

The 14.6 kd rubber elongation factor (Hev b 1) and 24 kd (Hev b 3) rubber particle proteins are recognized by IgE from patients with spina bifida and latex allergy

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Two major water-insoluble proteins are located on the surface of rubber particles in *Hevea brasiliensis* latex. A 14.6 kd protein (Hev b 1), found mainly on large rubber particles (>350 nm in diameter), and a 24 kd protein (Hev b 3), found mainly on small rubber particles (average diameter, 70 nm), are recognized by IgE from patients with spina bifida and latex allergy. Although Hev b 1 (also called the rubber elongation factor [REF]) has previously been reported as a major latex allergen, this conclusion has been disputed on the basis of results from other studies. The allergenicity of Hev b 1 is verified in this study by testing the recombinant protein generated from its gene. Because allergenicity is confined to patients with spina bifida and not observed in adults sensitive to latex, it is not a major latex allergen. The identification of Hev b 3 as another allergen originating from rubber particles is confirmed by immunogold labeling and electron microscopy. Observations with the monoclonal antibody USM/RC2 developed against Hev b 3 show that the protein has a tendency to fragment into several polypeptides of lower molecular weight (from 24 kd to about 5 kd) when stored at -20° C. There is also indication of protein aggregation from the appearance of proteins with molecular weights greater than 24 kd. Fragmentation of Hev b 3 is induced immediately on the addition of latex B-serum, which is normally compartmentalized in the luteoids in fresh latex. In the preparation of ammoniated latex (used for the manufacture of latex products), the luteoids are ruptured, and the released B-serum reacts with Hev b 3 on the rubber particles to give rise to an array of low molecular weight polypeptides that are allergenic to patients with spina bifida. (*J Allergy Clin Immunol* 1996;98:628-39.)

Key words: Latex, latex allergy, spina bifida, monoclonal antibodies, immunogold labeling, rubber particle, rubber particle protein, Hev b 1, Hev b 3, USM/RC2

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Type I hypersensitivity to latex from the commercial rubber tree, *Hevea brasiliensis*, has recently received great attention with the increase in reports of such incidences. Many latex allergens have been reported in the literature.^{1, 2} In the main, they have not been well characterized, but most are thought to be soluble, and therefore extractable in water.³ Although allergy to proteins present in natural rubber latex and in manufactured latex products has commonly been associated with persons who are frequently in contact with latex products, such as examination gloves (e.g., health care workers), patients with spina bifida who undergo multiple surgical procedures are another risk group.^{4, 5} Patients with spina bifida are especially sensitive to specific allergens that do not

Abbreviations used

EDTA:	Ethylenediaminetetraacetic acid
MBP:	Maltose-binding protein
PCR:	Polymerase chain reaction
REF:	Rubber elongation factor
SDS:	Sodium dodecylsulfate
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

usually affect adult patients without spina bifida who are otherwise sensitive to latex.⁵

Recently, Czuppon et al.⁶ announced that the "rubber elongation factor" (REF), a 14.6 kd protein found on the surface of rubber particles,⁷ was the major allergen in natural rubber latex. A subsequent note⁸ by the same group identified the 14-amino-acid N-terminus of the REF, which they designated Hev b 1, as the major epitope. This proposition was disputed by McCullough et al.⁹ who could not corroborate the findings of Czuppon et al.⁶ Sunderasan et al.,¹⁰ who extracted a 14 kd protein (believed to be the REF) from rubber particles derived from fresh natural rubber latex, were also unable to demonstrate allergenicity of the protein.

This study is aimed at investigating the allergenicity of the two major water-insoluble rubber particle proteins of *Hevea* latex, one of which is the REF. The allergenicity of the REF (Hev b 1) to patients with spina bifida is verified by testing the recombinant protein generated from its gene. The putative allergen so synthesized would be completely free from contamination by other latex proteins that might co-purify with REF, had the latter been isolated from latex. This report also identifies another rubber particle allergen (Hev b 3), which is specific to patients with spina bifida. It is further shown that the group of low molecular weight polypeptides, to which many patients with spina bifida are sensitized, are derived from the fragmentation of Hev b 3.

METHODS

Latex separation by high-speed centrifugation

Rubber trees (*Hevea brasiliensis*) of the clone RRIM 600, growing in the Rubber Research Institute of Malaysia Experiment Station in Selangor Darul Ehsan, were tapped; and the exuded latex was collected into chilled containers. The latex was centrifuged at 44,000 g in a Sorvall RC 5C high-speed centrifuge (Du Pont Company, Wilmington, Del.) for 1 to 2 hours, after which the latex separated into three main fractions: the

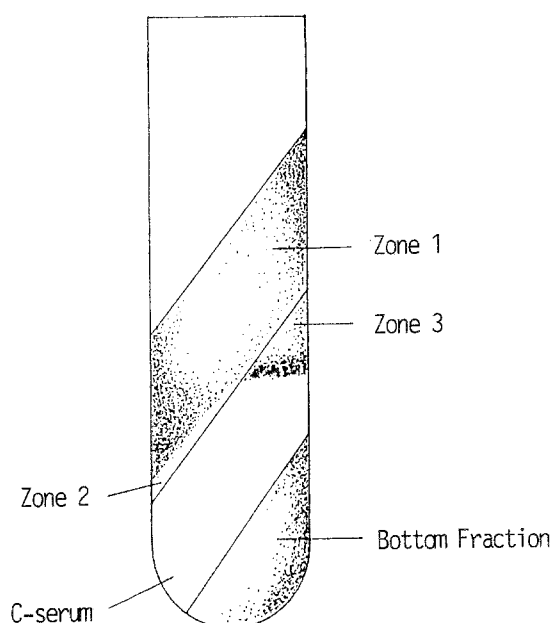


FIG. 1. High-speed centrifugation of natural rubber latex. Centrifugal zones are labeled according to Moir.¹¹

rubber cream at the top, the "bottom fraction," and the aqueous C-serum in between. The rubber cream comprised mainly spherical rubber particles containing rubber (cis-polyisoprene). Most of the rubber cream consisted of rubber particles of the larger species (generally >350 nm in diameter), found in Zone 1 of the centrifuged latex, according to Moir's¹¹ terminology (Fig. 1). Smaller rubber particles (average diameter, 70 nm) were found in the Zone 2 rubber cream and also as suspended particles in Zone 3 and in the C-serum (Fig. 1).

