IMMUNOLOGICAL AND MOLECULAR INVESTIGATIONS OF CHILDHOOD ASTHMA IN THE SUB-HIMALAYAN REGION OF WEST BENGAL, INDIA

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BY

MANOJ LAMA

SUPERVISOR

Prof. T.K. Chaudhuri

CO-SUPERVISOR

Prof. Mridula Chatterjee

DEPARTMENT OF ZOOLOGY

UNIVERSITY OF NORTH BENGAL

SEPTEMBER 2014

Dedicated

to

My Parents

DECLARATION

I declare that the thesis entitled "IMMUNOLOGICAL AND MOLECULAR INVESTIGATIONS OF CHILDHOOD ASTHMA IN THE SUB-HIMALAYAN REGION OF WEST BENGAL, INDIA" has been prepared by me under the guidance of Prof. T. K. Chaudhuri, Department of Zoology, University of North Bengal and Prof. Mridula Chatterjee, Department of Pediatrics, North Bengal Medical College & Hospital. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

Manoj Lama

(Manoj Lama)

Department of Zoology
University of North Bengal
Raja Rammohunpur, Distt. Darjeeling

Dated: 12-09-2014

UNIVERSITY OF NORTH BENGAL

DEPARTMENT OF ZOOLOGY
DST-FIST & UGC-SAP Sponsored

P.O. North Bengal University,
Raja Rammohunpur, Dt. Darjeeling,
West Bengal, India, PIN - 734 013

CERTIFICATE

This is to certify that the thesis entitled "IMMUNOLOGICAL AND MOLECULAR INVESTIGATIONS OF CHILDHOOD ASTHMA IN THE SUB-HIMALAYAN REGION OF WEST BENGAL, INDIA" is an original investigative study carried out by Mr. Manoj Lama for the award of Doctor of Philosophy (Ph.D.) degree in Science (Zoology) of the University of North Bengal, under our joint supervision. He has carried out the work in the Department of Zoology, University of North Bengal.

He is conversant with techniques and literature cited in the dissertation and carried out the work thoroughly. In character and disposition, Mr. Manoj Lama is fit to submit the thesis.

12/9/14

Co-Supervisor
Prof. Mridula Chatterjee
Professor
Department of Pediatrics
North Bengal Medical College & Hospital

T.K. Chaudhuri
12.9.14

Supervisor
Prof. T.K. Chaudhuri
Professor
Department of Zoology
University of North Bengal

Phone: (0353) 2776353, Fax: (0353) 2699 001, E-mail: zoology_nbu@rediffmail.com, Visit us at: www.nbu.ac.in

PREFACE

I started my research work in 2008 which has been documented in this dissertation entitled "IMMUNOLOGICAL AND MOLECULAR INVESTIGATIONS OF CHILDHOOD ASTHMA IN THE SUB-HIMALAYAN REGION OF WEST BENGAL, INDIA" under the joint supervision of Prof. T. K. Chaudhuri, Department of Zoology, University of North Bengal and Prof. Mridula Chatterjee, Department of Pediatrics, North Bengal Medical College and Hospital, Sushrutnagr, Dist. Darjeeling.

Asthma is a common chronic disorder that is characterized by recurrent symptoms of wheeze, breathlessness, chest tightness, variable airflow limitation and chronic inflammation of the airways. Narrowing of the airway is primarily caused by inflammation, excess mucus production and contraction of smooth muscle surrounding the airways. Due to the chronic nature of asthma the lives of sufferers are affected in a multitude of ways including sleeplessness, daytime fatigue, reduced levels of activity and work and school absenteeism. This can result in life-long detrimental effects including adverse outcomes on early education in children, reduced fitness, weight gain and the inability to concentrate while at work.

The prevalence of asthma in children in this Sub-Himalayan region of West Bengal, India, seems to be high and various environmental triggers may be responsible for this. Therefore, the present case-control study was designed with the broad objectives of understanding the basic immunological and molecular aspects of asthma. The study was conducted in asthmatic and control children of age group 3 to 12 years. Asthma was diagnosed by the physician and the subjects were recruited in the study from the Department of Pediatrics, North Bengal Medical College & Hospital, Sushrutnagar, Siliguri. Blood samples were collected from the participants under appropriate conditions and brought to the Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, where the further experiments were performed. The findings of the study are published in various research journals and are presented and discussed in details in the Results & Discussion part of this dissertation.

ABSTRACT

Asthma is the most common chronic disease of childhood and the leading cause of childhood morbidity as measured by school absences, emergency department visits, and hospitalizations. It is well known that the prevalence of asthma has been reported to increase in many places around the world during the last decades. The increased prevalence of asthma is multifactorial in etiology. Asthma is characterized by airway hyperresponsiveness and inflammation, in which various cells such as eosinophils, neutrophils, macrophages and T-lymphocytes, cytokines and mediators play a role. Beside local inflammation, systemic inflammation is present in asthma, as shown by increased levels of plasma fibrinogen and serum amyloid A. Serum levels of the well-known inflammatory marker C-reactive protein (CRP) can be simply and inexpensively measured in order to assess systemic inflammation. Asthma typically begins in early childhood, with an earlier onset in males than females. Atopy is present in the majority of children with asthma over the age of 3, and allergen-specific sensitization is one of the most important risk factors for the development of asthma. Immunoglobulin (Ig) E has been shown to be a major contributing factor for the development of bronchial hyperresponsiveness in asthma. An elevation in serum IgE levels contributes to asthma and is considered a potent predictor of the development of asthma. Immune and inflammatory responses, mediated by cytokines, play important roles in the pathophysiology of asthma. These responses are associated with over expression of T helper (Th)-2 cytokines, particularly interleukin (IL)-4, IL-5, and IL-13 and decreased expression of Th-1 cytokines, IL-2 and IFN-γ. Asthma is a heterogeneous disease for which a strong genetic basis is firmly established. It is a complex disorder influenced by gene-environment interaction. HLA genes have been shown to be consistently associated with asthma and its related phenotypes in various populations.

The overall objectives of the study were to estimate the prevalence of asthma in children aged between 3-12 years and to investigate the associated risk factors, to determine the serum CRP concentration in asthmatic children to understand the inflammatory process in asthma and to study the effect of corticosteroid on serum CRP level, to estimate the levels of total serum IgE in asthmatic and control subjects and to investigate the relationship of various demographic and clinical characteristics with the level of total serum IgE in asthmatics, to determine the serum levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines in order to investigate the alteration in Th1/Th2

balance in asthma, if any and to determine the frequency of some of the selected HLA class I and class II allelic groups in asthmatic and control groups.

For the prevalence study, we considered children who visited the Out-Patient Department of Pediatrics, North Bengal Medical College and Hospital, from May 2009 to April 2010. Asthma was diagnosed by the physician. The relevant data were collected using the questionnaire. In this hospital-based study, the mean prevalence of asthma among children in the age group between 3 to 12 years was found to be 3.06%. Further analysis of associated risk factors revealed that family history of asthma was significantly associated with asthma (33% versus 15.45% in asthmatics and controls respectively, $p<0.05$). The prevalence rate of childhood asthma in and around Siliguri seems to be comparable to the prevalence rates prevailing in other rural areas of the country as reported by various studies. Results of our study also indicated the association of family history of asthma/atopy with asthma suggesting that genetic predisposition may be an important etiology for the development of asthma.

The latex agglutination test was performed for determining serum CRP concentration among 87 asthmatic children. The limitation of detection of the test was <6mg/L. Among 87 asthmatic children, 15 children were ICS-naïve and 72 were ICS-inhaling. The elevated serum CRP concentration was detected in 13 (86.7%) ICS naïve-children and in only 3 (4.2%) ICS-inhaling children. The CRP concentration was significantly elevated in the serum of ICS-naïve asthmatic children compared to ICS-inhaling asthmatic children ($p< 0.001$). This study suggests that the asthmatic inflammation is associated with the elevation of serum CRP concentration and the ICS, which has the anti-inflammatory properties, might have played a role in reducing the CRP concentration in the ICS-inhaling children to the normal level.

The level of total serum IgE was measured using ELISA kits (AccuBind, Monobind Inc., USA). The absorbance was measured at 450nm in the ELISA plate reader (Bio-Rad). The sensitivity of the IgE AccuBind^{TM} ELISA test system was 1.0 IU/ml with the intra- and inter-assay precisions of 1.95–5.87% and 3.52–8.42%, respectively. The results showed that asthmatic children had significantly elevated level of total serum IgE compared to the control subjects. The levels of total IgE and IL-4 in sera of 44 asthmatic children showed a significant positive correlation. Total serum IgE>150IU/ml was found to be significantly associated with the age, exposure to cigarette smoke, and raised eosinophil count in asthmatic children. In conclusion, the elevated

level of total serum IgE may demonstrate the allergic etiology of asthma in the subjects studied. The higher age group, exposure to cigarette smoke and raised eosinophil count were associated with the elevated level of total serum IgE.

Serum levels of IL-4 and IFN-γ were determined among eighty children (18 steroid-naive, 30 steroid-treated children with asthma and 32 healthy controls) using commercially available ELISA kits (Endogen Human IL kit, Pierce Biotechnology, Inc., Rockford). Absorbance was measured at 450nm in a microtitre plate reader (Opsys MR, Dynex Tehnologies). Serum level of IL-4 was significantly higher in steroid-naive group of asthmatic children compared to the control subjects and was lower in steroid-treated group though the level was statistically not significant. In contrast, serum levels of IFN-γ were significantly lower in both steroid-naive and steroid-treated groups of asthmatic children compared to control subjects. The results of our study suggest that serum level of IL-4 may be elevated in concert with decreased level of IFN-γ in asthma. Determination of serum levels of IL-4 and IFN-γ may be a useful tool for understanding the disease processes in asthma.

Molecular typing of the selected HLA class I and class II allelic groups was performed by polymerase chain reaction using sequence-specific primers (PCR-SSP). The PCR products were electrophoresed in 2% pre-stained agarose gel and the result was interpreted for the presence of a specific band of the HLA allelic group. The results of the present study showed a significantly higher frequency of *HLA-DRB1*03* in asthmatics than in controls (11.43% versus 3.64%, OR=3.78, 95% CI=1.61 – 8.85, p=0.0025, p_{corr}<0.05). Analysis of HLA allelic groups in two groups of asthmatic children with high and low total serum IgE levels revealed no significant association. HLA-DRB1*03 may be implicated in the susceptibility to asthma in the pediatric population.

