Hydrophobic Allergens from *Hevea brasiliensis* Bottom Fraction Membrane

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ชื่อวิทยานิพนธ์ โปรตีนก่อการแพ้ชนิดไม่ละลายน้ำสกัดจาก บอททอมแฟรคชั่นเมมเบรนของน้ำยางพารา

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บทคัดย่อ

เป็นที่ทราบกันดีว่าโปรตีนในน้ำยางพารา (*Havea*) ซึ่งถูกนำไปทำผลิตภัณฑ์ต่างๆ เช่น ถุงมือ, ถุงยางอนามัย, อุปกรณ์ทางการแพทย์ เป็นสาเหตุให้เกิดปฏิกิริยาภูมิคุ้มกันไวเกิน และจากการศึกษาที่ผ่านมามีหลักฐานว่าโปรตีนจากน้ำยางพาราที่สามารถสกัดได้ด้วยน้ำ เป็นสาเหตุให้เกิดการแพ้ชนิดเฉียบพลัน (Type 1) จนกระทั่งปัจจุบันนี้มีการระบุโปรตีนจากน้ำยางพาราที่เป็นสาเหตุการแพ้แล้วทั้งหมด 13 ชนิด (Hev b 1-13) โดย International Allergen Nomenclature Committee ซึ่งการศึกษาโปรตีนก่อการแพ้ที่ผ่านมามุ่งเน้นไปที่โปรตีนชนิดที่ละลายน้ำได้ ที่ได้จาก B-serum และ C-serum ของน้ำยางพารา ส่วนเมมเบรนของอนุภาคใน bottom fraction (BF) ของน้ำยางพาราสดที่ได้จากการปั่นแยกด้วยความเร็วสูงเป็นแหล่งใหม่ที่สำคัญอีกแหล่งหนึ่งที่มีบทบาทต่อการก่อให้เกิดโรคภูมิแพ้ นอกเหนือจากแหล่งโปรตีนอื่นๆอันได้แก่ อนุภาคยาง C-serum และ B-serum ซึ่งเป็นที่รู้จักกันเป็นอย่างดีและได้มีการศึกษากันอย่างกว้างขวางมาก่อนหน้านี้แล้ว เมมเบรนของอนุภาคใน BF ที่เตรียมได้จากการศึกษาครั้งนี้ ประกอบด้วยโปรตีนชนิดต่างๆ คล้ายคลึงกับที่พบในเมมเบรนของอนุภาคลูทอยด์ ซึ่งเป็นองค์ประกอบหลักของ BF โปรตีนที่เป็นองค์ประกอบของเมมเบรนของอนุภาคใน BF จัดเป็นโปรตีนที่ไม่ละลายน้ำ สามารถสกัดออกจากเมมเบรนได้ด้วยน้ำแอมโมเนีย ดีเทอร์เจนต์ (เช่น Triton x-100) ในการศึกษาวิจัยในครั้งนี้ได้มุ่งเน้นศึกษาโปรตีนก่อการแพ้จากเมมเบรนของอนุภาคใน BF ซึ่งเป็นโปรตีนที่ไม่ละลายน้ำ ซึ่งโปรตีนชนิดนี้จะยังคงเหลือตกค้างในผลิตภัณฑ์ยางพาราที่ผ่านการล้างด้วยน้ำหลายครั้ง และการวิจัยในครั้งนี้ยังศึกษาถึงการจับจำเพาะของ IgE และ IgG จากเซรั่มเลือดของกลุ่มตัวอย่างกับบริเวณ conformational epitope ของโปรตีนก่อการแพ้

การใช้วิธีการปั่นแยกด้วยความเร็วสูง น้ำยางพาราสดจะถูกแยกเป็น 3 ส่วน ส่วนบนสุดจะเป็นส่วนของยาง ส่วนล่างสุดจะเป็นส่วนของ BF ส่วนกลางจะเป็นของเหลวใสเรียกว่า C-serum ในส่วนของ BF จะประกอบด้วยอนุภาคลูทอยด์ และ Frey-Wyssling อนุภาคลูทอยด์มีลักษณะเป็นรูปร่างทรงกลม มีเยื่อหุ้มชั้นเดียว เมื่อนำ BF ผ่านการทำแช่แข็งสลับกับละลาย

หลายๆ ครั้ง จะทำให้ได้ส่วนของของเหลวและตะกอน ส่วนของเหลวที่ได้เรียกว่า B-serum และในส่วนของตะกอนนี้เองที่เรียกว่า Bottom Fraction Membrane (BFM) หลังจากเติม 0.2% triton x-100 ลงไปเพื่อสกัดโปรตีนที่ไม่ละลายน้ำจาก BFM แล้วนำสารละลายไปปั่นแยกอีกครั้ง จะได้โปรตีน BFM อยู่ในส่วนของสารละลายที่เราจะนำไปศึกษาต่อไป จากการแยกโปรตีน BFM ด้วยโพลิอะคริลาไมด์เจลอิเล็กโทรโฟรีซิส แบบมีเอสดีเอส (SDS-PAGE) พบว่า BFM โปรตีนที่ได้มีหน่วยย่อยหลากหลายขนาดตั้งแต่ 10 จนถึง 70 กิโลดาลตัน และมีกลุ่มของโปรตีนหลักขนาด 30 และ 35 กิโลดาลตัน

จากการนำโปรตีน BFM ไปศึกษาการจับจำเพาะของ IgE และ IgG จากเซรั่มของเลือดกลุ่มตัวอย่างด้วยวิธี indirect ELISA พบว่าในเซรั่มเลือดกลุ่มคนงานจะมีค่าการจับจำเพาะของ IgE และ IgG สูงเมื่อเทียบกับกลุ่มบุคลากรทางการแพทย์ และกลุ่ม CAP^{+} และ CAP^{-} โดยเฉพาะคนงาน no.4 ที่มีประวัติการแพ้โดยมีอาการหอบหืด, ผิวหนังเป็นผื่นแดง, ตาอักเสบ เมื่อสัมผัสผลิตภัณฑ์ยาง มีค่าจับจำเพาะ IgE และ IgG สูงมาก จึงเป็นตัวอย่างที่น่าสนใจที่ถูกเลือกมาศึกษาด้วยวิธี co-immunoprecipitaion เพื่อที่จะวิเคราะห์หาโปรตีนก่อการแพ้ที่จำเพาะใน BFM

จากการทดลองพบว่า โปรตีนขนาด 55 กิโลดาลตัน ที่อยู่ใน BFM สามารถจับจำเพาะกับ IgE ในเซรั่ม คนงาน no.4 โดยใช้เทคนิค co-immunoprecipitaion พบว่าโปรตีน 55 กิโลดาลตัน ที่ได้มีขนาดใกล้เคียงกับโปรตีนก่อการแพ้ Hev b 4 แต่อย่างไรก็ตาม Hev b 4 เป็นโปรตีนก่อการแพ้ชนิดละลายน้ำได้ แต่ 55 กิโลดาลตันที่ได้จากการศึกษาในครั้งนี้ เป็นชนิดไม่ละลายน้ำ เพราะฉะนั้น โปรตีนชนิดนี้น่าจะเป็นโปรตีนก่อการแพ้ตัวใหม่ ซึ่งยังไม่ถูกค้นพบมาก่อน ในการนำไปใช้จะมีประโยชน์ต่อไปในอนาคต โดยการรวมโมเลกุลนี้ลงไปในชุดตรวจวินิจฉัยการแพ้ยางพาราเพื่อความถูกต้องและแม่นยำมากขึ้นในการวินิจฉัยผู้ป่วยที่มีอาการแพ้ยางพารา

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Abstract

It has been known that proteins from *Hevea* latex, when exist in latex products such as gloves, condoms, surgical aids etc. can cause hypersensitivity reaction. There is evidence suggesting that the water extractable proteins in latex are the cause of the immediate allergy (hypersensitivity Type 1 reaction). At present, a total of 13 latex allergens, Hev b1-13, have been designated by the International Allergen Nomenclature Committee. In the past numerous studies were concentrated on soluble proteins which located in B-serum and C-serum. Bottom fraction membrane (BFM) prepared from centrifuged fresh latex was found to be an important new source of latex proteins for identifying latex allergens besides those already known in rubber particles (RP), C-serum and B-serum. The protein composition of BFM was similar to those in the membrane of lutoid particles, the main constituents of bottom fraction (BF). The BFM proteins are hydrophobic and extractable by ammonia water, detergent (such as Triton X-100). This research aims at investigating the allergenic properties of the bottom fraction membranes (BFM), which are the hydrophobic proteins that remain after many washes within the latex products. This study focuses on the specific bindings of IgE and IgG in sera which are isolated because of their conformational epitopes for allergenicity.

Using ultracentrifugation, fresh latex separates into three layers with the rubber cream on the top, the bottom fraction at the bottom and the C-serum in between. The bottom fraction consists mainly of lutoid particles and Frey-Wyssling complexes. These particles are membrane bound. Lutoids are spherical in shape and are bound by a single osmosensitive membrane. Afer repeated freeze-thaw cycles and recentrifugation, tha bottom fraction renders

the supernatant (B-serum) and the pellet which is a mixture of BFM. By adding 0.2 % triton X-100 along with continuous shaking, the hydrophobic
proteins within the BFM were isolated. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the BFM proteins, which contained two major bands at 30 and 35 kDa, the rest were in the range of 10 to 70 kDa.

The extracted BFM proteins were used to detect specific-IgE and –IgG by indirect ELISA. Serum samples from latex glove factory workers (LGWs) had noticeably higher levels of both Ig isotypes in comparison to others i.e. health care worker, CAP^{+} and CAP^{-} groups. Clinical symptoms i.e. asthma, eczema, and conjunctivitis suggested that only subject LGW no. 4 was allergic. Modified co-immunoprecipitation was applied in an attempt to isolate the molecules recognized by specific IgE in LGW no.4. A reactive conformational epitope of 55 kDa was identified. The 55 kDa protein located is within the range of Hev b 4 molecular sizes. However, Hev b 4 was isolated from B-serum in water-soluble form. The 55 kDa hydrophobic protein, thus may be a novel allergic class. This finding suggests that the 55 kDa molecule should be incorporated to the immunoassay for latex specific IgE. Including this molecule will improve the diagnostic value for screening the risk of latex allergy.

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Kesajee Mengumpun

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List of Abbreviations

°A	=	Angstrom
APS	=	Ammonium persulfate
ATPase	=	Adenosine 5'- triphosphatase
BFM	=	Bottom fraction membrane
BSA	=	Bovine serum albumin
°C	=	Degree Celsius
CAP^{+}	=	Positive CAP
CAP^{-}	=	Negative CAP
cDNA	=	Complementary DNA
cm	=	Centimeter
DNA	=	Deoxyribonucleic acid
ECL	=	Electrogenerated chemiluminescence
ELISA	=	Enzyme-linked immunosorbent assay
FCA	=	Flow cytometric assay
FW	=	Frey-Wyssling
g (force)	=	Gravitation acceleration
g (mass)	=	gram
h	=	Hour
HCWs	=	Health care workers
HCl	=	Hydrochloric acid
HRP	=	Horseradish peroxidase
IgG	=	Immunoglobulin G
IgE	=	Immunoglobulin E
kDa	=	Kilodalton
LAWs	=	Latex glove factory workers
LM	=	Lutoidic membrane
M	=	Molar
mA	=	Milliampare

List of Abbreviations (continued)

mg	=	Milligram
µg	=	Microgram
min	=	Minute
ml	=	Milliliter
µl	=	Microliter
mM	=	Milli Molar
MW	=	Molecular weight
MHC	=	Major histocompatibility complex
NADH	=	Nicotinamide adenine dinucleotide, reduced form
nm	=	Nanometer
NRL	=	Natural rubber latex
OD	=	Optical density
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline
PVDF	=	Polyvinylidene fluoride
RAST	=	Radioallergosorbent test
RP	=	Rubber particle
rpm	=	Round per minute
RT	=	Room temperature
SB	=	Spina bifida
SDS	=	Sodium dodecyl sulphate
sec	=	Second
SPT	=	Skin prick test
Tris	=	Tris (hydroxymethyl) aminomethane
TMB/H_2O_2	=	Tetramethyl Benzidine/ hydrogen peroxide
V	=	Volt
UV-vis	=	Ultraviolet-visible
%	=	Percent

Chapter 1

Introduction

Latex is the milky sap produced by the tropical rubber tree *Hevea brasiliensis* (Euphorbiacea). Proteins from *Hevea* latex in latex products such as gloves, condoms, and surgical aids can cause a hypersensitivity reaction (Nutter, 1979; Turjanmaa *et al.,* 1984; Alexelsson *et al.,* 1987; Leynadier *et al.*, 1989). Latex allergy has become a serious problem worldwide since the early 1980s, (Charous *et al.,* 1994) due to the increasing use of natural rubber latex (NRL) products. Proteins present on various NRL products have been implicated in causing allergy in specific risk groups (Nutter, 1979; Owhby *et al.,* 1991; Slater, 1989; Spaner *et al.,* 1989; Sussman *et al.,* 1991; Tarlo *et al.,* 1990; Turjanmaa, 1987). One risk group of particular interest consists of health care workers (HCWs) exposed to powdered NRL gloves (Kelly *et al.,* 1993). Other important risk groups are workers in industries in which latex products are manufactured, including those collecting and processing latex from rubber trees (Tarlo *et al.,* 1990; Turjanmaa, 1987; Kelly *et al.,* 1993; Alenius *et al.,* 1994). Patients with congenital anomalies such as spina bifida, those undergoing multiple surgeries, and patients with atopy are more susceptible to latex protein-induced allergy and asthma (Kelly *et al.,* 1990). It has been reported that 2.8-8.8% of all health care workers and 29-72% of patients suffering from spina bifida are allergic to *Hevea* latex proteins (Lagier *et al.,* 1992; Turjanmaa, 1987; Arellano *et al.,* 1992; Michael *et al.,* 1996; Nieto *et al.,* 1996; Cremer *et al.,* 1998; Czuppon *et al*., 1993).

As described by Moir (1959), when fresh latex is centrifuged, it separates into three layers with the rubber cream on the top, the bottom fraction at the bottom and the C-serum in between. The bottom fraction consists mainly of lutoid particles and Frey-Wyssling complexes. These particles are membrane bound. 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 (Dickenson, 1964; Gomez and Moir, 1974; Pakianathan *et al.,* 1966). The bottom fraction is subjected to repeated freeze-

thraw cycles and recentrifuged that the supernatant and pellet are separated. The supernatant is the B-serum and the pellet is a mixture of bottom fraction membrane and other co-sedimenting materials. There is evidence suggesting that the water extractable proteins in latex are the cause of the immediate allergy (hypersensitivity type I reaction) (Turjanmaa *et al.,* 1988). Thus, previous studies have concentrated on soluble proteins located in B-serum and C-serum (Nel and Guijuluva, 1998). However, some allergens have been found in membrane-bound particles such as Hev b 1 (Rubber Elongation Factor) (Czuppon *et al.,* 1993). At present, a total of 13 latex allergens, Hev b1-13 (Wagner and Breiteneder, 2005), have been designated by the International Allergen Nomenclature Committee. According to the latex glove manufacturing processes, pre-vulcanisation and post-vulcanisation leaching usually includes extensive washing steps with water. Thus, water-soluble proteins from C-serum and B-serum can be leached out by washing when the latex is processed (Yip and Cacioli, 2002). However, hydrophobic proteins still remain in the latex products. Consequently, they may induce an allergic responses in certain glove-users i.e. HCWs.

Previous studies of latex allergy have focused on the B-serum as the source of allergenic proteins, whereas the residue or pellets have been omitted. The present study aims to isolate proteins from the bottom fraction membrane (BFM) in order to gain intact antigens that have conformational epitopes for allergenicity by focusing on specific binding of IgE and IgG in sera. As a consequence, identification of the novel immunoreactive molecules against IgE will benefit the improvement of latex allergic screening kit.

Literature Review

1. Natural Rubber Latex (NRL)

1.1 Introduction

There are many plants capable of producing latex; they belong to several different families but are mainly of the Dicoteledons (Metcalfe, 1967). Latex is the fluid, generally milky in appearance, which flows from these plants after the slightest wound; it is produced and then stored, rarely, in parenchyma cells or more frequently in the tube structure known as laticifers

Of some 12,500 species of laticiferous plants, approximately 7,000 produced polyisoprene. In most case the polyisoprene is with mixed resin, making the latex difficult to use when the content of the latter is high. Finally only the limited number of rubber-producing plants can be exploited and only a few species are cultivated and have economic importance (Bonner and Galstone, 1943). Among them, *Hevea brasiliensis* was soon revealed to be the best rubber producer. This tree species, which grow in the hot humid intertropical regions, is exploited by tapping the bark. *Hevea* possesses articulated laticifers in the barks, although it is thought that laticifers are also capable of apical intrusive growth in the cotyledons, inner seed coats, and in young leaves (Bobilioff, 1923). Laticifers form from procambial cells in young plantlets; they are found in the primary pheoem in shoots, roots, and the veins of young leaves, and later in flowers and fruits. As soon as the cambium has formed it produces a special laticiferous system in the secondary phloem. Articulated, anastomosing laticiferous vessel form successive vertical networks called rings or mantles. In *Hevea*, these secondary laticiferous vessels of the trunk are exploited by tapping the bark. The tree releases a large amount of latex at each tapping and can be exploited for more than a decade.

1.2 Fresh Hevea Latex

1.2.1 Major Constituents

The *Hevea* latex, as it flows out of the tree, is a complex cytoplasm containing a suspension of rubber and non-rubber particles in an aqueous medium (Southorn, 1961 and Archer *et al.,* 1969). Using high speed centrifugation (59,000g), Cook and Sekhar (1953) separated latex into four fractions. There were: an upper white fraction

of rubber cream, an orange or yellow layer containing Frey-Wyssling complexes, a colourless serum named C-serum and a greynish yellow gelatinous sediment, the “bottom fraction”, containing mainly of lutoids (Dickenson, 1969 and Southorn, 1966).

Moir (1959) using differential staining and high speed centrifugation techniques, showed that the sedimentable material in latex did not consist wholly of one species of particle. By treating the latex with trace amounts of Janus Green B or neutral red before centrifugation, he obtained eleven zones (Fig.1). Zone 1 correspond to the “top whitish fraction” of Cook and Sekhar (1953) which consists mainly of hydrocarbon particles. Zone 2 was a much smaller, translucent layer situated under the lowest portion of Zone 1. Zone 3 was a suspension of rubber particles in the serum. Zone 4 was the yellow and orange layer of Cook and Sekhar (1953). The aqueous Zone 5 correspond to C-serum and Zones 6-11 together were broadly equivalent to the “bottom fraction”.

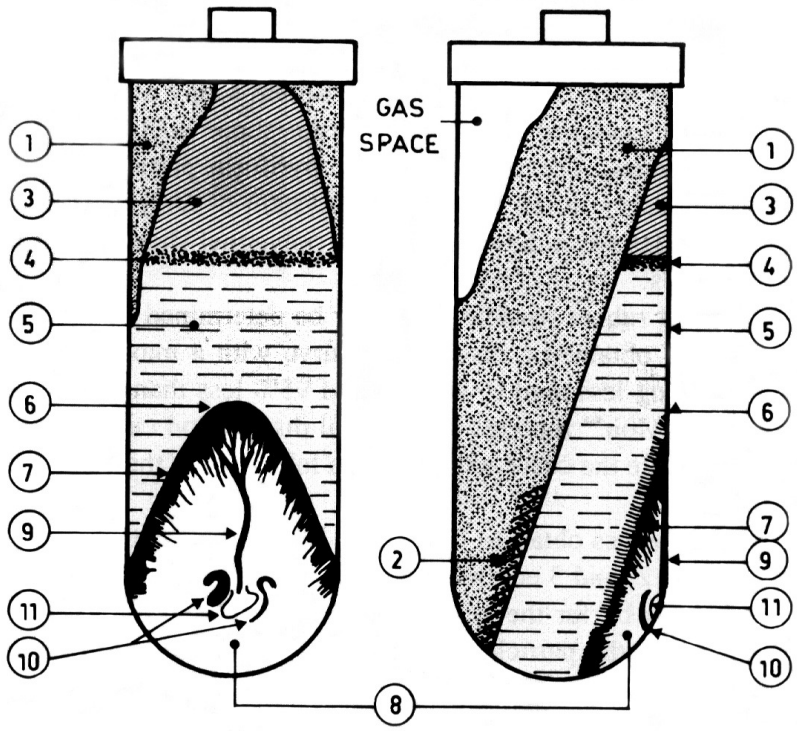

Fig. 1 Separation of fresh latex by ultracentrifugation (53,620 g max X 40 min). Fraction 1-3 correspond to white rubber phase. Fraction 4 is a yellow orange layer constituted by Frey-Wyssling particles. Fraction 5 is an almost clear serum (C-serum) corresponding to the latex cytosol. Fraction 6 to 11 constitute the "bottom fraction" in which highest in quantity is the lutoid fraction (Moir, 1959).

Rubber particles

The rubber particles usually have a size ranging from 50 A° to about 30,000 A° (3 μm), although in extreme cases particles of 5 or 6 μm are also found. These are spherical bodies in young trees but in mature trees the particles are large, often having a pear shape. The shape in certain cases seems to be a clonal character. Pear shape is reported to be very frequent in clones such as Tjir 1 and PR 107 (Southorn, 1961).

A rubber particle of average size, about 1,000 A°, contains hundreds of molecules of the hydrocarbon and is surrounded by a surface film of proteins and lipids. The rubber particles are also associated with triglycerides, sterols, sterol esters, tocotrienols and other lipids. Dupont *et al.* (1976) have confirmed the presence of phosphatidylcholine and small amount of phosphatidyl ethanolamine in the lipids associated with rubber particles. The protein envelope of rubber particles is visible in sections of osmium stained rubber particles and is approximately 100 A° thick (Fig. 2) (Andrews and Dickenson, 1961). The envelope carries a negative charge and confers colloidal stability to the rubber particles.

According to Dickenson (1969) there are rubber particles with variously stained regions. An osmiophilic region surrounded by a weakly stained periphery attributed to lack of uniformity when rubber particles are deposited on existing particles during biosynthesis. He has also suggested that the inner particulate inclusion, having 50-80 A° thickness, might be molecules of rubber of molecular weight about 100,000.

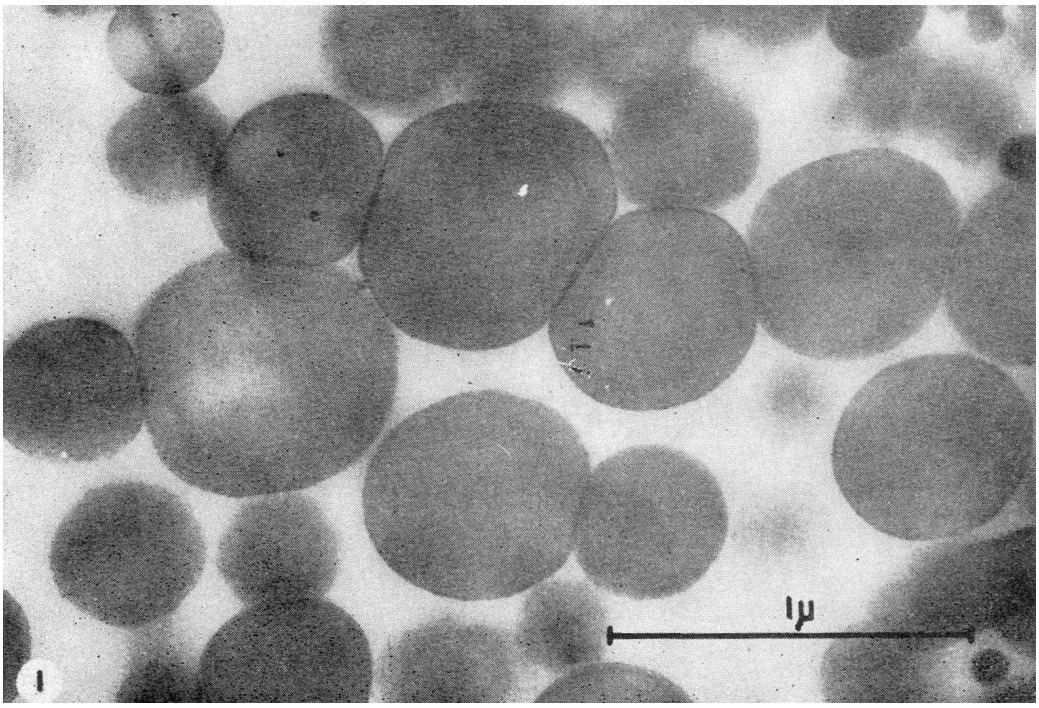

Fig. 2 Rubber particles in ultra-thin section showing, in some, the thin surrounding layer (Andrews and Dickenson, 1961).

Lutoids

Lutoids form the next major component of *Hevea* latex. They are membrane-bound bodies and mostly larger in size than the rubber particles. They are 2-5 μm in diameter bounded by a unit membrane of about 80 A° thickness (Fig. 3) (Dickenson, 1965; Gomez and Moir, 1979). It was Wiresum (1957) who first suggested that the lutoids behave like vacuoles due to stainability with neutral red. Though controversy existed in this regard, the work of Ribaillier *et al.* (1971) provided evidence for the vacuolar properties of lutoids.