A spatula was used to make an opening in the rubber cream layer of the centrifuged latex through which a Pasteur pipette was inserted to collect C-serum. Zone 1 rubber cream was sampled from the center portion of the zone after 1-hour centrifugation. To obtain Zone 2 rubber cream, the C-serum was drained away after 2-hour centrifugation, and the entire rubber fraction (Zones 1 and 2 together) was then scooped out. Zone 2 rubber cream at the bottom of the rubber fraction was skimmed off and collected with a spatula.

Protein preparation from rubber particles

The rubber cream from centrifuged latex was collected in its entirety. For some experiments the portions of the cream representing Zone 1 and Zone 2 were sampled separately. The rubber particles in the rubber cream samples were washed by resuspension in 30% sucrose and recovered by centrifugation. The proteins on the surface of the rubber particles were then solubilized and extracted by adding an equal volume of detergent comprising 0.1% Triton-X 100 and 1% sodium dodecylsulfate (SDS) to the rubber cream. The

mixture was vortexed and then centrifuged to obtain the supernatant that contained solubilized proteins.

RNA isolation from latex

Standard methods described elsewhere¹² were used in the preparation and purification of DNA, mini- and maxipreps, DNA purification, restriction endonuclease digestions, agarose gel electrophoresis, ligations, transformations, and poly(A)⁺ messenger RNA isolation by oligo(deoxythymidylic) acid cellulose column chromatography.

About 80 ml of latex was collected in 120 ml of RNA extraction buffer (0.1 mol/L Tris-HCl, 0.3 mol/L LiCl, 0.01 mol/L ethylenediaminetetraacetic acid [EDTA], 10% SDS, pH 9.5). The mixture was centrifuged in polyallomer tubes at 112,700 g for 30 minutes at 15° C. The rubber plug was then removed, and total RNA was isolated from the aqueous phase according to the method of Prescott and Martin.¹³ Poly(A)⁺ mRNA was isolated by oligo(deoxythymidylic) acid-cellulose affinity column chromatography of latex total RNA.

Synthesis of complementary DNA for the coding region of REF

REF cDNA synthesis. First-strand cDNA synthesis was prepared by reverse transcribing 50 ng of latex poly(A)⁺ mRNA in a volume of 50 μ l with the Riboclone cDNA Synthesis System (Promega Corp., Madison, Wis.) according to the vendor's instructions. Synthesis of cDNA was accomplished by polymerase chain reaction (PCR) amplification. Each reaction was performed in a volume of 50 μ l containing 50 ng of first-strand cDNA template, 0.04 mol/L Tris HCL (pH 7.2), 0.09 mol/L KCl, 0.005 mol/L MgCl₂, 0.005 mmol/L deoxyadenosine triphosphate, 0.005 mol/L deoxythymidine triphosphate, 0.005 mol/L deoxycytidine triphosphate, 0.005 mol/L deoxyguanosine triphosphate, 2.5 U Taq polymerase (Amplitaq; Perkin-Elmer Corp., Norwalk, Conn.), and 500 ng of each primer overlaid with paraffin (80 μ l). The 40-mer oligonucleotide primers used were: 5'CGATGGTACCATGGCTGAAGACGAAGACAACCAACAAGGG3' (REF₁) and 5'CGATGGATCCTCAATTCTCTCCATAAAACACCTTAGCAAG3' (REF₂).

The sequences of primers REF₁ and REF₂ corresponded to positions 39-68 and 426-455 (complementary strand), respectively, in the cDNA insert of the clone pREF1.¹⁴ Primers REF₁ and REF₂ also contained the *Asp*718 and *Bam*HI recognition sequences at their 5' and 3' ends, respectively. The reactions for PCR were programmed for 40 cycles on a DNA thermal cycler (Perkin-Elmer Corp.), each cycle being performed at a denaturing temperature of 94° C (duration, 1 minute), annealing temperature of 43° C (duration, 2 minutes), and extension at 72° C (duration, 1 minute). After PCR, the cDNA product was detected by electrophoresing a 5 μ l aliquot of the reacted mix on agarose (1% wt/vol,

Tris-borate-EDTA buffer)/ethidium bromide (5 μ g/ml) gels.

The cDNA was purified by chloroform-isoamyl alcohol (24:1) extraction and followed by proteinase K (100 ng) digestion for 30 minutes at 37° C. The cDNA band excised from the agarose (1.2%, Tris-acetate-EDTA buffer)/ethidium bromide gel was purified and double-digested to completion with *Asp*718 and *Bam*HI. The digested cDNA was purified by phenol-chloroform (50:50) extraction and precipitated in ethanol in the presence of one-tenth volume of 8 mol/L LiCl and 1 μ l of glycogen (Boehringer Mannheim GmbH, Mannheim, Germany).

Cloning of REF cDNA. The phagemid, pBluescript SK⁺ (Stratagene, La Jolla, Calif.), was linearized by double-digesting to completion with *Asp*718 and *Bam*HI. The cDNA for the coding region of REF was ligated to the phagemid by T4 DNA ligase, and 7 μ l of the ligate was used for the transformation of *Escherichia coli* DH5 α to ampicillin resistance. The resulting clones were screened by miniprep assay, and amplification of the recombinant plasmid (pLREF) was carried out by maxiprep.

Nucleotide sequencing and subcloning of REF cDNA. Both strands of the REF cDNA insert were sequenced by the Sanger's dideoxyribonucleotide chain termination method¹⁵ with the T3 and T7 primers (Stratagene), Taq DNA polymerase, and α -sulfur 35-labeled deoxyadenosine triphosphate.

Because the REF cDNA had originally been cloned for another purpose in an unrelated study, a series of restriction endonuclease digestions and subclonings was performed to ensure that the REF cDNA insert was ligated to the pMAL-c2 vector in the same translatable reading frame as the *malE* gene. Accordingly, the cDNA insert of pLREF was first excised by double-digestion of the fusion plasmid with *Asp*718 and *Bam*HI. It was then subcloned into the pUC18 plasmid at the same sites to produce the clone pUREF. The cDNA insert was subsequently excised from pUREF by double-digesting it with *Eco*RI and *Bam*HI and then subcloned into pMAL-c2 (New England Biolabs, Inc., Beverly, Mass.) at the same sites to produce the clone pMREF in the same translational reading frame as the *malE* gene of the vector. The pMAL-c2 protein fusion and purification system was used to overexpress REF from its cloned cDNA.