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Manoj Lama

Dated: 12-09-2014 *(Manoj Lama)*

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ABBREVIATIONS

AHR	:	Airway hyperresponsiveness
ALA	:	American Lung Association
APC	:	Antigen presenting cell
ASM	:	Airway smooth muscle
BAL	:	Bronchoalveolar lavage
BHR	:	Bronchial hyperresponsiveness
BMI	:	Body mass index
CI	:	Confidence interval
CREG	:	Cross reacting group
CRP	:	C-reactive protein
CTL	:	Cytotoxic T lymphocyte
dNTPs	:	Deoxyribonucleotides
EAR	:	Early-phase asthmatic reaction
EBF	:	Exclusive breastfeeding
ELISA	:	Enzyme Linked Immunosorbent Assay
EMTU	:	Epithelial-mesenchymal trophic unit
EpC	:	Epithelial cell
ETS	:	Environmental tobacco smoke
FH	:	Family history
GINA	:	Global Initiative for Asthma
GM-CSF	:	Granulocyte-macrophage colony stimulating factor
GWAS	:	Genome-wide association studies
HLA	:	Human Leukocyte Antigen
hs-CRP	:	High sensitive C-reactive protein

ICS	:	Inhaled corticosteroid
IFN-γ	:	Interferon gamma
IgE	:	Immunoglobulin E
IL-4	:	Interleukin-4
ISAAC	:	International Study of Asthma & Allergy in Childhood
LABAs	:	Long-acting inhaled β2-agonists
LAR	:	Late-phase asthmatic reaction
LPS	:	Lipopolysaccharide
MHC	:	Major histocompatibility complex
MPV	:	Metapneumovirus
NHLBI	:	National Heart, Lung, and Blood Institute
OD	:	Optical density
OPD	:	Out-Patient Department
OR	:	Odds ratio
PBMCs	:	Peripheral blood mononuclear cells
PCR	:	Polymerase chain reaction
PEF	:	Peak expiratory flow
PGs	:	Prostaglandins
SAA	:	Serum amyloid-A
SSP	:	Sequence specific primers
TAP	:	Transporter associated with antigen processing
TLC	:	Total leukocyte count
TNF-α	:	Tumor necrosis factor alpha
TNF-β	:	tumor necrosis factor-beta
TSLP	:	Thymic stromal lymphopoietin

CHAPTER – 1

INTRODUCTION, REVIEW OF LITERATURE & OBJECTIVES OF THE STUDY

1. 1 INTRODUCTION

Asthma is a chronic disorder, characterized by recurrent symptoms of wheeze, breathlessness, chest tightness and cough, often associated with bronchial hyperresponsiveness (BHR), variable airflow limitation and chronic inflammation of the airways (Johansson *et al.,* 2004). It usually begins in childhood, often in association with an inherited susceptibility to produce IgE to common environmental allergens, including house dustmite, animal protein, fungal spores, and pollens (Boushey, 1998). It is estimated that up to 80% of children with asthma may be atopic. Atopy is a personal or familial tendency to become sensitized and produce IgE antibodies in response to common environmental allergen exposures (Johansson *et al.,* 2004). Approximately, 300 million people are affected worldwide causing 250 000 annual deaths by this airway disease (Bateman *et al.,* 2008). Asthma reduces the quality of life for the affected individual, and places a burden on society as a whole due to elevated health care costs and decreased productivity of asthmatic individuals. The burden of asthma is heavy not only for the individual and the family but also for society (Cleemput and Kesteloot, 2002; von Mutius, 2000). Asthma can place considerable limitations on the physical, emotional, social, and professional lives of sufferers, and these may be greater when symptoms are not adequately controlled. Children can become very distressed by their disease, with considerable absences from school and reduced participation in family life.

The cardinal symptom of asthma is recurrent wheezing, but all that wheezes are not asthma and not all asthma wheezes. According to the National Heart, Lung, and Blood Institute (NHLBI) expert panel report, a history of recurrent wheezing, cough, breathlessness, or chest tightness suggests a possible diagnosis of asthma (NHLBI, 1997). Taking a good history of symptoms, precipitating factors, and development of the disease and its prior response to treatment are extremely important in understanding asthma. Several other demographic and environmental risk factors for asthma have been identified. Low socioeconomic status and non-white race have been linked to increased asthma prevalence. Male gender is a risk factor for asthma in early childhood. Viral infections of the lower respiratory tract resulting in wheezing during infancy, in particular respiratory syncytial virus, significantly increase the risk of developing asthma. Exposure to environmental

factors such as tobacco smoke, dust, or cockroaches plays a key role in the development of asthma. Prenatal exposure to environmental tobacco smoke has also been associated with recurrent wheezing and a physician diagnosis of asthma in young children, however, this may represent transient wheezing, as the association does not persist (Taussig *et al.,* 2003; Burrows *et al.,* 1989; Lannero *et al.,* 2006). Weather change is also a commonly reported precipitating factor of the symptoms of asthma.

The diagnosis of asthma in childhood is primarily based on frequency, quality, and severity of symptoms in addition to family history and other allergic co-morbidities. Response to therapy can be especially helpful as a diagnostic tool in younger children where pulmonary function testing can be a challenge. In a school-aged child, the diagnosis of asthma is accomplished by obtaining pertinent information regarding type, frequency, and severity of symptoms in addition to determining the presence of risk factors, such as a parent with asthma or the coexistence of atopic dermatitis. Additionally, airflow limitation that improves following bronchodilator in a child with lower respiratory symptoms strongly supports the diagnosis of asthma. Optimal treatment of asthma requires an understanding of the central concept of asthma control and how this is used to modify treatment. Environmental control and intermittent reliever therapy is all that is necessary for intermittent asthma. Persistent asthma requires regular anti-inflammatory controller therapy. Inadequate adherence and poor technique are more usual causes for treatment failure than incorrect drug selection (Levin and Weinberg, 2011).

It is well known that the prevalence of asthma has been reported to increase in many places around the world during the last decades (Manning *et al.,* 2007). The causes of asthma and why asthma seems to have increased is still not well understood. The increase in asthma prevalence has been suggested in some way to be related to western lifestyle factors, as most often increased prevalence rates are reported from westernized countries (Beasley *et al.,* 2000; Britton, 2003). In the developing countries although the prevalence of childhood asthma is reported to be lower, there is growing evidence to suggest that the prevalence is increasing alarmingly as it did in the western countries over 2-3 decades ago. The International Study of Asthma and Allergy in Childhood (ISAAC) has shown that the prevalence of asthma and atopy in children from affluent countries is higher than in low-

income countries (Asher *et al.,* 2006). The prevalence is variable in different regions and countries in the world. Aït-Khaled *et al.* (2007) described the prevalence of a wide range of atopic disorders throughout Africa. The highest prevalence of current asthma was observed in urban areas with a higher standard of living, but asthma also had a representative prevalence in endemic parasite and tuberculosis zones. In Latin America, the prevalence of asthma and allergic diseases in childhood is similar to that in industrialized countries, although great variability has been found. In a recent survey in Asia, a 16.1% prevalence of wheezing in the previous 12 months was found in rural children from Bangladesh; similar percentages were reported in other developing regions (www.isaac.auckland.ac.nz). In India, an investigation by Jain *et al.* (2010) in a cross sectional community based study on rural Indian children showed the prevalence of bronchial asthma to be 10.3%. Taken all together, the evidence shows the prevalence of asthma is high and is still increasing, mainly in developing countries, although a slightly upward trend has been also shown in high income countries (Pearce *et al.,* 2007).

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells. Jousilahti *et al.* (2002) showed the association of sensitive systemic inflammation markers (CRP, serum amyloid-A [SAA], and plasma fibrinogen) with asthma. That supports a hypothesis of persistent systemic inflammation in asthma in parallel with local inflammation. Several studies have shown the association of asthma with airway inflammation in which eosinophils and mast cells play an important role. The recent studies employing bronchoalveolar lavage in infants and young children have provided the supportive evidence of increased eosinophils in the lavage fluid of allergic asthmatics. Eosinophils are at relatively low levels in infants younger than 30 months. Furthermore, increased neutrophil numbers have been found in lavage fluid in children with asthma of greater persistence and longer duration. Inflammatory changes mainly affect the airway mucous membrane lining. In addition to the inflammatory changes, goblet cells in the mucous membrane produce increased amounts of thick sticky mucus. A third component influencing airway narrowing is smooth muscle contraction of the bronchial wall, leading to bronchospasm and bronchoconstriction. Airway inflammation is

responsible for the characteristic feature of bronchial hyperreactivity, making the child with asthma vulnerable to weather changes, humidity, cold air, mist, non-specific irritants, and exercise-induced bronchospasm (Levin and Weinberg, 2011).

C-reactive protein (CRP) is one of the most characteristic markers of the inflammatory process. The monitoring of CRP levels is a good diagnostic tool and is very useful for the assessment of early inflammation, treatment monitoring and acute-phase diseases (Tall, 2004). In recent years, there have been some reports concerning the measurement of serum levels of hs-CRP as a useful tool for detecting systemic inflammation in asthma (Takemura *et al.,* 2006; Fujita *et al.,* 2007). Several studies have indicated a positive correlation between asthma and increased CRP levels (Jousilahti *et al.,* 2002; Ford, 2003; Olafsdottir *et al.,* 2005).

Allergic diseases including asthma are characterized by an increase of serum IgE levels (Peng, 2009; Rage *et al.,* 2009). IgE plays a central role in the initiation and the propagation of the inflammatory cascade and thus the allergic response (Buhl, 2005). Exposure to environmental factors, particularly inhalant allergens is commonly reported as a precipitant of acute exacerbations of asthma (Bacharier *et al.,* 2003). IgE is implicated in airway inflammation and allergic reactions and may play a role in modulating the severity of asthma, because previous studies have found associations between high IgE levels and asthma severity, airway hyperresponsiveness, and lower baseline lung function (Naqvi *et al.,* 2007).

Cytokines play a critical role in the orchestration of chronic inflammation in many diseases, including asthma. Multiple cytokines and chemokines have been implicated in the pathophysiology of asthma (Barnes *et al.,* 1998; Chung and Barnes, 1999). With airway hyper-responsiveness being the physiological hallmark of asthma, it is also characterized by chronic inflammation of the respiratory tract, allergen-specific IgE production, infiltration of eosinophils, the recruitment of T cells into the airways, and alterations in the fine balance between type 1 helper T lymphocytes (Th1) and type 2 helper T lymphocytes (Th2) responses towards Th2 bias (Larche *et al.,* 2003). Th2 cells secret a panel of cytokines with several overlapping functions including Interleukin (IL)-4,

IL-5, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF). By mediating differentiation of the Th2 subpopulation and eosinophils, as well as modulating B-cell proliferation and IgE switching, the Th2 cytokines are thought to play a prominent role in asthma (Robinson *et al.,* 1992; Wills-Karp and Finkelman, 2008). The sentinel Th1 cytokine, interferon gamma (IFN-γ) and IL-12 reciprocally stimulate their production and function during cell-mediated immunity and development of naive T lymphocytes into Th1 cells. Evidence suggests a contributory role of Th1 cells and their cytokines in asthmatic inflammation and airway hyperresponsiveness (Cooper and Khader, 2007; Kumar *et al.,* 2006).

