Latex allergy is recognized as a serious health problem among health care workers and children with spina bifida. A number of IgE-reactive proteins have been identified in natural and processed latex products. One of the most acidic proteins in the cytoplasm of lacticifer cells of rubber trees (Hevea brasiliensis) is demonstrated to be a potent allergen in eliciting allergic reactions in humans. This protein, with pI = 3.5, has a molecular mass of 16 kDa with a blocked N terminus and an unusual amino acid composition. This acidic protein was found in extracts prepared from latex gloves, which were shown to be allergenic. The purified protein elicits histamine release from human basophils passively sensitized with serum from latex-allergic individuals in a dose-dependent manner.

From a latex cDNA library, the cDNA coding for this protein was isolated and sequenced. The deduced amino acid sequence shows a high degree of homology to another acidic protein identified in kiwifruit (Actinidia delicosa var. delicosa). The sequence homology (47% sequence identity) between these two acidic proteins suggests a molecular explanation for the high frequency of fruit hypersensitivity in latex-allergic patients.

Allergy to natural rubber products has been recognized as a serious medical problem especially among health care workers and children with spina bifida (1, 2). Five to 10% of surgeons and operating room nurses were reported to be allergic to latex gloves (3, 4), and as high as 34% of children with spina bifida were allergic to latex gloves or other rubber products (5). Agents responsible for eliciting the type I hypersensitivity have been demonstrated in native latex proteins with molecular sizes ranging from 5 to 200 kDa (6–11).

Latex of the rubber tree (Hevea brasiliensis) is produced by a group of specialized cells, lacticifers. The cytosol of these cells contain rubber particles and a large number of proteins. Upon wounding, the cytoplasmic content of these cells is expelled in the form of latex. For the manufacturing of rubber goods, the fresh milky latex is collected in strong ammonia solution (20% concentration) to prevent coagulation. Chemicals such as antioxidants and antidegradant are added, followed by vulcanization at a high temperature. Despite such harsh conditions, many proteins remain on the surface of latex products, even after leaching to remove excess chemicals and proteins (12). When proteins from the glove extracts were analyzed on gel electrophoresis, a smeared pattern, indicative of degraded protein, was observed (10). Nevertheless, strong allergic reactions can be elicited by the proteins/epitopes remaining on the surface of the latex product.

When latex is subjected to centrifugation, it can be separated into a floating white rubber particle layer and a clear pale yellow C-serum fraction. We have previously identified an acidic protein (pI = 3.5) in the C-serum fraction of the latex. The protein has an apparent molecular mass of 25 kDa on SDS-PAGE and was found by IgE immunoblotting to be reactive in 52% of latex-allergic patient sera (10). It has been shown that allergen recognition patterns differ between adults and children; this acidic protein, however, is recognized by both (10). Due to its acidic nature and possible association with isoprene residues, it migrates through the nitrocellulose membrane during the electroblotting process and thus escaped previous detection.

Patients with latex allergy are often reported to be allergic to fruits (13), particularly avocado, chestnut, banana, and kiwifruit (14). The cross-reactivity suggests that constituents of these fruits might share common antigenic determinants with some latex allergens, even though these fruits are botanically unrelated to latex. The purpose of this study was to determine the amino acid sequence of the acidic protein and to demonstrate its allergenicity based on its ability to induce histamine release. The sequence of this protein was compared to proteins in the Swiss Protein data bank for possible elucidation of the physiological roles of this protein in latex.

MATERIALS AND METHODS

Purification of the pI 3.5 Protein from Latex—Latex was collected from a H. brasiliensis (clone RRIM 600) from Malaysia in a glycercinated buffer solution as described previously (10). The mixture was centrifuged at 50,000 × g at 4 °C for 2 h to separate the rubber particles from the C-serum. The floating layer, consisting mostly of rubber particles, was carefully removed. Glycerol was added to the C-serum to a final concentration of 50% (v/v) to prevent degradation and the growth of microorganisms. The mixture was stored at 4 °C until use.