Overexpression of REF

The procedures used for the induction of fusion protein overproduction, affinity chromatography purification, cleavage of fusion protein by factor Xa protease, and purification of REF target protein by hydroxyapatite chromatography were done according to the vendor's instructions.

Induction for expression of maltose-binding protein (MBP)-REF fusion protein. The procedure used was according to instructions from the vendors for the

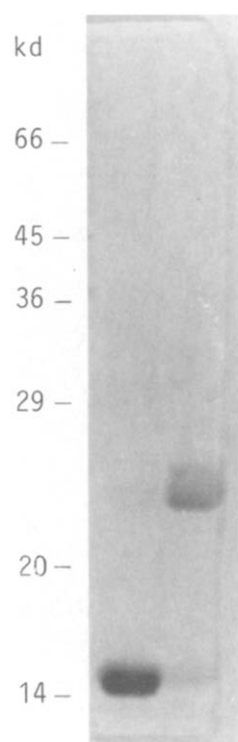


FIG. 2. SDS-PAGE (11% gel) of proteins extracted from Zone 1 (*left*) and Zone 2 (*right*) of the rubber cream of centrifuged latex. Molecular weight calibration is by markers from Sigma Chemical Company.

Fusion Protein and Purification Systems kit (New England Biolabs, Inc.). Isopropyl β -D-thiogalactopyranoside-induced *E. coli* cells were disrupted by a freeze (-20°C)–thaw cycle (ambient temperature) and sonication (Vibra Cell; Sonics & Materials, Inc., Danbury, Conn.) in an ice-water bath with 15-second pulses for 3 minutes. The release of fusion protein eluted from the amylose column was monitored by the Bradford assay.¹⁶ Cleavage of the MBP-REF fusion protein was by factor Xa protease¹⁷ in the presence of 0.04% (wt/vol) SDS.

Preparation of rubber particles and B-serum

To carry out immunogold labeling with IgE antibodies, Zone 2 rubber particles in 30% sucrose were chromatographed on a Sephacryl S-200 1.6×82 cm column (Pharmacia Biotech, Uppsala, Sweden), equilibrated with 0.05 mol/L Tris-HCl, pH 7.8. Fractions eluted from the chromatographic column were monitored by light scattering at 650 nm, which showed that most of the rubber particles had been recovered in the void volume. For immunogold labeling with the monoclonal antibody USM/RC2, small rubber particles that remained suspended in the C-serum after high-speed centrifugation were recovered by passing the reconstituted freeze-dried C-serum through a Sephadex G-150 chromatography column ($2.6 \text{ cm} \times 81 \text{ cm}$, Pharmacia Biotech) and eluted with 0.25

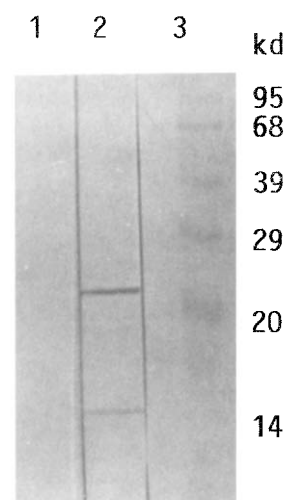


FIG. 3. Western blot of rubber particle proteins (14.6 kd and 24 kd) extracted from *Hevea* latex assessed for their binding with IgE by incubating with sera from adult latex-allergic patient (*lane 1*) and patients with spina bifida (*lane 2*). Molecular weight markers (Integrated Separation Systems, Natick, Mass.) are shown in *lane 3* (SDS-PAGE, 15% gel).

mol/L Tris-HCl, pH 8.0. The first distinct peak that was eluted represented the void volume of the column and contained considerable quantities of small rubber particles visible under the electron microscope.

B-serum was prepared as previously described.¹⁸ Briefly, the bottom fraction (consisting mainly of vacuole-like organelles called lutoids) was recovered and resuspended in 0.4 mol/L mannitol. The bottom fraction was resedimented by centrifugation and then subjected to repeated alternate freezing and thawing to rupture the lutoids.¹⁹ The fluid from the lutoids, the B-serum, was recovered by centrifugation.

Monoclonal antibodies

Spleen cells from a BALB/c mouse immunized with latex C-serum were fused with mouse myeloma cells according to protocols previously described by Kohler and Milstein.^{20,21} The resulting hybridoma cells were screened for antibodies specific to C-serum proteins. Selected hybridomas were recloned twice, and secreted monoclonal antibodies were used either in unpurified form in hybridoma cell supernatants or as preparations purified by affinity chromatography.

Electrophoresis and immunodetection

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out (usually on 12% or 15% gels) according to the method of Laemmli.²² Typically, a protein sample of 10 to 50 μg (depending on whether single proteins or protein mixtures were analyzed) was loaded in each well of the gel intended for

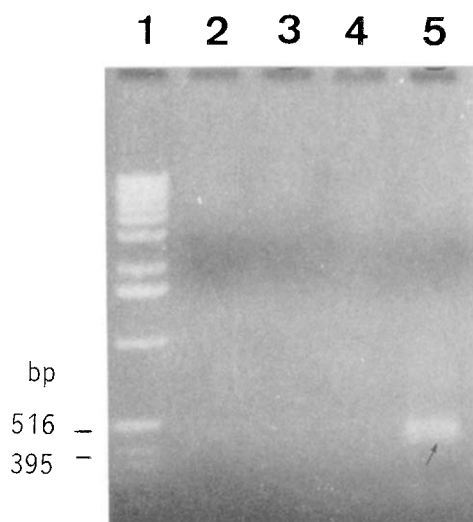


FIG. 4. Agarose gel (1%, Tris-borate-EDTA buffer) electrophoresis of cDNA (arrow) of REF's coding region as generated by PCR. Lane 1, 1 kb ladder DNA marker (Gibco BRL Life Technologies, Inc., Grand Island, N.Y.); lanes 2 to 4, PCR with 5, 25, and 50 ng of template DNA, respectively, in the absence of $MgCl_2$; lane 5, PCR with 50 ng of template DNA in the presence of 0.005 mol/L $MgCl_2$.