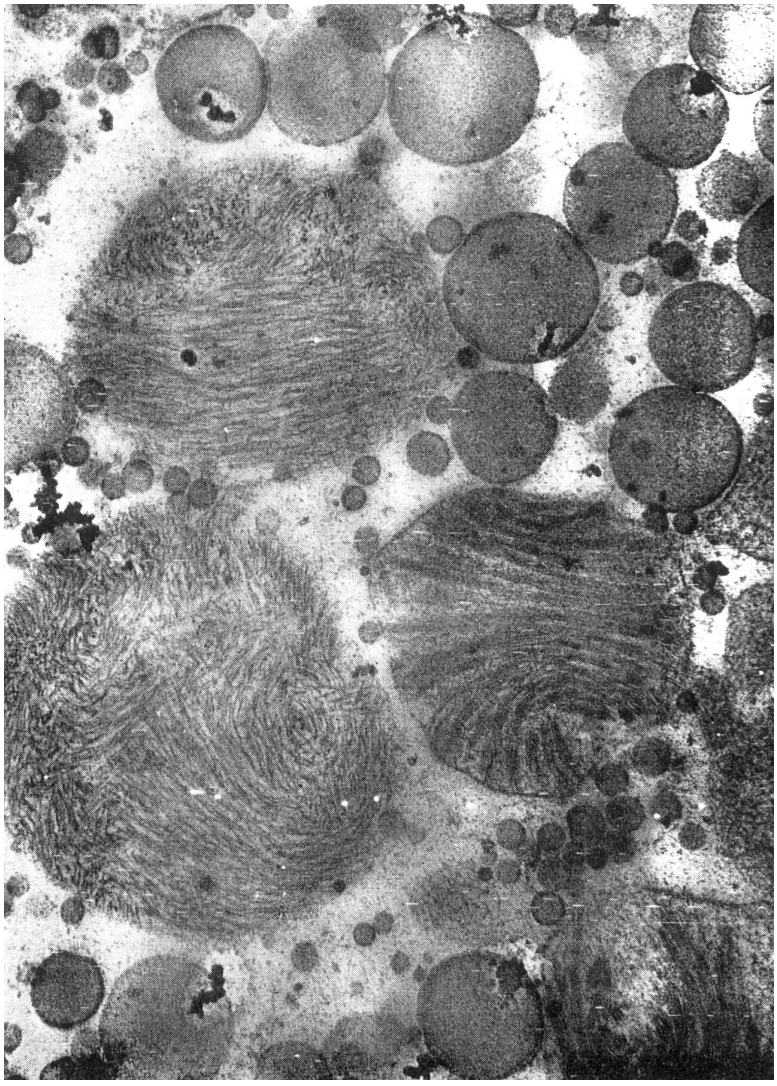

Fig. 3 Lutoid particles of two apparent morphological types in a young latex vessel of *Hevea brasiliensis* (Dickenson, 1965).

The content of lutoids (B-serum) has a very rapid flocculating action on aqueous suspension of rubber particles in lalex, resulting in the formation of microflocs (Southorn and Edwin, 1968). This activity is appearently moderated by the ambient C-serum and is much reduced if B-serum is boiled. Southorn and Yip (1968) demonstrated that this fast initial flocculating action of B-serum is an electrostatic one involving the interaction between the cationic contents of B-serum and the anionic rubber particle surface.

By phase contrast microscopy and application of suitable staining procedure the structure of lutoid particles have been studied in detail. Mainly two types of fibrillar structures (Fig. 4) have been described. The first type, known as microfibrils, are characteristics of latex vessels in young tissues (Dickenson, 1965, 1969; Audley, 1965, 1966). As seen by phase contrast microscopy of tapped latex from young tissue, the microfibrils are freely suspended in the fluid content of the lutoid B-serum. The microfibrils are seen usually as grouped together in bundles. Each bundle has a diameter of 450-500 A°. Individual microfibrils are several micron long and 70-80 A° in diameter. The microfibrils can be isolated from the sediments of latex from young tissues which on negative staining with phosphotungstic acid shows further details. Each microfibril is a tightly coiled continuous helix with hollow axis. The diameter of the helix is about 125 A° and that of hollow axis 30 A°. The microfibrils consist of an acidic protein while nucleic acid seem to be absent. Microfibrils however are not present in tissue or latex collected from the mature bark. It is believed that they disintegrated as the particles mature or else the young lutoids containing microfibrils themselves disintegrate as the tissue age and are replaced by a population of lutoids without microfibrils. However, the microfibrils do not seem to have vital role in rubber biosynthesis.

The second type of fibrilllar structures, observed in lutoids of latex from mature bark of stimulated trees, are known as ‘microhelices’, so named (Gomez and Yip, 1975) because of their spring like shape. These structures were first observed by Dickenson (1965, 1969). They are occasionally found in unstimulated trees and their number increases on dilution.

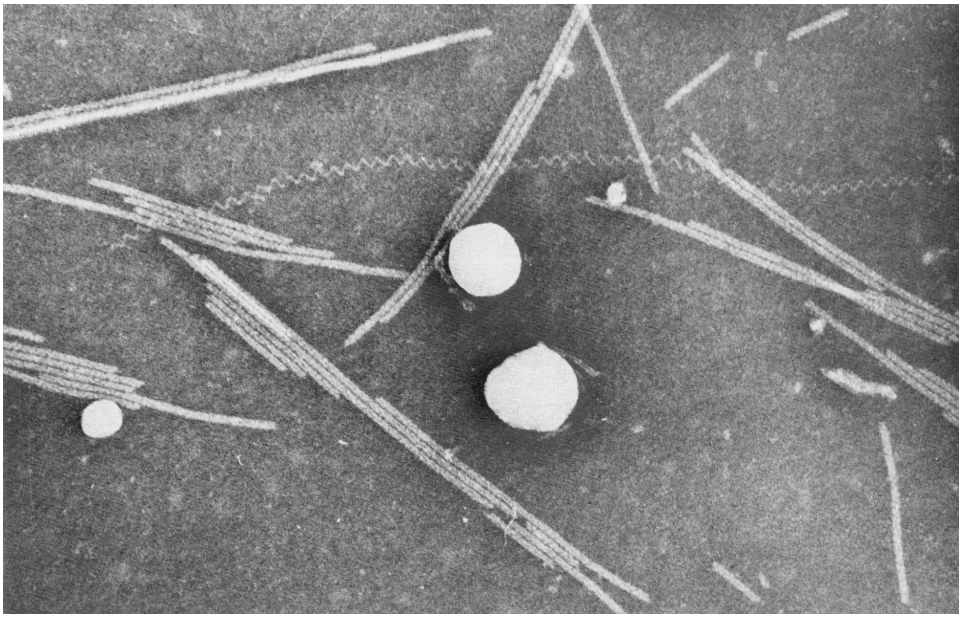

Fig. 4 Isolated microfibrils and one microhelix from latex of green stem.

However, microhelices are reported to be more frequent in lutoids of tapped latex than *in situ* latex (Southorn and Edwin, 1968; Gomez and Yip, 1975) and are occasionally observed in latex collected from young

tissue also.

As reviewed by Gomez and Moir (1979) the microhelices are approximately 1 μm in length with a diameter of 200 A°, having a fibre width of about 50 A° and an open hollow helix having a 300 A° wide pitch. Dickenson (1965) suggested the formation of microhelices from microfibrils but this has been questioned by Gomez and Yip (1975).

A third type of lutoid inclusion – minute spherical particles in Brownian movement – was observed by Schoon and Phoa (1956). Later Southorn (1960, 1961) found such particles in large number in the botttom fraction of ultracentrifuged latex of long rested trees and this was confirmed by Dickenson (1969). The role of such particles in latex is unknown.

Frey-Wyssling complexes

Yellow globules, in clusters in tapped latex were first noted by Frey-Wyssling. The existence of such particles in groups, associated with a vacuolar body was observed by Southorn (1969) in phase contrast microscopy. He found that the individual particles were covered by a membrane, this was confirmed by electron microscopy. Dickenson (1969) named these particles, enclosed as a single structure, as Frey-Wyssling complexes.

The Frey-Wyssling complexes are more or less spherical in shape in a size range of 3-6 μm (diameter) and are bounded by a double membrane (Fig. 5). Within the membrane there are two types of particles – large osmiophilic globules in variable numbers and a system of rope-like tubules of about 750 A° diameter, usually embeded in a membrane bound matrix of osmiophilic nature. The complex structure of Frey-Wyssling complexes has been elaborated by Dickenson (1969) who described a series of concentric lamellae of the double unit membrane and the system of tubules and also highly folded invaginations of the inner membrane.

The Frey-Wyssling complexes are considered to have vital role in metabolic activities. Though Dickenson (1969) opined that these structures may be possible sites of rubber biosynthesis, the double membrane and presence of carotene and polyphenoloxidase in the Frey-Wyssling complexes led to a tentative suggestion that it is a type of plastid.

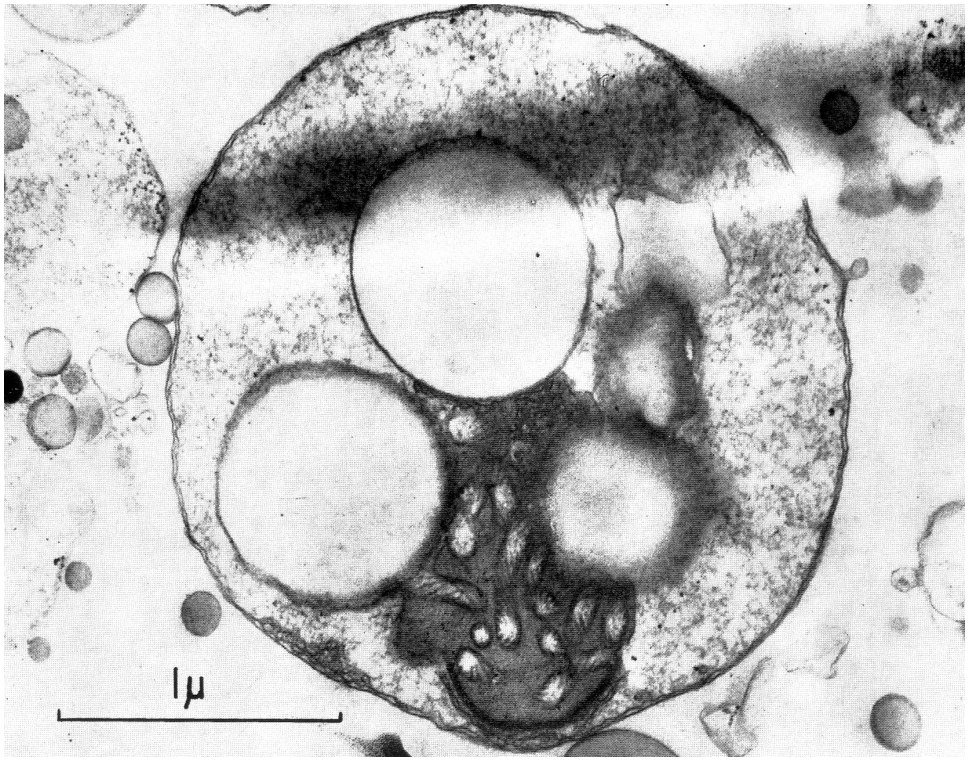

Fig. 5 A Frey-Wyssling complex showing the double membrane envelope; lipid inclusions; one system of tubules with interior spiral structures all contained in a membrane-bound osmiophilic matrix, with associated lamellae (Dickenson, 1969).

1.2.2 Organic Non-rubber Constituents

Proteins

The total protein content in latex has been estimated to be about 1% (Archer and McMullen, 1961; Archer *et al.,* 1963b; Tata, 1980a). However, discrepancies in the distribution of the proteins between the major phases of latex exist. Archer and McMullen (1961) reported that 20% of the total proteins was absorbed on the rubber surface, 66% in the C-serum and 14% in the bottom faction. Later, reports variously described the distribution as 20%, 60% and 20% (Archer et al., 1963b) and 27.2%, 47.5% and 25.3% (Tata, 1980a) for the rubber phase, C-serum and bottom fraction respectively.

Proteins on the surface of the rubber particle: The existence of proteins in association with phospholipids on the surface of rubber particles was recognized as early as 1953 by Bowler. He attributed that this protein-phospholipid layer imparted a net negative charge to the rubber particle, thereby contributing to the colloidal stability of these particles. By measuring the iso-electric points of various latex samples, he concluded that there was more than one protein adsorbed on the rubber surface and that the relative proportion of the adsorbed proteins varied with clones.

Apart from the estimation that the protein adsorbed on rubber surface accounted for about 1% of the weight of rubber (Cockbain and Philpott, 1963). The major protein on the rubber surface has been shown to be negatively charged and has a molecular weight of approx. 65,000 (RRIM, 1982). It migrates towards the anode at a higher rate than the major C-serum protein α-globulin and contrary to the earlier suggestion, is therefore not identical with the latter.

Of the numerous enzymes reported in *Hevea* latex, only two have been found to be associated with the rubber surface. These are isopentenyl pyrophosphate polymerase (Lynen, 1967; Archer *et al*., 1963a) and rubber transferase (Lynen, 1967; Archer *et al.,* 1963a; Archer and Cockbain, 1969; McMullen and McSweeney, 1966; Archer *et al*., 1966). Their presence on the rubber surface is not surprising, since they are involved in rubber biosynthesis.

Proteins in the C-serum: Nearly half of the enzymes examined in *Hevea* latex appeared to be located in the C-serum of latex. These include enzymes for the

glycolytic pathway (Bealing, 1969; d'Auzac and Jacob, 1969) as well as many of the enzymes for rubber biosynthesis (Archer and Audley, 1967). Twenty-seven enzymes were separated by electrophoresis by Jacob and co-workers, of which, seventeen were shown to exist in multiple forms (Jacob *et al.,* 1978).

The first protein to be isolated from *Hevea* latex was from C-serum. It was named α-globulin by Archer and Cockbain (1955). This protein is the major protein component of C-serum. It is readily adsorbed at a water-air or oil-water interface with a resulting fall in the interfacial tension. This led to the suggestion that α-globulin was one of the proteins on the surface of rubber particles and that it contributed to the colloidal stability of fresh latex (Archer and Cockbain, 1955). However, as mentioned earlier, α-globulin was later found not to be present on the surface of the rubber particles (RRIM, 1982).

With the introduction of more sensitive techniques, further discoveries on proteins of C-serum were made. Using starch gel electrophoresis, Tata and Moir (1964) reported the presence of twenty-two protein bands in C-serum. Seventeen of these were anionic at pH 8.2, whilst five were cationic and existed in much concentrations. A comparative study on the proteins in the C-sera from four clones viz. RRIM 501, GT 1, Tjir 1 and Pil A44, revealed very little differences between their general electrophoretic patterns (RRIM, 1963). There was also no significant difference in the proteins with seasonal variation within a single clone. Later, the list of proteins in C-serum was enlarged to twenty-four (Tata and Edwin, 1970) using the same starch gel electrophoretic technique. Using polyacrylamide gel electrophoresis, Yeang *et al.* (1977) reported 26 protein bands from C-serum at alkaline pH and 15 bands at acid pH.

Proteins in the bottom fraction: Proteins in the bottom fraction are essentially studied as the soluble proteins in B-serum. These have been examined with various techniques, including paper electrophoresis (Moir and Tata, 1960), starch gel electrophoresis (Tata, 1975; Tata and Edwin, 1969) and polyacrylamide gel electrophoresis (Yeang *et al.,* 1977). Irrespective of the techniques used, the proteins of B-serum were found to be marked by different from those of C-serum. Upon electrophoresis (Audley, 1965; Karunakaran *et al.*, 1961; Moir and Tata, 1960), the B-serum proteins were usually

separated into two major protein bands at the extreme anionic and cationic ends, with minor bands in between (Fig. 6).

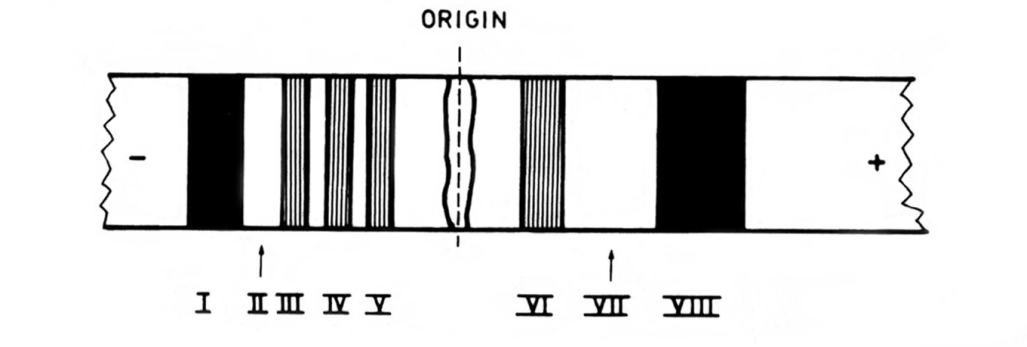

Fig. 6 Paper electrophoresis of dialysed proteins from lutoid fraction. The bands II and VII were not always visible. The anionic protein VI and the cationic proteins III, IV and V should be major constituents of microhelices. The principle cationic protein I is HEVAMINE which in fact is the lysozyme. The most important anionic protein VIII is HEVEIN (Moir and Tata, 1960).

Hevein: The major protein in B-serum is hevein, which accounts for about 70% of the water soluble proteins in the bottom fraction (Archer *et al*., 1969). Hevein is a low molecular weight anionic protein (approximate 5,000 daltons) with a higher (5%) sulphur content (Tata, 1975; Archer, 1960; Tata, 1976). All the sulphur in hevein exists as eight disulphide (S-S) bridges of cystine (Archer, 1960; Tata, 1976). Because of its low molecular weight and number of S-S bridges, hevein is heat stable, and is not precipitated by the common reagents for precipitating proteins eg. trichloroacetic acid (Tata, 1975; Tata, 1976). The molecular weight of hevein was first estimated to be about 10,000 ± 500 daltons by Archer (1960). Subsequent analysis showed that earlier preparations (Archer, 1960; Karunakaran *et al*., 1961) of hevein were mixtures containing hevein, traces of esterase and a protein with slighly less anionic mobility than hevein, termed pseudo-hevein (Tata, 1975; Tata, 1976). When pure hevein (free of pseudo-hevein) was isolated and characterised, it was found to be a single peptide chain with glutamic acid at the N-terminus and a molecular weight of approximately 5,000 daltons (Tata, 1975; Tata, 1976). Later, complete amino acid sequence of hevein was reported (Walujuno *et al.,* 1976). It contained 43 amino acid residues in a single polypeptide chain and an estimated molecular weight of 4,729 daltons.

The microfibrilar protein: Dickenson (1963, 1965, 1969) in his ultrastructural studied and electron microscopic investigations of lutoids, first described some fibrillar components having a tightly coiled helical structure, which he named microfibrils. These structures were observed within lutoid of young latex vessels but were absent from mature vessels. These microfibrils were later shown to be proteins containing upto 4% carbohydrate, and having an isoelectric pH about 4 (Audley, 1965; 1966). At ambient temperature (20°C), the microfibrils break up into smaller segments which reassemble on freezing (Audley, 1965; 1966).

The microhelices: These structures were first observed by Dickenson (1963) in lutoids from mature trees. However, Dickenson described them as stretched microfibrils. Later, Southorn and Yip (1968) and Gomez and Yip (1974; 1975; 1976) carried out detailed investigations and reported that these zig-zag structure differed from microfibrils in that they were larger in dimensions and were open helcies (not tigthly coiled

helices of the microfibrils). They were called "Microhelices" by Gomez and Yip (1975). Lowering of the ionic concentration of B-serum by dialysis against water or by dilution with water resulted in the formation of micohelices (Tata, 1975; Gomez and Yip, 1974; 1975; 1976). Furthermore, their formation required the combination of two glycoproteins in a certain ratio. These are an acidic "assembly factor" (molecular weight 160,000) and a slightly basic "pro-helical protein" (molecular weight 22,000) (Tata, 1975). A third glycoprotein termed the "building factor" (molecular weight 5,000) appeared to promote the combination of single microhelices into bundles (Tata, 1980b). The "pro-helical protein" has some flocculating activity on suspensions of rubber particles *in vitro.* Microhelices are rarely seen from young trees.

The basic proteins: The presence of basic proteins in B-serum was first demonstrated when B-serum or an aqueous extract of freeze-dried bottom fraction was electrophoresed (Tata and Edwin, 1970; Moir and Tata, 1960; Karunakaran *et al.,* 1961). Two basic proteins – a major and a minor basic protein – which account for about 4% of the total proteins in latex were found to have lysozyme and chitinase activities (Tata, Beintema and Balabaskaran, 1983). The major basic protein has been crystallised and its molecular weight (approx. 26,000) determined. Its first 21 amino acid residues were elucidated, and found to differ significantly from those of hen egg, duck egg, baboon milk and T4 phage lysozymes (Tata, Beintema and Balabaskaran, 1983). The major basic protein, also referred to earlier as "band (I) first peak protein" (Fig. 6) was found to be identical with hevemine A, a cationic protein described by Archer (1976), another basic protein in B-serum.

Proteins in the lutoid membrane

Several proteins are present in the lutoid membrane, and many of these are active enzymes. One well-characterized membrane enzyme is ATPase (Moreau *et al.,* 1975). The electron transport activity of lutoid has been linked to ATPase, which is activated by several anions, this in turn leading to an accumulation of anions within the lutoid compartment. ATPase also operates as a proton pump to maintain proton gradients between the lutoid and latex cytosol-a function which was demonstrated by Chrestin and Gidrol. Other membrane enzymes include NADH-cytochrome C reductase, this functions in

an outward proton-pumping redox system that tends to reduce the concentration of protons in lutoid and hence acidify the cytosol (Moreau *et al.,* 1975). NADH-quinone reductase (d'Auzac *et al*., 1986) has also been described as being responsible for the production of superoxide ions.

Recently, the enzyme HMG-CoA reductase has been purified from lutoid membrane by solubilization with mild detergent (Wititsuwannakul and Suwanmanee, 1990). Characterization of the purified enzyme was carried out by determining its molecular structure and properties. The native enzyme was found to be a tetramer of four 44 kDa subunits-as found for other plant specimens-and membrane bound (Bach, 1986).

Lipids and phospholipids

Lipids and phospholipids associated with the rubber and non-rubber particles in latex play a vital role in the stability and colloidal behaviour of latex. Earlier studies (Cockbain and Philpott, 1963; Blackley, 1966) demonstrated that the rubber particles are strongly protected by a complex film protein and lipid material. It is believed that some of the lipids are present within the rubber particle. The concentration and distribution of lipids between the rubber cream and the bottom fraction had been studied (Ho *et al.,* 1976). These lipids were isolated and divided into neutral lipids and phospholipids for further analysis. There appeared to be distinct variation in the amount of neutral lipids extractable from rubber cream and from the bottom fraction. Colloidal stability of latex was found related to the natural lipid content of rubber particles (Sherief and Sethuraj, 1978). Lutoids from different clones, however, were qualitatively similar. Triglycerides and sterols were the main components of the neutral lipids of rubber particles, whilst sterols and long-chain free fatty acids mainly made up the neutral lipids of the bottom fraction. A furanoid fatty acid containing a methylfuran group was found mainly in the triglyceride fraction of the neutral lipids (Hasma and Subramaniam, 1978). It constituted about 90% of the total esterified acids. It was suggested that the main triglyceride in *Hevea* latex contained three furanoid fatty acids, hence making it a rare triglyceride known in nature. The phospholipid content of the rubber particles (approx. 1% of the weight of rubber) was similar between different clones. The total phospholipid content of bottom fraction was much less (only about 10%) than that in the rubber cream. It

was suggested that the amount of neutral lipids (especially triglycerides) associated with the rubber particles was inversely related to the plugging index of the clone which the latex originated from (Ho *et al.,* 1976). Lutoid stability, as indicated by bursting index, was found to be negatively correlated with the phospholipid content of the bottom fraction of latex (Sherief and Sethuraj, 1978).

A systematic study of the glycolipids from natural rubber was reported (RRIM, 1980). The glycolipids fraction was found to consist mainly of esterified sterol glucoside (ESG), monogalactosyldiglyceride (MGDG), sterol glucoside (SG) and digalactosyldiglycerate (DGDG). The sterol attached to ESG and SG was mainly β-sitosterol, while the acid components of ESG, MGDG and DGDG were of 14:0; 16:1; 18:0; 18:1; 18:2; 18:3 and furanoic acids. The constituents of the phospholipids are mainly phophatidyl ethanolamine (PE), phosphatidyl choline (PC) and phosphatidyl inositol (PI).

2. Allergy

Allergic rhinitis, asthma, and atopic eczema are among the commonest causes of chronic ill health. These diseases are increasing in prevalence, and they add considerably to the burden of health care costs. In Sweden, for example, the number of children with allergic rhinitis, asthma, or eczema roughly doubled over a 12-year period (Aberg *et al*., 1995) and in the United States the annual cost of treating asthma is about $6 billion (Smith *et al.,* 1997).