The C-serum was desalted in a 20 mM citrate buffer, pH 3.0, by passing through a PD-10 column (Sephadex G-25M, Pharmacia Biotech Inc.). The fraction collected in the void volume was subjected to ion-exchange chromatography on a Pharmacia cation exchange column (HiTrap™ SP) using a FPLC system: buffer A, 20 mM sodium citrate

The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; HPLC, high performance liquid chromatography; LB, Luria-Bertani medium; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); PCR, polymerase chain reaction; MALDI, matrix-assisted laser desorption ionization.
buffer, pH 3.5; buffer B, 20 mM sodium citrate buffer, pH 3.5, and 1 mM NaCl, pH 8.0, and heated for 15 min at 60 °C. 5% buffer B contained the pl 3.5 protein. This fraction was further purified by a C-4 reverse phase column (Vydac) with a gradient of 0.12% trifluoroacetic acid in water, 0.1% trifluoroacetic acid in acetonitrile.

Trypsin and Endoproteinase Asp-N Digestions and Separation of Digested Fragments—For trypsin digestion, 50 μg of purified pl 3.5 protein was dissolved in 10 ml of 4 mM urea in 0.1 mM Tris-HCl, 2.0 mM CaCl₂, pH 8.0, and heated for 15 min at 80 °C. Tryptic digestion was performed at room temperature overnight (15). For endoproteinase Asp-N digestion, 50 μg of purified protein was dissolved in 0.1 mM Tris-HCl buffer, pH 8.0, and digestion was performed at 37 °C overnight with a substrate/enzyme ratio of 50/1. The resulting peptides were separated by reverse-phase HPLC using a C-4 or a C-18 column and eluted with a gradient of 0.12% trifluoroacetic acid, 0.1% trifluoroacetic acid in acetonitrile (15).

Amino Acid Analysis and Amino Acid Sequence Analysis—Amino acid analysis was carried out after hydrolysis in 6 N HCl for 90 min at 150 °C by using a Beckman model 6300 amino acid analyzer (16, 17). The amino acid sequences of several peaks from the trypptic digestion were sequenced by automated Edman degradation and analyzed on a model 476A gas-phase microsequencer (Applied Biosystems) connected to an on-line model 1200A phenylthiobenzamidoanalyzer. The endoproteinase Asp-N peptides were sequenced on a model LP 3000 Beckman protein sequencer with on-line phenylthiobenzamidoanalyzer.

Latex Glove Extract, Ammoniated Latex Extract, and Heat-treated Latex Extract—Glove extracts were generous gifts from Dr. Robert Hannum (The Johns Hopkins University, Baltimore, MD) and Dr. John Yunginger (Mayo Clinic, Rochester, MN). The extracts were prepared and concentrated as described previously (10, 18, 19). Ammoniated latex extract was from Dr. Robert Hamilton. To test the stability of the pl 3.5 protein at high temperature, a condition used in the manufacture of latex gloves, a preparation of the latex microspheres (Apliglo, BioPys) connected to an on-line model 1200A phenylthiobenzamidoanalyzer. The endoproteinase Asp-N peptides were sequenced on a model LP 3000 Beckman protein sequencer with on-line phenylthiobenzamidoanalyzer.

SDS-PAGE, IEF Gel Analysis, and Immunoblotting—SDS-PAGE was performed with a 16% gel under non-reducing conditions and proteins were visualized with silver stain as described previously (10). IEF gel electrophoresis was performed with Ampholine™ PAG plates, pH 3.5–9.5 (Pharmacia), at 500 V for 90 min. After completion of the electrophoresis, immunoblotting was carried out with passive capillary blotting by pressing a nitrocellulose membrane directly against the gel for 45 min. The membrane was reacted with a serum (31 years old, female) that reacts strongly to the pl 3.5 protein and followed by peroxidase-labeled anti-human IgE antibody. The IgE-reactive bands were detected by the chemiluminescence horseradish peroxidase system (Amersham Life Science) according to the manufacturer’s instructions.

