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Hydrophobic Allergens from the Bottom Fraction Membrane of *Hevea brasiliensis*

Kesajee Mengumpun[1], Chatchai Tayapiwatana[2,3], Robert G. Hamilton[4], Pasuree Sangsupawanich[5] and Rapepun Wititsuwannakul[1]

SUMMARY Several proteins of rubber latex have been recognized as allergens causing immediate hypersensitivity in humans. In this study, a bottom fraction membrane (BFM) protein preparation from *Hevea brasiliensis* trees grown in southern Thailand was used to detect specific IgE in four groups of serum samples. The first group included 170 samples of latex glove factory workers (LGWs); group 2 consisted of the sera of 35 health care workers (HCWs) who were repeatedly exposed to powdered latex gloves; groups 3 and 4 were 31 positive and 22 negative sera, respectively, obtained from Johns Hopkins University School of Medicine, Baltimore, USA, tested for IgE to latex allergen. It was found that 56/170 (33%), 5/35 (14%), 11/31 (35.5%) and 1/22 (4.5%) samples of the LGWs, HCWs, CAP^+ and CAP^- groups had significant IgE to the BFM proteins, respectively. However, of all subjects only one subject of group 1 had experienced allergic morbidity consisting of eczema, conjunctivitis and asthma. The IgE of this subject bound to a 55 kDa component in the rubber latex BFM preparation. Thus, this protein may be regarded as a novel, although minor, latex allergen. Further investigation is needed to characterize the component and to pinpoint its allergenic role.

Latex is a milky sap produced by the tropical rubber tree, *Hevea brasiliensis* (Euphorbiacea). Proteins of *H. brasiliensis* remaining in the latex products, such as gloves, surgical aids and condoms, can cause hypersensitive reactions in allergic prone individuals.[1] Latex allergy has become a growing health problem worldwide due to an increased use of natural rubber latex (NRL) products.[2] Specific risk groups for this allergy include health care workers (HCWs) who are repeatedly exposed to powdered NRL gloves such as operation room nurses, workers of latex product factories, as well as those who process latex from the rubber trees.[3-5] Moreover, a significant portion of patients with atopy and congenital anomalies such as spina bifida are allergic to *H. brasiliensis* latex proteins.[6] Latex protein induced-allergy and -asthma have also been found among patients who underwent multiple surgeries.[7]

When fresh latex is centrifuged, three separated layers are obtained.[8] The top layer contains rubber cream, the middle consists of C-serum and the bottom fraction mainly of Frey-Wyssling complexes and lutoid particles. The lutoids are spherical in shape, vary in size from 0.5-3 μm, and are bound by a single osmosensitive membrane about 8 nm thick.[9] Subjecting the latex bottom fraction to repeated freeze-thaw cycles and centrifugation sepa-

From the [1]Department of Biochemistry, Faculty of Science, Prince of Songkhla University, Songkhla 90112, Thailand, [2]Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai 50200, Thailand, [3]Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok 10120, Thailand, [4]Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21224, USA, [5]Department of Pediatrics, Faculty of Medicine, Prince of Songkhla University, Songkhla 90112, Thailand.
Correspondence: Rapepun Wititsuwannakul
E-mail: rapepun.w@psu.ac.th

rates it into a supernatant which contains the B-serum and the pellet containing a mixture of bottom fraction membrane (BFM) and some other co-sedimented materials. The B- and C-serum fractions have been shown to cause type I- (IgE-mediated) hypersensitivity.[10,11] The membrane-bound particles also contain allergens such as Hev b 1 (rubber elongation factor).[12] At present, a total of 13 latex allergens, *i.e.* Hev b 1-13, have been designated by the International Allergen Nomenclature Committee.[13]

During latex product manufacturing such as in a glove factory[14] the pre- and post-vulcanization leaching usually involves extensive washing of the latex with water which eventually leaches out the proteins including allergens in the B- and C-serum fractions. However, several hydrophobic proteins still remain in the finished latex products and consequently may induce allergic responses in some users.