The term "allergy" was introduced in 1906 by von Pirquet, who recognized that in both protective immunity and hypersensitivity reactions, antigens had induced changes in reactivity (von Pirquet, 1963). With the passage of time the word has become corrupted and is now frequently used synonymously with IgE-mediated allergic disease. It was von Pirquet's intent that the term should apply to the "uncommitted" biologic response, which may lead either to immunity (a beneficial effect) or allergic disease (a harmful effect). The term "atopy" (from the Greek *atopos,* meaning out of place) is often used to describe IgE-mediated diseases. Persons with atopy have a hereditary predisposition to produce IgE antibodies against common environmental allergens and have one or more atopic diseases (i.e., allergic rhinitis, asthma, and atopic eczema). Some allergic diseases, such as contact dermatitis and hypersensitivity pneumonitis, develop through IgE-independent mechanisms and in this sense can be considered nonatopic allergic conditions.

2.1 Atopy and Type 2 Helper T Cells

All of us inhale aeroallergens derived from pollen, house-dust mites, and cat dander. In general, adults and children without atopy mount a low-grade immunologic response; they produce allergen-specific IgG1 and IgG4 antibodies (Kemeny *et al.,* 1989) and *in vitro* their T cells respond to the allergen with a moderate degree of proliferation and the production of interferon-γ by type 1 helper T (Th1) cells (Romagnani, 1991; Ebner *et al.,* 1995; Till *et al.,* 1997). Persons with atopy, by contrast, have an exaggerated response characterized by the production of allergen-specific IgE antibodies; they have elevated serum levels of IgE antibodies and positive reactions to extracts of common aeroallergens on skin-prick tests. T cells from their blood respond to allergens *in vitro* by inducing cytokines produced by type 2 helper T (Th2) cells (i.e., interleukin-4, 5, and 13) (Romagnani, Human and Subsets, 1991; Till *et al.,* 1997) rather

than cytokines produced by Th1 cells (interferon-γ and interleukin-2). There are many exceptions to this rule, but the immunopathological hallmark of allergic disease is the infiltration of affected tissue by Th2 cells (Kay *et al.,* 1991; Robinson *et al.,* 1992; Durham *et al.,* 1992).

In utero, T cells of the fetus are primed by common environmental allergens that cross the placenta. As a result, the immune response of virtually all newborn infants is dominated by Th2 cells (Prescott *et al.,* 1998). It has been proposed that during subsequent development the normal (i.e., nonatopic) infant's immune system shifts in favor of a Th1-mediated response to inhaled allergens (a process termed "immune deviation") (Holt *et al.,* 1999) whereas in the potentially atopic infant there is a further increase in Th2 cells that were primed in utero. Microbes are probably the chief stimuli of protective Th1-mediated immunity. Macrophages that engulf microbes secrete interleukin-12, which induces Th1 cells and natural killer cells to produce interferon-γ, thereby shifting the immune system into an "allergyprotective" Th1-mediated response. Other factors may also influence whether Th1 or Th2 cells dominate the response, including the amount of allergen, the duration of exposure to the allergen, and the avidity of allergen-specific interactions between T cells and antigen-presenting cells (Constant and Bottomly, 1997; Rogers and Croft, 1999) (Fig. 7).

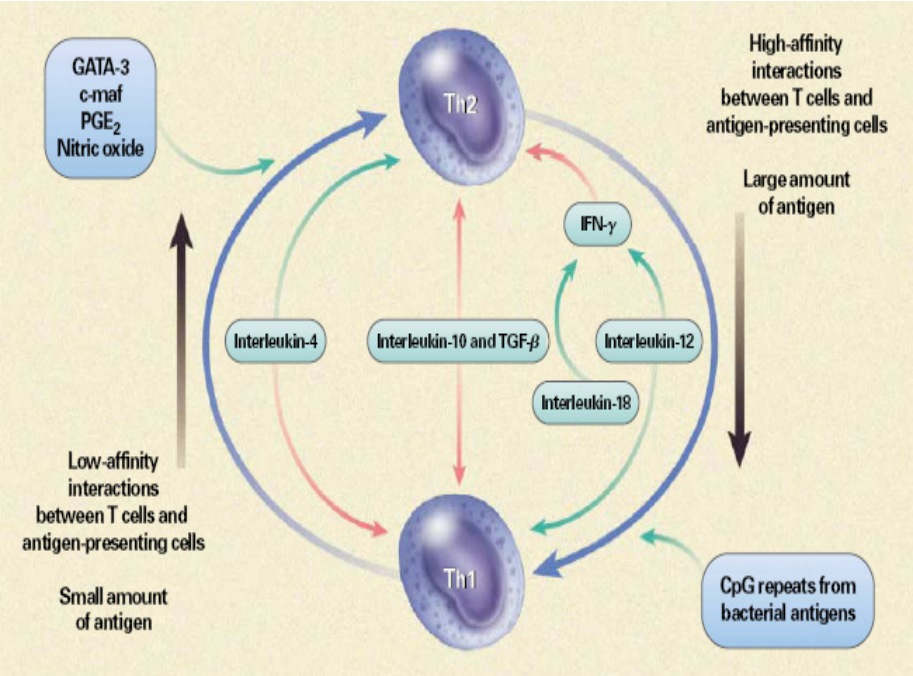

Fig. 7 Immunologic and Cellular Factors Regulating the Expression of Th1 and Th2 Cells (Kay, 2001). Whether the immune response is dominated by Th1 or Th2 cells is dependent on interleukin-12 and interleukin-4, respectively, as well as on the avidity of interactions between T cells and antigen-presenting cells and the amount of allergen to which the immune system is exposed (antigen) (Constant and Bottomly, 1997; Rogers and Croft, 1999). In addition, the presence of cytidine–phosphate–guanosine (CpG) repeats derived from bacteria favors the Th1 phenotype, whereas the presence of transcription factors such as GATA-3 favors the Th2 phenotype (Caramori *et al.,* 1999) as does the presence of c-maf and prostaglandin E_2 (PGE_2). Nitric oxide favors the expression of Th2 cells by being less inhibitory to Th2 cells than Th1 cells, whereas in humans interleukin-10 and transforming growth factor β (TGF-β) generally dampen the responses of both types of cells. Interferon-γ (IFN-γ) inhibits Th2-mediated responses; both interleukin-12 and interleukin-18 release interferon-γ from T cells. Interleukin-4 inhibits the expression of Th1 cells and promotes Th2-mediated responses. Green arrows indicate stimulatory effects, and red arrows inhibitory effects, of the cytokines.

2.2 Rising Incidence of Allergic Disease

The marked increase in the prevalence of atopic disease in western Europe, the United States, and Australasia during recent years indicates the importance of environmental influences. An informative example is the change in the incidence of seasonal allergic rhinitis and asthma after the reunification of Germany. These disorders were less common in East Germany than West Germany before reunification (von Mutius *et al.,* 1994) whereas since reunification, the prevalence of atopy and hay fever, but not asthma, has increased among children who spent their early childhood in East Germany (von Mutius *et al.,* 1998). This phenomenon raises the possibility that a Western lifestyle accounts for the increases in prevalence. Perhaps in Western countries the developing immune system is deprived of the microbial antigens that stimulate Th1 cells, because the environment is relatively clean and the use of antibiotics for minor illnesses in early life is widespread (Rook and Stanford, 1998).

The results of epidemiologic studies support this theory. Evidence that the bacteria that colonize the gastrointestinal tract prevent atopic sensitization was found in studies of one-year-old infants in countries with a low prevalence of atopy (Estonia) and a high prevalence (Sweden). Lactobacilli and eubacteria predominated in Estonian infants, whereas clostridia were more frequent in Swedish infants (Sepp *et al.,* 1997). When studied one year later, the children with atopy were colonized less often by lactobacilli and had higher levels of aerobic bacteria (such as coliforms and *Staphylococcus aureus*) than children without atopy (Bjorksten *et al.,* 1999). Moreover, atopy and allergic asthma were less frequent in populations exposed to *Helicobacter pylori, Toxoplasma gondii,* and hepatitis A virus. By producing an environment rich in interleukin-12, these microbes could drive a Th1- mediated response. This mechanism may explain why in Europe and Africa, farming or living in a rural community, which increases the likelihood of exposure to bacteria found in barns, protects against atopic disease (Braun-Fahrlander *et al.,* 1999).

Other factors that may favor the Th2 phenotype in infants include diet and being when pollen counts are high (Cookson, 1999). Furthermore, atopic allergic diseases are less common in younger children who have three or more older siblings and among children who have had measles or hepatitis A — another indication that repeated immune stimulation may protect against atopic allergy (Openshaw and Hewitt, 2000). This view is supported by the study by Ball

et al. (2000) who provided evidence that exposure of young children to older children at home or to other children at day-care centers protected against the development of asthma and frequent wheezing in childhood (Ball *et al.,* 2000).

This "hygiene" hypothesis is not easily reconciled with the increased prevalence among poor blacks in the United States of atopic asthma associated with sensitization to cockroaches and house-dust mites (Schwarta *et al*., 1990; Call *et al*., 1992). However, we need more data on the rates of infection by foodborne and orofecal microbes in inner cities in the United States: the compounding effect of gut flora that does not protect against atopy and heavy exposure to allergens may explain this paradox. The development of specific allergic diseases may be related to alterations in the target organ. For example, the cofactors required for an asthma attack may include respiratory virus infections and exposure to allergens, tobacco smoke, and air pollutants (Holgate, 1999). These factors, alone or in combination, may alter immunoregulatory mechanisms at mucosal surfaces in ways that promote a Th2-mediated allergic inflammatory response (Fig. 8).

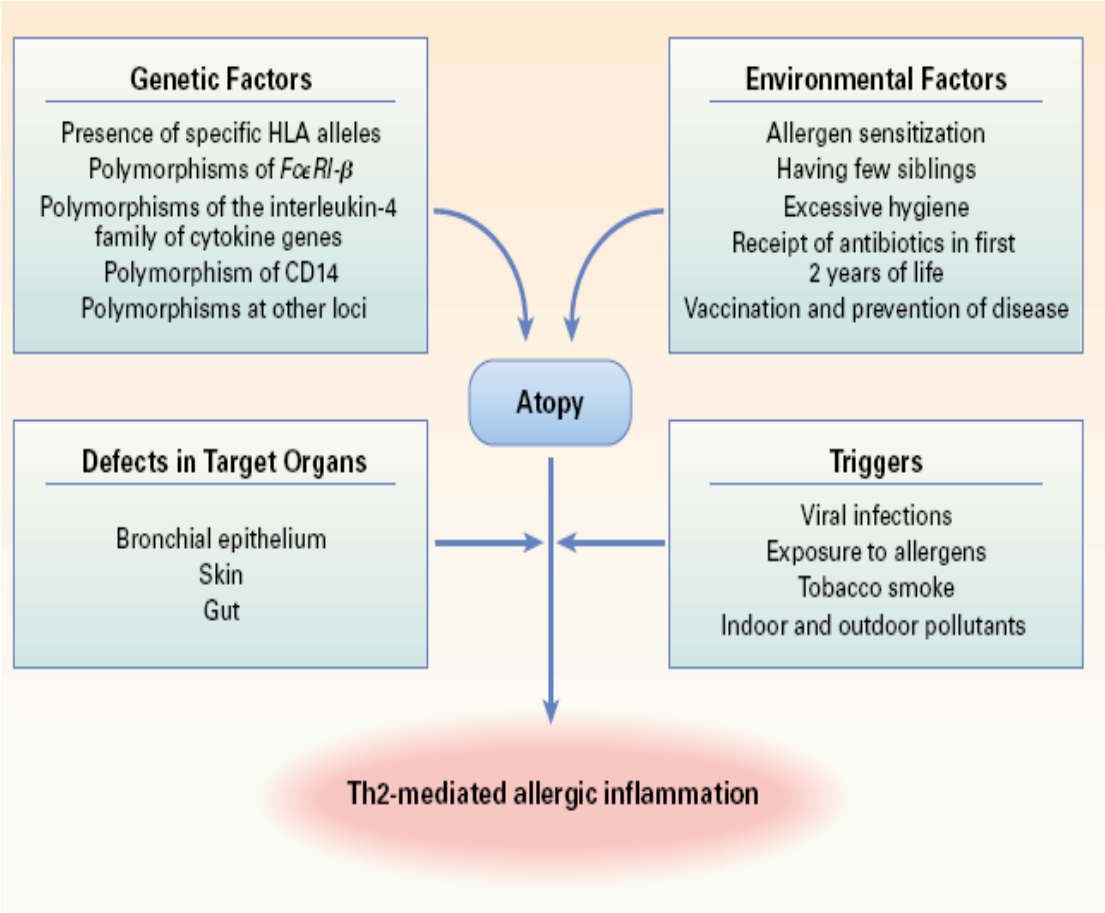

Fig. 8 Factors Influencing the Development of Atopy and Allergic Inflammation Mediated by Th2 Cells (Atopic Allergic Disease) (Kay, 2001).

The induction of atopy is dependent on interactions between genes and the environment. The induction of atopic allergic disease may require further interactions between defects in the target organ and various environmental triggers. FcεRI-β denotes the gene for the β chain of the high-affinity receptor for IgE.

2.3 Allergens

Many allergens are soluble proteins that function in their natural state as enzymes, by, for example, inducing proteolysis. Allergenic properties may be related to the enzymatic activity (e.g., increased mucosal permeability) and to aerodynamic properties, which in turn depend on the size of the particle. The major allergens of Western developed countries are Der p 1 and Der p 2, from the house-dust mite (*Dermatophagoides pteronyssinus*); Fel d 1, from the cat (*Felis domesticus*); several tree allergens, including Bet v 1 from the birch tree (*Betula verrucosa*); and many grasses, such as Phl p 1 and Phl p 5 from timothy grass (*Phleum pratense*). The ragweed allergens Amb a 1, 2, 3, 5, and 6 from short ragweed (*Ambrosia artemisiifolia*) and Amb t 5 from giant ragweed (*Ambrosia rifida*) are important seasonal allergens in North America. Allergies to Hev b 1 through 7 from latex, the milky sap harvested from the rubber tree (*H. brasiliensis*), and Ara h 1, 2, and 3, which are highly allergenic peanut proteins, are increasingly important problems (Burks *et al.,* 1998).

2.4 Genetics

Atopic allergic diseases are familial and have a genetic basis. The difficulties of conducting genetic studies of allergy are due in part to the multiple markers for atopy and allergic diseases. For instance, atopy (manifested by positive skin-prick tests and elevated serum IgE levels) and asthma (manifested by airway hyperresponsiveness) are not always inherited together. Techniques used to identify genes that are relevant to allergy and asthma include the candidate-gene approach, which depends on the identification of polymorphisms in a known gene, and positional cloning, which links the inheritance of a specific chromosomal region with the inheritance of a disease (Cookson, 1999). Such studies have linked several loci to atopy, but the clinical relevance of these findings is unclear. Examples are the associations between an allele of the HLA-DR locus and reactivity to the ragweed allergen Ra 529 (Marsh *et al.,* 1982) and the linkage of atopy to a polymorphism of the gene for the β chain of the high-affinity receptor for IgE (FcεRI-β) 30 (Hill and Cookson, 1996) and to the interleukin-4 family of cytokine genes on chromosome 5 (Marsh *et al.,* 1994). By contrast, certain alleles of the tumor necrosis factor gene complex, although linked to asthma, are independent of serum IgE levels and other measures of atopy (Moffatt and Cookson, 1997).

Polymorphisms of the FcεRI-β gene appear to be associated with equal frequency to severe atopy, asthma, and eczema. Also, positional cloning indicates that chromosomes 2q, 5q, 6q, 12q, and 13q contain loci linked to both asthma and atopy (Cookson, 1999). Polymorphisms in the gene encoding the high-affinity receptor for bacterial lipopolysaccharide (CD14) have been linked to total serum IgE levels and may help explain the association between childhood infections and the development of atopy (Baldimi *et al.,* 1999).

Several of the genes and genetic regions that have been linked to atopy and asthma have also been implicated in rheumatoid arthritis (chromosome 2) and inflammatory bowel disease (chromosomes 2 and 12) (Cookson, 1999). There has been recent interest in loci with pharmacologic relevance. Polymorphisms within the promoter region of the 5-lipoxygenase gene (Drazen *et al.,* 1999) and in the β-adrenergic receptor gene may regulate the response to inhibitors of 5-lipoxygenase or β-adrenergic agonists, respectively (Cookson, 1999; Drazen *et al.,* 1999). These findings raise the possibility that genotyping will become useful in planning therapy for asthma and other allergic diseases.

2.5 IgE and Its Receptors

Acute allergic reactions result from the release of preformed granule-associated mediators, membrane derived lipids, cytokines, and chemokines when an allergen interacts with IgE that is bound to mast cells or basophils by the α chain of the high-affinity IgE receptor (FcεRIα) (Kinet, 1999). This receptor also occurs on antigen-presenting cells, where it can facilitate the IgE-dependent trapping and presentation of allergen to T cells (Stingl and Maurer, 1997). Eosinophils also possess FcεRI-α, but in these cells it is almost entirely intracellular; after being released by degranulation of the eosinophil, it may help regulate local levels of IgE (Smith *et al.,* 2000).

The most important inducers of the production of IgE are interleukin-4 and interleukin-13. These cytokines initiate transcription of the gene for the epsilon class of the constant region (C_e) of the immunoglobulin heavy chain. The production of IgE also requires two transcription factors, nuclear factor kB and STAT-6; the former pathway involves the co-stimulatory molecules CD40 and the CD40 ligand (CD154), and the latter is activated when interleukin- 4 binds to the high-affinity α chain of the interleukin- 4 receptor (Corry and Kheradmand, 1999).

Allergens, including the products of some infectious microorganisms (e.g., *Aspergillus fumigatus*) and helminthic parasites, evoke Th2-mediated responses that are characterized by high serum levels of IgE, whereas other bacterial antigens (such as those associated with *Listeria monocytogenes* and *Mycobacterium tuberculosis*) elicit a Th1-mediated response that is dominated by cellular immunity (the appearance of cytotoxic T cells and delayed hypersensitivity). In this latter class of organisms, the DNA contains repeating sequences of cytosine and guanosine nucleosides called CpG repeats which can bind to receptors on antigen-presenting cells and trigger the release of interleukin-12. This cytokine, which is produced almost exclusively by antigen-presenting cells, drives and maintains the Th1-mediated response. Furthermore, the interferon-γ produced by activated Th1 cells (Robinson *et al.,* 1997) and interleukin-18, produced by macrophages, (Robinson *et al.,* 1997) join forces to suppress the production of IgE antibodies (Yoshimoto *et al.,* 1998). Therefore, at least theoretically, interferon-γ, interleukin-12, and interleukin-18, either alone or in combination, have therapeutic potential for inhibiting the synthesis of IgE. Furthermore (as discussed below), CpG repeats may redirect allergens to produce a Th1-mediated, rather than a Th2-mediated, immune response.

The physiologic relevance of the low-affinity IgE receptor (CD23) remains speculative. It may be involved in antigen trapping and presentation, thereby augmenting the production of interleukin-4 or interleukin-13 (Squire *et al.,* 1994). It can, however, override the positive effects of antigen presentation by combining with excess IgE and antigen under conditions in which high levels of interleukin-4 have caused the up-regulation of this type of receptor (Gustavsson *et al.,* 1994).

2.6 Allergic inflammation

In a person with atopy, exposure of the skin, nose, or airway to a single dose of allergen produces a cutaneous wheel-and-flare reaction, sneezing and runny nose, or wheezing within minutes. Depending on the amount of the allergen, these immediate hypersensitivity reactions are followed by a late-phase reaction, which reaches a peak six to nine hours after exposure to the allergen and then slowly resolves. In the skin, late-phase reactions are characterized by an edematous, red, and slightly indurated swelling; in the nose, by sustained blockage; and in the lung, by further wheezing. Immediate hypersensitivity is the basis of acute allergic reactions. It is caused by molecules released by mast cells when an allergen interacts with

membrane-bound IgE. The complex of allergen, IgE, and FcεRI on the surface of the mast cell triggers a noncytotoxic, energy-dependent release of preformed, granule associated histamine and tryptase and the membranederived lipid mediators leukotrienes, prostaglandins, and platelet-activating factor. These mast-cell mediators have a critical role in anaphylaxis, rhinoconjunctivitis, and urticaria. The role of histamine in chronic asthma and eczema is probably minimal, however, as shown by the relative ineffectiveness of histamine antagonists in controlling these conditions.

Mast cells produce the three cysteinyl leukotrienes C_4, D_4, and E_4, which cause the contraction of smooth muscles, vasodilatation, increased vascular permeability, and the hypersecretion of mucus when they bind to specific receptors (Drazen *et al*., 1999).

Eosinophils, macrophages, and monocytes are also major sources of cysteinyl leukotrienes. Mast cells also contain tryptase, a four-chain neutral protease that activates the protease-activated receptors on endothelial and epithelial cells. The activation of these receptors initiates a cascade of events, including the up-regulation of adhesion molecules that selectively attract eosinophils and basophils (Holgate, 1999).

In the cutaneous late-phase reaction, eosinophils and neutrophils accumulate, and then CD4+ T cells and basophils infiltrate the site (Ying *et al.,* 1999). Late-phase asthmatic (Robinson *et al.,* 1993) and nasal10 reactions have a similar pattern of cellular infiltration, although basophils are not prominent in the lower airways (Macfarlane *et al.,* 2000).

Depending on the target organ, late-phase reactions can be provoked by the activation of mast cells or T cells. In the skin of atopic subjects and normal subjects, cross-linking of mast-cell–bound IgE with an antibody against IgE provokes both immediate hypersensitivity and late-phase reactions (Dolovich *et al.,* 1973). Late-phase reactions can be induced in patients with atopic asthma in the absence of immediate hypersensitivity involving mast cells. These reactions were induced in patients with asthma who were allergic to cats by an intradermal injection of peptides derived from a cat allergen (Haselden, Kay and Larche, 1999). The fact that these late-phase reactions were independent of IgE and were major-histocompatibility-complex (MHC)–restricted indicates that the activation of T cells alone is sufficient to initiate airway narrowing in patients with allergic asthma.

Antigen-presenting cells are critical in initiating and controlling allergic inflammation. Dendritic cells and cutaneous Langerhans' cells are particularly important in asthma and atopic eczema, respectively. They present antigen to CD4+ Th2 cells in an MHC class II–restricted fashion. Overproduction of the granulocyte–macrophage colony-stimulating factor in the airway mucosa of patients with asthma enhances antigen presentation and increases the local accumulation of macrophages (Holt *et al.,* 1999). Alveolar macrophages obtained from patients with asthma by bronchoalveolar lavage present allergen to CD4+ T cells and stimulate the production of Th2-type cytokines, (Larché *et al.,* 1998) whereas alveolar macrophages from control subjects do not.

Th2-type cytokines such as interleukin-4, 5, 9, and 13 influence a wide range of events associated with chronic allergic inflammation. Interleukin-4 and interleukin-13 stimulate the production of IgE and vascular-cell adhesion molecule 1; interleukin-5 and interleukin-9 are involved in the development of eosinophils; interleukin-4 and interleukin-9 promote the development of mast cells; interleukin-9 and interleukin-13 help promote airway hyperresponsiveness (Wills-Karp *et al.,* 1998); and interleukin-4, interleukin-9, and interleukin-13 promote the overproduction of mucus (Table 1). Eosinophils can injure mucosal surfaces by releasing toxic basic proteins, cysteinyl leukotrienes, and platelet activating factor. They also damage inhibitory M2 muscarinic receptors, which may allow unchecked cholinergic responses in patients with asthma (Adamko *et al.,* 1999). By contrast, eosinophils may also repair damage, since they produce fibrogenic growth factors and matrix metalloproteinase, which remodel airway tissue in asthma (Levi-Schaffer *et al.,* 1999).

Table 1. The Role of Cytokines Produced by Th2 Cells in Chronic Allergic Inflammation (Kay, 2001).

Event	**Th2-Type Cytokines Involved**	**Other Factors Involved**
Production of IgE	Interleukin-4, interleukin-9, and interleukin-13	Interleukin-γ, interleukin-12, and interleukin-18
Development and accumulation of eosinophils and basophils	Interleukin-4, interleukin-5, interleukin-9, and interleukin-13	Interleukin-3, granulocyte-macrophage colony-stimulating factor, eotaxin-1, eotaxin-2, eotaxin-3, RANTES, monocyte chemotactic protein 3, monocyte chemotactic protein 4, and vascular-cell adhesion molecule 1
Development of mast cells	Interleukin-4, interleukin-9, and interleukin-13	Interleukin-3 and stem-cell factor
Airway hyperresponsiveness	Interleukin-9, and interleukin-13	Interleukin-11 and growth factors involved in remodeling
Overproduction of mucus	Interleukin-4, interleukin-9, and interleukin-13	Histamine, leukotriene C_4, leukotriene D_4, substance P, and calcitonin-gene-related peptide

Interleukin-5 releases both mature and immature eosinophils from the bone marrow (Palframan *et al*, 1998) regulates the expression of the transmembrane isoform of its own receptor, (Tavernier *et al.,* 2000) and is essential for the terminal differentiation of committed eosinophil precursors (Clutterbuck *et al.,* 1989). The preferential accumulation of osinophils occurs through the interactions between selective adhesion molecules ($\alpha_4\beta_1$ integrin and vascular-cell adhesion molecule), the migration of eosinophils toward receptors for CC chemokines as a result of recruitment by eotaxin-1, eotaxin-2, eotaxin-3, monocyte chemotactic protein (MCP) 3 and MCP-4; prolonged survival (delayed apoptosis) under the influence of interleukin-5, interleukin-3, and granulocyte–macrophage colonystimulating factor; and the local differentiation of tissue-infiltrating eosinophil precursors induced by interleukin-5 (Denburg, 1998).