Mass Spectrometry—To accurately determine the molecular weight of the pl 3.5 protein, HPLC-purified pl 3.5 protein was analyzed on a Kratos (Shimadzu) MALDI III mass spectrometer using matrix-assisted laser desorption ionization (MALDI). Approximately 5 pmol (0.5 μl) of protein was dissolved in 0.1% trifluoroacetic acid and applied to the target with 0.5 μl of sinapinic acid (Sigma) matrix solution on top. The sample/matrix mixture was dried under vacuum and then analyzed by MALDI.

Histamine Release from Human Basophils—For passive sensitization of human basophils (20), venous blood was collected from a volunteer who is not allergic to latex. Twenty ml of heparinized blood was mixed with 25 ml of 154 mM NaCl solution containing 6% dextran for 1 h. After sedimentation of red cells, the polynuclear leukocytes in the supernatant were subjected to histamine measurement by an automated histamine analyzer (Toosh Manufacturing Co. Ltd., Tokyo, Japan) with sensitivity of 0.1 ng/ml. Spontaneous histamine release was measured with FACSTM CM-Tracker of XL1-Blue MRF’ cells and subtracted from each point. Percent histamine was calculated by: % specific release = challenged release/total histamine × 100.

Determination of pl 3.5-specific IgE in Patient Serum by ELISA—The amount of pl 3.5-specific IgE in allergic patient sera was determined by direct ELISA using purified pl 3.5 protein to coat the 96-well plate and incubation with allergic patient sera. The attached IgE was determined by horseradish peroxidase-conjugated anti-human IgE.

Complementary DNA Library, Preparation of cDNA Phagemids, and Purification of Phagemid DNA—A cDNA library from latex constructed in AzaphI (21) was a gift from Dr. A. Kush of the University of Singapore. In vivo excision of phagemid from the vector was performed using a Stratagene ExAssist/SOLAR System according to a protocol provided by the manufacturer. Briefly, overnight cultivated XL1-Blue MRF’ cells was diluted 1:100 with LB and grown at 37 °C for 2–3 h to a mid-log phase. Cells were gently spun down and resuspended in 10 ml MgSO₄ to an A60₀ of 5.0. In a 50-ml conical tube, 1 × 10¹¹ XL1-Blue MRF’ cells, 10% ExAssist helper phage, and 10% plaque-forming units of amplified latex cDNA library were combined and incubated at 37 °C for 15 min. To this mixture, 25 ml of LB was added and incubated for another 2–2.5 h at 37 °C with shaking and cells were spun down for 15 min at 2000 × g. The supernatant was transferred to a fresh tube and heated at 70 °C for 15 min, then spun for 15 min at 4000 × g. The supernatant that contained the excised phagemid plBluescript, packaged as filamentous phage particles, was decanted into a sterile tube. To this mixture MoSO₄ was added to a final concentration of 7% and stored at 80 °C. For this bound phagemids were harvested on 0.45 μm filter, diluted with 100 μl of LB-ampicillin plates using SOLAR cells and incubated overnight at 37 °C. The confluent cells were washed off the plates, and DNA was prepared using Qiagen plasmid DNA extraction kit.

Isolation of cDNA Encoding the pl 3.5 DNA by Polymerase Chain Reaction (PCR)—Based on the amino acid sequences of a tryptic fragment, (EETKTEEP), a degenerate 23-base oligonucleotide was synthesized. Using the degenerate oligomer, T7 primer, and the phagemid DNA as a template, a 550-base pair DNA fragment was generated by PCR. The fragment was gel-purified and cloned into a Bluescript plasmid for sequence analysis. This was found to be the 3′ portion of the cDNA coding for the latex pl 3.5 protein. The cDNA that contains the entire coding sequence of the latex pl 3.5 protein was generated by PCR using a T3 primer and a specific 22-base oligomer (GAG AGT AGT TAC TGT GAG GTC T) from the 3′-end of the cDNA sequence. A 800-base pair DNA fragment identified by Southern blot was isolated and cloned into a Bluescript plasmid. Five cDNA clones containing coding sequences for pl 3.5 protein were isolated and sequenced.