The present study was performed to determine the allergenicity of the proteins in the latex BFM among Thai subjects who worked in a latex glove factory and Thai health care workers who were repeatedly exposed to powdered latex gloves.

MATERIALS AND METHODS

Bottom fraction membrane (BFM) proteins of rubber latex

Fresh latex was collected from regularly tapped rubber trees belonging to clone RRIM 600 grown at the Songkhla Rubber Research Center, Songkhla province, southern Thailand. The trees were tapped at 06:00 a.m. and the exuded latex was collected in a beaker chilled on ice. The collection time of each tapping was ~60 minutes. The chilled latex was filtered through four layers of cheese-cloth to remove the bark tissue debris and particulate materials. The filtrate was centrifuged at 59,000 × *g* and 4°C for 45 minutes. The bottom fraction was collected, suspended in five volumes of Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4 containing 0.9% NaCl) and washed three times with the TBS by centrifugation. The preparation was then subjected to four alternative rounds of freezing and thawing. It was centrifuged and the BFM pellet was washed three times with TBS. The washed BFM was re-suspended in 10 volumes of extraction buffer (50 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100) and kept with stirring overnight at 4°C. The preparation was centrifuged and the supernatant containing the BFM proteins was incubated with SM2 absorbent (1:10 w/v) for 15 minutes to remove residual Triton X-100. The protein content of the preparation was determined using BCA Protein Assay Reagent (Pierce Biotechnology; Rockford, IL).

A portion of the BFM protein preparation was labeled with biotin using Sulfo-NHS-LC-Biotin (Pierce Biotechnology).

Subjects and serum samples

Four groups of serum samples were included in this study. The sera of group 1 were from 170 Thai individuals who worked in a latex glove factory (LGWs). The sera of group 2 were obtained from 35 health care workers of Chulalongkorn Hospital in Bangkok (provided by Dr. Porntip Puvabunditsin). This part of the work was approved by the Medical Ethics Committee of Songklanakarin Hospital, King Chulalongkorn Memorial Hospital, Thailand, and each participating subject gave a written informed consent. Groups 3 and 4 were 31 and 22 sera, respectively, obtained from Johns Hopkins University School of Medicine, Baltimore, USA. Group 3 sera were positive for specific IgE to latex allergen coated beads as tested by a CAP assay using Latex ImmunoCAP (Pharmacia Biotech Inc.; San Francisco, CA.) (CAP^+), and the sera of group 4 were CAP^-.

Determination of the serum levels of IgE and IgG that bound to BFM proteins by indirect ELISAs

Indirect ELISAs were used for determining specific IgE and IgG that bound to BFM proteins in all serum samples. Each well of a 96-well plate (Immunomaxi high binding,TTP; Stafa-Zurich, Switzerland) was coated with 50 µl of 10 µg/ml BFM proteins at 4°C for 18 hours. Unbound antigens were removed by washing with a washing buffer (PBS, pH 7.2 containing 0.05% Tween-20; PBST). The unoccupied sites in each well were blocked by incubation with 200 µl of 2% skim milk in PBS (blocking solution). After the blocking proteins were removed by washing, individual serum samples (diluted 1:5 in the blocking solution) were added to the wells; the plate was incubated at 25ºC for 1 hour and then all wells were washed. Goat anti-

human immunoglobulin isotype-HRP conjugate and a chromogenic HRP substrate were used for developing the color reaction. After the enzymatic reaction was stopped by adding 100 µl of 1N HCl to each well, the optical density (OD) of the content of each well was determined at an absorbance of 450 nm (A_{450nm}).