Asthma and allergy are complex conditions often present in the same family or closely related subjects. Genetic factors undoubtedly contribute to disease susceptibility but the expression of the disease can be modulated by environmental exposures and the interactions between the two. Candidate-gene and linkage studies followed by positional cloning have already provided a large number of susceptibility genes (Vercelli, 2008a). The last decade has been marked by the publication of more than 20 genome-wide association studies (GWASs) in asthma or allergy phenotypes. GWASs have reported novel and interesting genes but have also confirmed the role of some functionally relevant genes previously described. However, heritability of allergic diseases has not been elucidated completely so far (Kabesch, 2010).

The HLA genes map on chromosome 6p21 play an important role in the regulation of the immune system (Shiina *et al.,* 2004). Many studies have documented that 6p21 region is strongly linked to atopic phenotype and asthma and it is considered a major locus influencing allergic diseases (Cookson, 2004; Moffatt *et al.,* 2003; Hakonarson and Wjst, 2001). Numerous studies have investigated the association of HLA alleles and/or haplotypes with asthma. Some of the earlier studies have reported the association of various HLA class I alleles/haplotypes (Turton *et al*., 1979; Morris et al., 1980; Huang *et al*., 1981; Bondarenko *et al*., 1991; Blumenthal *et al*., 1992; Kim *et al*., 2006), while large number of studies have investigated the association of HLA class II alleles/haplotypes (Soriano *et al*., 1997; Lara-Marquez *et al*., 1999; Guo *et al*, 2001; Woszczek *et al*., 2002;

Torío *et al*., 2003; Movahedi *et al*., 2008; Hanchard *et al*., 2010) with childhood asthma in different populations.

Childhood asthma was associated with the *HLA-DP* locus (*HLA-DPA1* and *HLA-DPB1*) in Japanese and Korean populations (Noguchi *et al.,* 2011). In that study, modest associations were shown for the 17q21 locus containing *ORMDL3/GSDMB/GSDMA* and 5q31 (*IL5/RAD50/IL13*), whereas there were no associations with *PDE4D, DENND1B, IL18R1*, and *IL2RB* (Binia and Kabesch, 2012). GWAS in Asian populations also confirmed genetic heterogeneity between children and adults. The largest GWAS so far published on an Asian population identified the most significant associations between adult asthma and the major histocompatibility complex region (Hirota *et al.,* 2011).

1.2. REVIEW OF LITERATURE

1.2.1 Definition of asthma

Asthma is a common chronic disorder of the airways that is complex and characterized by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation. The interaction of these features determines the clinical manifestation and severity of asthma and the response to treatment (NHLBI, 2007).

1.2.2 Childhood Asthma

Asthma is the most common chronic disease of childhood and the leading cause of childhood morbidity from chronic disease as measured by school absences, emergency department visits, and hospitalizations (Masoli *et al.,* 2004). Asthma typically begins in early childhood, with an earlier onset in males than females (Bisgaard and Szefler, 2007; Kuehni *et al.,* 2007). Atopy is present in the majority of children with asthma over the age of 3, and allergen-specific sensitization is one of the most important risk factors for the development of asthma (Sly *et al.,* 2008).

1.2.3 Symptoms of asthma

Wheeze, Cough, breathlessness (typically manifested by patterns of activity limitation) and nocturnal symptoms/awakenings are the common symptoms of asthma.

1.2.3.1 Wheeze

Wheeze is the most common symptom associated with asthma in children. It has been strictly defined as a continuous high-pitched sound, sometimes with musical quality, emitting from the chest during expiration (Elphick *et al.,* 2001). Wheezing occurs in several different patterns but a wheeze that occurs recurrently, during sleep, or with triggers such as activity, laughing, or crying is consistent with a diagnosis of asthma.

1.2.3.2 Cough

Cough due to asthma is recurrent and/or persistent, and is usually accompanied by some wheezing episodes and breathing difficulties. Nocturnal cough (occurring when the child is asleep) or cough occurring with exercise, laughing, or crying in the absence of an apparent respiratory infection, strongly supports a diagnosis of asthma.

1.2.3.3 Breathlessness

Breathlessness that occurs during exercise and is recurrent increases the likelihood of the presentation being due to asthma. In infants and toddlers, crying and laughing are an exercise equivalent (GINA, 2009).

1.2.4 Risk Factors of Asthma in Children

The risk factors for the development of asthma in children can be divided as allergic and nonallergic environmental triggers.

1.2.4.1 Allergic Environmental Triggers

The allergic environmental triggers for the development of asthma in children include, house dust mites, Cockroaches, animal allergens and indoor fungi.

1.2.4.1.1 House Dust Mites

The house dust mites are the predominant indoor allergens. Their bodies and feces are the sources of indoor allergen. House dust mites infest fabrics, including mattresses, bedding, rugs, upholstered furniture, and carpets (Arlian and Platts-Mills, 2001). It is estimated that it takes 100 mites per gram of dust to produce sensitivity and 500 per gram of dust to cause wheezing. 50% of perennial asthma is due to dust mites (Paramesh, 2002).

1.2.4.1.2 Cockroaches

Exposure to cockroach allergen in the living quarters is associated with the development of sensitization, and sensitization to cockroach allergen is associated with an increased risk of developing asthma (Morgan *et al.,* 2004).

1.2.4.1.3 Companion Animal Allergens

The relationship between exposure and sensitization to allergens from companion animals is not clear, and there are insufficient data to recommend for, or against, the presence of a pet in the home unless the child has become sensitized to the pet species (Bufford and Gern, 2007; Ownby *et al.,* 2002; Platts-Mills *et al.,* 2005; Platts-Mills *et al.,* 2001).

1.2.4.1.4 Fungi

Sensitization to *Alternaria* is a major risk factor not only for the development of asthma in children, but also for its severity (O'Hollaren *et al.,* 1991; Salo *et al.,* 2006).

1.2.4.2 Nonallergic Environmental triggers

Nonallergic triggers of asthma exacerbations in children affect both atopic and non-atopic children. The triggers having the most concern for children: environmental tobacco smoke (ETS), viral infections, endotoxin, pollutants and microbes and their products are discussed here along with the other two risk factors viz. maternal diet during pregnancy and/or lactation and the psychosocial environment.

1.2.4.2.1 Environmental Tobacco Smoke (ETS)

ETS is a common indoor exposure, which can be assessed by measuring cotinine, a metabolite of nicotine, in urine or saliva. Thus, this exposure is unique in that there is a feasible, inexpensive means of measuring personal exposure over time. According to an Institute of Medicine report, smoking in the home is causally related to exacerbations of asthma in preschool aged children (Stark *et al.,* 2003). ETS is associated with asthma in older children also (Johnston *et al.,* 2000).

1.2.4.2.2 Viral Infections

Viruses are the most important cause of acute infection-induced wheezing in infants and children (Apter, 2003; Stein *et al.,* 1999). Children with severe viral respiratory infections, particularly RSV infections, are at risk for the development of asthma (Apter, 2003; Castro-Rodriguez *et al.,* 1999).

1.2.4.2.3 Endotoxin

Endotoxin, lipopolysaccharide (LPS), is a major component of the outer membrane of gram-negative bacteria. Exposure to endotoxin in infancy is theorized to be protective of the development of allergy and asthma (Gereda *et al.,* 2001; Gehring *et al.,* 2002). In light of endotoxin's Th-1-inducing activity, this theory is consistent with the Hygiene Hypothesis. However, exposure later in life is proposed to increase acute and chronic inflammation (Braun-Fahrlander *et al.,* 2002).

1.2.4.2.4 Pollutants

The pollutants cause the oxidative stress, airway inflammation and asthma in those who are genetically susceptible to oxidant stress exposures in addition to causing the direct toxicity on the lungs (Gauderman *et al.,* 2005; Millstein *et al.,* 2004). The effect of air pollution caused by traffic or industry on pediatric asthma has been extensively studied (Hirsch *et al.,* 1999; D'Amato *et al.,* 2005).

1.2.4.2.5 Microbes and their products

The impact of bacterial products and their relationship to the development of asthma is increasingly a focus of interest and forms part of the so called "hygiene hypothesis". Exposure to farming environment in early life has been associated with a reduced risk of asthma and allergy in children compared to those who have not grown up on a farm (Braun-Fahrlander *et al.,* 2002; von Mutius and Radon, 2008). Wheezing in early childhood is predominantly linked to viral infections, especially those due to rhinovirus, respiratory synctial virus (RSV), Boca virus, and metapneumovirus (MPV) (Heymann *et al.,* 2005; Jackson *et al.,* 2008; Lee *et al.,* 2007).

1.2.4.2.6 Maternal Diet during Pregnancy and/or Lactation

There are insufficient data to support a protective effect of any dietary intervention during pregnancy or lactation in preventing asthma atopic disease (Greer *et al.,* 2008; Kramer and Kakuma, 2006). Although breastfeeding decreases early childhood wheezing associated with upper and lower respiratory infections, there is little evidence that breastfeeding

prevents development of persistent asthma (Gdalevich *et al.,* 2001; Sears *et al.,* 2002; Takemura *et al.* 2001; Wright *et al.,* 2001).

1.2.4.2.7 Psychosocial Factors

A child's social environment may play a role in the development and severity of asthma (Chen *et al.,* 2004; Wright *et al.,* 2002). Stress in family or other primary caregivers during the first year of life is associated with an atopic profile and wheeze in infants, and is also associated with asthma at age 6 to 8 years (Wright *et al.,* 2005).

Besides, various other risk factors are responsible for the development of asthma. Children born by Cesarean section have a higher risk of asthma than those born by vaginal delivery (Tollanes *et al.,* 2008), particularly children of allergic parents (Roduit *et al.,* 2009). Paracetamol (acetaminophen) use during pregnancy (Rebordosa *et al.,* 2008) and for fever in the child's first year of life (Beasley *et al.,* 2008) has been associated with increased prevalence of asthma in children.

1.2.5 Diagnosis of asthma in children

The diagnosis of asthma is challenging in preschool children for many reasons. There are no specific diagnostic tools or surrogate markers for detecting asthma in infancy. Many preschool children with wheezing will not persist to be diagnosed with asthma. Large birth cohorts have shown that approximately 50% of preschool children with recurrent wheezing episodes will have only transient wheezing of childhood (Martinez *et al.,* 1995). The diagnostic evaluations using spirometry, exhaled nitric oxide, and sputum samples are not feasible in the preschool children.