Western blotting. Molecular weight reference markers were run simultaneously in all experiments. Separated proteins were transferred electrophoretically to a nitrocellulose membrane (Western blotting), which was then blocked with phosphate-buffered saline–nonfat milk and incubated with the primary antibody being tested. Detection of allergen-antibody binding was achieved by using an enzyme (horseradish peroxidase)–conjugated secondary antibody against human IgE to generate an insoluble colored product. To detect the binding of proteins to rabbit or mouse immunoglobulins, nitrocellulose membranes were incubated with serum from appropriately immunized animals, followed by incubation with the respective enzyme-conjugated secondary antibody. Matching gels were stained with either Coomassie Blue R250 or silver according to the vendor's instructions (Bio-Rad, Microscience Div., Hercules, Calif.).

Two-dimensional electrophoresis was carried out with isoelectric focusing for the first dimension and SDS-PAGE for the second dimension with the Mini-Protean II apparatus (Bio-Rad Laboratories, Life Science Group, Hercules, Calif.), based on a modified method of O'Farrell²³ and according to the manufacturer's instructions. Briefly, tube gels used for isoelectric focusing were pre-electrofocussed by running at 200 V for 10 minutes, 300 V for 15 minutes, and 400 V for 15 minutes before addition of the antigen samples. Samples containing a mixture of C- and B-sera (5:2) were applied to 4% polyacrylamide tube gels containing ampholyte (pH 3 to 10). Isoelectric focusing was performed at constant voltage of 500 V for 10 minutes and thereafter at 750 V for 4 hours. After isoelectric focusing, tube gels were

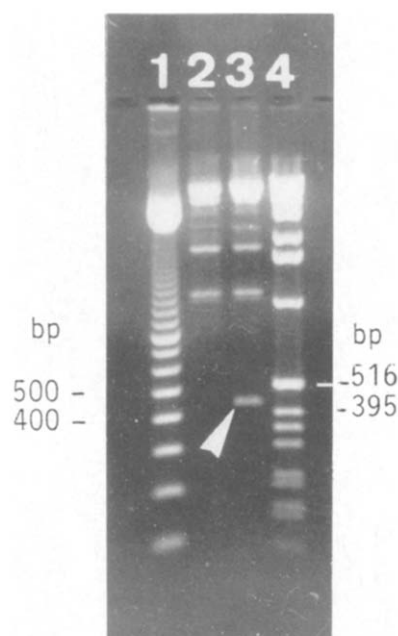


FIG. 5. Agarose gel (0.7%, Tris-borate-EDTA buffer) electrophoresis of pMREF (MBP-REF fusion plasmid) after double-digesting with *EcoRI* and *BamHI*. Lane 1, 100 bp ladder DNA marker (Pharmacia Biotech Norden, Uppsala, Sweden); lane 2, pMREF (undigested); lane 3, pMREF double-digested with *EcoRI* and *BamHI* to release the REF cDNA insert fragment (arrowhead); lane 4, 1 kb ladder DNA marker (BRL).

loaded on 12% SDS-polyacrylamide slab gels for electrophoresis for 45 minutes.

Dot blots were carried out by pipetting 2 to 10 μ g of test protein contained in 2 μ l directly on to nitrocellulose membrane. The immunologic reaction was carried out in the same manner as with the Western blot.

Immunogold labeling and electron microscopy

Rubber particles prepared from centrifuged latex or gel-filtered C-serum were fixed briefly in 0.25 mol/L osmium tetroxide and deposited on to Formvar carbon-coated nickel grids. The grids were first blocked for 30 minutes with phosphate-buffered saline, pH 7.2, containing 1% bovine serum albumin. To detect Hev b 3, incubation was carried out with USM/RC2 as the primary antibody for 15 minutes. Labeling was then carried out with 5 or 10 nm colloidal gold conjugated to goat anti-mouse antibody. To detect patient IgE, incubation was carried out with patient plasma and then with a monoclonal mouse anti-human IgE antibody (Sigma Chemical Co., St. Louis, Mo.). Gold labeling was performed as for Hev b 3. Control samples, in which the primary antibody was omitted or substituted with an irrelevant antibody, were also prepared. The test and control samples were then examined under a Philips CM 12 electron microscope (Philips, Eindhoven, The Netherlands).

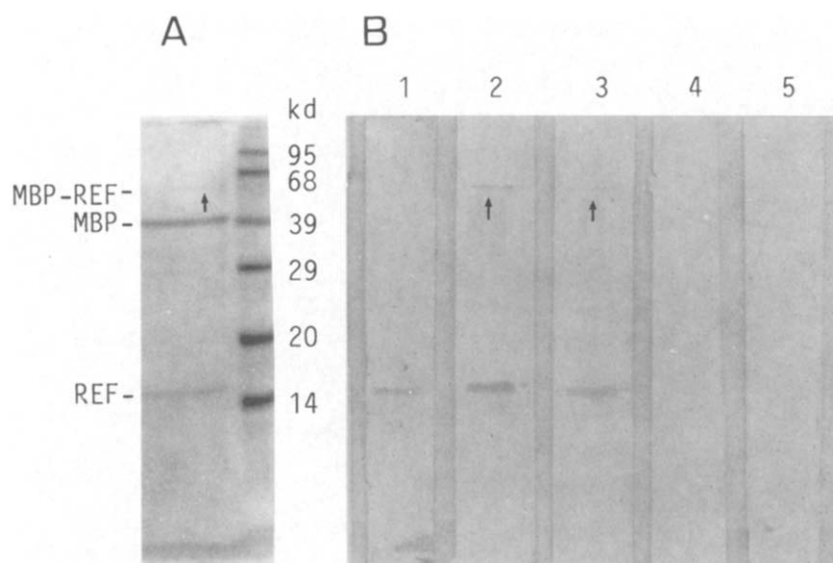


FIG. 6. Allergenicity of REF synthesized by *E. coli*. **A**, Electrophoretic separation of the fusion maltose-binding protein (MBP), REF, and the fusion protein MBP-REF (15% gel, SDS-PAGE, Coomassie Blue staining). **B**, Western blots from a similar gel incubated with patient or rabbit serum to show binding of REF and MBP with patient IgE or with rabbit IgG. Lane 1, Rabbit immunized against latex glove eluate; lanes 2 and 3, two spina bifida pools; lane 4, pool obtained from pediatric patients without spina bifida; lane 5, adult latex-allergic patient. Molecular weight markers (Integrated Separation System) are shown. Bands corresponding to MBP-REF are marked with arrows.