Co-immunoprecipitation

Co-immunoprecipitation was performed as follows: the Protein A gel (Seize X Immunoprecipitation Kit; Pierce Biotechnology) was conjugated with IgG_{2a} subclass monoclonal antibody (mAb) specific to human Fcε (Biodesign International; Saco, ME) using a disuccinimidyl suberate (DSS) cross-linker, and the preparation was incubated at 25ºC for 1 hour. After washing five times with a washing buffer, 350 µl of the serum sample to be tested for the presence of IgE to BFM proteins were added to the mAb-conjugated protein A beads. After appropriate incubation to allow binding of the serum IgE to the anti-□-chain-affinity protein A beads, unbound serum proteins were washed away and the biotinylated BFM proteins were added to the IgE-bound-affinity protein A beads. The mixture was rotated for 1 hour at 25ºC, washed five times, and the biotinylated BFM protein-IgE complexes were eluted from the affinity beads by using an elution buffer (glycine buffer, pH 2.8). The pH of the eluted fraction was adjusted to 7.4 before the preparation was resolved by 15% SDS-PAGE under reducing conditions. The separated components were trans-blotted onto a PVDF membrane and the blot was incubated with HRP-conjugated streptavidin. The biotinylated proteins were revealed by using an ECL Western blot analysis system (Amersham Health; Buckinghamshire, UK) and autoradiography.

Competitive ELISA for the detection of serum IgE that bound to BFM proteins

A competitive ELISA was used to demonstrate the presence of specific IgE in the eluted fraction (BFM proteins-IgE complexes) obtained from the co-immunoprecipitation process. For competitive inhibition, 200 µl of the eluted fraction were mixed with 1.2 µg of unlabeled BFM proteins (inhibitor) and the final volume of the mixture was rounded to 400 µl by adding 2% BSA in PBS, pH 7.4. The mixture was incubated at 37ºC for 1 hour. The eluted fraction (200 µl) mixed with 2% BSA in PBS (without inhibitor) served as negative competition control. Both mixtures were appropriately added to ELISA wells pre-coated with egg white avidin (Sigma Chemical Co.). After incubation at 25ºC for 1 hour and washing, 100 µl of the chromogenic substrate TMB (KPL, Inc.) were added to the individual wells and the plate was kept in the dark at 25ºC for 30 minutes. The enzymatic reaction was stopped with 100 µl of 1N HCl and the OD of the content in each well was determined at A_{450nm}. The IgE competitive result was determined by comparing the OD of the test well (with inhibitor) to the OD of the negative competition control (well without inhibitor). The % inhibition value of the sample was calculated with the formula: *% inhibition = (OD of negative competition control-OD of the test well) × 100/OD of the negative competition control***.**

Statistical analysis

GraphPad Prism 4 (GraphPad Software, Inc.; San Diego, CA) was used for statistical analysis of the differences of the ELISA OD among groups. Probability (p) values (paired t-tests) less than 0.05 were considered significantly different.

RESULTS

The indirect ELISA results for detecting specific IgE to BFM proteins in the sera of all four groups are shown in Fig. 1. The mean ± SD of the OD of the specific IgE in the serum samples of group 1 (LGWs) was 0.10 ± 0.26. The mean ± SD of the 35 HCWs of group 2 was 0.04 ± 0.13, which was not significantly different from group 1 (p = 0.19). The mean ± SD of the serum samples of groups 3 (CAP^+) and 4 (CAP^-) were 0.10 ± 0.15 and 0.02 ± 0.02, respectively. These values were also not significantly different from those of group 1 (p = 0.50 and 0.13, respectively). When the arbitrarily chosen mean + 2 SD of group 4 (0.02+0.04 = 0.06) was used as cut-off level between positive and negative IgE to BFM proteins, it was found that 56 (33%), 5 (14%), 11 (35.5%) and 1 (4.5%) subjects of LGWs, HCWs, CAP^+, and CAP^- were positive.