Multiple Sequence Alignment—DNA sequencing was performed using an Amer sham/ U. S. Biochemical Corp Sequenase™ version 2.0 DNA sequencing kit. The initial sequencing was carried out using M13 (−18) and T7 primers for the C-terminal portion of the cDNA. The complete coding sequence of latex pl 3.5 cDNA was achieved by using specific internal primers 17-m (GAG AGT AGT TAC TGT GAG GTC T), N5–1R (CAG CAT CGC AAG AGG AGC CCA AG), N5–2R (GCA CAA CCG GCC GCT GCA CCT G), and N5–2R (AGC TGG ACG TTC CTC TGG).

Computer Search for Sequence Homology—The FASTA program provided with PCGene was used to search for protein sequence homology of the pl 3.5 protein with other proteins of known sequence in the Swiss Protein sequence bank.

RESULTS

Purification of the pl 3.5 Protein—Fractions collected from the cation-exchange column were analyzed by SDS-PAGE and IEF immunoblotting. The fraction containing the pl 3.5 protein was purified to homogeneity by HPLC reverse-phase chromatography (Fig. 1). This acidic protein could not be stained by Coomassie Blue and had poor staining using alkaline phosphatase-labeled antibody, but it could easily be detected by silver stain and by chemiluminescence using peroxidase-labeled antibody.

Latex Gloves, Heat-treated Latex, and Ammoniated Latex—The pl 3.5 protein can be readily detected by passive transfer of an IGE gel to a nitrocellulose membrane, and by treating the membrane with latex-allergic patient serum. The IEF immunoblot of extracts from eight brands of commercial latex gloves, heat-treated latex, and ammoniated latex is shown in Fig. 2.
Most of the glove extracts contained this protein in varying concentrations. The pI 3.5 protein appeared to be unaffected by autoclaving, while most of other proteins formed aggregates that remained near the origin of the sample loading zone (Fig. 2, lane 3). To demonstrate that the acidic proteins from the glove extracts were the pI 3.5 protein, an inhibition immunoblot experiment was performed. The serum was treated with the purified pI 3.5 protein prior to immunoblotting. Once the IgE specific for this acidic protein was removed, the band at pI 3.5 was no longer detectable (data not shown).

**Amino Acid Analysis and Amino Acid Sequence**—The amino acid composition derived from the amino acid analysis of the purified protein is comparable with that from the sequence deduced from the cDNA. The protein contains unusually high amounts of glutamic acid (46 residues), threonine (21 residues), alanine (29 residues), and proline (22 residues) and no aromatic amino acids, methionine or cysteine/cystine. Amino acid sequence analysis indicated that the N terminus of the pI 3.5 protein was blocked. Several amino acid sequences were obtained from HPLC-purified, trypsin-generated, and endopeptidase Asp-N-generated peptides of the purified protein (Fig. 3). The deduced amino acid sequence from the cDNA was nearly identical to that obtained by amino acid sequencing, with the exception of two amino acids at positions 67 and 103 (Fig. 3). At position 81, both Ser and Pro were found in different cDNA clones while amino acid sequence indicated a Pro at this position.

**Molecular Mass Determination by Mass Spectroscopy**—The molecular mass of the purified pI 3.5 protein as determined by mass spectroscopy was 16001.2 Da. The molecular mass calculated from the deduced amino acid sequence was 15957.5 Da. The discrepancy of 43 Da is mostly likely from an acetyl group (43 Da) attached to the N-terminal alanine residue.