Allergic inflammation may also follow the release of neuropeptides from nerve cells by the action of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 (Bonini *et al.,* 1999; Braun *et al.,* 2000). These neurotrophins are secreted by macrophages, T cells, eosinophils, and mast cells (Braun *et al.,* 2000). Neuropeptides, particularly substance P, calcitonin-gene–related peptide, and neurokinin A (all of which are located predominantly in sensory neurons, but also in inflammatory cells), cause characteristic features of allergic inflammation, including vasodilation, increased vascular permeability, and in the lung, contraction of the smooth muscles of the airway and hypersecretion of mucus (Belvisi and Fox, 1997). They also release histamine from mast cells in the lungs (Forsythe *et al.,* 2000). Tryptase can also trigger nerve cells to release neuropeptides by binding to protease-activated receptors. Further amplifications of chronic allergic reactions may be mediated by histamine-releasing factor or factors (Steinhoff *et al.,* 2000). Pathways leading to acute and chronic allergic reactions are shown in Fig. 9.

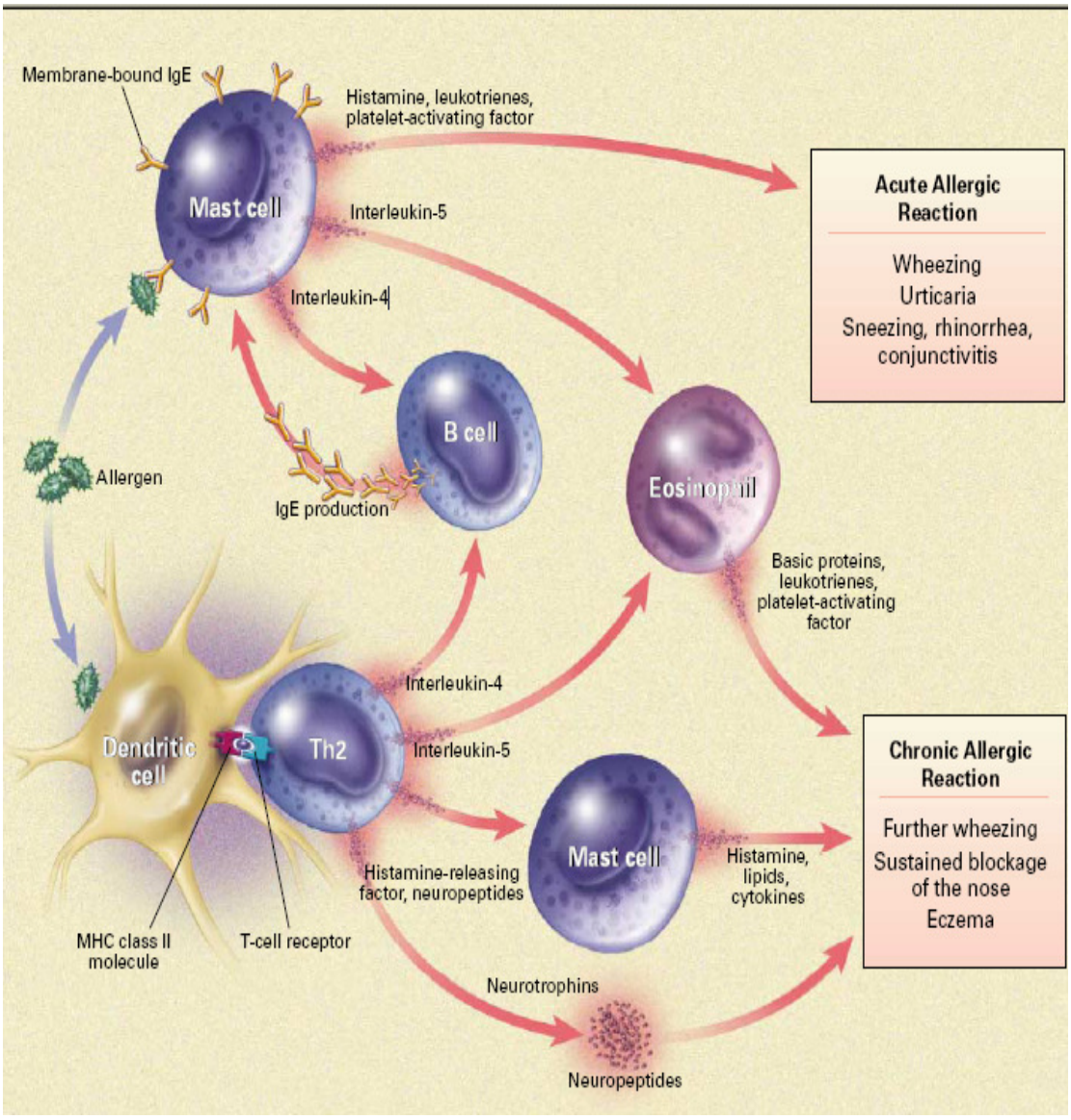

Fig. 9 Pathways Leading to Acute and Chronic Allergic Reactions (Kay, 2001).

Acute allergic reactions are due to the antigen-induced release of histamine and lipid mediators from mast cells. In the skin and upper airways, basophils (not shown) may also participate in allergic tissue reactions. Chronic allergic reactions, including the latephase reaction, may depend on a combination of pathways, including the recruitment of eosinophils, the liberation of mast-cell products by histamine-releasing factors (MacDonald *et al.,* 1995) and neurogenic inflammation involving neurotrophins and europeptides. MHC denotes major histocompatibility complex.

3. Natural Rubber Latex Allergy

3.1 Definition and Symptoms

Latex allergy is an immunoglobulin IgE mediated hypersensitivity to protein present in natural rubber latex with contact urticaria to latex gloves as paradigm. In contrast, delayed hypersensitivity to rubber latex is caused by accelerators or antioxidants added to cure natural latex or synthetic rubber with allergic contact dermatitis to rubber gloves as the paradigm (Slater, 1992; Taylor, 1993).

The typical allergic reaction to latex protein is characterized by pruritis, erythema and edema, as is common with other allergen-IgE, mast cell sensitivities (Forstrom, 1980; Granady and Slater, 1995; Ownby, 1995). As sentivity and exposure to allergen increases, urticaria may also develop, initially restricted to the site of the latex contact, but it may eventually spread to the contiguous area of the skin and finally become systemic (Frostrom, 1980; Meding and Fregert, 1984; Pecquet *et al.*, 1990; Ownby, 1995). Airborne exposure may lead to nasal, ocular and pulmonary symptoms (Sondheiner *et al.*, 1989; Turjanmaa and Reunala, 1989; Lagier *et al.*, 1990; Baur *et al.*, 1993; Tomazic *et al.*, 1994; Kujala *et al.*, 1996). The ocular symptoms usually start with pruritis and progress to tearing, chemosis and edema (Sussman *et al.,* 1991; Swanson *et al.*, 1994; Granady and Slater, 1995; Ownby, 1995). Frequently, direct contact with latex products leads to sudden swelling of the eyelids. Nasal symptoms include sneezing, watery rhinorrhea and congestion. Patients may develop sore throat, irritation of the larynx or cough (Lagier *et al.*, 1990; Kujala *et al.*, 1996). Pulmonary symptoms may range from coughing to life-threatening asthma (Forstrom, 1980; Granady and Slater, 1995). Lung function changes and chest symptoms may or may not be present in patients exposed to airborne latex allergens (Tarlo *et al.*, 1990). Gastrointestinal, cardiovascular and genitourinary symptoms from latex allergy have been well documented (Morales *et al.*, 1989; Nguyen *et al.*, 1991; Sussman *et al.*, 1991; Ownby, 1995). The severe forms of latex allergy are characterized by wheezing, stridor, sneezing rhinorrhea, ocular itching, urticaria, hypertension and anaphylaxis (Ownby, 1995). Although not very common, latex-induced anaphylaxis occurs soon after exposure to the allergens and even with prompt intervention latex-induced

anaphylaxis can be fatal (Gerber *et al.*, 1989; Leynadier *et al.*, 1989; Schwartz and Zurowski, 1993).

3.2 Epidemiology and Risk Factors

At present, the incidence of NRL allergy is still unknown, but several prevalence studies have been published. In European health-care workers screened with skin prick testing (SPT), the prevalence of NRL allergy has range from 2.8% to 10.7% (Turjanmaa, 1987; Arellano *et al.*, 1992; Yassin *et al.*, 1994; Turjanmaa *et al.*, 1995a). In agreement with SPT screening, a serologic study based on RAST found a 5.5% prevalence of NRL allergy among 381 hospital workers in the USA (Kaczmarek *et al.*, 1996). Children with spina bifida have shown the highest prevalence of NRL allergy. The frequency has ranged from 32% to 51% in SPT screenings (Kelly *et al.*, 1993; Moneret-Vautrin *et al.*, 1993; Slater, 1994). In contrast to these studies, a low prevalence rate of 4.3% was reported for Venezuenlain children with spina bifida (Capriles-Hulet *et al.*, 1995). The occurrence of NRL allergy has not been systematically surveyed among the general population, but the prevalence seems to be clearly less than 1% (Arellano *et al.*, 1992; Moneret-Vautrin *et al.*, 1993; Hadjiliadis *et al*., 1995; Turjanmaa *et al.*, 1995b).

In addition to repeated exposure to gloves and other NRL products, atopy seems to be the principle determinant for the development of NRL sensitization. In agreement with this, NRL-allergic health care workers are atopic 2.2-4.2 times more often than their coworkers without NRL allergy (Turjanmaa, 1987; Arellano *et al.*, 1992; Moneret-Vautrin *et al.* 1993; Yassin *et al.*, 1994). Hand eczema disrupts the barrier and, together with personal atopy, this condition is one of the main predisposing factors of NRL allergy (Wrangsjo *et al.*, 1988; Jaeger *et al.*, 1992; Charous *et al.*, 1994; Taylor and Praditsuwan, 1996). In addition to contact with gloves, the patients may also react to airborne NRL in their working environment (Tarlo *et al.*, 1990).

Children with spina bifida form a well-known, prominent risk group for NRL allergy (Slater, 1989; Moneret-Vautrin *et al.,* 1993; Swanson *et al.*, 1994). Many of these NRL-allergic children have had associated food allergy to cereals, banana and other fruits (Yhitalo *et al.*, 1996). These findings suggest that a pre-existing food allergy could be an additional risk factor for NRL allergy in children.

3.3 Diagnosis of NRL Allergy

3.3.1 Skin Tests

In Europe, the open patch test and scratch-chamber test were the first methods used in the diagnosis of NRL allergy. Later, prick testing became more popular, and in addition to glove pieces and eluates, ammoniated and non-ammoniated NRL were used as skin prick test material (Turjanmaa, 1988). In one report, nonstandardized allergic preparations were compared in prick testing, and a good correlation was found between the glove eluates, non-ammoniated latex and crushed rubber-tree leaves (Turjanmaa *et al.*, 1988b).

Wrangsjo *et al.* (1988), investigating 30 patients with contact urticaria, obtained positive skin tests with a solution of rubber latex that had been extracted in saline (1:9 w/v), centrifuged, sterized by filtration and diluted 1:100 w/v in saline containing 0.03% human serum albumin.

Turjanmaa *et al.* (1988a) performed skin prick tests in 40 latex allergic subjects with extracts of 19 brands of latex surgical and cleaning gloves. Twenty-six of the subjects had been sensitized by surgical gloves and the remaining 14 by cleaning gloves. The results confirm that allergic proteins persist in various sorts of latex glove after being manufactured from natural rubber latex and that these allergens can cause contact urticaria. However, the frequency of positive test reactions varied from 8 to 100%, depending on the source of the extract, indicating that there are differences in the quality or the amount of latex allergens eluting from different brands of gloves.

There are several commercially available nonstandardized allergens in Europe and two of these NRL allergens have been compared with an allergenic glove eluate (Triflex, Baxter). A group of 110 patients with NRL allergy was tested; the sensitivities of the commercial allergens were 88% (Staller-gènes) and 54% (ALK), and that of a reference glove eluate was 92% (Turjanmaa *et al.*, 1994). The specificity of all test materials was 100% because none of the 200 control patients not allergic to NRL had positive reactions.

3.3.2 Use or Challenge Tests

In Finland and Sweden (Turjanmaa *et al.*, 1995b), the use test with latex gloves is performed when there is a discrepancy between SPT results and clinical history or

when *in vitro* studies give positive reactions and the patient has not noticed any symptoms after using rubber products.

Turjanmaa *et al.* (1988b) reported that a use test in which a latex glove was worn on a damperred hand for 15 minutes gave positive results in 12/13 cases; the thirteenth patient, who had not worn latex gloves during the previous 4 years, had a positive response only after wearing the glove for 4 hours. None of these allergic patients reacted to contact with a vinyl glove, and control employees without contact urticaria did not react to the latex glove.

Wrangsjo *et al.* (1988) tested 10 patients with a history of systemic symptoms by placing 1 cm^2 pieces of surgical glove latex material moistened with saline on the skin of the forearm, or by having them wear one glove finger, for 20 minutes. A positive reaction was observed in 9/10 patients, two of whom experienced only distant symptoms (conjunctivitis, rhinitis, dyspnea, facial redness) during the test.

Jaeger *et al.* (1992) tested seven patients allergic to latex gloves with six different brands of sterile gloves, including three "hypoallergic" brands and two latex-free brands. The latex-free gloves were tolerated, but the "hypoallergic" gloves were not.

These authors also pointed out that it is difficult to standardize provocation tests because of different reports from patients and the unknown allergen content in rubber materials, as well as differences in the penetration of allergens into the skin (Turjanmaa *et al.*, 1988b; Wrangsjo *et al.*, 1988; Jaeger *et al.*, 1992).

Inhalation provocation tests have been done with latex to confirm the presence of respiratory symptoms induced by contact with latex or airborne latex particles. Jaeger *et al.* (1992) performed such tests with 18 patients who had experienced dyspnea or rhinitis/conjunctivitis related to latex contact. Inhalation of cornstarch from latex gloves in a small inhalation chamber during 5-60 minutes induced reactions in all 18 patients tested. The most frequent reactions during the test were rhinitis, cough and conjunctivitis. A significant increase in airway resistance was seen in five cases. One patient developed severe facial angioedema and hypotensis; another complained of generalized malaise. These symptoms were not induced by handling of cornstarch-powdered, latex-free gloves.

3.3.3 Blood or *in vitro* Tests

A latex radioallergosorbent test (RAST) is available commercially for the *in vitro* detection of latex IgE antibodies (Latex, k82, Phamacia Diagnostic), but, it is less sensitive than skin tests and provocation challenge, detecting these antibodies in only about 50-70% of skin test-positive, latex allergic patients (Frosch *et al.*, 1986; Axelsson *et al.*, 1987; Turjanmaa *et al.*, 1988b; Wrangsjo *et al.*, 1988; Jaeger *et al.*, 1992).

Jaeger *et al.* (1992) found that in 16% of their allergic patients, specific IgE antibodies were detected by RAST only when the test was made with latex-coupled disks prepared in their laboratory.

Sandberk *et al.* (1992) reported that 39% of a group of 31 in a spina bifida clinic had a positive RAST. Three of these children had had an allergic reaction to latex, whereas no child with a negative test had a clinical history of hypersensitivity.

A new latex-specific fluorescent enzyme immunoassay for the detection of latex-specific IgE (Phamacia CAP System, PCS) was evaluated by Dolen *et al.* (1992). Mathew *et al.* (1992) obtained a positive result with this assay in 4/10 skin test positive, latex sensitive spina bifida patients.

Kwittken *et al.* (1992) have developed a flow cytometric assay (FCA) for antilatex IgE antibodies and report an excellent correlation between FCA and RAST and between FCA and ELISA values.

A patients' IgE response to NRL antigens has also been evaluated with immunoblotting (Alenius *et al.*, 1991; Alenius *et al.*, 1993; Alenius *et al.*, 1994a; Alenius *et al.*, 1994c) and immunoelectrophoretic methods such as cross radioimmunoelectrophoresis (Makinen-Kiljunen *et al.*, 1992). A sensitivity of 81% has been demonstrated with the immunoblot method (Alenius *et al.*, 1994c). In addition, the histamine-release test has been found to be sensitive in the evaluation of latex-specific IgE antibodies *in vitro* (Carrillo *et al.*, 1986; Turjanmaa *et al.*, 1989). In one study, 13 of 14 (93%) samples were found to be positive (Turjanmaa *et al.*, 1989) and another study reported positive results in 11 of 16 (69%) cases (Leynadier and Dry, 1991).

3.4 Latex Allergens

The latex allergy epidemic has come and gone, at least in the highly industrialized countries of Europe and North America. It was caused in part by the adoption of precaution measures in response to the increase in viral infections such as hepatitis and HIV in the late 1980s and 1990s (Ownby, 2002). Present-day low prevalence rates in the USA and in Finland suggest that the peak of the latex allergy epidemic is over at least for health care workers (HCWs) (Reunala *et al.,* 2004). This is by and large due to the preventive measures implemented following the recommendations of several reputable research teams as well as task forces of European and American Allergy Associations.

Outside the health care field, the situation seems to be different. Eighteen percent of flower greenhouse workers reported immediate symptoms associated with wearing latex gloves (Carrillo et al, 1995). A study from Spain involving skin prick test screening showed that 6% of construction workers had latex allergy (Conde-Salazar *et al.,* 2002). Latex allergy is also more frequent in patients with food allergy. A higher percentage of latex allergy (10.4%) was found in latex-exposed individuals with food allergy than in those without (5.6%) (Kanny *et al.,* 2001). In general, atopic patients are more susceptible to latex allergy than nonatopic individuals (Nettis *et al.,* 2003). It is quite possible that the prevalence of latex allergy in such risk groups outside the medical field has developed in a different way. Nevertheless, forefront research on latex allergy and the individual latex allergens has decreased and slowed down considerably.

There are population-dense countries around the world that are striving to attain the economical and technological status that is the norm in Europe and the USA. So the question arises whether the history of latex allergy will repeat itself. Recent data on the high prevalence of latex allergy in Taiwan (Chen and Lan, 2002), Turkey (Ozkan and Gokdogan, 2003), and Poland (Dudek *et al.,* 2003) show that the increased use of latex products in these fast developing countries may lead to the emergence of a new epidemic. Although the occurrence of latex allergy among health care workers and workers in the diverse branches of industry with frequent latex exposure is still not documented in the literature, China, India and Mexico can be expected to encounter increased incidences of latex allergy. Education and prevention measures will need to be installed, but this will only be possible with a time lag. Therefore, improved surveillance and testing in these countries will be necessary to evaluate any new emergence of latex allergy.

Although latex allergy represented a hot topic in allergy research for a rather long period of time, specific immunotherapy must still be considered as an experimental treatment (Pereira *et al.,* 2003; Sastre *et al.,* 2003). Accurate diagnosis of latex allergy, whenever possible in a component-resolved way, is of great importance for the development and application of any specific immunotherapy. At present, the skin prick test (SPT) is considered the most reliable test for the diagnosis of latex allergy (Ebo *et al.,* 1997; Palosuo *et al.,* 1998; Kim *et al.,* 1998). It is in this context that the current status of knowledge about the thirteen officially accepted latex allergens (www.allergen.org) and present available data on their frequency of recognition or reactivity in different study populations have been reviewed.

3.4.1 Hev b 1 (Rubber Elongation Factor)

Hev b 1, a small protein of 137 amino acid residues with a molecular mass of 14.6 kDa, was the first latex allergen characterized on the molecular level (Czuppon *et al.,* 1993). Alenius *et al.* (1996) reported a relatively low frequency of IgE antibodies to purified Hev b 1 with the exception of spina bifida (SB) patients. In IgE immunoblot studies, these authors observed that 67% of sera of SB patients displayed Hev b 1-specific IgE wheras none of the sera of latex-allergic individuals showed any reactivity. Using nHev b 1 in ELISA, a prevalence of 18% could be observed in latex-allergic individuals. Yeang et al. (1996) produced a recombinant Hev b 1 in *Escherichia coli* that did not bind IgE of 19 latex-allergic individuals in immunoblot studies. There is only one study that recognizes Hev b 1 not only as a major allergen for SB patients but also as an important latex allergen for HCWs. In this study, 81% of the SB patients and 50% of the HCWs showed IgE reactivity to Hev b 1 (Chen *et al.,* 1997). More recent studies performed with natural and recombinant Hev b 1 clearly demonstrated that Hev b 1 was one of the major allergens of *Hevea* latex for SB patients with prevalences ranging from 54 to 100%, but that it was less important for HCWs and other latex-allergic individuals with prevalences ranging from 13 to 32% (Kurup *et al.,* 2000; Raulf-Heimsoth *et al.,* 2002; Bernstein *et al.,* 2003).

3.4.2 Hev b 2 (β-1, 3-Glucanase)

The basic β-1, 3-glucanase isolated from *Hevea* latex was first described by Sundersan *et al*. (1996) as the Hev b 2 allergen which appeared as a doublet of 34 kDa in SDS-PAGE and was recognized by IgE antibodies of a latex-allergic patient. The cDNA of Hev b 2 codes for a protein of 374 amino acid residues with N-terminal and C-terminal extensions that are

cleaved off, resulting in a mature protein of 35.1 kDa with one putative N-glycosylation site (Chye and Cheung, 1995). Hev b 2 seems to be a major allergen of *Hevea* latex with prevalences ranging from 38 to 65% (Kurup *et al.,* 2000; Bernsetein *et al.,* 2003). Yagami's group. (Yagami *et al.,* 2002) described that the carbohydrate structures of Hev b 2 were the major IgE-binding epitopes. Based on these findings, it is not surprising that in one study performed with a recombinant Hev b 2 expressed in *E. coli* only 7% positive responses were detectable in SPTs (Yip *et al.,* 2000). Nor did Raulf-Heimsoth *et al*. (Raulf-Heimsoth *et al.,* 2003) observe any responses to their recombinant Hev b 2.

Wagner and colleaqnes (2004) recently observed on a molecular level that Hev b 2 cross-reacted with a homologous protein of bell pepper, indicating that Hev b 2 is one of the latex allergens involved in the latex-fruit syndrome.

3.4.3 Hev b 3

Hev b 3 is one of the latex allergens that is mainly associated with latex allergy in SB patients. A cDNA clone encoding a protein of 204 amino acid residues with a predicted molecular mass of 22.3 kDa has been described (Wagner *et al.,* 1999). Hev b 3 showed 47% sequence identity with Hev b 1 (Wagner *et al.,* 1999). The protein was produced as a recombinant protein in *E. coli* and a prevalence of 83% in SB patients was found in IgE immunoblot experiments (Wagner *et al.,* 1999). Cross-reactivity of Hev b 3 and Hev b1 was described as well (Wagner *et al.,* 1999). Natural and recombinant Hev b 3 proteins were used in several studies revealing similar prevalences. For SB patients, prevalences ranged from 77 to 100% and from 7 to 32% for HCWs and latex-allergic individuals (Kurup *et al.,* 2000; Raulf-Heimsoth *et al.,* 2002; Bernstein *et al.,* 2003; Yip *et al.,* 2000). Interestingly, prevalence data of Hev b 3 highly correlate with the data obtained for Hev b 1, indicating that these proteins contain very similar IgE epitopes.

3.4.4 Hev b 4 (Cyanogenic Glucosidase)

Hev b 4 comprises a triplet of IgE-binding proteins of molecular masses of 50-57 kDa. Sunderasan *et al*. (2002) have recently described the component with the highest molecular mass of this protein complex as a cyanogenic glucosidase. Studies with natural Hev b 4 have demonstrated that this protein was an important allergen for HCWs as well as SB patients. Kurup *et al*. (2000) performed ELISA and RAST studies with nHev b 4. The prevalences

obtained with RAST assays were lower than those obtained with ELISA. RAST data ranged from 30 to 46% for SB patients and from 23 to 61% for HCWa versus 77% for SB patients and 65% for HCWs with ELISA. Natural Hev b 4 was also used in SPTs with latex-allergic HCWs revealing a prevalence of 39% (Bernstein *et al.,* 2003).

3.4.5 Hev b 5

Hev b 5 was characterized as an IgE-binding protein by two research groups at the same time. Both groups described a cDNA encoding a protein consisting of 151 amino acid residues with a predicted molecular mass of 16 kDa (Akasawa *et al.,* 1996; Slater *et al.,* 1996). Slate *et al*. (1996) produced Hev b 5 as a recombinant protein with the maltose-binding protein as fusion component and performed RAST assays revealing a prevalence of 92% for HCWs and 56% for SB patients. Further studies were performed with recombinant Hev b 5 molecules. In CAP-FEIA tests, 68% of HCWs and 33% of SB patients showed reactivity to Hev b 5 (Raulf-Heimsoth *et al.,* 2002). In SPT studies prevalences of 62 and 65% were determined (Bernstein *et al.,* 2003; Yip *et al.,* 2000).

Hev b 5 is one of the most important allergens of Hevea latex and therefore indispensable for the diagnosis of latex allergy. Pharmacia Diagnostics has reported a lower sensitivity of their latex ImmunoCAP due to the lack of Hev b 5. Retesting of patients' sera with the ImmunoCAP preparation spiked with a recombinant Hev b 5 showed that some of the responses were stronger and some sera testing negative became positive (Lundberg *et al.,* 2001).