Therefore, the diagnosis of asthma in young children can be done based on symptom patterns and on a careful clinical assessment of family history and physical findings. The presence of atopy or allergic sensitization provides additional predictive support, as early allergic sensitization increases the likelihood that a wheezing child will have asthma (Sly *et al.,* 2008).

1.2.5.1 Clinical History

A clinical diagnosis of asthma is often prompted by symptoms such as episodic breathlessness, wheezing, cough, and chest tightness (Levy *et al.,* 2006). For young children having a history of recurrent respiratory symptoms, a strong family history of asthma in first degree relatives (especially the mother), and/or atopy presenting as atopic dermatitis, food allergy, and/or allergic rhinitis also make a diagnosis of asthma more likely.

1.2.5.2 Therapeutic trial

A trial of treatment with short-acting bronchodilators and inhaled glucocorticosteroids for at least 8 to 12 weeks may provide some guidance as to the presence of asthma. Marked clinical improvement during the treatment and deterioration when it is stopped supports a diagnosis of asthma (GINA, 2009).

1.2.5.3 Test for IgE-mediated Allergy (Atopy)

It has been shown that allergic sensitization is the major risk factor for the development of asthma and for its persistence and severity (Illi *et al.,* 2006; Sears *et al.,* 2003). Sensitization to allergens can be assessed using either immediate hypersensitivity skin testing or an *in vitro* method that detects antigen-specific IgE antibody.

1.2.5.4 Chest Radiograph (X-ray)

A plain chest radiograph may help to exclude structural abnormalities of the airway (congenital malformations such as congenital lobar emphysema, vascular ring), chronic infection (e.g. tuberculosis), or other diagnoses. Radiographic studies such as chest X-rays are often performed in children with suspected asthma mainly to rule out other causes of cough or wheeze and have little diagnostic utility (Spahn *et al.,* 2009).

1.2.5.5 Lung Function Testing

The diagnosis of asthma is usually based on the presence of characteristic symptoms. However, measurements of lung function, and particularly the demonstration of reversibility of lung function abnormalities, greatly enhance diagnostic confidence. This is because patients with asthma frequently have poor recognition of their symptoms and poor perception of symptom severity, especially if their asthma is long-standing (Killian *et al.,* 2000).

1.2.5.5.1 Peak Expiratory Flow

The peak expiratory flow (PEF) is the maximum flow obtained within the first 200 milliseconds of a forced expiratory maneuver after inhalation to total lung capacity (TLC). Peak expiratory flow measurements are made using a peak flow meter and can be an important aid in both diagnosis and monitoring of asthma. Modern PEF meters are relatively inexpensive, portable, plastic, and ideal for patients to use in home settings for day-to-day objective measurement of airfow limitation. However, measurements of PEF are not interchangeable with other measurements of lung function such as FEV_1 in either adults (Sawyer *et al.,* 1998) or children (Eid *et al.,* 2000).

1.2.5.5.2 Spirometry

Spirometry is the recommended method of measuring airflow limitation and reversibility to establish a diagnosis of asthma. Measurements of FEV_1 and FVC are undertaken during a forced expiratory maneuver using a spirometer. The degree of reversibility in FEV_1 which indicates a diagnosis of asthma is generally accepted as 12% and 200 ml from the pre-bronchodilator value (Pellegrino *et al.,* 2005). Spirometry is reproducible, but effort-dependent. Therefore, proper instructions on how to perform the forced expiratory maneuver must be given to patients, and the best of three recordings should be considered.

1.2.6 Treatment of Asthma

For all patients with a confirmed diagnosis of asthma, the goal of treatment is to achieve control of the clinical manifestations of the disease and maintain this control for prolonged

periods. Medications currently available for childhood asthma include: reliever medications (Short-acting inhaled β2-agonists and other bronchodilators) and controller medications (ICS, LTRA, LABAs, Sustained-release theophylline, Cromolyn sodium, Oral steroids and Anti-IgE antibodies).

1.2.6.1 Reliever medications

1.2.6.1.1 Short-acting β2 agonists

Rapid-acting inhaled β2-agonists are the most effective bronchodilators available and therefore the preferred reliever treatment for asthma in children of 5 years and younger. An MDI with spacer is, in the most cases, an effective way for delivering reliever therapy (Castro-Rodriguez and Rodrigo, 2004; Cates *et al.,* 2006). When delivery is not optimal because of lack of cooperation or distress, or when the child is hypoxic, nebulizer therapy is also an option. Oral therapy is not recommended due to its slower onset of action and its tendency to produce more side effects. The safety margin for dose range is wide and determination of the optimal dose can be difficult. The lowest effective dose that provides adequate clinical control and minimizes side-effects, such as tachycardia, dizziness and jitteriness, is recommended. Salbutamol, the most commonly used drug, has a favorable safety and efficacy profile in patients aged 2–5 years (Skoner *et al.,* 2005). Terbutaline and formoterol also have safety and efficacy profiles comparable to that of salbutamol.

1.2.6.1.2 Ipratropium bromide

The only other reliever of any relevance is Ipratropium bromide. In acute asthma its combined use with β2-agonists may result in favorable outcomes in children (Rodrigo and Castro-Rodriguez, 2005), although results were ambiguous in those less than 2 years of age (Everard *et al.,* 2005).

1.2.6.2 Regular controller therapy

The main goal of regular controller therapy should be to reduce bronchial inflammation.

1.2.6.2.1 Inhaled corticosteroid (ICS)

Inhaled corticosteroid (ICS) is a first-line treatment for persistent asthma. It reduces the frequency and severity of exacerbations and should be introduced as initial maintenance treatment (200µg BDP equivalent) when the patient has inadequate asthma control. Atopy and poor lung function predict a favorable response to ICS (Szefler *et al.,* 2005). If control is inadequate on a low dose after 1–2 months, reasons for poor control should be identified. If indicated, an increased ICS dose or additional therapy with LTRAs or LABAs should be considered. A low-dose inhaled glucocorticosteroid is recommended as the preferred initial treatment to control asthma in children 5 years and younger (Guilbert *et al.,* 2004; Szefler *et al.,* 2007).

Table 1. Low Daily Doses[*] of Inhaled Glucocorticosteroids for Children 5 Years & Younger

Drug	**Low Daily Dose (µg)**
Beclomethasone dipropionate	100
Budesonide MDI+Spacer Budesonide nebulized	200 500
[†]Ciclesonide	NS
Fluticasone propionate	100
[†]Mometasone furoate	NS
[†]Triamcinolone acetonide	NS

[*]A low daily dose is defined as the dose which has not been associated with clinically adverse effects in trials including measures of safety.
[†]NS = Not studied in this age group. *(Table adapted from GINA, 2009)*

1.2.6.2.2 Leukotriene Receptor Agonist (LTRA)

It is an alternative first-line treatment for persistent asthma. Evidence supports use of oral montelukast as an initial controller therapy for mild asthma in children (Knorr *et al.,* 2001), as it provides bronchoprotection, and reduces airway inflammation as measured by nitric oxide levels in some preschool children with allergic asthma (Straub *et al.,* 2005). It is a therapy of choice for those who cannot or will not use ICS. LTRA is suggested as

treatment for viral-induced wheeze and to reduce the frequency of exacerbations in young children aged 2–5 years (Bisgaard *et al.,* 2005).

1.2.6.2.3 Long-acting inhaled β2-agonists (LABAs)

Long-acting inhaled β2-agonists (LABAs) are bronchodilators, but as long-term therapy for asthma they are usually prescribed in combination with an inhaled glucocorticosteroid and are therefore considered controller medications. Efficacy is not well documented in children in contrast to adults, and use should be evaluated carefully (Verberne *et al.,* 1998; Sorkness *et al.,* 2007). Combination products of LABA and ICS may be licensed for use in children over 4–5 years, however, the effect of LABAs or combination products has not yet been adequately studied in children of 5 years and younger. Formoterol and salmeterol have shown long-lasting bronchodilatory and bronchoprotective in this age group (Nielsen and Bisgaard, 2001).

1.2.6.2.4 Oral theophylline

Theophylline is inexpensive, and in some countries, it is used for children whose families cannot afford ICS, LTRAs, or LABAs. There is anecdotal evidence that low-dose theophylline may be of benefit in select groups of children who remain uncontrolled on ICS, LTRAs or LABAs. Although a few studies in children 5 years and younger suggest clinical benefit from regular use of theophylline, the effects are small and mostly non-significant (Seddon *et al.,* 2006).

1.2.6.2.5 Cromolyn sodium (nedocromil)

Cromolyn sodium can be prescribed for children as young as 2 years of age. It is less effective than ICS. It must be used frequently (four times per day), and may take up to 4 weeks to work (Guevara *et al.,* 2006). It is free of side-effects. Cromolyn sodium is available as oral or nasal inhalers, nebulizer solution, and eye drops. In a Cochrane review, it has been shown that the cromolyn therapy has no beneficial effect on the preschool children (van der Wouden *et al.,* 2003).

1.2.6.2.6 Anti-IgE antibodies

Patients aged ≤12 years may benefit if they have moderate-to-severe persistent atopic asthma that is inadequately controlled despite treatment with other therapies (Walker *et al.,* 2006). Mode of application and cost will limit this intervention to patients who fail to respond to currently available therapies.

1.2.6.2.7 Oral glucocorticosteroids

Because of the side effects associated with prolonged use, oral glucocorticosteroids in young children with asthma should be restricted to the treatment of acute severe exacerbations. If used, oral glucocorticosteroids (syrup or tablets) are preferred to systemic (intramuscular or intravenous) administration, but are most effective when administered early in an exacerbation. A dose equivalent to prednisolone 1-2mg/kg/day, with a maximum of 20 mg in children under 2 years of age and 30 mg for children 2-5 years, is recommended. A 3-5 day course is sufficient in most children and can be stopped abruptly (GINA, 2009).

1.2.7 Pathophysiology of asthma

Asthma is an inflammatory disorder of the conducting airways which undergo distinct structural and functional changes, leading to non-specific BHR (bronchial hyperresponsiveness) and airflow obstruction that fluctuates over time (Holgate *et al.* 2010). The development of clinical asthma results from a complex biologic interaction between multiple gene products (one or more containing genetic variations that enhance susceptibility) and at least one environmental toxin (Los *et al.,* 1999; Cookson, 1999).