**Complementary cDNA Coding for the pI 3.5 Protein**—Fig. 3 is the result of DNA sequence analysis of five independent clones of cDNAs coding for the pI 3.5 protein. The DNA sequences for the coding region of all clones were identical with the exception of position 312, which is either a C or a T. This change gave rise to a different amino acid, either proline or serine. The noncoding regions at the 3' end adjacent to the poly(A) tail were quite variable. Fig. 3 depicts the sequence of a cDNA clone with the shortest sequence at the 3' end.

**Sequence Homology Analysis**—The computer search identified an acidic protein (pI 3.5) from kiwifruit that has a substantial sequence homology to the latex acidic protein (22). Fig. 4 is the sequence comparison of the latex pI 3.5 protein with a kiwifruit acidic protein, which has a molecular mass of 18.9 kDa with a pI of 3.5. The latex acidic protein has a truncated middle region when the sequences of these proteins are aligned with maximal sequence homology. The homology is most striking in the segments around the N and C termini.

**Histamine Release Induced by Purified Latex pI 3.5 Protein**—To analyze the allergenic properties of the pI 3.5 protein, histamine release was performed using passively sensitized human basophils. Fig. 5 demonstrates that the purified pI 3.5 protein induced a dose-dependent histamine release when cells were first incubated with sera from allergic patients. The amount of histamine release also correlated with the amount of pI 3.5-specific IgE present in the individual serum. (The higher the level of pI 3.5-specific IgE, the better the responses of dosage of pI 3.5 proteins.) Patient 6 was allergic to latex but with a very low level of pI 3.5-specific IgE in the serum. When this serum was used for sensitization of basophils, no detecta-
its presence in most of the glove extracts tested demonstrates the potential importance of this protein in allergy to latex products. The fact that the purified pI 3.5 protein induced histamine release in IgE sensitized-basophils confirms that it is an allergen, Hev b 5. Histamine release could be detected at an allergen concentration as low as 10 ng when cells were sensitized with serum from patients who had a high IgE titer to this specific protein.

In a previous report (23), rubber elongation factor (molecular mass, 14.5 kDa), Hev b 1, was shown to react with sera from all 13 latex-allergic patients in the study. In our experience with 50 latex-allergic patient sera, only 22% reacted to the rubber elongation factor, while 52% of latex-allergic sera reacted with the pI 3.5 protein (10). Due to the large number of allergens in latex, it does not seem feasible to use a single allergen for immunotherapy or for diagnostic purposes.

The pI 3.5 protein in latex is composed of only 14 of the 21 naturally occurring amino acids, with unusually high numbers of glutamic acid and proline residues in the repeated motif of XEEX or XEEE (X can be any amino acid, but most frequently Lys or Ala residues). The molecular mass determined by MALDI mass spectrometry (16001.2 Da) agrees well with that calculated from the amino acid composition deduced from the cDNA (15957.5 Da). The difference can be explained by the presence of an acetyl group, which blocked the N terminus. There were sequence heterogeneities among the five cDNA clones, suggesting the possible existence of a family of genes coding for the proteins.

Recently a high percentage of latex allergy patients have been reported to also have food allergy (13). Fruits frequently have been reported to have cross-reactivities with latex including chestnut, banana, papaya, avocado, and kiwifruit (14). A health care worker who developed allergy to latex gloves after working in the hospital later also developed allergy to avocado and kiwifruit. The most likely explanation for the cross-reactivities is the existence of constituents with common antigenic determinants in latex and various fruits. In our study, the acidic latex protein shares a 47% sequence identity with the kiwifruit pI 3.5 protein. The homology is most striking in the N- and C-terminal segments. The pI 3.5 protein in kiwifruit is one of the proteins that appears in the early stage of fruit development (21), but its biological function is not known. The highest level of mRNA coding for the acidic protein in kiwifruit appears on day 6 after anthesis. The biological role of the pI 3.5 protein in latex is also unknown and needs further investigation. The molecular bases of allergenic cross-reactivity between latex and other fruits are currently under investigation.

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Acidic Latex Allergen

A Novel Acidic Allergen, Hev b 5, in Latex: PURIFICATION, CLONING AND CHARACTERIZATION
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