The ELISA results determining the levels of specific IgG to the BFM proteins of all groups are

shown in Fig. 2. The mean ± SD values of LGWs, HCWs, CAP^+, and CAP^- were 0.20 ± 0.34, 0.03 ± 0.04, 0.09 ± 0.13, and 0.02 ± 0.04, respectively. The mean IgG to BFM proteins of group 1 was significantly higher than those of groups 2-4 (p = 0.01, 0.07 and 0.02, respectively).

Among all sera of all four groups, only three serum samples of group 1 (LGWs) had both IgE and IgG to the BFM proteins elevated (Fig. 3). They were samples no. 4, no. 248, and no. 253. The IgE and IgG ELISA optical densities at A_{450nm} of these three subjects were 2.71, 0.78 and 1.40 and 2.64,

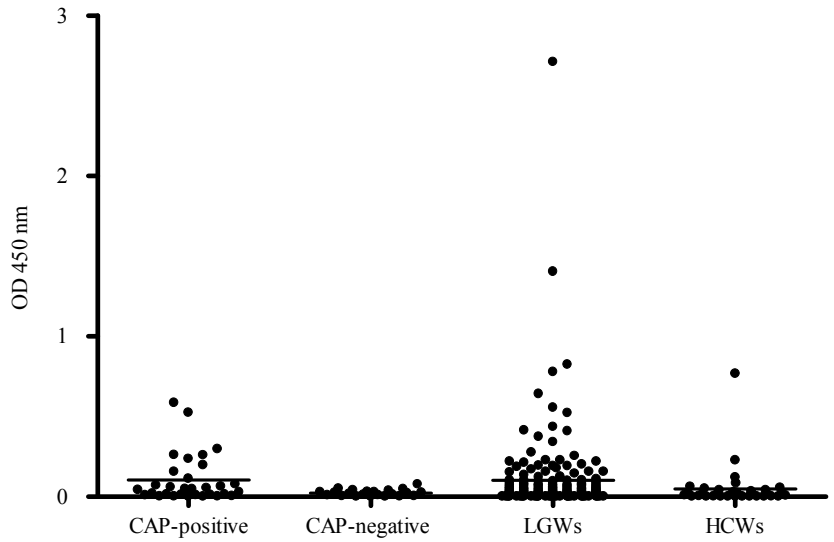

Fig. 1 Indirect ELISA results of three deliberately repeated experiments, for determining IgE to BFM proteins in the sera of subjects of all study groups. Solid horizontal lines represent the mean values of the sera of each group.

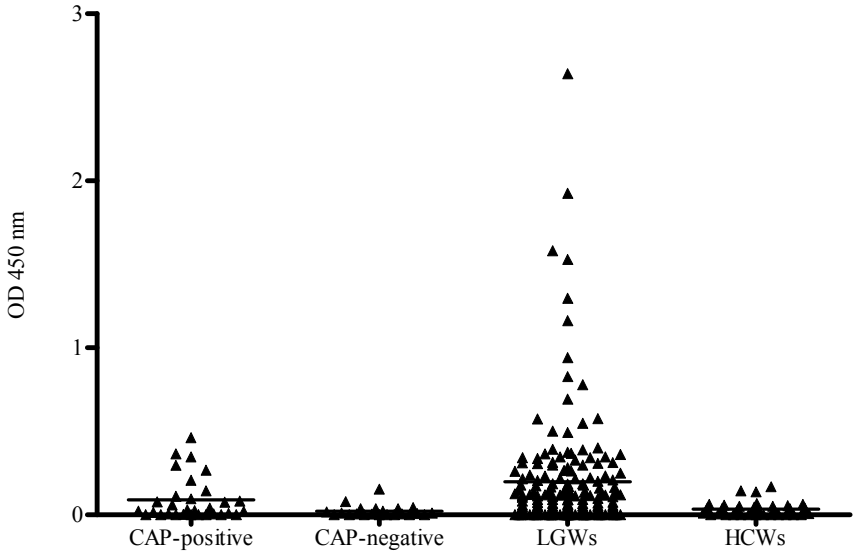

Fig. 2 Indirect ELISA results for determining specific IgG to BFM proteins in the sera of all groups. Mean OD values at A_{450nm} are shown by solid horizontal lines. Data from three experimental repeats are shown.