Patient sera and plasma

Thirteen blood serum samples from latex IgE-positive adult patients were obtained from the Johns Hopkins University School of Medicine and from Diagnostics Products Corporation (Los Angeles, Calif.). In addition, six plasma samples from adult latex-allergic patients were obtained from PlasmaLab International (Everett, Wash.). All adult patients had clinical symptoms of latex allergy, and the plasma and serum samples had anti-latex IgE confirmed by RAST or ELISA. Two IgE-positive serum pools from children with spina bifida were tested. Pool 1 comprised sera from 53 patients with spina bifida with a variety of clinical sensitivities, and pool 2 comprised a mixture of 101 serum samples from patients with confirmed spina bifida and from patients who were latex-positive but in whom a diagnosis of spina bifida had not been confirmed. Control plasma samples from adult patients who did not have a record of frequent contact with latex were obtained from the Kuala Lumpur General Hospital. For control pediatric sera, Bangkok Pooled Convalescent Sera combined from more than 200 patients with serologically confirmed dengue from the Children's Hospital, Bangkok, was used. All adult serum and plasma samples (from control and latex-allergic patients) were not pooled but were tested for immunoreaction individually. Sera and plasma used for incubations in Western and dot blots were diluted 1:5 with the blocking buffer.

RESULTS

Allergens on the surface of rubber particles in *Hevea* latex

Two main proteins of approximately 14.5 and 24 kD were observed when the extract of the washed rubber cream of centrifuged latex was analyzed by SDS-PAGE, as noted previously.²⁴ The smaller protein (more accurately, 14.6 kD) has previously been named the *rubber elongation factor* (REF) by Dennis and Light.⁷ When rubber particles from Zone 1 and Zone 2 of the centrifuged latex cream were isolated and extracted separately, the 14.6 kD protein was found principally on the larger Zone 1 particles (>350 nm in diameter), whereas the 24 kD protein was extracted mainly from the smaller Zone 2 particles (average diameter, about 70 nm) (Fig. 2). There were traces of 14.6 kD protein in the Zone 2 extract, just as there were traces of the 24 kD protein in the Zone 1 extract. Another protein of approximately 73 kD was frequently seen in the Zone 2 extract but was absent from the Zone 1 extract. Other faint protein bands were sometimes observed, but it was not clear whether these were minor rubber particle surface proteins or contaminants from the C-serum. Dot blots and Western

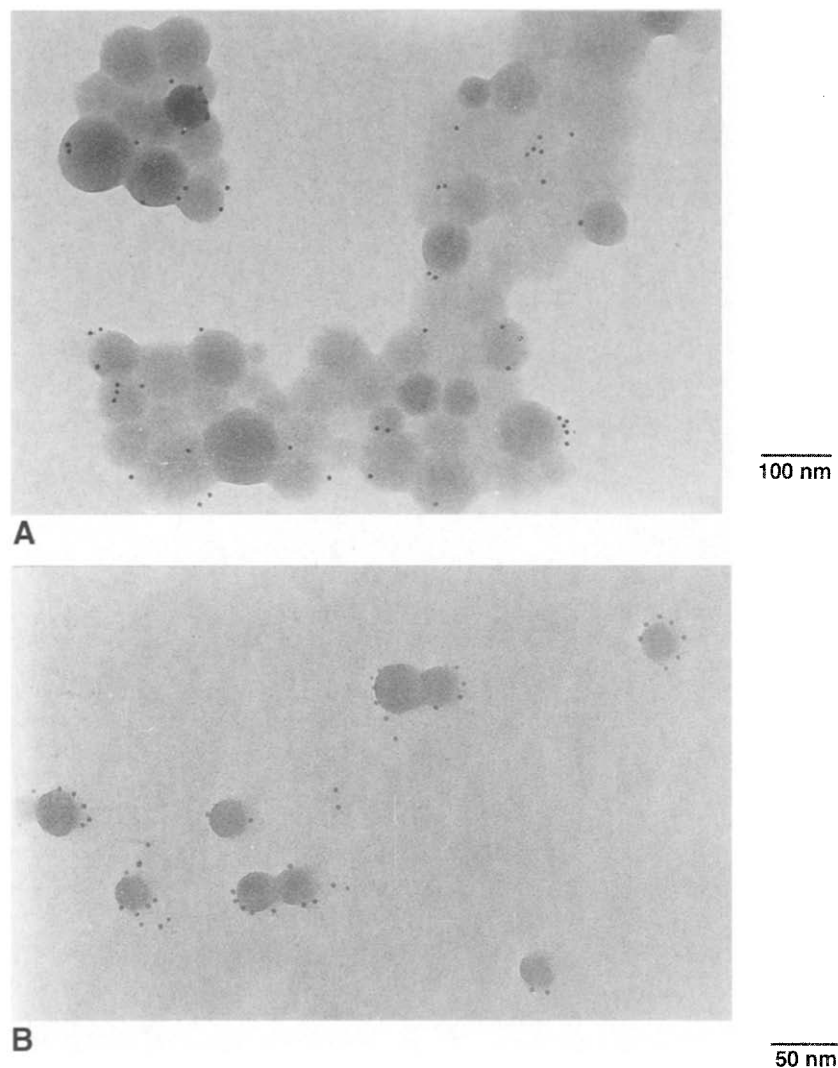


FIG. 7. Immunogold labeling of small rubber particles from the rubber cream of centrifuged latex (**A**) and gel-filtered C-serum (**B**). Rubber particles were labeled first by incubation with the monoclonal antibody USM/RC2 as the primary antibody and goat anti-mouse antibody conjugated to colloidal gold as the secondary antibody. Colloidal gold of 10 nm and 5 nm in diameter was used in **A** and **B**, respectively. Labeling is visible mainly on the periphery of the whole mounted rubber particles because of the narrow focusing depth of field.

blots of rubber particle proteins on nitrocellulose membranes were incubated with various plasma and serum samples of patients with histories of latex allergy. The membranes were then incubated with a conjugated secondary antibody with specificity for the epsilon heavy chain of human IgE. Control samples from nonallergic patients were also tested. As exemplified in Fig. 3, none of the 20 plasma and serum samples from latex-allergic adult patients were positive against the 14.6 and 24 kd rubber particle proteins. Sera from pediatric patients without spina bifida were similarly negative (result not presented). On the other hand, sera from both the spina bifida pools were positive for

both the 14.6 and 24 kd proteins. The results of the Western blots were confirmed with dot blots, which produced similar results for the two spina bifida serum pools and for the 20 individual serum and plasma samples. The 24 kd rubber particle protein is designated Hev b 3. The 14.6 kd protein, the REF, has previously been reported as a latex allergen by Czuppon et al.,⁶ who designated it Hev b 1.