1.2.7.1 Airway inflammation

Inflammation has a central role in the pathophysiology of asthma. As noted in the definition of asthma, airway inflammation involves an interaction of many cell types and multiple mediators with the airways that eventually results in the characteristic

pathophysiological features of the disease: bronchial inflammation and airflow limitation that result in recurrent episodes of cough, wheeze, and shortness of breath (Figure 1). This allergic inflammatory response is characterized by an infiltration with eosinophils and resembles the inflammatory process mounted in response to parasitic and worm infections. The inflammatory response not only provides an acute defense against injury, but is also involved in healing and restoration of normal function after tissue damage as a result of infection of toxins. In asthma, the inflammatory response is activated inappropriately and is harmful rather than beneficial (Barnes, 2003).

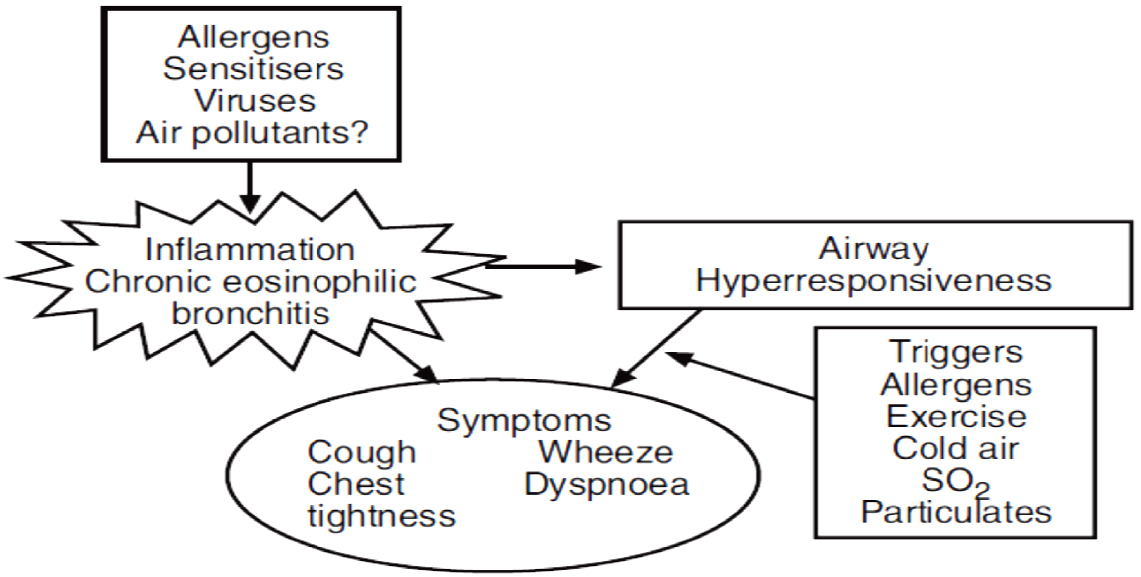

Figure 1. Inflammation in the airways of asthmatic patients leads to airway hyperresponsiveness and symptoms. (*Figure taken from Barnes, 2003).*

1.2.7.2 Bronchial hyperreactivity

Airway inflammation also leads to bronchial hyperresponsiveness, described as excess airway narrowing in response to stimuli. Depending on the degree of inflammation, the airways can close. The more severe the asthma, the more hyperreactive the airways. The ultimate result and significance is the degree of airflow obstruction resulting from trigger exposure (Conboy-Ellis, 2006). BHR is a fundamental abnormality in asthma which increases in proportion to disease severity and is functionally antagonized by β2-

adrenoceptor agonists. The mechanisms underlying BHR are still not known for certain, but an increase in airway smooth muscle alterations to its physicochemical properties (An and Fredberg, 2007) and mast cell infiltration (Begueret *et al.,* 2007) are considered important.

1.2.7.3 Airflow obstruction

Bronchospasm, edema, and mucus hypersecretion lead to airflow obstruction, but it is often reversible. Variable airflow obstruction is demonstrated by measuring forced expiratory volume (FEV1), peak expiratory flow (PEF), or hyperresponsiveness to methacholine challenge (Boulet *et al.,* 1999). However, as the disease becomes more persistent and inflammation more progressive, other factors further limit airflow (Figure 2). These include edema, inflammation, mucus hypersecretion and the formation of inspissated mucus plugs, as well as structural changes including hypertrophy and hyperplasia of the airway smooth muscle. These latter changes may not respond to usual treatment.

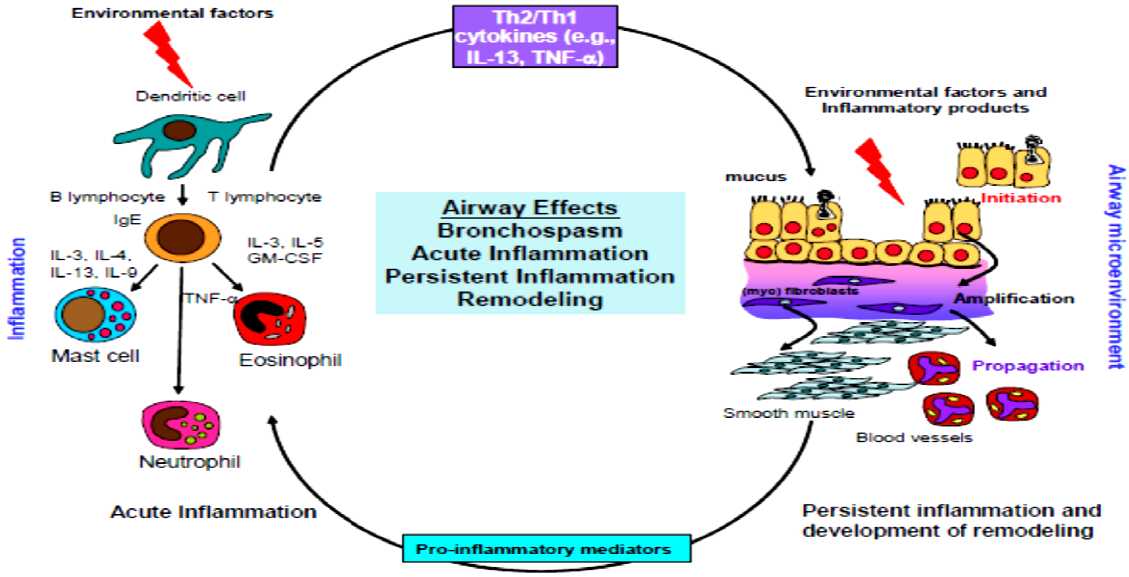

Figure 2. Factors limiting airflow in acute and persistent asthma. Key: GM-CSF, granulocyte-macrophage colony-stimulating factor; IgE, immunoglobulin E; IL-3, interleukin 3 and TNF-α, tumor necrosis factor-alpha. (*Taken from: Holgate & Polosa, 2006).*

1.2.7.4 Airway Remodeling

Airway remodeling involves an activation of many of the structural cells, with consequent permanent changes in the airway that increase airflow obstruction and airway responsiveness and render the patient less responsive to therapy (Holgate and Polosa 2006). These structural changes can include thickening of the sub-basement membrane, subepithelial fibrosis, airway smooth muscle hypertrophy and hyperplasia, blood vessel proliferation and dilation, and mucous gland hyperplasia and hypersecretion. On the basis of a large number of converging observations, it is suggested that in asthma a structurally and functionally defective lower airways epithelium underlies abnormal responses to the inhaled environment leading to enhanced signalling between the airway epithelium and underlying structural (the epithelial–mesenchymal trophic unit, EMTU) and immune cells. This would promote a microenvironment that facilitates allergic sensitization, supports different types of inflammation and predisposes the airways to exacerbations leading to persistence of asthma during childhood (Holgate *et al.,* 2010). Activation of the EMTU might also be responsible for driving tissue remodelling that progressively leads to a loss of reversibility, reduced lung function and refractoriness to treatment in adults (Figure 3).

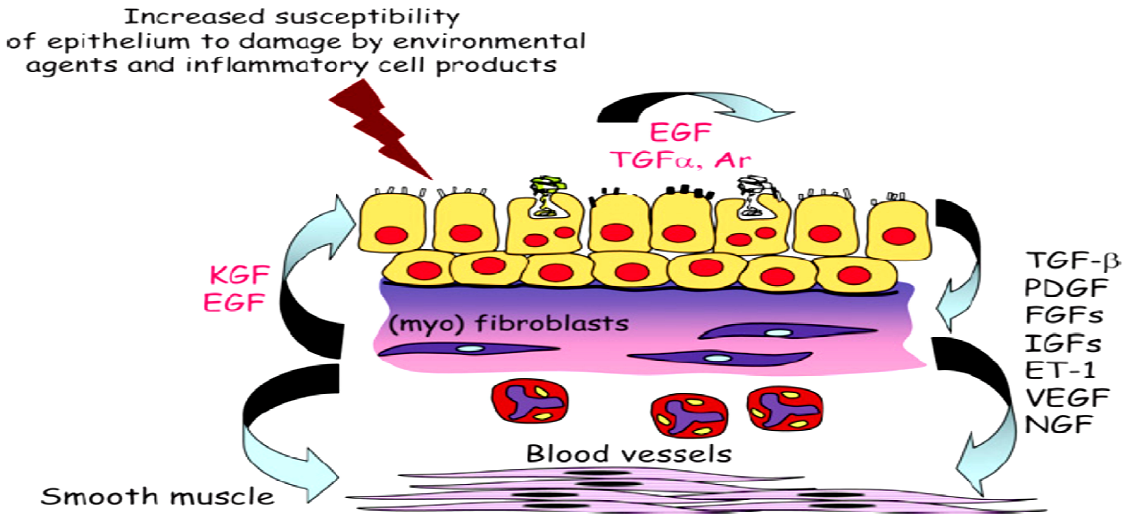

Figure 3. Chronic asthma is characterized by enhanced epithelial–mesenchymal communication with the release of a range of different growth factors linked to remodeling. *(Taken from: Holgate et al., 2010).* Key: Ar, amphiregulin; EGF, epidermal growth factor; ET-1, endothelin-1; FGF, fibroblast growth factor; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; PDGF, platelet-derived growth factor; NGF, nerve growth factor; VEGF, vascular endothelial growth factor.

1.2.7.5 Effector Cells of Inflammation and Remodeling in Asthma

Normally there is a fine balance between immune cells, the epithelium, and the host immune response in the healthy human airway. Airway inflammation in asthma reflects a distortion of this balance and is orchestrated through complex interplay between multiple effector and target components (Murphy and O'Byrne, 2010).

1.2.7.5.1 Mast Cells

Mast cells are critical in mediating the acute response in asthma. While classically, mast cell activation occurs following the binding of antigens to FcεRI-bound, antigen-specific IgE, they may also be activated through other mechanisms, including stimulation of complement receptors, FcγRI, and via TLRs (Nigo *et al.,* 2006). Activated mucosal mast cells release bronchoconstrictor mediators (histamine, cysteinyl-leukotrienes, prostaglandin D2) (Robinson, 2004). Mast cells also can release a large number of cytokines to change the airway environment and promote inflammation even though exposure to allergens is limited. Mast cells are generally considered proinflammatory and mediators of tissue destruction, they may conversely help limit airway damage (Kalesnikoff and Galli, 2008).