1.16 and 1.53, respectively (Fig. 3). However, only subject no. 4 had actually experienced allergic morbidity consisting of eczema, conjunctivitis and asthma. Skin prick tests of the three subjects were all negative to the BFM preparation.

The biotinylated BFM proteins which bound with the IgE obtained from the co-immunoprecipitation process (eluted fraction from affinity protein A beads) were separated by 15% SDS-PAGE under reducing conditions; the separated components were probed with HRP-streptavidin and the reactive bands were revealed by chemiluminescence and autoradiography. Lane 1 of Fig. 4A shows the components in the co-precipitated BFM preparation. When the separated components were probed with serum of subject no. 4 of group 1 (LGW), a reactive band of ~55 kDa was seen (lane 2, Fig. 4A). Lanes 3 and 4 of Fig. 4A show negative reactions when the SDS-PAGE separated-biotinylated preparations were probed with representative sera of CAP+ (lane 3) and CAP- (lane 4) subjects, respectively. No serum samples of other subjects were positive for binding to the SDS-PAGE separated-biotinylated BFM proteins from the co-immunoprecipitation assay (data not shown). Fig. 4B shows protein bands/smears when the unlabelled BFM proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The protein components ranged from ~10 to ~70 kDa with two distinct bands at ~30 and 34 kDA.

To confirm the presence of IgE to BFM proteins in the eluted complexes from the anti-IgE-affinity protein A beads, an avidin-biotin capture inhibition ELISA was performed. The eluted fraction, pre-incubated with or without un-labeled BFM proteins (inhibitor), was added into the ELISA well coated with egg white avidin. The captured complexes containing IgE specifically bound to biotinylated BFM proteins were observed using HRP-conjugated goat anti-human IgE. Fig. 5A shows the OD at A_{450nm} of the eluted fraction obtained by using serum of the subject no. 4. A reduction of ~27% of the OD was seen by incubating the eluted fraction with un-labeled BFM proteins before adding it to the ELISA well containing immobilized avidin (Fig. 5B), implying the presence of specific IgE to BFM proteins in the eluted fraction.