Verifying the allergenicity of Hev b 1

To resolve the previous discrepant findings^{6, 8, 9} regarding the allergenicity of the REF, the recombinant REF protein was synthesized from its gene

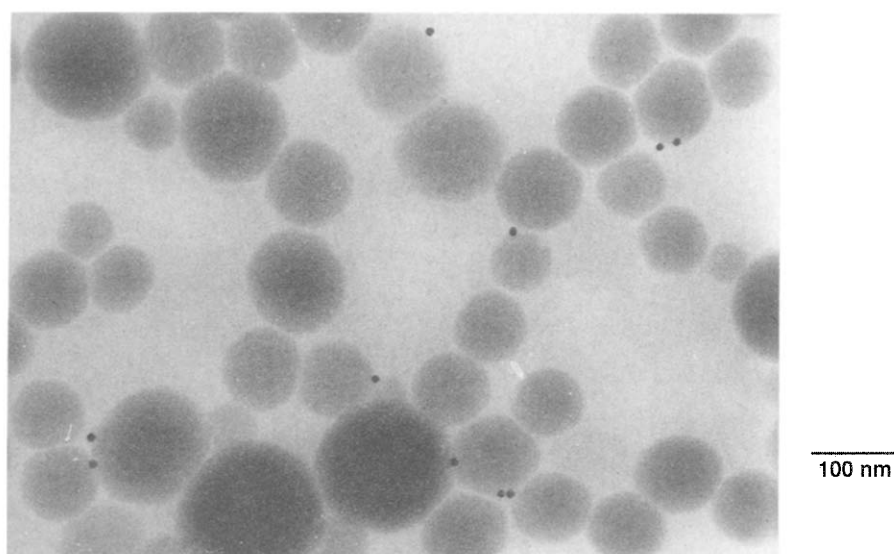


FIG. 8. Immunogold labeling of centrifuged latex Zone 2 rubber particles. Rubber particles were incubated with blood serum from patients with spina bifida, followed by incubation with a monoclonal anti-human IgE antibody. Labeling was carried out with goat anti-mouse antibody conjugated to 10 nm colloidal gold.

and tested for recognition by IgE of latex-allergic patients.

The cDNA of the coding region for REF was generated by PCR (Fig. 4). The incorporation of restriction sites into the 5' ends of the PCR primers REF₁ and REF₂ resulted in the generation of the REF cDNA having the *Asp*718 and *Bam*HI sites at its 5' and 3' ends, respectively. This facilitated its forced cloning into the corresponding sites of the phagemid pBluescript SK⁺ to produce the clone pLREF. Because the REF protein was to be expressed from its cDNA, the establishment of a correct nucleotide sequence for the coding region was deemed to be important before any attempt at fusion plasmid construction could be initiated. For this, the bidirectional nucleotide sequencing of the REF cDNA insert in pBluescript SK⁺ was performed by using the primers for the T3 and T7 promoters that flank the multiple cloning sites of the vector. The nucleotide sequence obtained for the cDNA of REF's coding region was 417 bp in length and corresponded exactly with those found in published literature.^{14, 25} The REF cDNA was subcloned into the pUC18 plasmid, double-digested with *Eco*RI and *Bam*HI, and then further subcloned into pMAL-c2 at the same sites to produce the clone pMREF (Fig. 5).

On induction of the recombinant *E. coli* cultures by isopropyl β -D-thiogalactopyranoside for 3 hours, the fusion protein was detected by SDS-

PAGE to be mainly in the supernatant from centrifugation of sonicated cells. The MBP-REF fusion protein, which had been cleaved by factor Xa protease, was separated by SDS-PAGE on a 15% gel to produce three main protein bands on Coomassie Blue staining: the uncleaved MBP-REF, MBP, and REF (Fig. 6, A). The proteins in a similar gel were electrophoretically blotted on to a nitrocellulose membrane (Western blot), which was then incubated in plasma or sera from individual adult latex-allergic patients and the two serum pools obtained from patients with spina bifida. Incubations with a serum pool obtained from latex IgE-negative pediatric patients, with polyclonal antibodies against MBP (supplied by the manufacturer of the MBP kit) and with polyclonal antibodies developed against latex glove eluate were also carried out. After the enzymic reaction of the conjugated secondary antibody (anti-IgE), it was seen that IgE in the two serum pools from patients with spina bifida recognized the REF and the MBP-REF bands (Fig. 6, B). This was clear evidence of REF's allergenicity. REF was also recognized by polyclonal antibodies from rabbits immunized against proteins eluted from latex gloves (Fig. 6, B). As expected, the remaining protein band (the MBP fragment with REF cleaved away) was nonallergenic. None of the three major proteins (MBP-REF, MBP or REF) reacted with IgE in the serum or plasma from any of the 20 adult

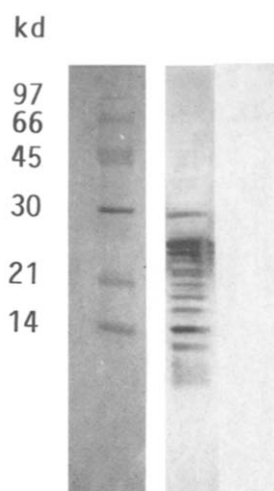


FIG. 9. Fragmentation of Hev b 3 on storage at -20°C . Western blot incubated with USM/RC2, followed by incubation with goat anti-mouse antibody conjugated to horseradish peroxidase (SDS-PAGE, 15% gel). Molecular weight markers (BioRad) are shown.

latex-allergic patients tested in this study or with IgE in the serum pool obtained from pediatric patients without spina bifida. Hence, allergenicity of Hev b 1 was confined to patients with spina bifida in this study.