3.4.6 Hev b 6

Hev b 6.01 (prohevein) is a precursor protein consisting of 187 amino acid residues that contains two domains, a 4.7-kDa amino-terminal domain (Hev b 6.02 or hevein), and a 14-kDa carboxy-terminal domain (Hev b 6.03). The precursor protein and both domains are present as distinct proteins in *Hevea* latex. Alenius *et al*. (1995) identified prohevein as a major latex allergen for HCWs as well as SB patients. Banerjee *et al*. (1997) produced all three Hev b 6 molecules as recombinant proteins with the maltose-binding protein as fusion component and demonstrated, based on ELISA experiments, that most of the IgE-binding domains were located on Hev b 6.02 with additional IgE epitopes on Hev b 6.03. For SB patients, prevalences up to 50% were obtained indicationg that Hev b 6 was also an important allergen for those children. Ylitalo *et al*. (1998) tested nHev b 6.01 and nHev b 6.02 with sera of SB patients or other latex

allergic children with prevalences from 58 to 86%. Additional studies also revealed high prevalences for SB patients ranging from 30 to 69% (Kurup *et al.,* 2000; Raulf-Heimsoth *et al.,* 2002). From these data one might conclude that Hev b 6 is as important for SB patients as Hev b 1 or Hev b 3. For HCWs and other latex-allergic adults, Hev b 6 is the most important allergen besides Hev b 5 with prevalences ranging from 40 to 88% obtained in several studies (Kurup *et al.,* 2000; Raulf-Heimsoth *et al.,* 2002; Bernstein *et al.,* 2003; Yip *et al.,* 2000; Banerjee *et al.,* 1997).

Hev b 6.02 shows sequence identities of more than 50% to hevein domains of class I chitinases from fruits. This may explain why Hev b 6 seems to be the most important allergen involved in the latex-fruit syndrome. Cross-reactivity of Hev b 6.02 with various fruit class I chitinases such as those from avocado and banana has been described (Wagner and Breiteneder, 2002).

3.4.7 Hev b 7 (Patatin-Like Protein)

Beezhold *et al*. (1994) first described a 46-kDa protein from fresh natural rubber latex that was recognized by IgE of HCWs. Kostyal *et al*. (1998) and Sowka *et al*. (1998) have cloned and expressed Hev b 7 in both *E. coli* and *Pichia pastoris*. The two Hev b 7 proteins of 43 kDa can be described as two isoforms because they differ in their sequences by only seven amino acid residues. In IgE immunoblot studies, Sowka *et al*. (1998) reported a prevalence of 11% for their recombinant protein. Sepplal *et al*. (2000) observed that 49% of latex-allergic individuals and less than 1% of latex-allergic children displayed IgE to purified Hev b 7. They concluded from their results that Hev b 7 is not an important latex allergen for children. In following studies using sera of SB patients it could be demonstrated that Hev b 7 is an important allergen for SB patients, with prevalences ranging from 15 to 77% (Kurup *et al.,* 2000; Wagner *et al.,* 2001). Recent studies have shown that for HCWs and other latex-allergic individuals Hev b 7 seems to be an important allergen, with prevalences ranging from 23 to 45% (Kurup *et al.,* 2000; Bernstein *et al.,* 2003; Yip *et al.,* 2000).

Hev b 7 shows about 50% sequence identity with the important potato allergen patatin (Sola t 1), indicating a potential cross-reactivity to this plant-derived food. Cross-reactivity between Hev b 7 and potato patatin was demonstrated with latex-and potato-allergic individuals

(Schmidt *et al*., 2002). Furthermore, proteins of 44-46 kDa in tomato, potato and *Hevea* latex, possibly patatin and patatin-like proteins, showed cross-reactivity (Reche *et al.,* 20001).

3.4.8 Hev b 8 (Profilin)

Plant profilins are important pan-allergens but there is an ongoing discussion about the clinical relevance of IgE binding to profilins that has to be distinguished from crossreactive IgE binding without evoking allergic symptoms. Hev b 8 was first identified as an IgE-binding protein by IgE inhibition studies (Vallier *et al.,* 1995). Two Hev b 8 isoforms were then described and produced as recombinant proteins in *E. coli* (Rihs *et al.,* 2000; Ganglberger *et al.,* 2001). One cDNA was derived from Hevea leaf mRNA that encoded a protein of 131 amino acid residues with a predicted molecular mass of 14.2 kDa (Rihs *et al.,* 2000) and another from Hevea latex also encoding a protein of 131 amino acid residues but a predicted molecular mass of 14 kDa (Ganglberger *et al.,* 2001). Both recombinant Hev b 8 isoforms revealaed similar IgE reactivity. The 'leaf' Hev b 8 was recognized by sera of 20% of HCWs and 12% of SB patients in CAP assays (Rihs *et al.,* 2000), the 'latex' Hev b 8 by sera of 24% of HCWs and 6% of SB patients in Western blot analyses (Ganglberger *et al.,* 2001). In a third study, a recombinant Hev b 8 with the maltosebinding protein as fusion component was used for SPT studies yielding a prevalence of 3% (Yip *et al.,* 2000).

Hev b 8 seems to be involved in the latex-fruit syndrome as cross-reactivity could be demonstrated to profilins from celery tuber (Ganglberger *et al.,* 2001), banana and pineapple (Reindl *et al.,* 2002), and bell pepper (Wagner *et al.,* 2004).

3.4.9 Hev b 9 (Enolase)

Hev b 9 was first identified as an IgE-binding protein of *Hevea* latex by microsequencing of protein spots after two-dimensional electrophoresis and Western blot with latex-allergic patients' sera (Posch *et al.,* 1997). The complete cDNA of this protein encoding an enolase of 445 amino acid residues with a calculated molecular mass of 47.6 kDa was described (Wagner *et al.,* 2000). The cDNA showed sequence identities of about 60% to Alt a 11 and Cla h 6, important allergens of the molds *Alternaria alternate* and *Cladosporium herbarum*. Hev b 9 was produced as a recombinant protein in *E. coli* and showed a prevalence of 14.5% in Western blot analyses using sera of 110 latex-allergic subjects (Wagner *et al.,* 2000). Furthermore, it could be demonstrated in the same study that Hev b 9 shared IgE epitopes with Alt a 11 and Cla h 6,

indicating a possible cross-reactivity. Hev b 9 was also produced as a recombinant protein in *E. coli* with the maltose-binding protein as fusion component (Raulf-Heimsoth *et al.,* 2002). Coupled to ImmunoCAPs , sera of SB patients and HCWs were investigated, resulting in no response for the SB patients and 10% positive responses for the HCWs (Raulf-Heimsoth *et al.,* 2002).

3.4.10 Hev b 10 (Manganese Superoxide Dismutase)

Hev b 10 was first identified as an IgE-binding protein of *Hevea* latex in the same study as Hev b 9 (Posch *et al.,* 1997). The cDNA encoding a protein of 206 amino acid residues with a predicted molecular mass of 22.9 kDa was then described and showed 49% identity with Asp f 6, the MnSOD from *Aspergillus fumigatus* (Wagner *et al.,* 2001). A recombinant Hev b 10 produced in *E. coli* showed a prevalence of 27% when used in Western blot analysis with sera of latex-allergic subjects. In addition, cross-reactivity to Asp f 6 was described (Wagner *et al.,* 2001). Hev b 10 was also produced as a recombinant protein in *E. coli* with the maltose-binding protein as fusion component (Rihs *et al.,* 2001). This protein was used in EASTs with sera of SB patients and HCWs and showed a prevalence of 10% for SB patients and of 0% for HCWs. The same protein was also used in CAP-FEIA experiments using sera of SB patients and HCWs. In this study, the prevalence for the SB patients was 0% and 3% for the HCWs (Raulf-Heimsoth *et al.,* 2002).

3.4.11 Hev b 11 (Class I Chitinase)

Fruit class I chitinases containing a small N-terminal hevein domain are described as the most important panallergens associated with the latex-fruit syndrome (Wagner and Breiteneder, 2002). Two cDNAs of *Hevea* latex class I chitinases have been described, one derived from *Hevea* latex coding for a protein of 295 amino acid residues and a molecular mass of 33 kDa (O'Riordain *et al.,* 2002) and another derived from *Hevea* leaves (Hev b 11.0102) also encoding a protein of 295 amino acid residues but with a predicted molecular mass of 31.6 kDa (Rihs *et al.,* 2003). Comparison of the amino acid residues revealed differences in eight positions. In sequence analysis, the latex sap Hev b 11 showed 70% identity with Pers a 1, the class I chitinase from avocado, and 58% identity of its chitin-binding domain with havein (Hev b 6.02) (O'Riordain *et al.,* 2002). The chitin-binding domain of the 'leaf' Hev b 11 showed 56% sequence identity with Hev b 6.02 (Rihs *et al.,* 2003). Both proteins were produced as

recombinant proteins in *E. coli* with the maltose-binding protein as fusion component (O'Riordain *et al.,* 2002; Rihs *et al.,* 2003). In Western blot analyses the 'latex' Hev b 11 showed a prevalence of 19% (O'Riordain *et al.,* 2002), whereas the 'leaf' Hev b 11 showed a prevalence of 29% in CAP assays (Rihs *et al.,* 2003). O'Riordain *et al*. (2002) could demonstrate that the 'latex' Hev b 11 was weakly cross-reactive with Hev b 6.02. but there are no data about a possible cross-reactivity with fruit class I chitinases.

3.4.12 Hev b 12 (Lipid Transfer Protein)

Lipid transfer proteins are important cross-reactive allergens, especially in plant-derived foods. Recently, Beezhold *et al*. (2003) described the cDNA of a lipid transfer protein of *Hevea* latex encoding a protein of 116 amino acid residues with a calculated molecular mass of 9.3 kDa. The same research group produced Hev b 12 as a recombinant protein in *E. coli* with glutathione S-transferase as fusion component. In Western blot analysis, 24% of latex-allergic individuals displayed Hev b 12-specific IgE. There are no data about potential cross-reactivity.

3.4.13 Hev b 13 (Early Nodule-Specific Protein)

Recently, Arif *et al*. (2004) have characterized an early nodule-specific protein (ENSP) as the main IgE-binding protein in the 42- to 46-kDa range of *Hevea* latex allergens. The cDNA of Hev b 13 contained two possible open reading frames. The cDNA thus coded for two possible proteins, one consisting of 391 amino acid residues and a calculated molecular mass of 43.3 kDa, and the second consisting of 372 amino acid residues and a calculated molecular mass of 41.2 kDa. With the longer open reading frame, a signal peptide was predicted resulting in a mature protein of 40.4 kDa. The molecular mass of the native Hev b 13 was 42.9 kDa as described by mass spectrometry (Arif *et al.,* 2004). The authors speculated that the discrepancy between these molecular masses was due to glycosylation as the Hev b 13 sequence contained three putative N-glycosylation sites. Hev b 13 seems to be a very important allergen for SB patients as well as HCWs. In one study, EAST experiments were performed, with nHev b 13 yielding prevalences of 75% in SB patients and 87% for HCWs (Raulf-Heimsoth *et al.,* 2003). In another study using nHev b 13 in SPTs, a prevalence of 63% was found in HCWs (Bernstein *et al.,* 2003). To date, no data are available on the influence of possible N-glycans on IgE binding.

Table 2. Panel of latex allergens and frequency of reactivity of SB patients and other latex-allergic children (Wagner, 2005).

Allergen	Patients	Source	Method	Frequency %
Hev b 1	6 SB	n	IB	67
	62 SB	n	EAST	81
	58 SB	r	CAP	93
	13 SB	n	ELISA	100
			RAST	54-85
	9SB	r	CAP	100
Hev b 2	13 SB	n	ELISA	54
			RAST	38-46
Hev b 3	35 SB	r	IB	83
	13 SB	n	EAST	100
			RAST	77-85
	9 SB	r	CAP	78
Hev b 4	13 SB	n	EAST	77
			RAST	30-46
Hev b 5	57 SB	r	RAST	56
	9 SB	r	CAP	33
Hev b 6	25 SB	r(6.01)	ELISA	48
	25 SB	r(6.02)	ELISA	56
	25 SB	r(6.03)	ELISA	28
	30 LAC	n(6.01)	ELISA	86
	12 SB	n(6.01)	ELISA	58
	30 LAC	n(6.02)	ELISA	63
	12 SB	n(6.02)	ELISA	58
	13 SB	r(6.01)	ELISA	69
			RAST	30-38
	9 SB	r(6.01)	CAP	44

Allergen	Patients	Source	Method	Frequency %
Hev b 7	35 LAC	n	ELISA	<1
	13 SB	r	ELISA	77
			RAST	15-23
	38 SB	n	CAP	40
Hev b 8	17 SB	r	CAP	12
	34 SB	r	IB	6
	9 SB	r	CAP	0
Hev b 9	9 SB	r	CAP	0
Hev b 10	20 SB	r	EAST	10
	9 SB	r	CAP	0
Hev b 11	no data			
Hev b 12	no data			
Hev b 13	11 SB	n	EAST	75

SB = Spina bifida

LAC = latex-allergic children

n = natural

r = recombinant

IB = IgE immunoblot

Table 3. Panel of latex allergens and frequency of reactivity of HCWs and other latex-allergic individuals (Wagner, 2005).

Allergen	Patients	Source	Method	Frequency %
Hev b 1	30 LAI	n	IB	<1
	45 LAI	n	ELISA	18
	103 HCW	n	EAST	50
	71 HCW	r	CAP	23
	31 HCW	n	ELISA	32
			RAST	13-19
	40 HCW	r	CAP	23
	62 HCW		SPT	23
Hev b 2	31 HCW	n	ELISA	65
			RAST	48-61
	29 LAI	r	SPT	7
	62 HCW	n	SPT	63
Hev b 3	15 LAI	r	IB	27
	31 HCW	n	ELISA	32
			RAST	19
	29 LAI	r	SPT	7
	40 HCW	r	CAP	12.5
	62 HCW	n	SPT	24
Hev b 4	31 HCW	n	ELISA	65
			RAST	23-61
	62 HCW	n	SPT	39
Hev b 5	13 HCW	r	RAST	92
	29 LAI	r	SPT	62
	40 HCW	r	CAP	68
	62 HCW	r	SPT	65

Allergen	Patients	Source	Method	Frequency %
Hev b 6	25 HCW	R(6.01)	ELISA	84
	25 HCW	r(6.02)	ELISA	88
	25 HCW	r(6.03)	ELISA	40
	31 HCW	r(6.01)	ELISA	55
			RASY	45
	29 LAI	r(6.01)	SPT	66
	40 HCW	r(6.01)	CAP	75
	62HCW	n(6.01)	SPT	63
Hev b 7	36 LAI	r	IB	11
	35 LAI	n	ELISA	49
	31 HCW	r	ELISA	42
			RAST	23-45
	29 LAI	r	SPT	41
	62 HCW	n(7.01)	SPT	45
Hev b 8	29 LAI	r	SPT	3
	25 HCW	r	CAP	20
	50 HCW	r	IB	24
	40 HCW	r	CAP	10
Hev b 9	110 LAI	r	IB	15
	40 HCW	r	CAP	10
Hev b 10	15 LAI	r	IB	27
	20 HCW	r	EAST	0
	40 HCW	r	CAP	3
Hev b 11	57 LAI	r	IB	19
	58 LAI	r(11.0102)	CAP	29
Hev b 12	37 LAI	r	IB	24
Hev b 13	53 HCW	n	EAST	87
	62 HCW	n	SPT	63

HCW = Health care worker

LAI = latex-allergic individual

n = natural

r = recombinant

IB = IgE immunoblot

This review of the available knowledge on 13 *Hevea* latex allergens (summarized in tables 2, 3) clearly shows that there are opportunities for further research. Two-dimensional gel electrophoresis of *Hevea* proteins followed by IgE immunblotting has revealed the existence of further allergens (Posch *et al.,* 1997; Posch *et al.,* 1997; Yagami *et al.,* 2004). Progress in diagnosis using recombinant allergens may only be limited by the ability to express these allergens for use in high throughput diagnostic screening assays.

On the other hand, a change of paradigm may favor the use of partially enriched or purified natural allergens. No reliable recombinant proteins are available for Hev b 2, 4 and 13. In addition, the role and importance of glycosylaion for allergenicity remains an unanswered question for Hev b 2 and 13. Therefore, a recent SPT study of latex-allergic HCWs relied on the use of purified natural *Hevea* latex allergens (Bernstein *et al.,* 2003).

The performance and reproducibility of diagnostic assays are highly dependent on the allergen content of the reagents used. Low diagnostic sensitivity was related to poorly represented and/or denatured allergens such as Hev b 5 (Chen *et al.,* 2000). Although the quality of latex extracts has improved in recent years, there is still room for further developments (Hamilton *et al*., 2002). In the future, the reagents of choice may well consist of a combination of recombinant and natural allergens.

In the next few years, it is possible that large new markets for latex allergy diagnostics and specific immunotherapy will open up. It is reasonable to assume that this will provide a new incentive for basic as well as applied latex allergy research.

Objective

The objectives of this study are:

1. To isolate the hydrophobic proteins within the bottom fraction membrane (BFM) from fresh latex *Hevea brasiliensis*.
2. To study the allergenicity of BFM proteins by focusing on specific binding sites of IgE and IgG in sera.
3. To modify the co-immunoprecipitation for detection of latex allergen.
4. To identify a hydrophobic protein within the BFM that possibly causes latex allergy determining the size by co-immunoprecipitation.

Chapter 2

Materials and Merhods

Materials

Chemicals and equipments used in this study are shown in Appendix A, B

Methods

Serum samples

Serum samples were obtained from 170 latex glove factory workers (LGWs) residing in Songkhla province, Thailand; 35 health care workers (HCWs) samples were generously provided by Dr. Porntip Puvabunditsin, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; 31 CAP-positive (CAP^+) and 22 CAP-negative (CAP^-) samples were generously provided by Dr. Robert G. Hamilton, Johns Hopkins University School of Medicine, Baltimore, USA. The medical ethics committees of Songklanakarin Hospital, King Chulalongkorn Memorial Hospital and Johns Hopkins Hospital approved the study protocol, and subjects gave written consent for their participation in the study.

Allergic skin test

A Skin prick test was performed using the bottom fraction membrane proteins preparation as described below. A 50% glycerol in saline solution (diluent) served as a negative control, whereas the positive control was histamine hydrochloride (1 mg/ml in diluent). The Skin test response was assessed as positive if the maximum wheal diameter was at least 3 mm greater than observed for the negative control, and the skin index was greater than 0.6. The skin index was calculated as the ratio of the diameter of the allergen wheal to the histamine wheal.

ImmunoCAP

Latex ImmunoCAP (Pharmacia Biotech) was used to test for latex specific IgE in the sera of patients and controls according to the instruction of the manufacturer. The protocol of the manufacturer was followed, and a value of 0.35 U/ml or more was considered positive.

Statistics

Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). Probability (P) values (paired t-tests) less than 0.05 were considered significant.

Collection of latex from rubber trees

Fresh *Hevea* latex used throughout this study was obtained from regularly tapped trees clone RRIM 600, which was grown at Songkhla Rubber Research Center, Songkhla, Thailand. The trees were tapped starting at 06.00 hours, and the exuded latex was collected in beakers and chilled on ice. Latex was collected for sixty minutes at each tapping.

Preparation of BFM proteins

The chilled latex was filtered through 4 layers of cheesecloth to remove the particulate materials and bark tissue debris. The filtrate was collected and centrifuged at 59,000 × g for 45 minutes at 4°C in an ultracentrifuge (Beckman, Model: L 8-70M). After centrifugation, the latex was separated into three distinct layers with the rubber cream and Frey-Wyssling complexes on the top, the bottom fraction at the bottom and C-serum in between. The bottom fraction was isolated and washed three times by suspending in 5 volumes of Tris-buffered saline, (TBS: 50 mM Tris-HCl, pH 7.4, containing 0.9% NaCl), stirred for 30 minutes at 4°C and recovered by centrifugation at 30,000 × g for 30 minutes. It was then subjected to bursting by alternative freezing and thawing (4 times) and the bottom fraction membrane (BFM) pellet was separated and washed 3 times with TBS. The washed BFM was suspended in 10 volumes of extracting buffer (50 mM Tris-HCl, pH 7.4, containing 0.2% Triton X-100) and extracted by stirring overnight at 4°C. The supernatant containing extracted BFM proteins was separated after centrifugation at 10,000 × g for 25 minutes and incubated with SM2 absorbent (1:10 w/v) for 15 minutes to remove residual Triton X-100.

Verification of IgE and IgG in sera by indirect enzyme-linked immunosorbent assay (ELISA)

To analyze the response of IgE in serum samples, each well of a 96-well immunomaxi high binding plate (TTP, Stafa-Zurich, Switzerland) was coated with 50 µl of 10 µg/ml of BFM proteins at 4°C for 18 hours. Unbound antigens were removed from the plate by washing five times with washing buffer (0.05% tween 20/PBS, pH 7.2). The coated plate was filled with 200 µl blocking solution (2% skim milk/PBS, pH 7.2) and incubated at room temperature for 2 hours to block unoccupied sites on the plate. After washing five times with

washing buffer, 50 µl of the sera (diluted 1:5 in 2% skim milk/PBS) were added to each well, and incubated at room temperature for 1 hour. Then, the wells were washed and further incubated with 50 µl of horseradish peroxidase (HRP) conjugated goat anti-human immunoglobulin E (IgE)(Sigma Chemical Co., St. Louis, MO). After incubation for 1 hour at room temperature, all wells were washed as above, 100 µl of the substrate 3, 3', 5, 5'- tetramethylbenzidine (TMB), (KPL, Inc., Gaithersburg, ML) were added and incubated in the dark at room temperature for the optimal period. The enzymatic reaction was stopped with 100 µl of 1N HCl and the optical density (OD) was measured at a wavelength of 450 nm using a microtiter plate reader.

A similar procedure for detecting IgG was applied as described earlier. The dilution of individual serum used was 1:1,000 in 2% skim milk/PBS. The HRP conjugated mouse monoclonal anti-human IgG (generous provided by Dr. Robert G. Hamilton) was added to monitor the immune-complex captured in the ELISA well.

Biotinylation of BFM proteins and co-immunoprecipitation

BFM proteins were biotinylated with 10 mM Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL) at 4°C for 2 hours. The biotinylation was quenched on ice for 2 hours. The binding/wash buffer (modified PBS buffer pH 7.4 (0.14 M NaCl, 8 mM sodium phosphate, 2 mM potassium phosphate and 0.01 M KCl)) was exchanged to remove excess biotin reagent using a desalting column with binding/wash buffer five times in a microcentrifuge at 4,000 × g for 1 min. Co-immunoprecipitation was performed using Seize X Immunoprecipitation Kit (Pierce Biotechnology). The Seize X Protein A gel was conjugated with mAb (IgG_{2a} subclass) to human Fcε (Biodesign International, Saco, ME) using disuccinimidyl suberate (DSS) cross-linker and incubated at room temperature for 1 hour. After washing five times with washing buffer, 350 µl of serum sample were added to mAb to human Fcε conjugated protein A bead, and washed with binding/wash buffer five times in a microcentrifuge at 4,000 × g for 1 min. Subsequently, the labeled BFM proteins were added to the washed beads and rotated for 1 hour at room temperature. After incubation, mixtures were washed five times and biotinylated BFM proteins were dissociated from protein A beads by elution buffer (pH 2.8). The immuno-precipitated proteins were resolved by 15% SDS-PAGE under reducing conditions and subsequently transferred to a PVDF membrane. Occupation of the free membrane surface was

performed in 5% skim milk/PBS at 4°C, overnight. The blocked membrane was incubated for 1 hour at room temperature with HRP-conjugated streptavidin. The biotinylated proteins were visualized by chemiluminescence with the ECL western blotting analysis system (Amersham Health, Buckinghamshire, UK), then exposed to Kodak X-ray film.

Indirect ELISA for validation of biotinylated BFM proteins

To validate IgE in sera by indirect ELISA, each well of a 96-well polystyrene microtiter plate (Corning, Inc.) was coated with 50 µl of 100 µg/ml egg white avidin (Sigma Chemical Co.) at 4°C for 18 hours. Unbound avidin were removed from the plate by washing five times with washing buffer (0.05% Tween 20/PBS, pH 7.2). The coated plate was filled with 200 µl blocking solution (2% skim milk/PBS, pH 7.2) and incubated at room temperature for 2 hours to block unoccupied sites on the plate. After washing five times with washing buffer, 50 µl of the biotinylated BFM proteins (diluted 1 µg/ml, 10 µg/ml) were added to each well, and the plates were incubated at room temperature for 1 hour. After washing five times with washing buffer, 50 µl of the sera (diluted 1:5 in 2% skim milk/PBS) were added to each well, and incubated at room temperature for 1 hour. The wells were washed and further incubated with 50 µl of HRP conjugated goat anti-human IgE (Sigma Chemical Co.). After incubation for 1 hour at room temperature, all wells were washed as above and 100 µl of the TMB, (KPL, Inc.) were added. The plates were incubated in the dark at room temperature for the optimal period. The enzymatic reaction was stopped with 100 µl of 1N HCl and the OD was measured at a wavelength of 450 nm using a microtiter plate reader.

