding of C-Ag to 14.3 kd-specific anti-latex IgE in the sera of patients with spina bifida. These results are consistent with a previous report from this laboratory, in which IgE from patients with spina bifida was shown to bind to a 14 kd band, and to a lesser degree, to 18 and 28 kd bands in NAL.<sup>3</sup>

CRI-C may be useful in advancing several studies of the human immune response to latex products. It can be used to purify C-Ag, which in turn may be useful in improving the safety and predictive value of diagnostic studies in patients with latex allergy and could facilitate strategies for the immunotherapy of this potentially fatal disorder.<sup>11</sup> CRI-C may also be used for the in situ localization of the molecule in sections of plant tissue from *H. brasiliensis*. Finally, CRI-C may be of value in the quantification of clinically relevant extractable antigen in latex products. Such studies are in progress.

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