Verifying the location of Hev b 3 in latex

The location of Hev b 1 on rubber particles is not in serious dispute because this allergen has been designated the REF, a known rubber particle protein.^{6,7} This is not so much the case for Hev b 3 because an allergen of closely similar molecular weight (27 kd) associated with spina bifida has been extracted as a presumed soluble latex protein.⁵ The location of Hev b 3 was therefore verified by electron microscopy of the rubber particles after immunogold labeling.

Rubber particles prepared from the rubber phase of centrifuged latex and from gel-filtered C-serum were incubated with the monoclonal antibody USM/RC2 (specific for Hev b 3) and subsequently labeled with colloidal gold conjugated to antibodies against mouse immunoglobulin. The rubber particles were seen to be labeled with colloidal gold when examined under the electron microscope. Labeling was much more active on the surface of the small rubber particles (Fig. 7) as compared with the larger ones. The rubber particles were also labeled when incubated with the spina bifida pool serum and labeled with colloidal gold conjugated to antibodies against human IgE

(Fig. 8). These results confirmed that USM/RC2 had specific binding to a protein located on the surface of rubber particles, especially the small particles, and that this protein, Hev b 3, was allergenic.

Fragmentation and aggregation of Hev b 3

Although Hev b 3 appeared as a single protein of 24 kd when freshly prepared from field latex, storage in the freezer (-20°C) resulted in its fragmentation into several polypeptides of lower molecular weight (between 24 kd and about 5 kd). Proteins of molecular weight greater than 24 kd were also observed, suggesting the occurrence of protein aggregation (Fig. 9). Fragmentation of freshly prepared Hev b 3 could be induced immediately by the addition of B-serum to the protein, whether prepared from centrifuged latex or from C-serum. The resulting Hev b 3 fragments, separated by SDS-PAGE, were visualized by incubation with the monoclonal antibody USM/RC2, which does not recognize any B-serum protein (Fig. 10).

The protein Hev b 3 was further characterized by two-dimensional polyacrylamide gel electrophoresis of a mixture of B-serum and C-serum (ratio 2:5). Isoelectric focusing was used in the first dimension, and SDS-PAGE was used in the second dimension. Silver staining revealed a number of proteins of different isoelectric points and molecular weights. A matching two-dimensional gel was electroblotted on to a nitrocellulose membrane (Western blot), which was then incubated with USM/RC2. The latex proteins binding to the antibody were revealed by the reaction of an enzyme-linked anti-mouse antibody. A cluster of polypeptides binding specifically to Hev b 3 were found mainly in the molecular weight range of 14 to 24 kd with isoelectric point values from 4.3 to 5.7 (Fig. 11).

DISCUSSION

Although the greater part of the natural rubber latex proteins are found in the C-serum and B-serum of centrifuged latex, about a quarter of the total latex proteins are in the rubber cream that comprises mainly the rubber particles.²⁵ Dennis and Light⁷ reported that the major protein on the surface of rubber particles was a 14.6 kd protein, which they termed the *rubber elongation factor* (REF). In the same article, they also reported the presence of a minor rubber particle protein of 24 kd that was sometimes encountered, an observation confirmed by Hasma²⁶ and by Sunderasan et al.¹⁰ The results in this study indicate that the 14.6 and 24 kd proteins are located principally on different rubber particles: the large and small

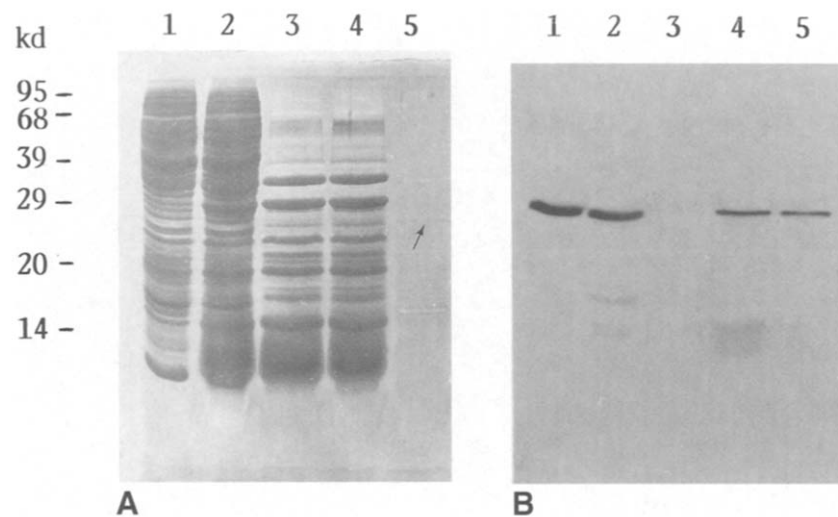


FIG. 10. Effect of adding B-serum to C-serum (which contains Hev b 3) and to Hev b 3 prepared from Zone 2 rubber cream of centrifuged latex (SDS-PAGE, 15% gel). **A**, Coomassie blue staining. Hev b 3 is denoted by arrow. **B**, Matching Western blot incubated with USM/RC2, followed by incubation with goat anti-mouse antibody conjugated to horseradish peroxidase. Molecular weight calibration is by markers from Integrated Separation Systems. Lane 1, C-serum; lane 2, C-serum + B-serum; lane 3, B-serum; lane 4, Hev b 3 from Zone 2 + B-serum; lane 5, Hev b 3 from Zone 2.

rubber particles, respectively. The difference in surface proteins of these two species of rubber particles is not surprising because they have dissimilar staining reactions,¹¹ suggesting differences in their surface chemistry. The large protein (>300 kd) and an array of at least 30 proteins from rubber particles reported by Cornish et al.²⁷ were not observed in this study.

In the isolation of REF, the possibility of a contaminating allergen or its fragmented derivative co-purifying with REF cannot be entirely ruled out. Czuppon et al.,⁶ who named the REF as a major latex allergen, asserted that it also existed as a 58 kd tetramer. Latex allergens of 50 to 60 kd have indeed been reported,^{10, 28} but Sunderasan et al.¹⁰ showed that patient IgE that recognized such a protein in their study did not bind with REF extracted from rubber particles. Moreover, they found that polyclonal antibodies that were developed against the latter did not recognize this allergen (results not presented).