Competitive ELISA for immune-complex detection

Competitive ELISA was carried out in order to demonstrate the occurrence of IgE specifically interacting with the biotinylated BFM proteins in the eluted fraction collected from the co-immunoprecipitation process. The eluted fraction was preincubated with 1.2 µg of unlabelled BFM proteins at 37°C for 1 hour. The ELISA well was precoated with 50 µl of 100 µg/ml egg white avidin (Sigma Chemical Co.). Unbound egg white avidin was removed from the plate by washing five times. The well was filled with 200 µl blocking solution (2% BSA/PBS, pH 7.2) and incubated at room temperature for 2 hours. After extensive washing, 50 µl of the reaction

mixture were applied into the egg white avidin-coated well and incubated at room temperature for 1 hour. Then, the wells were washed and 50 µl of HRP conjugated goat anti-human IgE (Sigma Chemical Co.) were added. After incubation for 1 hour at room temperature, all wells were washed as above and 100 µl of the chromogenic substrate TMB (KPL, Inc.) were added. The plates were incubated in the dark at room temperature for the optimal period. The enzymatic reaction was stopped with 100 µl of 1N HCl and the OD was measured at a wavelength of 450 nm using a microtiter plate reader. The competitive result was determined in comparison with the control well in which the eluted fraction was not preincubated with the unlabelled BFM proteins. The % inhibition value of sample was calculated with the formula.

$$\% \text{ inhibition} = ([\text{OD of no inhibitor} - \text{OD of inhibitor}] \times 100 / \text{OD of no inhibitor})$$

Chapter 3

Results

Verification of IgE in sera

Four sample groups were used to evaluate the level of specific IgE (Fig. 10). A total of 170 sera from LGWs were assayed by indirect ELISA. The mean value of specific IgE was 0.10 OD. Only 38 individuals had a specific IgE level above the mean value. The mean value obtained from CAP^+, 31 subjects, was equal to that of the LGWs group. Nine CAP^+ samples had a specific IgE level above the mean value. Whereas 11 out of 22 CAP^- samples exhibited specific IgE level higher than mean (0.02 OD). Seven sera from the HCWs group (n=35) had an OD greater than the mean (0.04 OD). The cut-off value was arbitrarily defined as the mean +2 S.D. of CAP^- group (0.02 + 0.04). Regarding the decision line of 0.06 there were 56, 11, 1 and 5 samples in LGWs, CAP^+, CAP^-, and HCWs classified as positive for IgE against BFM proteins. The three highest levels of specific IgE in LGWs group from sera no. 4, 248, and 253 were 2.71, 1.40, and 0.78 OD respectively. Considering the difference in the means between LGWs and the others, there were no significant differences (CAP^+, CAP^-, and HCWs; P=0.50, 0.13, and 0.19 respectively, by paired t-tests).

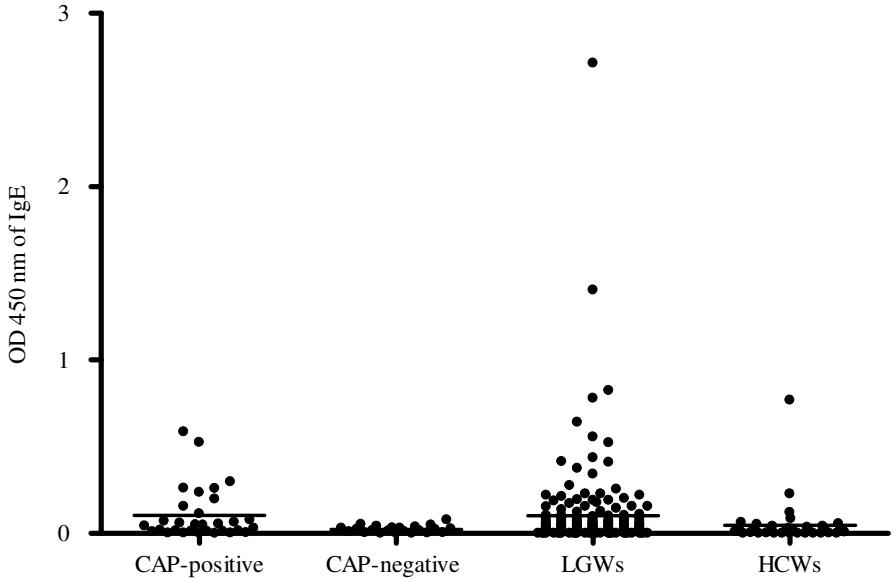

Fig. 10 Relative comparison of specific IgE levels from 31 CAP^{+}, 22 CAP^{-}, 170 LGWs and 35 HCWs samples by indirect ELISA. The serum samples were incubated with BFM proteins coated wells. Bound specific IgE was detected by HRP conjugated goat anti-human IgE. Each data point represents the specific IgE level in relation to the absorbance value at OD 450 nm. Solid horizontal lines represent the means of individual categories. These data are representative of three independent experiments.

Verification of IgG in sera

In addition to the level of specific IgE, the level of specific IgG were determined in individual groups expounded above Figure 11 show plot of the analyzed mean values of LGWs, CAP^{+}, CAP^{-}, and HCWs were 0.20, 0.09, 0.02, and 0.03 OD accordingly. Samples in each group that had an OD higher than the referred mean values were 50, 9, 7, and 15. The cut-off value was calculated from the mean and S.D. obtained from the CAP^{-} group as described earlier. According to the cut-off value, 0.02 + 0.07=0.09, there were 86, 9, 1, and 3 subjects defined as positive for IgG against BFM proteins. Comparison of CAP^{+}, CAP^{-}, and HCWs means against LGWs mean showed significant differences with the latter a groups (P=0.07, P=0.02*, and P=0.01*, by paired t-tests).

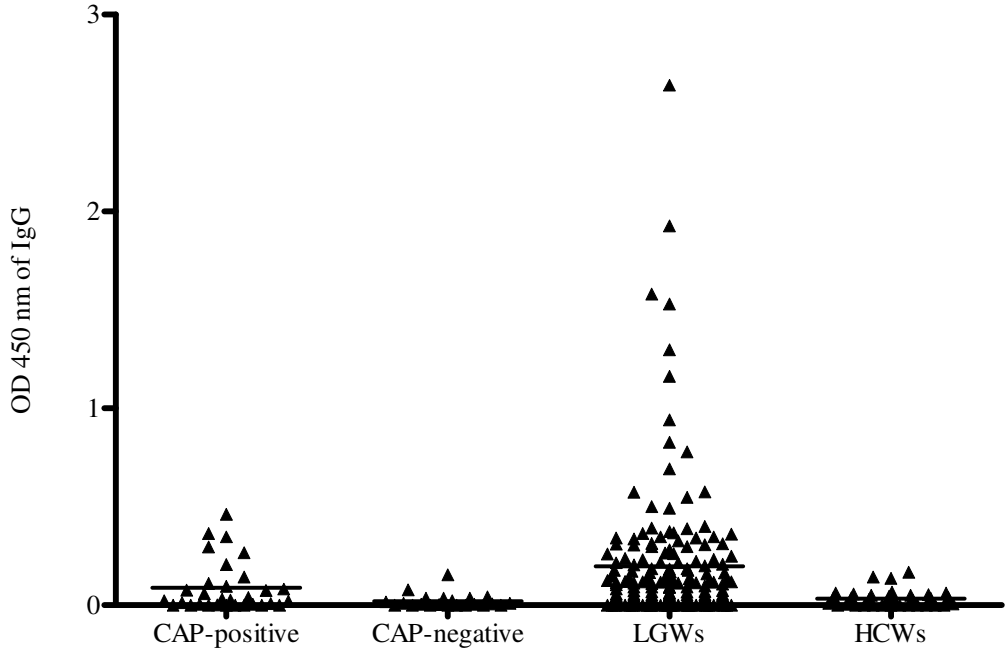

Fig. 11 The IgG level from 31 CAP^{+}, 22 CAP^{-}, 170 LGWs and 35 HCWs samples was assayed by indirect ELISA. Wells were coated with BFM proteins and reacted with serum samples. Bound specific IgG was detected by HRP conjugated mouse monoclonal anti-human IgG. The absorbance value at OD 450 nm is used to compare the level of specific IgG in each sample group. Solid horizontal lines represent the means of individual categories. These data are the representative of three independent experiments.

Correlation of specific IgE and IgG

To compare the levels of IgE and IgG, the OD of each sample were plotted in scatter format (Fig. 12). The sera from samples 4, 248, and 253 had the highest levels of specific IgE with the OD of specific IgG of 2.64, 1.16, and 1.53 respectively (Table 5). These LGWs subjects were selected for further validation of clinical symptoms and for the skin prick test. Clinical symptoms i.e. asthma, eczema, and conjunctivitis suggested that only subject no. 4 was allergic. Skin prick tests were negative for all subjects.

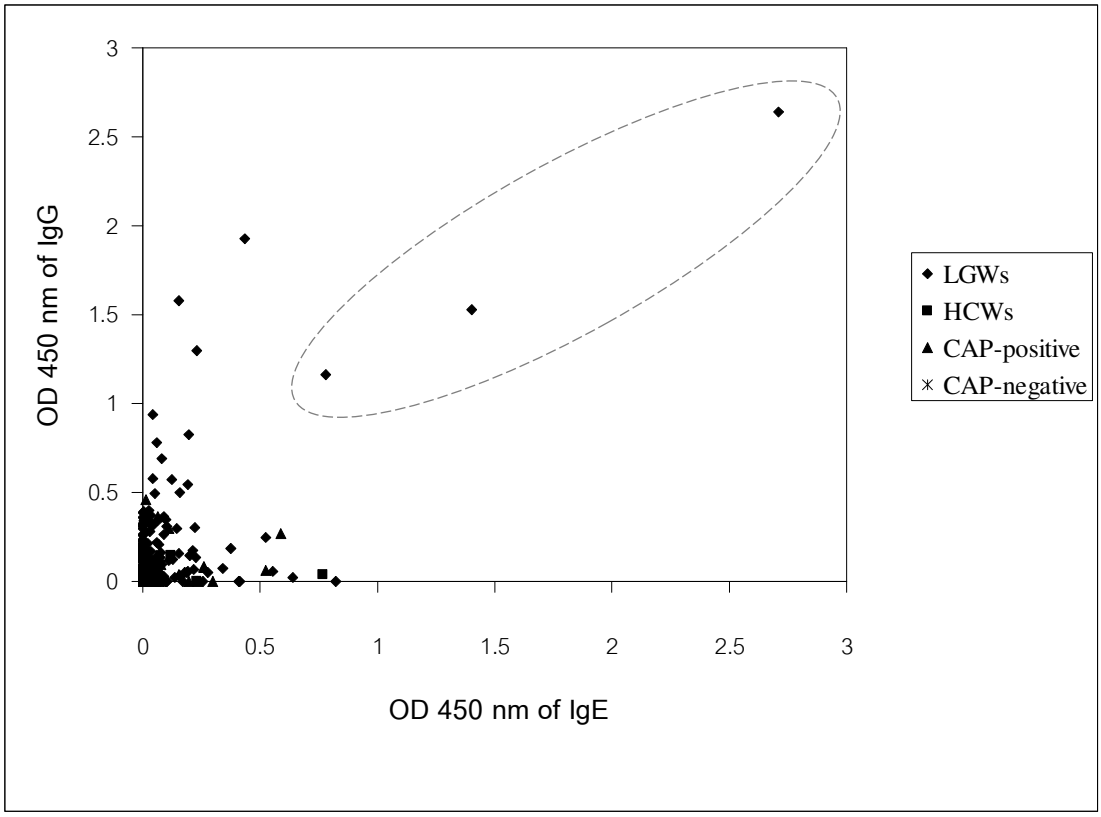

Fig. 12 Correlation of specific IgE and IgG levels obtained from indirect ELISA (Fig. 10 and 11, respectively). Each data point represents an individual sample from 31 CAP^{+}, 22 CAP^{-}, 170 LGW and 35 HCW groups. LGWs no. 4, 248, 253 were shown in the ellipse.

Table 4. Characteristics of three selected samples from LGW group demonstrating highest level of specific IgE and IgG against BFM proteins. Clinical symptoms and results of skin prick test are included.

Latex glove factory workers (no.)	Sex	Age (years)	Working period (months)	Symptom	Skin prick test	IgE ELISA (450 nm)	IgG ELISA (450 nm)
4	female	51	22	asthma, eczema, conjunctivitis	negative	2.71	2.64
248	female	29	24	no	negative	0.78	1.16
253	female	34	120	no	negative	1.40	1.53

The efficiency of biotinylation process

The quality of labeled BFM proteins was analyzed using the avidin capturing system (Fig. 13). The biotinylated BFM proteins at 10 μg/ml showed positive immunoreactivity with serum from LGW no. 4 when HRP conjugated goat anti-human IgE was used as a detector (OD 450 nm = 1.95). This specific formation of the immune-complex was demonstrated by dilution effect when 1 μg/ml biotinylated BFM proteins were introduced (OD 450 nm = 0.39).

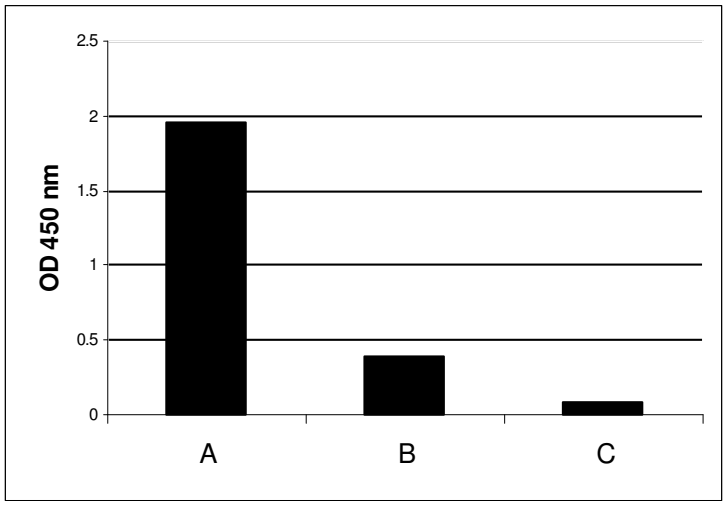

Fig. 13 The efficiency of the biotinylation process was validated using avidin capturing ELISA. Egg white avidin-coated wells were exposed with 10 µg/ml (A), 1 µg/ml (B) and 0 µg/ml (C) of the biotinylated BFM proteins. The serum sample from LGW no. 4 was applied to each individual well. Following the addition of HRP conjugated anti-IgE, the TMB substrate was applied to monitor the immunoreaction at OD 450 nm. These data are the representative of three consecutive experiments.

SDS-PAGE and co-immunoprecipitation

The separated BFM proteins by 15% SDS-PAGE under reducing condition contained two major bands at 30 and 35 kDa by Comassie Brilliant Blue 250 (Fig. 14B). The rest were in the range 10 to 70 kDa. The segregated polypeptides were transferred to a PVDF membrane and probed with serum no. 4, which had the strongest specific IgE reactivity against BFM proteins in indirect ELISA. No reactive band could be observed by Western immunoblotting (data not shown). Co-immunoprecipitation was subsequently performed to the specific IgE anti-BFM components in native conformation. Serum no. 4 was blended with Protein A gel covalently linked with mAb (IgG_{2a}) anti- human IgE. After extensive washes, the biotinylated BFM proteins were mixed with IgE-trapped gel. The precipitated fraction was eluted from the gel and further applied to SDS-PAGE under reducing conditions. The proteins were blotted to a PVDF membrane and probed with avidin-HRP. A 55 kDa band was observed using the ECL chemiluminescence visualization system (Fig. 14A). No binding activity was found in CAP^{+} and CAP^{-} samples with negative OD for specific IgE.

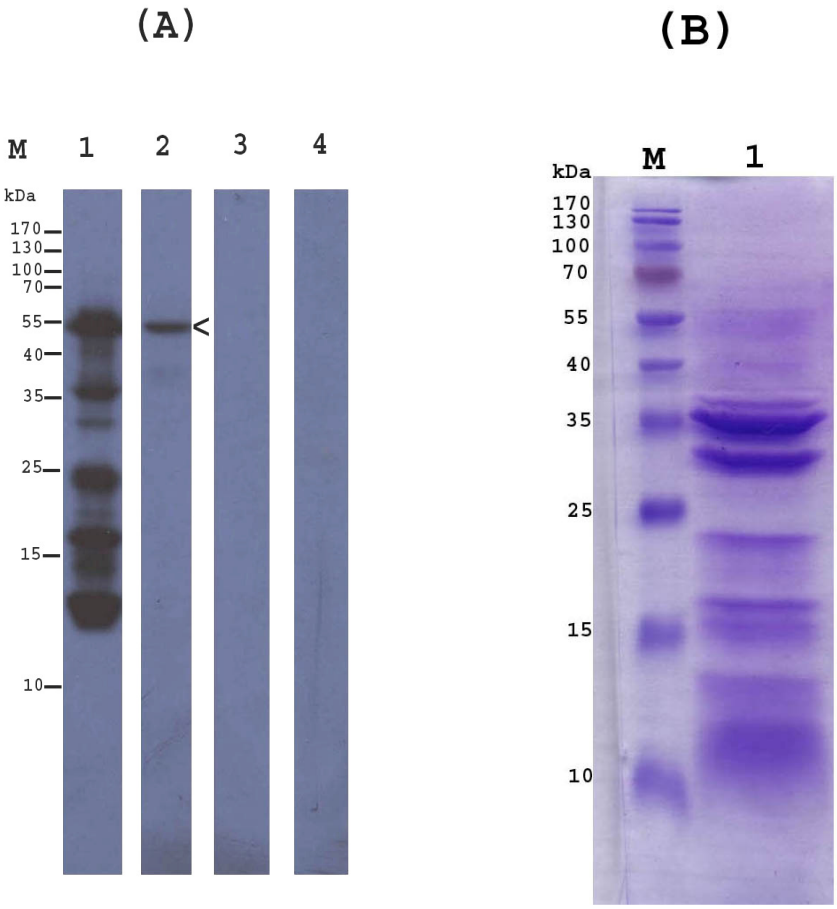

Fig. 14 Identification of immunoreactive allergen by co-immunoprecipitation assay (A). Serum sample was separately incubated with Protein A gel cross-linked with mouse monoclonal anti-human IgE. The captured IgE were further reacted with biotinylated BFM proteins. Subsequently, the specifically bound components were separated from solid matrix using pH 2.8 elution. The collected fraction was separated under denaturing condition by SDS-PAGE, electroblotted to PVDF membrane and probed with streptavidin conjugated HRP. The reactive bands were visualized by chemiluminescent substrate system after exposing with X-ray film. Protein mixtures of biotinylated BFM proteins are shown in lane 1. Fractions collected from incubation with LGW sample no. 4, CAP^{+} sample no. P1396 and CAP^{-} sample no. P2136 are shown in lanes 2, 3, and 4 respectively. Whole protein extract is shown by Coomassie Blue R250 staining in polyacrylamide gel under denaturing condition (B). BFM proteins were shown in lane 1.

The competitive ELISA for detecting immune-complex

To confirm the presence of IgE anti-BFM protein complex in the purified eluate from the co-immunoprecipitation process, avidin-biotin capture inhibition ELISA was performed (Fig. 15). After neutralizing the eluate with 2% BSA/PBS pH 7.4 to promote the rebinding of immune-complexes, the mixture was added into the ELISA well coated with egg-white avidin. The captured complex containing IgE specifically unite to biotinylated BFM proteins were observed using HRP conjugated goat anti-human IgE. In parallel, the specific binding of IgE antibodies was verified by mixing the neutralized eluate with unlabelled BFM proteins. The OD diminished 26.89% compared to the non-inhibition well, demonstrating the competition between biotinylated and unlabelled BFM proteins for specific IgE.

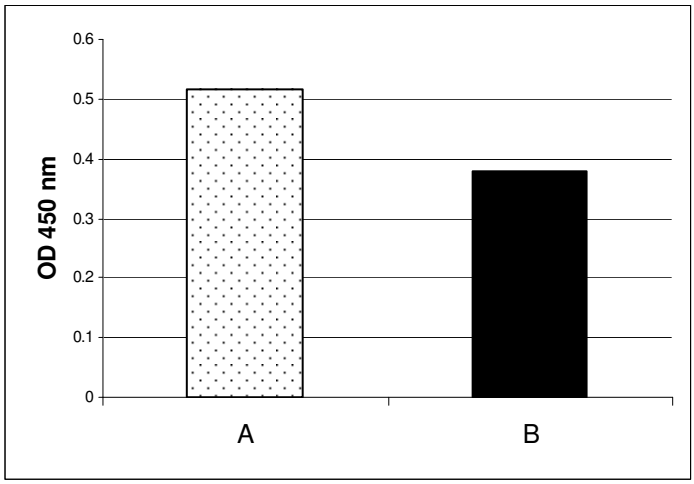

Fig. 15 The competitive ELISA for detecting immune-complex in the eluted fraction of co-immunoprecipitation experiment. The eluted fraction was added to egg white avidin-coated wells (A). The eluted fraction was blended with unlabelled BFM proteins before applying into the egg white avidin-coated well (B). The avidin bound immune-complex was detected with HRP conjugated goat anti-human IgE. The absorbance was measured at OD 450 nm. These data are the representative of three independent experiments.

Chapter 4

Discussion

The incidence of immediate hypersensitivity to natural rubber latex has increased since the early 1980s (Charous *et al*., 1994). This has been caused in part by the institution of mandatory universal precautions for handling bodily fluids. Prevalence of latex sensitization and allergy is reported to be 3% to 17% in HCWs, 11% in LGWs and 1% to 6.5% in the general population (Binkley *et al*., 2003). In addition to presenting symptoms, several immunological assays have been developed, e.g. specific IgE determination by CAP-RAST, basophil histamine release assay, ELISA and immunoblotting to diagnose latex sensitization. Allergy to natural rubber latex (NRL) involves sensitization to multiple constituent proteins; therefore different groups of patients respond to specific latex proteins in various ways. Soluble components reside in latex glove are majority claimed involving in the allergic reactions. Although the total protein content of an extract provides an indication of latex glove allergenicity, the assay is time-consuming, and its analytical sensitivity is limited. Moreover, it cannot discriminate between allergenic and nonallergenic proteins extracted from latex gloves. For this reason, having pure identified allergens is necessary to make the standard for immunologic diagnosis (Wagner and Breitender, 2005). Currently, thirteen Hev b proteins have been recognized by the International Union of Immunological Societies (IUIS) as latex allergens (Wagner and Breitender, 2005). Whereas most of the C-serum and B-serum proteins are water soluble, those of the rubber particles are in generally insoluble, i.e. Hev b 1 (Yeang *et al*., 2002). The Hev b 2, 4, 6 and 10 were isolated from B-serum (Yeang *et al*., 2002) while allergens residing in the BFM have never been reported. Although in the glove manufacturing process rubber latex has to pass through extensive washing, there are still cases reported of allergic reaction to rubber gloves (Yip and Cacioli, 2002). Therefore, it was necessary to investigate whether the causative allergens are less water-soluble and whether novel allergens have hydrophobicity property. The BFM proteins were extracted from BFM using 0.2% triton X-100 for solubilizing of membrane proteins (Gincel and Shoshan-Barmatz, 2002; Hagen *et al*., 1979).

1. Verification of IgE and IgG in sera

The results of indirect ELISA for specific-IgE and -IgG against BFM proteins demonstrated that the LGW group had a higher level of immunoreactivity compared to the HCW group (Fig. 10, 11). In addition, the CAP^{+} group showed comparatively low response to BFM proteins. This result supported the suggestion that the different rubber components which these study groups experience give rise to different diagnostic patterns. The LGWs are often exposed to BFM proteins in the production process.

2. Correlation of specific IgE and IgG

In the correlation study of IgE and IgG (Fig. 12), LGWs 4, 248, and 253 were selected from their comparatively high IgE. Recently, Kraft *et al*. (2006) described the influence of IgG level in the anti-allergic response. If the FcγRIIB forms co-aggregation with FcεRI through the allergen cross-linking via IgG and IgE, the pro-allergic signal will be diminished. Activation of the high-affinity receptor for IgE (FcεRI) on allergic effector cells induces a multitude of positive signals via immunoreceptor tyrosine-based activation motifs, which leads to the rapid manifestation of allergic inflammatory reactions. As a counterbalance, the coaggregation of the IgG receptor FcγRIIB mediates inhibitory signals via immunoreceptor tyrosine-based inhibition motifs. Interestingly, only LGW number 4 showed clinical symptoms (Table 5), although the corresponding IgG level was high. The optimal ratio of IgG/IgE should be further examined to predict the patient's status. However, the BFM proteins were prepared in crude form; the IgG level probably does not reflect the specific binding to the same allergic molecules recognized by the elevated IgE. Considering the result of skin prick test, all selected LGWs 4, 248 and 253 gave negative allergic skin reaction (Table 5) although the proteins used were from BFM. A number of investigators have also reported lack of correlation between *in vitro* (ELISA, RAST, and immunoblotting) and *in vivo* (skin test) measures of IgE using double-blind, placebo-controlled, food challenges (DBPCFCs; the standard for food allergy diagnosis). (Bernhisel-Broadbent and Sampson, 1989; Bernhisel-Broadbent *et al*., 1989; Burks *et al*., 1989; Eigenmann *et al*., 1996) Moreover, the quality of these methods is difficult to be controlled. Physicians should realize that a positive result from allergen-specific IgE does not always indicate allergy. The standard for the diagnosis of allergic disease remains a combination of positive

double-blind challenge, the presence of specific IgE, and demonstration that the symptoms are the result of IgE-mediated inflammation (Ownby, 1988). The clinician has a choice of two confirmatory tests that have been extensively used clinically to identify a state of sensitization: the skin test and the blood test for latex-specific IgE antibody. Detection of latex-specific IgE antibody in the skin is attractive because it is rapid, is sensitive, and involves a clinically observable and biological relevant response in the skin of the individual. However, since no FDA-approved latex skin testing extract is available in the United States, serological tests for latex-specific IgE have assumed greter importance as an alternative diagnostic confirmatory test. At last resort, *in vivo* provocation test may be used, especially in cases where there is a convincingly positive clinical history but a negative skin test and/or blood test for latex-specific IgE antibody (Hamilton, 2002). A standardized diagnostic protocol for latex allergy is still lacking. Accordingly, identification of allergic molecules presented in BFM will support the development of immunodiagnostics.