1.2.7.5.2 Basophils

Basophils have a crucial role in initiating allergic inflammation through the binding of antigen-specific IgE antibodies at the FcεRI (Obata *et al.,* 2007). Using a basophil-specific marker a small increase in basophils has been documented in the airways of asthmatic patients, with an increased number after allergen challenge. However, these cells are far outnumbered by eosinophils (approximately 10:1 ratio) and their functional role is unknown (Macfarlane *et al.,* 2000).

1.2.7.5.3 Eosinophils

Increased numbers of eosinophils exist in the airways of most, but not all, persons who have asthma (Chu and Martin, 2001; Williams, 2004). These cells contain inflammatory

enzymes, generate leukotrienes, and express a wide variety of pro-inflammatory cytokines. Increases in eosinophils often correlate with greater asthma severity. Increased numbers of eosinophils present in the airways release basic proteins that may damage airway epithelial cells. Eosinophils may also have a role in the release of growth factors and airway remodeling (Larche *et al.,* 2003).

1.2.7.5.4 Neutrophils

Neutrophils are increased in the airways and sputum of persons who have severe asthma, during acute exacerbations, and in the presence of smoking. Inhaled corticosteroids reduce airway eosinophils, but increase airway neutrophils and increase the expression of the neutrophil chemoattractant IL-8, which is associated with loss of asthma control (Maneechotesuwan *et al.,* 2007).

1.2.7.5.5 T lymphocytes

Increased numbers of T lymphocytes in the airways release specific cytokines, including IL-4, IL-5, IL-9, and IL-13, that orchestrate eosinophilic inflammation and IgE production by B lymphocytes (Akbari *et al.,* 2006). An increase in Th2 cell activity may be due in part to a reduction in regulatory T cells that normally inhibit Th2 cells.

1.2.7.5.6 Dendritic Cells

These cells function as key antigen-presenting cells that interact with allergens from the airway surface and then migrate to regional lymph nodes to interact with regulatory cells and ultimately to stimulate Th2 cell production from naïve T cells (Kuipers and Lambrecht, 2004).

1.2.7.5.7 Platelets

There is some evidence for the involvement of platelets in the pathophysiology of allergic diseases. After allergen challenge there is a significant fall in circulating platelets (Sullivan *et al.,* 2000) and circulating platelets from patients with asthma show evidence of increased activation and release the chemokine RANTES (Moritani *et al.,* 1998).

1.2.7.5.8 Macrophages

Macrophages are the most numerous cells in the airways and they can be activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines that amplify the inflammatory response (Peters-Golden, 2004). Macrophages may both increase and decrease inflammation, depending on the stimulus. Alveolar macrophages normally have a suppressive effect on lymphocyte function, but this may be impaired in asthma after allergen exposure (Spiteri *et al.,* 1994). One anti-inflammatory protein secreted by macrophages is IL-10 and its secretion is reduced in alveolar macrophages from patients with asthma (John *et al.,* 1998). Macrophages from normal subjects also inhibit the secretion of IL-5 from T-lymphocytes, probably via the release of IL-12, but this is defective in patients with allergic asthma (Tang *et al.,* 2001). Macrophages may therefore play an important anti-inflammatory role, by preventing the development of allergic inflammation.

1.2.7.5.9 Epithelial cells

Airway epithelium is another airway lining cell critically involved in asthma (Polito and Proud, 1998). The generation of inflammatory mediators, recruitment and activation of inflammatory cells, and infection by respiratory viruses can cause epithelial cells to produce more inflammatory mediators or to injure the epithelium itself. The repair process, following injury to the epithelium, may be abnormal in asthma, thus furthering the obstructive lesions that occur in asthma.

1.2.8 Prevalence of Childhood Asthma

International Study of Asthma and Allergies in Childhood (ISSAC) is a systematic approach to recording the worldwide prevalence of pediatric asthma. Key findings from ISAAC Phase One (1994–1996) included large variations in the worldwide prevalence of symptoms of asthma which were found even among genetically similar populations (ISAAC Steering Committee, 1998a; ISAAC Steering Committee, 1998b) suggesting that environmental factors play an important role. Further study of the global prevalence and

severity of asthma symptoms was undertaken in ISAAC Phase Three, conducted between 2000 and 2003, involving 798,685 adolescents from 233 centres in 97 countries, and 388,811 children from144 centres in 61 countries (Lai *et al.,* 2009). As in ISAAC Phase One, wide variations in prevalence were found around the world. The prevalence of wheeze in the past 12 months in adolescents varied from 32.6% in Wellington (New Zealand) to 0.8% in Tibet (China), and in children from 37.6% in Costa Rica to 2.4% in Jodhpur (India).The prevalence of symptoms of severe asthma (defined as ≥4 attacks of wheeze, or ≥1 night per week sleep disturbance from wheeze, or wheeze affecting speech in the past 12 months) varied from 16% in Costa Rica to 0.1% in Pune (India) in adolescents, and from 20.3% to 0% in the same two centres in children (Asher, 2010).

The prevalence of asthma is increasing in both the developed and developing countries of the world. Prevalences are high (>10%) in developed countries and also increasing in developing regions as they become more westernized. The highest asthma prevalences are found in the United Kingdom (>15%), New Zealand (15.1%), Australia (14.7%), the Republic of Ireland (14.6%), Canada (14.1%), and the United States (10.9%) (Masoli *et al.,* 2004).

In developing regions (Africa, Central and South America, Asia, and the Pacific), asthma prevalence continues to rise sharply with increasing urbanization and westernization. High prevalences have been reported in Peru (13.0%), Costa Rica (11.9%), and Brazil (11.4%). In Africa, asthma prevalence is highest in South Africa (8.1%), perhaps the most westernized of the African countries (Masoli *et al.,* 2004).

In India, large numbers of studies have reported the varying rates of asthma prevalence in pediatric population. A study has reported a wide variation (4-19%) in the prevalence of asthma in school-going children from different geographic regions of India (ISAAC Steering Committee, 1998a). A recent study reported the prevalence of bronchial asthma to be 10.3% in rural Indian children (Jain *et al.,* 2010). Another study on school-going children in Lucknow showed the prevalence of asthma to be 2.3% in age the group of 6-7 years and 3.3% in age group of 13-14 years (Awasthi *et al.,* 2004).

1.2.9 Economic Burden of Asthma

The economic burden of pediatric asthma may be divided into the direct costs and indirect costs of care. Data from the National Heart, Lung and Blood Institute (NHLBI) and the American Lung Association (ALA) estimated the total direct cost of asthma in 2010 at more than $15 billion, with prescriptions accounting for more than one-third of expenditures. Indirect costs from lives lost and lost productivity exceed $5 billion, with total costs related to asthma recently reaching $20.7 billion (American Lung Association, 2010). In addition, Kamble & Bharmal calculated total expenditures per person with asthma, estimating an annual cost to treat the disease of $1,004.60 per child (Kamble and Bharmal, 2009).

1.2.10 Social Impact of Asthma

Asthma attacks and exacerbations pose strain on the health care system and affect school and job performance. Because of the chronic nature of asthma the lives of sufferers are affected in a multitude of ways including sleeplessness, daytime fatigue, reduced levels of activity and work and school absenteeism. This can result in life-long detrimental effects including adverse outcomes on early education in children, reduced fitness, weight gain and the inability to concentrate while at work. A study of caregivers of children with asthma in France indicated that during a 12-month period 30% of caregivers missed work overall and 13% missed more than 5 days of work because of their child's asthma (Laforest *et al.,* 2004).

1.2.11 C-Reactive Protein (CRP)

C-reactive protein is considered as the prototypic marker of inflammation in humans and a member of a highly conserved family of proteins called the pentraxins (Figure 4). The human *crp* gene is located on chromosome 1q23 (Walsh *et al.,* 1996) and consists of two exons and one intron (Lei *et al.,* 1985; Woo *et al.,* 1985). CRP is synthesized as a 206 amino acid polypeptide and secreted by hepatocytes as an approximately 23 kDa, non-

glycosylated monomer, which non-covalently associates to form the homopentameric ring structure characteristic of pentraxin family members (Thompson *et al.,* 1999).

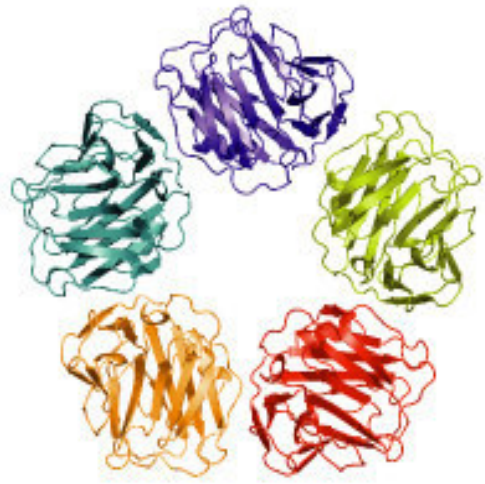

Figure 4. Three-dimensional structure of human C-Reactive Protein (CRP). CRP is synthesized as a 206 amino acid polypeptide that folds to form a flattened jellyroll structure, which then assembles into a radially symmetrical pentamer. *(Taken from: Thompson et al., 1999).*

C-reactive protein (CRP) is an acute-phase reactant secreted in response to circulating inflammatory cytokines. Interleukin-6 plays a critical role in CRP induction (Castell *et al.,* 1990). CRP was initially described in 1930 by Tillet and Francis as the serum factor responsible for the precipitation of acute phase sera with the C-substance (C-polysaccharide, CPS) of pneumococcal cell walls. Although CRP is structurally distinct from the immunoglobulins, it shares with them the ability to initiate several biological functions including precipitation (Tillet & Francis, 1930), opsonization (Ganrot & Kindmark, 1969), capsular swelling (Hedlund, 1961) and agglutination (Patterson & Higginbotham, 1965). Two major biological activities of CRP have been well defined: first, it is able to bind several biological substrates that are distributed widely in nature (Gotschlich *et al.,* 1982). Second, it has significant activation capabilities, in particular to activate the complement system (Kaplan & Volanakis, 1974) and to bind to and modulate the function of phagocytic leukocytes (Kindmark, 1971). These effects support the concept that this serum protein may have a potentially central role in host defense mechanisms.