To assess the allergenicity of REF while completely eliminating any possible interference from other contaminating latex allergens, the protein cDNA was cloned into a protein-expressing vector and then overexpressed in *E. coli*. Although McCullough et al.⁹ disputed that the REF N-terminus was allergenic, there was still the possibility that the epitope site was present elsewhere on the

molecule. In this study the entire protein was synthesized, and there was no presumption of the epitope site on the protein. Ample verification was obtained to show that the protein synthesized by *E. coli* was REF. First, the cloned cDNA was sequenced to check for fidelity with the published REF sequence. Further confirmation of the identity of the protein was obtained by its interaction with polyclonal antibodies developed against REF isolated from latex (results not presented).

Assays for allergenicity showed that REF (Hev b 1) is indeed a latex allergen. However, these results do not really corroborate the proposition of Czuppon et al.^{6, 8} that Hev b 1 is a major latex allergen because allergenicity was confined to patients with spina bifida. On the other hand, IgE in the sera of a cross-section of latex-sensitive adults in this study did not react to the rubber particle proteins. These observations are consistent with reports concerning a 14 kd protein from latex that is allergenic to patients with spina bifida.^{5, 29, 30}

Although the rubber particles of natural rubber latex are found mainly in the rubber cream when latex is centrifuged, some of the very small rubber particles (approximately 50 nm in diameter) can also be found in the C-serum where they remain suspended after high-speed centrifugation. That the small rubber particles from the C-serum and

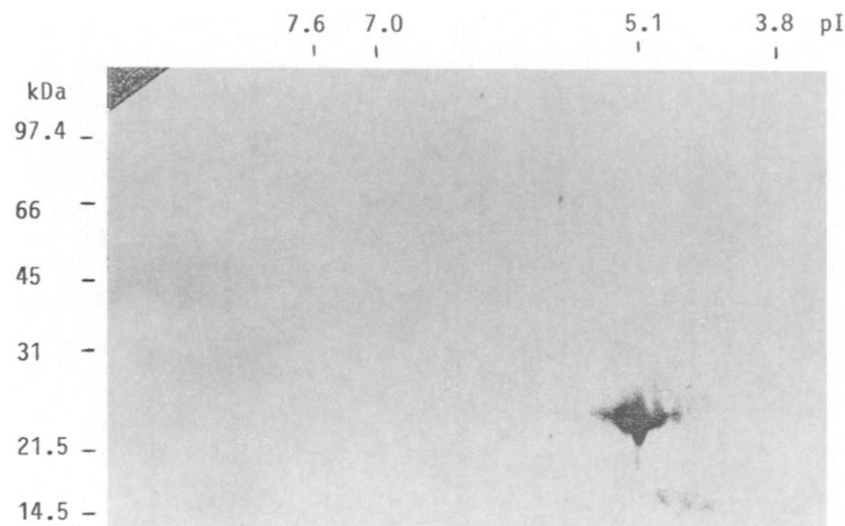


FIG. 11. Two-dimensional polyacrylamide gel electrophoresis of a mixture of C-serum and B-serum (5:2). Isoelectric focusing was used in the first dimension, and SDS-PAGE was used in the second dimension. Western blot was incubated with USM/RC2 as the primary antibody to detect Hev b 3. Antigen-antibody binding was revealed by using goat anti-mouse antibody conjugated to alkaline peroxidase as the secondary antibody. Molecular weight and isoelectric point calibrations are by markers from BioRad.

from Zone 2 of centrifuged latex carry the latex allergen Hev b 3 was demonstrated by immunogold labeling (Figs. 7 and 8). It is possible that protein allergens attributed to the latex serum (and by inference, that are soluble) might include proteins originating from the small rubber particles. Rubber particle proteins are generally insoluble in water, although they are solubilized slightly in the presence of the ammonia, which is universally used to stabilize and preserve commercial latex.²⁷ Ammonia has another effect on *Hevea* latex: it ruptures organelles present in the latex called *lutoids*, resulting in the release of B-serum that the lutoids contain. As shown in the results from this study, B-serum fragments Hev b 3 on the rubber particles, giving rise to an array of low molecular weight polypeptides, which are recognized by IgE from patients with spina bifida. On two-dimensional PAGE, the spacial distribution of these polypeptides (governed by their molecular weights and isoelectric points) was very similar to that for the cluster of latex serum polypeptides, reported by Alenius et al.,³⁰ to be allergenic to patients with spina bifida. The binding of USM/RC2 to these polypeptides showed that they were of common origin, being derived from Hev b 3. The fact that the Hev b 3 fragments, resulting either from storage or by reaction with B-serum, are electrophoretically discrete bands (Figs. 9 and 10) indicates that fragmentation occurs at predetermined

sites on the molecule and is not a consequence of a generalized protein degradation.

The fact that sera from pediatric patients without spina bifida do not recognize Hev b 1 or Hev b 3 indicates that allergy to the protein is specific to spina bifida and is not generalized for pediatric patients. Why should patients with spina bifida be especially sensitive to these two rubber particle proteins, whereas adult latex-sensitized patients are less affected? There are various possible explanations: for example, this may be due to a genetic predisposition or an age-related increase in sensitivity to allergens. Although this may be true, other explanations are possible. Many latex-induced allergic reactions are believed to be caused by water-soluble proteins.³ Although small amounts of Hev b 1 and Hev b 3 are solubilized in ammoniated latex,²⁶ most would not survive the latex manufacturing process in which high temperature, chemicals, and other denaturing conditions are encountered. Unlike solubilized rubber particle proteins, the proteins in their unsolubilized form are ubiquitously present in latex products but are not transmissible through water (or sweat). Hence, allergy to rubber particle proteins among health care workers and users of gloves and other latex products is relatively uncommon. On the other hand, patients with spina bifida undergo numerous surgical procedures, beginning with the neonatal meningomyelocele repair to neural, uro-

logic, and orthopaedic procedures. The body cavity mucosal membranes of patients with spina bifida frequently come into direct contact with catheters or surgeons' gloves. Accordingly, it is possible that allergy is induced in this manner^{4,31} even though the causative allergens, Hev b 1 and Hev b 3, from the surface of rubber particles are normally insoluble in water.

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