3. Co-immunoprecipitation

The CAP^{+} (sample no. P1396) which showed the high level of CAP unit, 9.75 U/ml, and noticeably low levels of IgE and IgG against BFM proteins, was selected for the co-immunoprecipitation. In contrast with LGW no. 4, this serum sample contained no specific IgE for allergic components in biotinylated BFM proteins, suggesting the absence of BFM proteins in the CAP assay. Accordingly, combining of the purified 55 kDa protein (Fig. 14A) with BFM proteins in the CAP assay should be validated for its diagnostic value.

The major goal of the study, given the results, was to determined the specific molecule captured by specific IgE in LGW no. 4. The protein had a molecular weight of 55 kDa. There were no immunoreactive bands observed using Western immunoblotting. Individual protein allergens can be complicate in structure, with various antibody recognition sites (epitopes). Epitopes can be either linear form or have a more complex conformational structure, and glycoprotein, may contain or be influenced by sugar moieties (Rihs *et al*., 1999). This suggests the recognition of conformational epitopes by specific IgE which was previously reported in soybean profiling (Rihs *et al*., 1999) and grass pollen (Hantusch *et al*., 2004) allergens. The 55 kDa protein located is within the range of Hev b 4 molecular sizes (Wagener

and Breiteneder, 2005). However, Hev b 4 was isolated from B-serum in water-soluble form (Yeang *et al*., 2002). The 55 kDa proteins, thus may be a novel allergic class. The negative result obtained by CAP assay in latex-sensitized patients since 55 kDa is absent. Including this molecule in the assay panel will improve the diagnostic value for screening the risk of latex allergy. It is essential to have pure and standardized allergens to have consistent immunologic assays. A number of allergenic proteins have been produced by molecular cloning and expression of their genes. Although a large number of NRL allergens have been identified, it may not be necessary to use all these allergens for diagnosis of latex allergy.

Chapters 5

Summary

The result of this investigation could be summarized as follows:

1. The mean value of specific IgE was 0.10 OD, which verified that there was IgE is sera.
2. The cut-off value was 0.06 (mean +2 S.D. of CAP^- group); there were 56, 11, 1, and 5 samples in LGWs, CAP^+, CAP^-, and HCWs classified as positive for IgE against BFM proteins.
3. Considering the difference in mean between LGWs and the others there were no significant differences (CAP^+, CAP^-, and HCWs; P=0.50, 0.13, and 0.19 respectively, paired t-tests).
4. The level of specific IgG in individual groups was the same as the level of specific IgE, which verified that there was IgG in sera.
5. The cut-off value was 0.09 (obtained from CAP^- group as described earlier); there were 86, 9, 1, and 3 subjects defined as positive for IgG against BFM proteins.
6. Comparison of CAP^+, CAP^-, and HCWs means against LGWs mean showed significant differences with the latter group.
7. Skin prick tests were given to the subjects that had the highest level of specific IgE with OD of 2.64, 1.16, and 1.53 which were plotted in a scatter format (Figure 3).
8. Table 1 suggested that only subject no. 4 was allergic even though that skin prick tests were negative for all subjects.
9. The quality of labeled BFM proteins was analyzed and the biotinylated BFM proteins showed positive immunoreactivity with serum from LGW no. 4 when HRP conjugated goat anti-human IgE was used as a detector (OD 450 nm = 1.95).
10. The dilution effect demonstrated the specific formation of the immune-complex when 1 μg/ml biotinylated BFM proteins were added (OD 450 nm = 0.39).
11. 15% SDS-PAGE was used to separate the BFM proteins, which contained two major bands at 30 and 34 kDa, the rest were in the range of 10 to 70 kDa.

12. The segregated polypeptides were transferred to PVDF membrane and probed with serum no. 4, which had the strongest specific IgE reactivity against BFM proteins in indirect ELISA. No reactive bands could be observed by Western-blotting.
13. A co-immunoprecipitation was performed to specific IgE anti-BFM in native conformation. Through ECL chemiluminescence visualization system, a 55 kDa band was observed.
14. No binding activity was found in CAP^{+} and CAP^{-} samples, with negative OD for specific IgE.
15. Competitive ELISA showed that the captured complex containing IgE specifically attach to biotinylated BFM proteins due to the usage of HRP conjugated goat anti-human IgE.
16. This specific binding was assured by mixing the neutralized eluate with unlabelled BFM proteins and the OD diminished 26.89%, compared to the non-inhibition well.
17. This displays the competition between biotinylated and unlabelled BFM proteins for specific IgE.
18. The 55 kDa proteins found in the BFM (in range of Hev b 4 molecule size that belong to a water-soluble class) can be used as diagnostic tool to assess for possible latex allergy.

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Appendix A

Chemicals and equipment

1. Chemicals and antibodies

All chemicals used in this study were analytical grade reagents

Chemicals/Antibodies	Source
Acrylamide	BDH Laboratory Supplies, UK
Agarose (electrophoresis grade)	Sigma-Aldrich Co., USA
Ammonium sulfate	Sigma-Aldrich Co., USA
Ammonium persulfate	Amersham Biotech, Sweden
Bis-acrylamide	BDH Laboratory Supplies, UK
Egg white avidin	Sigma Chemical Co, USA
ECL western blotting analysis system	Amersham Health, Buckinghamshire, UK
Ethanol	Merck, Darmstadt, Germany
EZ-Link Sulfo-NHS-LC-Biotinylation Kit	Pierce, IL,USA
Fraction V bovine serum albumin	Sigma-Aldrich Co., USA
HCl	Sigma-Aldrich Co., USA
HRP conjugated goat anti-human IgE	Sigma Chemical Co., St. Louis, MO,USA
HRP conjugated goat anti-human IgG	Hybridoma reagent laboratory,Johns Hopkins, US
Methanol	Merck, Darmstadt, Germany
mAb (IgG_{2a} subclass) to human Fcε	Biodesign International, Saco, ME
NaCl	BDH Laboratory Supplies, UK
NaOH	BDH Laboratory Supplies, UK
Polyvinylidene-fluoride (PVDF) membrane	PALL, East Hill, NY, USA
Seize X Protein A Immunoprecipitation Kit	Pierce, IL,USA
Sodium dodecyl sulfate	Sigma-Aldrich Co., USA
Skim milk	Difco Laboratories, USA
TEMED	Sigma-Aldrich Co., USA

Tris base	Sigma-Aldrich Co., USA
Triton x-100	Sigma-Aldrich Co., USA
Tween 20	Fluka, Buchs, Switzerland
TMB/H_2O_2 sustrate solution	Fermentas, Lithuania

2. Equipment

Instrument-Models	**Source**
37 ^{0}C incubator	JP Selecta, Barcelona, Spain
96-well immunomaxi high binding plate	TTP, Stafa-Zurich, Switzerland
Desiccator cabinet	Structure Probe, Inc., USA
Electrophoretic power supply	Amersham Pharmacia Biotech, Sweden
Magnetic stirrer with hot plate	BOECO, Germany
Melting-point meter	A. KRÜSS Optronic GmBH, Germany
Microcentrifuge	Eppendorf AG, Hamburg, Germany
Sunrise microplate reader	Tecan, Austria
MRX-150 refrigerated microcentrifuge	TOMY, Japan
Rotatory evaporator	BÜchi, Switzerland
RT6000 D refrigerated centrifuge	Sorvall, Germany
Ultrasonic bath	Jencons (Scientific) Ltd, UK
Shaking platform	Tecnorama, Switzerland
UV2450 Spectrophotometer	Shimadzu, Japan

Appendix B

Reagent preparations

1. 1 mM phosphate buffered saline (PBS), pH 7.2

NaCl	8.00	gm
KCl	0.20	gm
Na_2HPO_4	1.15	gm
KH_2PO_4	0.20	gm

Dissolved all ingredient in distilled water (DW) until the volume 900 ml

Adjusted the pH to 7.2 with 1N HCl or 1N NaOH

Added DW until the volume was reached 1000 ml and stored at 4 $^{\circ}$C

2. Reagents for using in ELISA

2.1 0.05% Tween 20 in PBS (Washing buffer)

1 mM PBS	500	ml
Tween 20	0.25	ml

Mixed well and stored at room temperature (25 $^{\circ}$C).

2.2 Carbonate/bicarbonate coating buffer

Na_2CO_3	1.59	gm
$NaHCO_3$	2.93	gm
NaN_3	0.20	gm

Dissolved all ingredients in DW and filled up to 1,000 ml and kept at 4 $^{\circ}$C

2.3 Blocking buffer (1% Skim milk/PBS)

Skim milk	1.00	gm
PBS pH 7.2	100	ml

Mixed well, freshly prepared before use

2.4 Blocking buffer (2% Skim milk/PBS)

Skim milk	2.00	gm
PBS pH 7.2	100	ml

Mixed well, freshly prepared before use

2.5 Diluent buffer (2% BSA/PBS)

Bovine serum albumin	2.00	gm
PBS, pH 7.2	100	ml

Mixed well, freshly prepared before use

2.6 Stop reaction solution (1N HCl)

38 % v/v HCl	6.14	ml

Adjusted the total volume to 100 ml with distilled water

3. Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

3.1 1.5 M Tris-HCl, pH 8.8

Tris-base	18.15	gm
Deionized DW	75	ml

Adjusted the pH to 8.8 with concentrated HCl

Adjusted the volume to 100 ml with deionized DW and stored at 4 $^{\circ}$C

3.2 0.5 M Tris-HCl, pH 6.8

Tris-base	6.0	gm
Deionized DW	80	ml

Adjusted the pH to 6.8 with concentrated HCl

Adjusted the volume to 100 ml with deionized DW and stored at 4 $^{\circ}$C

3.3 Running buffer

Tris-base	1.51	gm
Glycine	7.20	gm
Sodium dodecyl sulfate	0.50	gm

Dissolved in 500 ml of distilled water and kept at 4 °C

3.4 Blotting buffer

Tris-base	3.03	gm
Glycine	14.41	gm
Sodium dodecyl sulfate	0.5	gm
Dissolved all gradients with DW	700	ml
Methanol	200	ml

Dissolved all ingredients in DW and filled up to 1,000 ml and kept at 4 °C

3.5 Copolymerization of 4% stacking gel (5 ml)

Stock acrylamide 30%	0.665	ml
Gel buffer pH 6.8	1.25	ml
SDS 10%	0.05	ml
Distilled water	3.01	ml
10% ammonium persulfate	0.1	ml
TEMED	0.01	ml

3.6 Copolymerization of 12% separatinging gel (10 ml)

Stock acrylamide 30%	4.0	ml
Gel buffer pH 8.8	2.5	ml
SDS 10%	0.1	ml
Distilled water	3.3	ml
10% ammonium persulfate	0.1	ml
TEMED	0.01	ml

3.7 Blocking buffer (5% Skim milk/PBS)

Skim milk	5.00	gm
PBS pH 7.2	100	ml

Mixed well, freshly prepared before use

3.8 Diluent buffer (5% BSA/PBS)

Bovine serum albumin	5.00	gm
PBS, pH 7.2	100	ml

Mixed well, freshly prepared before use

Appendix C

New latex allergens identified in bottom fraction membrane proteolipids

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Introduction

Latex is the milky sap produced by the laticiferous cells of the tropical rubber tree *Hevea brasiliensis* of the family Euphorbiacea. It is used in the manufacturing of medical tools used for examination and surgery. Latex allergy has become a serious problem worldwide since the late 1980s ().[1] Due to the increasing use of natural rubber latex (NRL) products, type I allergy to *Hevea brasiliensis* latex has become an important and increasing health problem. Proteins present on various NRL products have been implicated in causing allergy in specific risk groups.[2-8] One risk group of particular interest consists of health care workers (HCWs) exposed to powdered NRL gloves.[9] Other important risk groups are workers in industries in which latex products are manufactured, including those collecting and processing latex from rubber trees.[7-10] Patients with congenital anomalies such as spina bifida, those undergoing multiple surgeries, and patients with atopy are more susceptible to latex protein – induced allergy and asthma.[11] It has been reported that 2.8-8.8% of all health care workers and 29-72% of patients suffering from spina bifida (SB) are allergic to *Hevea* latex proteins.[12-18]

At present , a total of 13 latex allergens Hev b 1 [18,19](rubber elongation factor), Hev b 2 [20,21] (beta-1,3-glutcanases), Hev b 3 [19,22,33,34](small rubber protein), Hev b 4 [20,39](microhelix component), Hev b 5[23,24](acidic latex protein), Hev b 6.01 (prohevein), Hev b 6.02 (mature hevein), Hev b 6.03 (c-domain of prohevein)[21,25,26], Hev b 7.01(patatin homologue from B-serum), Hev b 7.02 (patatin homologue from C-serum)[27-29,38], Hev b 8 [30,31,32] (latex profilin), Hev b 9 [35](latex enolase), Hev b 10 [36](Mn –superoxide dismutase, Hev b 11 [20] (class I endochitinase), Hev b 12 [40](lipid transter pratein), Hev b 13 [41](latex esterase) are designated by the International Allergen Nomenclature Committee. Although purified NRL allergens have been isolated and characterized, more reseacrh is needed for the development of standardized reagents to be used for reliable diagnosis of latex allergy.The major difficulty is that the latex from the rubber plant

Hevea brasiliensis contains a large number of protein, which constitutes about 1% of the total weight.[42] It is essential to have pure and standardized allergens to have consistent immunologic assays. Although a large number of NRL allergens have been identified, it may not be necessary to use all these allergens for diagnosis of latex allergy.[10,43,44] However, by careful evaluation, the minimum number of essential allergen will be identified and selected for use in skin tests and in vitro assays.[45]

Currently available serological assays for latex-specific IgE display less than ideal diagnostic sensitivity.This suggests that certain allergenic epitopes are either missing or can not be fulfilled from presently available multiple Hev b allergens. Our study revealed a presence of additional novel latex allergens in proteolipids prepared from the bottom membrane, isolated from bottom fraction of ultracentrifuged fresh latex.

Methods

Subjects

Serum samples from 30 health care workers (HCWs) who are allergic to latex (HCW group : 8 men/22 women) were included in this study. In the HCWs group, the mean age of the subjects was 37.8 years (range, 26 to 50 years). The thirty latex-allergic patients were given a diagnosis of latex allergy on the basis of clinical history.

Total serum IgE (in ng/ml) and IgE anti-Latex (kU/L) were performed with the Pharmacia CAP system. Skin prick tests were performed with latex extract. NaCl served as a negative control and histamine (10 mg/ml) as a positive control. Skin test responses were assessed as positive if the maximum wheel diameter was 3 mm or greater without reaction of negative control and the skin index was greater than 0.6. The skin index was calculated as the ratio of the diameter of the allergen wheel to the histamine wheel. Phadiatop is a multi-allergen screen for allergen specific IgE antibody to 15 common aeroallergens. One atopic donor with no reaction to skin prick testing was used as negative control.

Preparation of allergen

Fresh *Hevea* latex from the clone RRIM 600 was collected in chilled containers and centrifuged at 109,000 g max for 45 min at 4 ^{0}C. The rubber cream and latex serum (C-serum) were carefully separated and discarded while the bottom fraction was later scooped off using a long spectula. The bottom fraction obtained from centrifuged tube was suspended in one volumes of distilled water containing 0.7% ammonium hydroxide with overnight stirring at 4 ^{0}C. The remaining insoluble bottom fraction membrane (BFM) was collected by centrifugation at 10,000 g for 30 min and further washed 3 times with isotonic buffer (50 mM tris-HCI, pH 7.4 containing 0.9% NaCI) to eliminate residual B-serum.

BFM proteolipids were prepared as decribed by Folch and Lees.[46] The washed BFM was added into five volume of a continuously stirred chloroform methanol mixture (2:1, v/v). The extract separated from the BFM was washed overnight with 0.6% aqueous NaCI. A thin whitish interfacial layer of a “fluff” was separated, precipitated with acetone (80% saturation) centrifuged and dried. The proteolipids were extracted by mixing the dry fluff precipitale with

DW containing 2% SDS. BFM fluff was further purified fllow by Model 491 Prep Cell instruction manual (BIO-RAD, U.S.). Purified 17 kDa protein was eluted with 50 mM Tris-HCI, pH 7.4. The protein content was determined by Bradford's method.[47]

Electrophoresis, immunoblotting, and immunoblot inhibition

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting were carried out basically to Leammli[48] and Towbin.[49] Electrophoresis was carried out with 7-15% gradient gels at constant current of 14 mA. Separated proteins were detected by staining with comassie Brilliant Bule R 250. After electrophoresis, the protein on the gel were transferred onto 0.45 μm pore size nitrocellulose membrane in blotting buffer (25 mM Trisma base, 192 mM glycine and 20% methanol, pH 8.3) for 1 hour at contant voltage of 100 V. After blocking with a solution consisting of 1% BSA in phosphate buffer saline (PBS) and washing with PBS, membranes were cut into in dividual 0.5 cm wide strips. By immunoassay, the strips were incubated individually with 1000 μl serum samples diluted 1:10 in blocking buffer (1% BSA in PBS) for 18 hours at 4^{o}c. The binding of specific IgE antibody to the protein was detected with peroxidase-conjugated anti-human IgE antibodies in blocking buffer (diluted 1:1000 for 2 hours at room temperature), and bands were visualized with a substrate colour development solution.

The procedure of immunoblot inhibition assay was the same as immunoblotting described above except that latex-sensitized serum sample (positive clinical latex history but without SPT data) was incubated with purified 17 kDa protein (40, 80, 120, 160, 200, 250 and 300 μg) for 18 hours at 4^{0}C before incubated with the blotted membrane.

Chemical N-terminal sequencing of purified 17 kDa protein

Purified 17 kDa protein (1 mg) was reconstituted in the mixture of 50μl 6M GuHCI 100mM Tris, 100μl 0.1%TFA and 100μl CAN sonicated 100μl 0.1%TFA, applied to Prosorb, washed with 300μl 0.1%TFA, the membrane was cut out and 25% placed in the cartridge and analyzed.

N-terminal amino acid sequencing was done on a pulsed liquid-phase ABI-Procise HT protein sequencer.

Results

Prevalence of sensitization to 17 kDa and 33 kDa proteins

Two main BFM proteolipids possessed molecular weight at 17 kDa and 33 kDa were used as the immunogens (Fig 1A). To analyze the prevalence of sensitivities to 17 kDa and 33 kDa proteins in patients with latex allergy, 30 serum samples (n=30) from HCWs allergic to latex were used. They had a median age of 37.8 years (Table I). Total IgE ranged from 41 ng/ml to 6367 ng/ml (median, 789 ng/ml. Latex-spectic IgE in serum ranged from 1.14 kU/L to 40.1 kU/L (Median, 9.5 ku/L). One serum without a history of latex allergy and negative skin prick test was used as negative controls. As shown in Fig1B, The prevalence of IgE antibody to the isolated 17 kDa and 33 kDa proteins from BFM proteolipids found among latex allergenic HCWs with positive clinical history and skin prick test were 100% (30/30) and 46% (14/30) respectively.

IgE recognition patterns of latex-allergic HCWs

The immunoblots of sera of all the 30 patients presented data on the IgE recognition of 17 kDa and 33 kDa from BFM proteolipid. In Fig 2, the possible combinations of the 2 latex allergens (17 kDa and 33 kDa) as recognized by IgE of latex-allergic HCWs are illustrated. The frequency of recognition of the 17 kDa and 33 kDa proteins were 46% (14/30). None of the patients showed IgE against only 33 kDa protein. Latex-allergic patients who recognized only 17 kDa protein were 53.3% (16/30).

Immunoblot and inhibition studies of 17 kDa protein

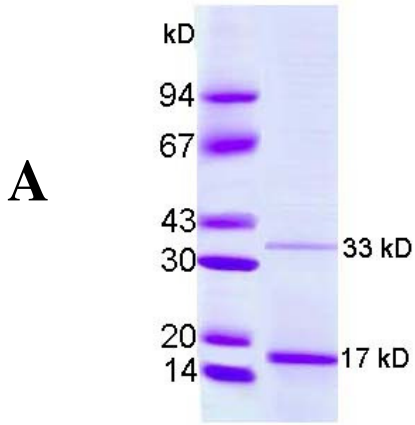
By immunoblot analysis, all 30 sera described in Table I were screened for specific IgE againt BFM proteolipid allergens, which included 17 kDa and 33 kDa proteins. As shown in Fig 1, all of latex-allergic HCWs revealed IgE binding to 17 kDa protein. From these data, we identified 17 kDa protein IgE binding band by immunoblotting and inhibition experiments using purified 17 kDa protein by Model 491 Prep Cell. In Fig 4, which shows one patient with a positive clinical history and skin prick test with the IgE antibodies to latex be completely inhibited by preincubation of serum with 120 μg of purified 17 kDa protein.

TABLE I. Characterization of the study population, which consisted of 30 latex-allergic HCWs

Code	Age(y)	Sex	SPT reult	Total IgE ng/ml	IgE anti-Latex kU/L	Phadiatop	17 kD	33 kD
I0133	41	F	+	1441	12.3	+	+	+
I1726	36	F	+	83	1.14	-	+	+
I4136	33	F	+	239	8.84	-	+	+
I4744	39	M	+	224	10.2	+	+	+
I4739	40	F	+	2017	11.2	+	+	+
I4141	39	M	+	168	3.68	+	+	+
I4139	50	F	+	428	2.57	+	+	+
I1531	35	F	+	211	3.2	+	+	+
I1781	26	F	+	354	5.21	+	+	-
I4134	42	M	+	41	3.4	+	+	-
I0635	42	F	+	170	4.75	+	+	-
I0761	35	F	+	304	11.2	+	+	-
I1421	26	F	+	205	3.05	+	+	-
I1092	49	F	+	209	7.29	+	+	+
I4133	33	M	+	609	8.69	+	+	-
I3182	49	F	+	75	2.75	-	+	-
I4743	46	F	+	158	3.27	+	+	+
I4740	49	M	+	328	2.72	+	+	-
I2363	38	M	+	2295	6.66	+	+	-
I6550	34	F	+	1156	4.84	+	+	+
I3286	40	F	+	1387	27.9	+	+	+
I4072	35	M	+	6367	55.2	+	+	+
I3439	35	F	+	681	18.5	+	+	-
G8655	31	F	+	1787	NA	+	+	+

Code	Age(y)	Sex	SPT reult	Total IgE ng/ml	IgE anti-Latex kU/L	Phadiatop	17 kD	33 kD
I6544	26	F	+	567	1.69	+	+	+
I4145	34	M	+	237	1.69	+	+	-
I6549	46	F	+	474	1.96	-	+	-
J2583	30	F	+	NA	NA	+	+	+
I4163	38	F	+	586	40.1	+	+	+
I4746	38	F	+	80	3.48	+	+	-

SPT, Skin prick test; M, Male; F, Female



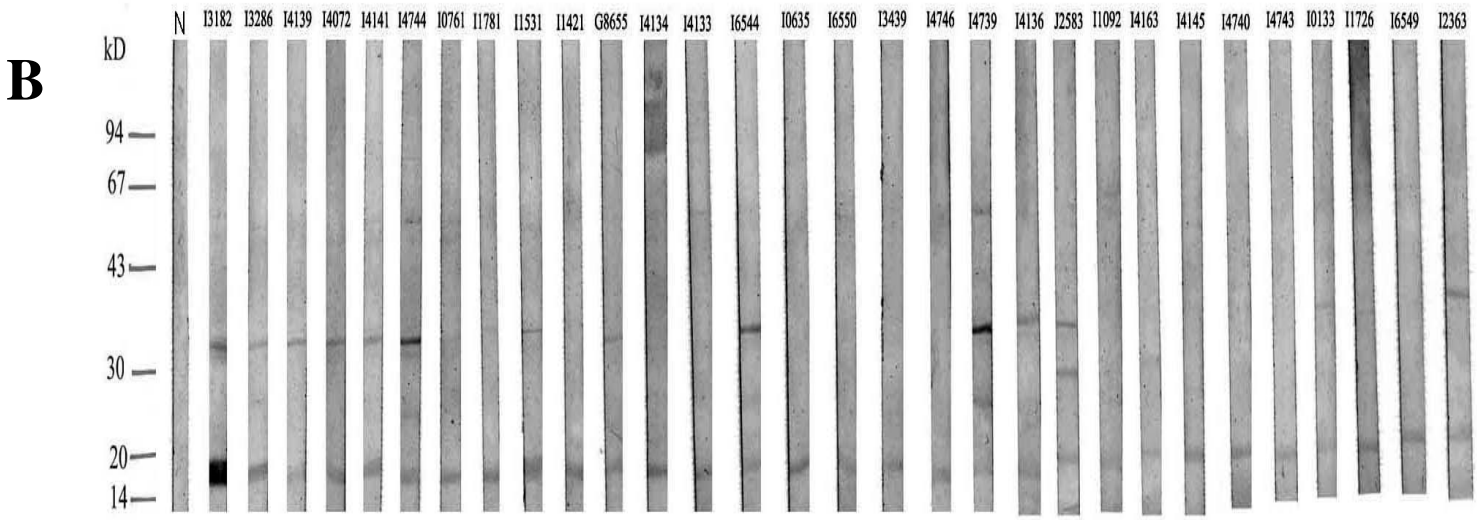


FIG1. (A) SDS-PAGE 7% to 15% gradient of bottom fraction membrane (BFM) proteolipids. (B)Immunoblot analysis of 30 latex-allergic HCWs Patient numbers refers to Table I. Controls consist of a negative control serum of 1 atopic donor without latex allergy (lane N).