CRP has now emerged as a circulating marker for cardiovascular diseases and asymptomatic atherosclerosis (Ridker *et al.,* 2000; Wang *et al.,* 2002). It has long been used clinically to evaluate the presence and degree of inflammation because CRP blood levels increase as much as 1,000-fold within 24 hours after the onset of inflammation (Kushner *et al.,* 1981). It has been demonstrated that CRP levels are associated with age, sex, race (African-American), body mass index (BMI), smoking, serumlipids, blood pressure, presence of diabetes mellitus, 2-h post-challenge glucose, frequency of exercise, and cardio-respiratory fitness (Folsom *et al.,* 2002; Hashimoto *et al.,* 2004).

1.2.11.1 C-reactive protein in asthma

An epidemiological study showed that elevated levels of hs-CRP correlate significantly with respiratory symptoms and with prevalence of non-allergic asthma (Olafsdottir *et al.,* 2005). Various publications suggest that CRP could be taken into consideration as a simple, cheap and reliable marker for monitoring asthmatic inflammation (Kony *et al.,* 2004; Olafsdottir *et al.,* 2005). In a study, the higher plasma CRP level was reported in asthma independent of various other factors (Kasayama *et al.,* 2009). Fujita *et al.* (2007) reported that increased hs-CRP levels may be associated with allergic inflammation, particularly eosinophilic inflammation, and the degree of airway obstruction in asthmatic patients. In another study, Szalai *et al.* (2002) suggested that an increase in CRP concentration may accompany the acute phase of allergic inflammation.

Elbehidy *et al.* (2010) showed significantly higher concentration of hs-CRP in three different groups of asthmatics than in controls. Further, serum hs-CRP levels were significantly higher in patients with uncontrolled asthma than in the two groups with controlled disease and hs-CRP correlated negatively with FEV1% and positively with sputum eosinophil%. A recent study has shown significantly higher level of serum hs-CRP in patients with acute asthma compared to controls (Razi *et al.,* 2012). Kilic *et al.* (2012) have also shown significantly higher levels of serum hs-CRP in asthmatic than in controls. They have also shown significantly higher level of hs-CRP in moderate asthmatics compared to controls.

1.2.12 Immunoglobulin E (IgE)

Immunoglobulin E (IgE) is set apart from other immunoglobulin isotypes because of its very short half-life (<1day) and very low concentrations in the circulation (Oettgen and Geha, 1999). In part, this is because some proportion of the circulating amount is continually removed and destroyed in endosomes (in the endosomes IgG is protected by FcγRn). IgE is extremely biologically active despite the low concentrations in the circulation. This is because IgE antibodies bind to high-affinity receptors on the surface of mast cells and basophils, so that these cells may be highly sensitive to allergens even when the concentration of IgE in the circulation is very low. In addition, the expression of the high-affinity receptors is upregulated during allergen-induced rhinitis (Rajakulasingam *et al.,* 1997), probably by IgE itself (Yamaguchi *et al.,* 1997; Williams and Galli, 2000). In addition to triggering immediate-hypersensitivity reactions and late-phase responses, there is accumulating evidence that preformed IgE can augment humoral and cellular immune responses to allergens (Oettgen and Geha, 1999).

1.2.12.1 Pathophysiologic Role of IgE in Asthma

IgE plays a key role in the pathogenesis of allergy. The genetic predisposition to mount a local mucosal IgE response, known as atopy, is one of the strongest risk factors for developing asthma (Nelson, 2001; Karjalainen *et al.,* 2003). The majority of asthma is associated with atopy (Pearce *et al.,* 1999), however there are also clinically defined variant forms of the disorder which are independent of atopy, i.e. do not mount an IgE response to environmental allergens (Macfarlane *et al.,* 2000). Th2 associated inflammation and IgE production are also the features of non-atopic or intrinsic asthma, although what drives this process remains unknown (Humbert *et al.,* 1999). Once synthesized, IgE antibodies circulate in the blood before binding to the high-affinity IgE receptor FcεRI that is present on mast cells in tissue or on peripheral blood basophils. Subsequent allergen exposures cause inflammatory-cell recruitment, activation and mediator release. IgE-sensitized mast cells expressing the high affinity IgE receptor (FcεRI) degranulate, releasing both pre-formed and newly synthesized mediators including histamine, leukotrienes, prostaglandins (PGs) and cytokines (Figure 5). Chemokines

released by inflammatory and resident cells direct recruitment of inflammatory cells, eosinophils and Th2 cells. Eosinophils release an array of pro-inflammatory mediators, including leukotrienes and basic proteins and mediators such as, IL-5 (Murdoch and Lloyd, 2010). These mediators cause the so-called early-phase asthmatic reaction (EAR), which is characterized by constriction of airway smooth muscle (ASM) cells, vascular leakage, mucus production, enhanced airway hyperresponsiveness (AHR) and recruitment of inflammatory cells (Bradding and Holgate, 1999). This EAR is immediate, lasting 30–60 min and 4–6 h later followed by the late-phase asthmatic reaction (LAR) (Baraniuk, 1997). The late-phase is characterized by excessive inflammation of the airways, resulting in structural changes, including airway wall thickening, subepithelial fibrosis, goblet cell hyperplasia, myofibroblast hyperplasia, ASM cell hyperplasia and hypertrophy, and epithelial hypertrophy. This is collectively known as airway remodeling (Bloemen *et al.,* 2007).

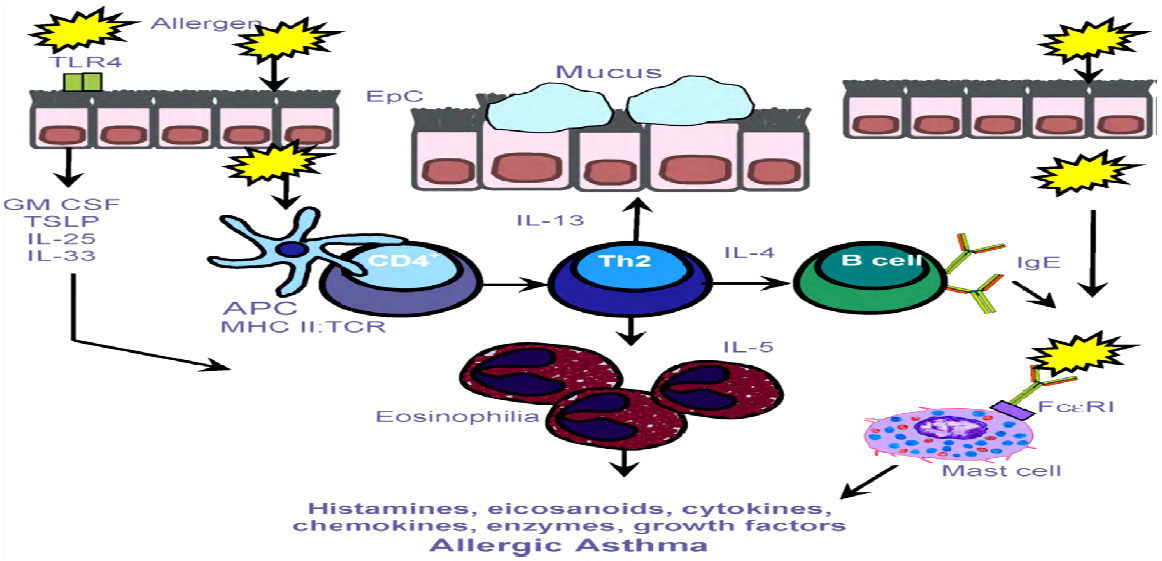

Figure 5. Immune cells and the inflammatory cascade in asthma. Initial exposure to allergen leads to the activation of allergen-specific Th2 cells and IgE synthesis (sensitization). Subsequent allergen exposures cause inflammatory-cell recruitment, activation and mediator release. IgE-sensitized mast cells expressing the high affinity IgE receptor (FcεRI) degranulate, releasing both pre-formed and newly synthesized mediators including histamine, leukotrienes and cytokines, which promote vascular permeability, smooth muscle contraction and mucus production. Key: APC, antigen-presenting cell; ASM, airway smooth muscle; EpC, epithelial cell; GM-CSF, granulocyte monocyte colony stimulating factor; MHC, major histocompatibility; TCR, T cell receptor; TSLP, thymic stromal lymphopoietin. *(Taken from: Murdoch & Llyod, 2010)*

1.2.12.2 IgE in asthma

Atopy is a nearly universal finding in children with asthma which is described as a tendency to produce excessive amounts of IgE antibodies when exposed to allergens (Burrows *et al.,* 1989). Estimation of total IgE level provides evidence in support of atopy. IgE concentration at birth is about 0.22 IU/ml. It reaches the adult value at 14 years of age. Markedly raised IgE levels have been reported in cases of parasitic infestations, wiskott–Aldrich syndrome, alcoholism, HIV and severe burns cases etc. Healthy, non allergic adults have an expected IgE concentration of up to 120 IU/ml (Witting *et al.,* 1980). IgE concentrations may vary as a result of diet, genetic background, geographical location and other influences (Chowdary, 2003). The concentration of IgE in serum is age dependent and normally remains at levels less than 10 IU/ml in most infants during the first year of life. There is a wide distribution of expected serum IgE values in healthy individuals of same age group (Kjellman *et al.,* 1976).

Allergic diseases including asthma are characterized by an increase in serum IgE levels (Peng, 2009; Rage *et al.,* 2009). IgE plays a central role in the initiation and the propagation of the inflammatory cascade and thus the allergic response (Buhl, 2005). Exposure to environmental factors, particularly inhalant allergens is commonly reported as a precipitant of acute exacerbations of asthma (Bacharier *et al.,* 2003). IgE is implicated in airway inflammation and allergic reactions and may play a role in modulating the severity of asthma, because previous studies have found associations between high IgE levels and asthma severity, airway hyperresponsiveness, and lower baseline lung function (Naqvi *et al.,* 2007).

Various population studies have shown an association between the prevalence of asthma/bronchial hyperresponsiveness and the total serum IgE levels (Freidhoff and Marsh, 1993; Grainger *et al.,* 1990; Sears *et al.,* 1991), independent of specific reactivity to common allergens or symptoms of allergy. Burrows *et al.* (1989) found a close correlation between serum IgE levels and the self-reported asthma. Borish *et al*. (2005) have reported higher IgE levels in severe asthmatics compared to moderate and mild asthmatics. Afshari *et al*. (2007) have reported considerably higher levels of serum IgE and IL-4 in asthmatics

than in non-asthmatic controls. In addition, several investigators have reported the elevated levels of total serum IgE in asthmatics (Anupama *et al.,* 2005; Sharma *et al.,* 2006; Sandeep *et al.,* 2010).