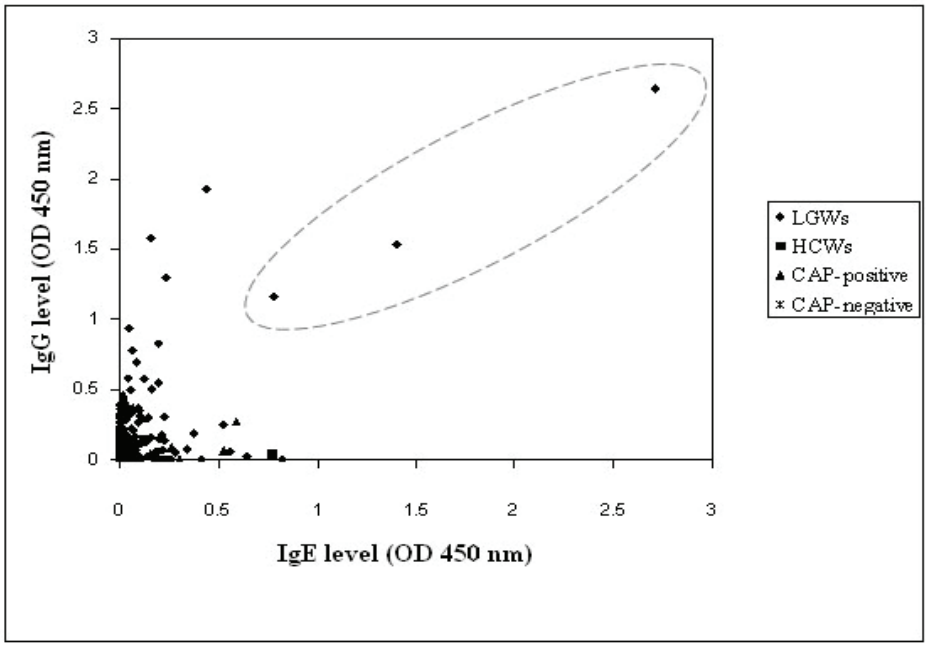

Fig. 3 Correlation of specific IgE and IgG levels of all serum samples. Only three subjects of group 1 (LGWs), *i.e.* no. 4, no. 248 and no. 253 shown in the circled area had correlating high values of both IgE and IgG isotypes to the BFM proteins.

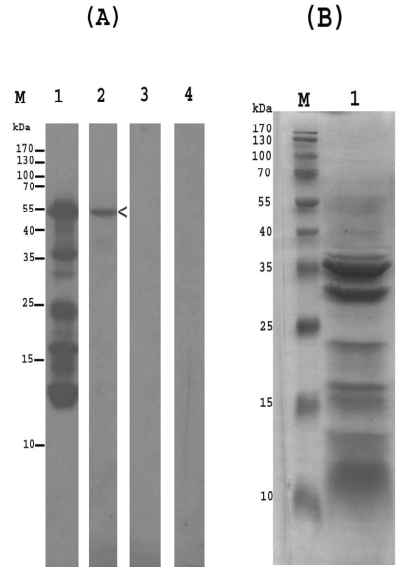

Fig. 4 (A), SDS-PAGE separated protein components of the biotinylated BFM preparation co-precipitated with IgE obtained from affinity-protein A beads (lane 1). The SDS-PAGE separated-biotinylated preparation probed with sera of subject no. 4 of group 1 is shown in lane 2, and representative sera of group 3 and 4 probed to the BFM blots are shown in lanes 3 and 4, respectively. (B), Separated components of a BFM protein preparation stained by Coomassie Brilliant Blue R- 250.

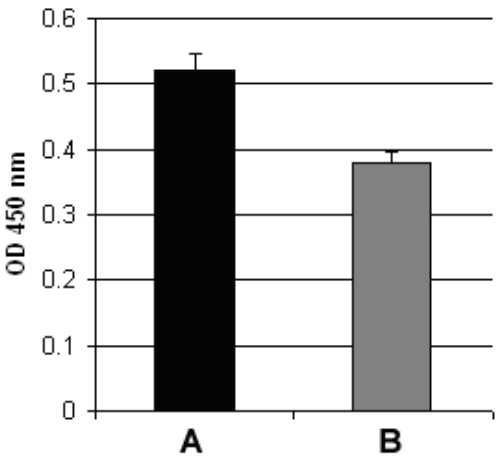

Fig. 5 Competitive ELISA results for detecting IgE in the eluted fraction (biotinylated BFM-IgE complexes) from the affinity-protein A beads. The eluted fraction was directly added to egg white avidin-coated ELISA wells (A). The eluted fraction was pre-incubated with unlabeled BFM proteins before adding to egg white avidin-coated ELISA wells (B). The average values of three independent experiments are shown.

DISCUSSION

The proteins in natural rubber latex are known allergens causing immediate hypersensitivity in some repeatedly exposed persons, such as health care and rubber industry workers. The incidence of latex allergy has increased since the early 1980s.[2] This was, in part, caused by the adoption of mandatory universal precautions for handling human specimens in response to the increase of highly contagious pathogens such as HIV and hepatitis. The prevalence of latex allergy was reported to be 3% to 17% in HCWs, 11% in LGWs, and 1% to 6.5% in the general population of North America and Germany.[15]

Patients with latex allergy, similar to those with other types of allergy, are diagnosed based on a history of allergen exposure, sensitization and clinical symptoms. Nevertheless, several immunological assays have been developed to confirm the diagnosis, such as specific IgE determination by CAP-Rast, basophil histamine release assay, IgE-ELISA and IgE-immunoblotting.