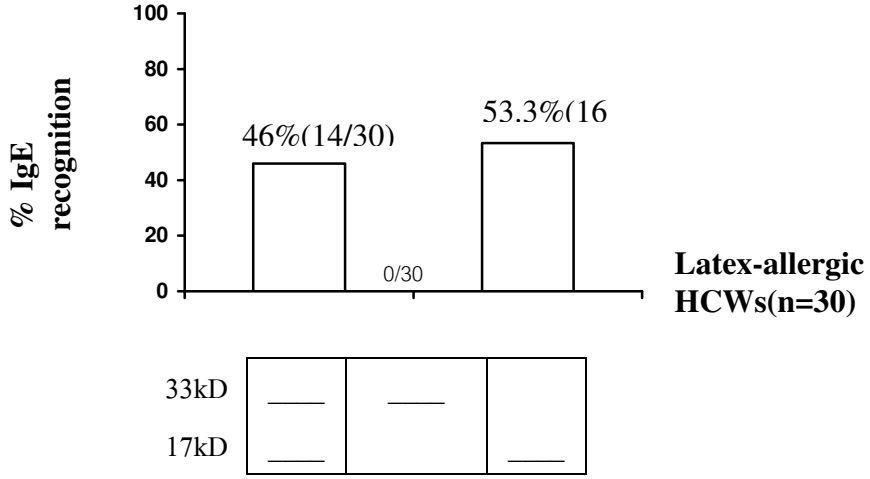
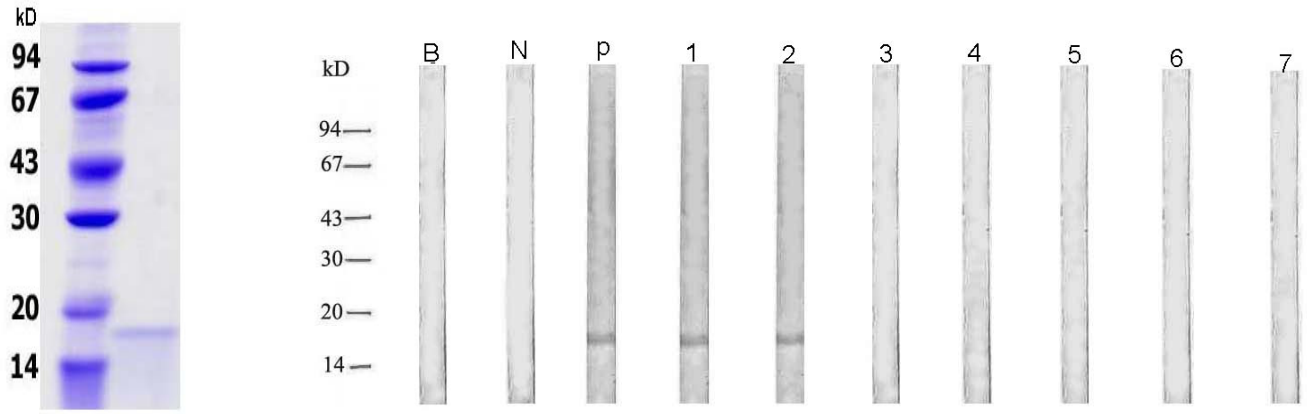


FIG2. IgE recogrition pattern of latex allergic HCWs. Lower panel shows the different possible combinations of IgE binding to the 2 latex allergens from BFM proteolipids (17 kDa and 33 kDa). Upper panel shows the percentage of IgE recognition of the possible combinations shown in the lower panel.

Purified 17 kD : 1 DLDVFVTGSF 10

FIG3. N-terminal sequencing of purified 17 kDa protein



A **B**

FIG4. (A) SDS-PAGE 7% to 15% gradient of purified 17 kDa. (B) Inhibition experiment. One sera donor latex allergy who was strong bound on 17 kDa without pretreatment (lane P) and after preincubation with purified 17 kDa protein 40, 80, 120, 160, 250 and 300 µg (land1-7). Controls consist of a negative control serum of 1 atopic donor without latex allergy (lane N) and a buffer control (lane B).

Discussion

Most of the studies reported on latex allergen were related to proteins in three fraction from ultracentrifuged latex namely rubber particles (RP), C-serum and bottolm fraction, particularly lutoids which contain B-serum.[45] The complete identification of latex allergens from those studies are not yet possible. This also includes unknown background of native allergenic protein the latex finished products such as latex glove. Hence, limitation for the study focuses on an important allergen which is a reliable standardized reagent. At present, most of allergens are not presently available as an identification tool for patients with latex allergy. Allergic reaction to natural rubber latex (NRL) proteins has increasingly been reported. In spite of ongoing active research,[50, 51] a reliable diagnostic test method which gives 100% sensitivity and specifcity is still not available. A skin prick test remains the most reliable method for diagnosis of NRL protein allergy. The inability to come up with the standard diagnosis for latex protein allergy is partly due to the absence of good standard allergenic protein allergens in the standard testing reagent or if present, its amount was too low for the diagnostic purpose. Although several NRL allergens had been identified, studies on IgE allergenic protein binding revealed only a limited number of allergens NRL protein .[52] In this study, proteolipids from BFM was extracted by chloroform-metanol in the from of "fluff".[53] The proteins associated with fluff could be further extracted by SDS. The SDS-PAGE of the SDS-extracted BFM proteolipids revealed two major components, 17 kDa and 33 kDa insoluble proteins (Fig 1.A). The 17 kDa protein was highly recognized by 100% and 33 kDa protein by 46% of the HCWs with positive clinical history and skin prick test (Table I; Fig 1.5 B). The high percentage (100%) of 17 kDa protein bound IgE recognition by sera from 30 HCWs patiens. Therefore we conclude that 17 kDa and 33 kDa insoluble protein has been indentified as an important allergen for latex-allergic patients, whereas, Hev b 1 and Hev b 3 are insoluble allergens. The prevalene of Hev b 1 was recognized only 82% and Hev b 3 79% of the latex-allergic children with SB.[54] In Fig 2, the possisble combination of the new insoluble allergen-17 kDa and 33 kDa proteins-as recognized by IgE of latex-allergic HCWs are illustrated. The high frequency of recognition of the 17 kDa was 100% both 17 kDa and 33 kDa 46%. And the high fequency of only 17 kDa proteins were 53.3%. Moreover, sequence alignment of 17 kDa

proteins was not similar to other proteins in Genebank (Fig3). Therefore, 17 kDa latex allergen will be good representative for diagnosis and to identify patients with latex allergies.

In the future, 17 kDa latex allergen will be use for good standardized reagent, improved testing, and perhaps finding way to desensitize people with severe latex allergy.

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Appendix D

Protein Allergens from Fresh Latex: Cross-Reactivity and Possible Prevention

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SUMMARY

Freshly tapped latex was fractionated by ultracentrifugation into rubber layer, C-serum and bottom fractions. The bottom fraction was used for B-serum, bottom membrane protein (BMP) and BM proteolipid (BMPL) preparations. An ELISA test was performed to evaluate IgE-binding capacity of the proteins present in C-serum, B-serum and BMP, by using 178 sera of local latex-glove factory workers (LGW). The most reactive IgE-binding fraction was identified to be BMP. SDS-PAGE immunoblotting revealed the binding of 33 kD BMPL to IgE antibodies in sera of subjects with positive puncture skin test and latex allergy history and sera of LGW with high ELISA OD. Similarly, the IgE antibodies with high BMPL-binding capacity in sera of LGW were also shown to bind a 33 kD proteolipid isolated from kiwi fruits. Cross-reactivity between 33 kD latex and kiwi proteolipids was revealed from SDS-PAGE immunoblotting inhibition experiment. Moreover, proteins of similar molecular weight around 30-33 kD, known as predominantly cross-reactive allergens among banana, latex and avocado, were also isolated as proteolipids in banana, avocado and chestnut. These findings suggest a common presence of (cross-reactive) proteolipid allergens. Hence, a hydrophobic means to eliminate these lipid-soluble allergens seems to be an important solution in preventing latex allergy as well as latex-fruit syndrome.

INTRODUCTION:

Allergic reactions to natural rubber latex (NRL) are becoming increasingly more common. In spite of on going active research inputs (1, 2), a diagnostic test method for NRL allergy giving 100% sensitivity and specificity is still unavailable. A puncture skin test (PST) remains the most reliable method in screening and diagnosis of NRL allergy. The inability to come up with definite diagnosis for latex allergy may due to a missing of critical allergen in the standard testing reagent or if present, the amount was too low for the diagnostic purpose. Although several NRL allergens had been identified and registered, studies on IgE-binding (allergenic) proteins revealed only a limited number in finished latex products as compared with NRL (3). This suggests that several potential NRL allergens had already been eliminated along various steps prior to and during manufacturing process. The latex proteins surviving these harsh treatments were likely to be very stable and have high affinity to rubber molecules. Accordingly, a stable 28 kD latex protein was suggested to be involved in cross-reactivity between latex allergy and food (4). The aim of present study was to identify major site of IgE-reactive proteins in fractionated fresh latex, to study their stability and cross-reactivity with kiwi fruit as well as to find proper means for elimination.

MATERIALS AND METHODS:

Latex and fruit proteins preparation:

The rubber layer, C-serum and bottom fractions were obtained from ultracentrifuged freshly tapped latex of RRIM 600 clone. The B-serum and bottom membrane protein (BMP) fractions were separated by centrifugation after repetitive freeze-thawing of the bottom fraction. The proteolipids were extracted from BMP or dry fruit homogenates prepared from banana, avocado, kiwi and chestnut by using chloroform/methanol (2:1) as described by Folch and Lee (5)

Serum samples:

To examine the IgE antibody pattern to NRL allergens in fractionated fresh latex, sera were sampled at local NRL glove factory from 178 workers. In immunobloting, sera samples of subjects with NRL allergy history and positive PST were obtained from HCW at the Songklanakarin and Siriraj Hospitals.

Alkaline treatment of BMP:

Isolated BMP was incubated at room temperature for various periods as indicated by suspending in 0.7% ammonia solution.

ELISA:

IgE-specific ELISA was carried out by coating 96-well polystyrene microtiter plate with 100 μl of assay protein solution (1 μg/ml) and incubated overnight at 4^{O}C. The wells were then emptied and postcoded 37 ^{O}C for 1 h with 5% skim milk in PBS-Tween (0.1 M phosphate-buffered saline pH 7.4 with 0.05% Tween 20, PBS-T). After three washings with PBS-T, an individual LGW sera was added to each well. It was incubated at 37 ^{O}C for 1 h and washed thrice. Biotinylated antihuman IgE was then added and incubated for 1 h. The wells were washed and incubated with streptavidin-conjugated alkaline phosphatase for 1 h. After washing, the substrate development solution was added and the optical density (OD) was read at 405 nm by an ELISA reader.

SDS-PAGE immunoblotting and inhibition test:

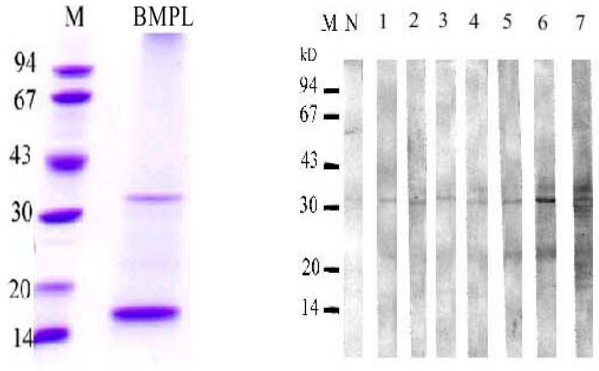
NRL proteins were electrophoresed through 7-15% gradient or 12% SDS-PAGE, according to the method described by Laemmli (6). The resolved proteins were transferred to nitrocellulose membranes (7) and cut into strips before incubated with individual tested serum. The peroxidase conjugated goat antihuman IgE was added to the strips and incubated overnight at room temperature. The strips were then washed before adding peroxidase substrate development solution to visualized IgE-reactive protein band. For the SDS-PAGE immunoblotting inhibition experiment, the sera containing high BMP reactive IgE was incubated overnight at 4^{o}C with KPL. The preincubated sera were then used in SDS-PAGE immunoblotting of BMPL as described above.

RESULTS:

The screening for reactive IgE binding proteins in fractionated fresh latex by using latex glove factory workers' (LGW) sera, revealed the highest presence of IgE reactive proteins in the BMP fraction. This was indicated by higher frequency of moderate to high ELISA OD values as compared to those obtained with proteins in C- and B-serum, respectively.(Table 1). Upon using selected LGW sera with high ELISA OD in SDS-PAGE immunoblotting of BMPL, the 30-33 kD proteins were revealed to have strong binding affinity towards IgE antibodies (Fig.1). Moreover, the SDS-PAGE immunoblotting of BMPL revealed the presence of IgE reactive 33 kD in all individual sera of subjects with latex allergy history and positive PST (Fig.2). The BMP was shown to be very stable, at least up to 2 months, under the alkalinity (0.7% ammonia solution) used for aging latex concentrate (Fig. 3). Kiwi fruit proteolipid (KPL) of about 33 kD was also similarly shown to bind IgE antibodies in selected LGW sera with high ELISA OD (Fig.4). The cross-reactivity between 33 kD proteolipid of BMP and kiwi was revealed from SDS-PAGE immunoblotting inhibition experiment (Fig.5). Besides the 33 kD KPL, proteolipids under similar molecular range (30-33 kD) as those reported for cross-reactive allergens among banana, latex and avocado were also detected in banana, avocado, chestnut under SDS-PAGE (Fig.6)

Table 1. ELISA screening on IgE reactive proteins in fractionated fresh latex

OD. ELISA	**Samples**		
	BMP	**B-serum**	**C-serum**
≥0.1 - 0.2	**31**	**44**	**67**
>0.2-0.4	**13**	**2**	**11**
>0.4	**4**	**-**	**1**
Total	**48/178** **27.0%**	**47/178** **25.9%**	**69/178** **38.8%**



(A) **(B)**

Fig. 1 SDS-PAGE (A) and IgE immunobloting (B) of BMPL using LGW sera with high ELISA OD.

M = Molecular weight markers (kDa)

(A) BMPL = Bottom fraction membrane proteolipid

(B) N = Negative control serum

1-7 = LGW sera with ELISA OD. of 0.6, 0.7, 0.8, 0.9, 0.9, 2.0 and 2.1 units, respectively.

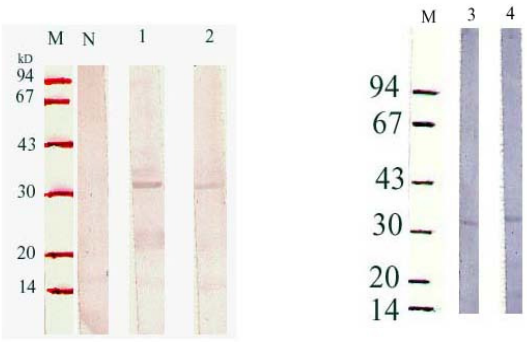


Fig. 2 SDS-PAGE immunobloting of BMPL using sera of subjects with latex allergy and positive PST.

M = Molecular weight markers (kDa)

N = Negative control serum

1-4 = +PST latex allergy sera

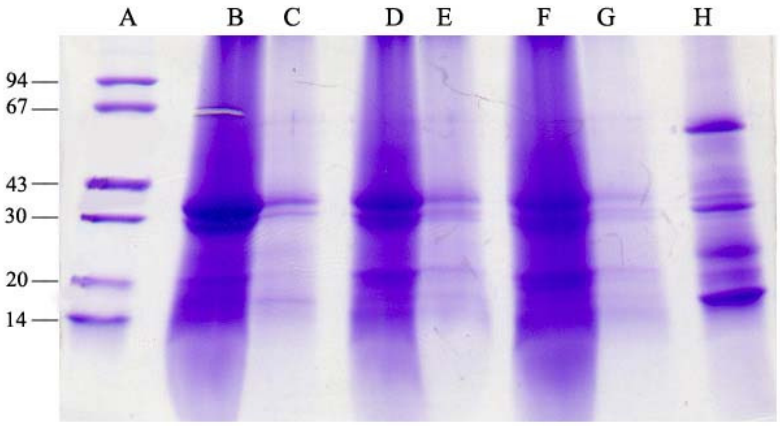


Fig. 3 SDS-PAGE of BMP after incubation in 0.7 % ammonia solution for various periods, as indicated.

A = Molecular weight markers (kDa)

B,D,F = BMP proteins released into the solution after alkaline treatment in ammonia solution for 15, 30 and 60 days, respectively.

C,E,G = Remaining proteins in the sediment BMP after alkaline treatment in ammonia solution for 15, 30 and 60 days, respectively.

H = Freshly prepared BMP

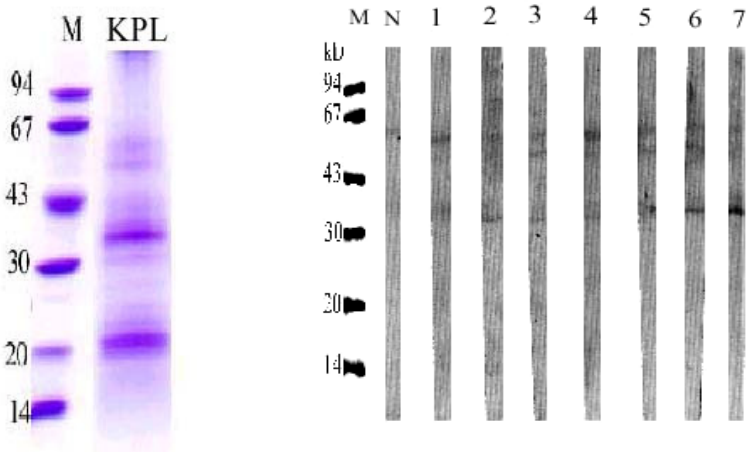


Fig.4 SDS-PAGE (A) and IgE immunoblotting (B) of kiwi proteolipid using LGW sera with high ELISA OD.

M = molecular weight markers (kDa)

(A) KPL = kiwi proteolipid

(B) N = negative control serum

1-7 = LGW sera with ELISA OD. of 0.6, 0.7, 0.8, 0.9, 0.9, 2.0 and 2.1 units, respectively.

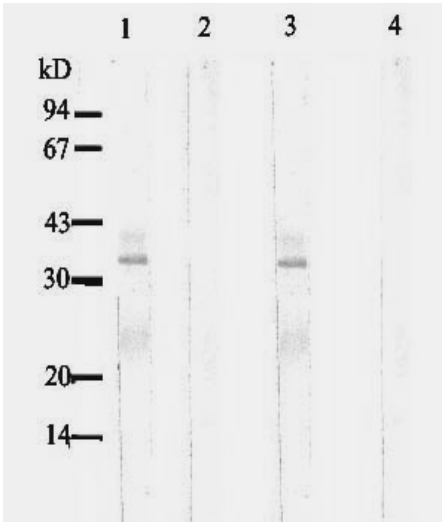


Fig. 5 SDS-PAGE immunoblotting inhibition experiment of BMPL, using LGW sera with high ELISA OD either without (1,3) or with (2,4) subjecting to 4 h preincubation with kiwi proteolipid.

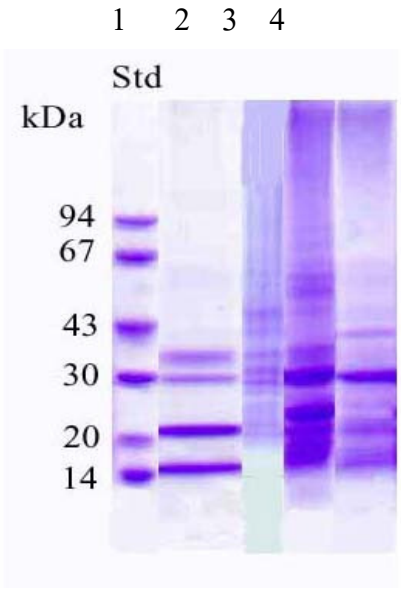


Fig. 6 SDS-PAGE of isolated proteolipids from banana (1), avocado (2), chestnut (3) and kiwi (4).

DISCUSSION:

Many investigators in western countries have been unable to obtained quantities of fresh latex material and limited to the use of finished products or ammoniated latex with a highly variable antigen content for their latex allergy studies. This, in part, may contribute to different immunoreactivity profiles reported among many IgE-binding latex proteins (8-11). Being surrounded by rubber plantations with ample freshly tapped latex, the immunoreactivity of IgE reactive (allergenic) proteins was investigated in this study by using various native protein fractions obtained from ultracentrifuged fresh latex of RRIM 600 clone. The result indicated a common presence of IgE reactive proteins in all latex protein fractions, similar to earlier finding on the wealth of IgE-binding proteins in non ammoniated latex (3). However, a different immunoreactivity profile was observed among these native protein fractions. The frequency of samples giving moderate to high ELISA OD units was found to be highest in BMP and lowest in B-serum fractions. The result suggests lutoid (bottom fraction) membrane as a native major site of rubber latex allergens. Accordingly, a 33 kD proteolipid of BMP was identified to be a major latex allergen. This was revealed from its IgE-affinity binding not only in sera of all subjects with latex allergy and positive PST but also in sera of LGW with high ELISA OD. The 30-33 KD BMP was demonstrated to survive through alkaline treatment period required for aging concentrate latex before using in making dipping products. Hence, it is likely to remain associated with rubber in latex finished products. Supporting evidence may be found in several previous immunoblot studies where proteins around 30 kD were most frequency found with IgE affinity in latex finished products (12). Moreover, the IgE in LGW sera, exhibiting high binding capacity to alkaline-stable BMP, was shown to bind a 33 kD proteolipid isolated from kiwi fruits (KPL). The result obtained from SDS-PAGE immunoblotting inhibition suggests 33 KPL and 33 BMPL as the causative cross-reactive allergens and a stable latex protein involvement in the cross-reactivity. These cross-reactive latex and kiwi allergens share similarity in hydrophobic property and similar molecular weight range (30-33 kD) as those predominantly cross-reactive allergens reported among banana, latex and avocado. The cross-reactive 30 kD allergens reported for banana and avocado (13-14) may similarly belong to lipid-soluble proteins since proteins of 30-33 kD were revealed under SDS-PAGE in the isolated proteolipids of these fruits. Moreover, a corresponding 30-33 kD proteolipids were also found in banana, avocado and chestnut,

suggesting similar cross-reactive implications in latex allergy. Therefore, in preventing latex allergy and latex-fruit syndrome, a hydrophobic means in eliminating the cross-reactive lipid-soluble allergens is suggested from this study.

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Vitae

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Education Attainment

Degree	Name of Institution	Year of Graduation
B.Sc. (Biotechnology)	Prince of Songkla University	1999

Scholarship Awards during Enrolment:

The Royal Golden Graduate Program from the Thailand Research Fund.

List of Publication and Proceeding

1. **Mengumpun, K**., Patthiya, A., Wititsuwannakul, D., Wititsuwannakul, R., Plant proteolipid lectins. 27th Congrees on Science and Technology of Thailand, Hat Yai, Songkla, Thailand : p 570(16-18 October 2001).
2. **Mengumpun, K.**, Jewtragoon, P., Sangsupawanich, P., Balachandra, K., Wititsuwannakul, R., Allergenic cross-reactivities between Hevea latex and vegetative foods. The Royal Golden Jubilee Ph.D. Congress I, Kanchanaburee, Thailand : p 140(23-25 April 2001).
3. **Mengumpun, K**., Jewtragoon, P., Sangsupawanich, P., Balachandra, K., Wititsuwannakul, R., Allergenic cross-reactivities between Hevea latex and vegetative foods. RGJ Seminar Series XVI : Bioscience and Biotechnology II, Hat Yai, Songkla, Thailand : p2(15 February 2002)**.**
4. Witiitsuwannakul, R., Jewtragoon,P., **Mengumpun, K**., Wititsuwannakul ,D., Balachandra, K., ,Sangsupawanich, P.,and Vichayanon, P. (2002) Protein Allergens from Fresh Latex: Cross-Reactivity and Possible Prevention: *International Latex Conference 2002 Papers Book* , Rubber & Plastics News and Akron Dispersions pp. 175-182. (Appendix D)

Vitae

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