Usually, different subjects respond to latex proteins differently; while some may be sensitized and upon re-exposure to the allergen, develop IgE-mediated clinical manifestations, others may respond to the same proteins by producing non-reagenic types of antibodies. Moreover, different allergic individuals may be allergic to different latex proteins, and also, in a different magnitude. For this reason, a battery of all pure and standardized allergens is necessary for screening the allergic status of individuals. Currently, 13 Hev b proteins have been recognized by the International Union of Immunological Societies (IUIS) as latex allergens.[13] Many of these proteins, such as Hev b 2, 4, 6 and 10, are contained in the water-soluble parts of the latex, *i.e.* the B- and, the C-serum fractions while others are associated with insoluble rubber particles, such as the Hev b 1.[16] Although the rubber latex has to pass through extensive washing with water during the glove-manufacturing process which eventually removes much of the water soluble allergens, there are still

case reports of allergic reactions to rubber gloves.[14] This led us to investigate the allergenic proteins in the BFM fraction that are probably retained in the finished products even after extensive water washing of the latex during the manufacturing process.

In this study, 56 (33%), 5 (14%), 11 (35.5%), and 1 (4.5%) subjects of the LGW, HCW, CAP^+ and CAP^- groups, respectively, had significant levels of specific IgE to BMF proteins in their sera. It can be seen that the proportion of subjects positive for specific serum IgE to the BFM proteins was higher in the LGW group than in the HCW group. Moreover, only 11 of 31 (35.5%) subjects of group 3 who were positive for latex specific IgE by the CAP system and 1 of the 22 (4.5%) subjects who were CAP-negative had significant serum IgE to the BFM proteins of the rubber trees grown in Thailand. These results reflect that latex of rubber trees from different areas may contain different kinds and/or amounts of allergens. Moreover, the findings support the notion that exposure to different rubber components stimulate different immunological responses in different subjects. Some rubber components may be allergenic to one and not to another and *vice versa.*

Recently Kraft *et al.*[17] have described the anti-allergic role of allergen specific IgG. When the FcγRIIB becomes co-aggregated with the FcεRI through allergen cross-linking of IgG and IgE on the cell surface, the pro-allergenic signal was diminished. Usually, activation of the high-affinity receptor for IgE (FcεRI) on allergic effector cells induces a multitude of positive signals *via* the immunoreceptor tyrosine-based activation motifs (ITAMs), which leads to the rapid manifestation of allergic inflammatory reactions. As a counterbalance, the co-aggregation of the IgG receptor, FcγRIIB, mediates inhibitory signals *via* phosphorylation of tyrosine in the immunoreceptor tyrosine-based inhibition motifs (ITIMs) located at the cytoplasmic tail of the receptor by the tyrosine kinase of the FcεRI pathway. Interestingly, only subject no. 4 of the LGW group among the three subjects who had high levels of both specific IgE and IgG to the BFM proteins in the serum had clinical manifestations while the other two subjects were normal, implying that the optimal IgE / IgG ratio might play an important part on the clinical outcome as has been previously suggested.[18] Moreover, IgG-blocking antibody seem to play a role in the interception of IgE allergen.[19] An increase of IgG, in particular IgG_4 antibodies, diminished IgE-mediated anaphylaxis.[20] Accordingly, further identification of IgG subclasses could correlate the immune status with the symptoms of latex allergic individuals.

In this study, only the serum of subject no. 4 of the LGW group contained specific IgE to the 55 kDa component in the BFM protein fraction. Thus, this component seems to be a minor allergen. Nevertheless, it may be regarded as a novel allergen located in the latex BFM. This finding, therefore, refines the allergenic protein profile of latex. The nature and biological activity of the 55 kDa component should be further investigated.

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