1.2.13 Cytokines

Cytokines are low-molecular weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to a number of stimuli. Their secretion is typically transient. The cytokine network is a complex and dynamic system, involved in numerous biological responses in the human body (Joseph *et al.,* 2002). Although an understanding of cytokine biology may appear daunting, it has many worthwhile utilities. Role of cytokines in disease pathogenesis unfolds the disease course in an explicit way. It also highlights the impact of molecular pathology on the practice of medicine and it has many practical therapeutic applications. Understanding cytokines physiology is an important step in optimizing their therapeutic use and furthering our knowledge of the biogenesis of different disorders (Ikram *et al.,* 2004).

1.2.13.1 CD4+ T cell subsets and Cytokine profile

In 1986, Mosmann *et al*. described two types of T helper (Th) clones in mice, Th1 and Th2 cells, which were distinguishable by the profile of cytokine production. Effector Th1 cells are involved in delayed-type hypersensitivity through their production of interferon (IFN)-γ and interleukin (IL)-2, whereas Th2 cells secrete IL-4, IL-9, IL-10 and IL-13, and promote antibody-mediated humoral immune responses (Brown and Ennis, 2005). Several studies suggest that polarized human Th1 and Th2 cells produce a relatively similar pattern of cytokines, compared to their mouse analogs (Romagnani, 1991), and numerous immunological diseases in humans have been associated with a Th1/Th2 cytokine imbalance (Romagnani, 1994). The cytokine profile of human CD4+ Th cells is elaborated in Table 2. Upon activation, naïve T helper cells become an uncommitted T cell termed Th0 cell. These Th0 cells secrete multiple varieties of cytokines, and in response to stimulation, differentiate into either Th1 or Th2 cells, distinguishable by their cytokine repertoire.

Table 2. Cytokine profiles of human CD4+ T cell subsets

Th0	Th1	Th2
IL-2	IL-2	*IL-2*
IL-3	IL-3	IL-3
IL-4		IL-4
IL-5		IL-5
IL-6		IL-6
IL-9		IL-9
IL-10		IL-10
IL-13		IL-13
IFN-γ	IFN-γ	
TNF-α	TNF-α	TNF-α
TNF-β	TNF-β	
GM-CSF	GM-CSF	*GM-CSF*

*Cytokines highlighted in italics represent very low levels (if present at all). *(Taken from: Brown and Ennis, 2005).*

1.2.13.2 Role of cytokines in the pathogenesis of asthma

Asthma is a common respiratory disorder characterized by recurrent episodes of coughing, breathlessness and wheezing. Cytokines play a key role in orchestrating the chronic inflammation of asthma and chronic obstructive pulmonary disease (COPD) by recruiting, activating, and promoting the survival of multiple inflammatory cells in the respiratory tract (Figure 5). Epithelial cells play an important role in orchestrating the inflammation of asthma through the release of multiple cytokines, including SCF (which maintains mast cells in the airways), TSLP (which acts on DCs to release the Th2 chemoattractants CCL17 and CCL22, which act via CCR4), and several chemokines that attract eosinophils by activating CCR3. Th2 cells orchestrate the inflammatory response in asthma through the release of IL-4 and IL-13 (which stimulate B cells to synthesize IgE), IL-5 (which is necessary for eosinophilic inflammation), and IL-9 (which stimulates mast cell proliferation). Mast cells are thus orchestrated by several interacting cytokines and play an important role in asthma through the release of the bronchoconstrictor mediators

histamine, cysteinyl-leukotrienes (Cys-LTs), and PGD2 (Barnes, 2008). Type 1 cytokines (IFN-γ, IL-12 and TNF-β) promote pro-inflammatory immune responses, whereas type 2 cytokines (IL-4, IL-5, IL-10, and IL-13) promote anti-inflammatory, antibody-dependent immune responses (Mosmann and Coffman, 1989). Dysregulated type 1/type 2 cytokine production and skewed development of memory Th1 or Th2 subsets, which secrete type 1/type 2 cytokines, respectively, have been implicated in the progression of multiple immune disorders including asthma (Steinke and Borish, 2001; Mazzarella *et al.,* 2000), leukemia (Zhang *et al.,* 2000), and other cancers (Skinnider and Mak, 2002). As a result, there is a great interest in using type 1 and type 2 cytokines as markers of human immune function. It is well established that a strong correlation exists between the presence of eosinophils and the presence of Th2 cells in the asthmatic airways and that classical Th2 cell-derived cytokines, namely IL-4, IL-5, IL-9 and IL-13, play critical roles in orchestrating and amplifying allergic inflammation in asthma (Nakajima and Takatsu, 2007). However, accumulating evidence suggests that the regulation of allergic inflammation is more complex. Although insight into the pathophysiology of asthma has increased substantially over recent years, a number of issues remain to be further clarified. These include a better understanding of the exact functional role of each cytokine in the sensitization process and in the complex relationship between inflammation, remodeling and altered airway behavior (Kips, 2001).

1.2.13.3 Role of Th2 Cytokines in Allergic Inflammation

1.2.13.3.1 IL-4

Eosinophilic inflammation is the characteristic of allergic disorders including asthma. Allergic diseases including asthma are characterized by inflammation with pronounced infiltration of eosinophils (Kay, 2001; Cohn *et al.,* 2004). An essential biological activity of IL-4 in the development of allergic inflammation is to drive the differentiation of naïve Th0 cells into Th2 cells, which secrete IL-4, IL-5, IL-9 and IL-13 but not IFN-γ (O'Garra and Arai, 2000; Murphy and Reiner, 2002). Various studies have suggested that IL-4 is essential for the initial differentiation and/or expansion of antigen-specific Th2 cells but

may not be essential for the induction of allergic airway inflammation at an effector phase (Coyle *et al*., 1995; Brusselle *et al.,* 1994). On the other hand, some studies have indicated the importance of IL-4 in promoting allergic inflammation at an effector phase by inducing the recruitment of Th2 cells in part via vascular cell adhesion molecule-1/very late antigen-4-dependent mechanisms (Cohn *et al.,* 1997). Therefore, IL-4 could play a role in the induction of allergic inflammation in a sensitized individual, but the relative importance of IL-4 depends on the state of sensitization and/or genetic background.

1.2.13.3.2 IL-5

IL-5 is a key Th2-type cytokine that plays an important role in the differentiation, maturation, and survival of eosinophils (O'Byrne, 2007). It has been demonstrated that the expression of IL-5 mRNA in bronchial biopsies of asthmatic patients is increased as compared with healthy volunteers and that the predominant source of IL-5 mRNA is CD4+ T cells (Robinson *et al.,* 1992). Indeed, CD4+ T cell activation in asthma is accompanied by increased serum concentrations of IL-5 (Corrigan *et al.,* 1993). IL-5 mRNA and protein are also found in mast cells located within allergen-challenged tissues.

1.2.13.3.3 IL-9

Over-expression of IL-9 in mice induces inflammation mediated by eosinophils, mucus hyperplasia, mastocytosis, AHR, and increased expression of other Th2 cytokines and IgE. Asthmatic patients show increased expression of IL-9 and its receptor in the airways (Zhou *et al.,* 2001). IL-9 plays an important role in differentiation and proliferation of mast cells and interacts synergistically with SCF (Barnes, 2008).

1.2.13.3.4 IL-13

Accumulating evidence suggests that IL-13 plays a key role in the allergic response via its actions on epithelial and smooth muscle cells and not through traditional effector pathways involving eosinophils and IgE-mediated events (Wills-Karp, 2004; Wynn, 2003). The importance of IL-13 was evidenced by the finding that neutralization of endogenously released IL-13 with a soluble form of IL-13Rα2, which binds IL-13 but not IL-4, during

antigen exposure largely inhibited the characteristics of asthma in murine asthma models (Wills-Karp *et al.,* 1998). The cytokines involved in asthma are shown in Figure 6.

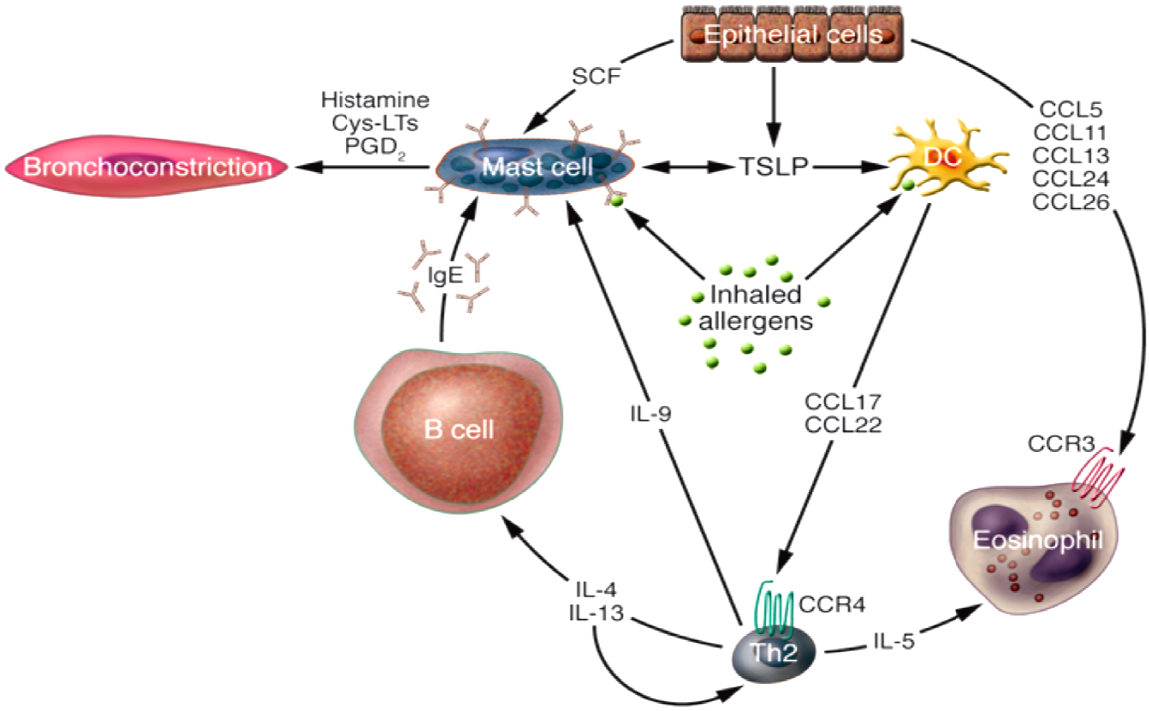

Figure 6. Cytokines involved in asthma. *(Taken from: Barnes, 2008).*

1.2.13.4 Cytokines in asthma

With airway hyper-responsiveness being the physiological hallmark of asthma, it is also characterized by chronic inflammation of the respiratory tract, allergen-specific IgE production, infiltration of eosinophils, the recruitment of T cells into the airways, and alterations in the fine balance between type 1 helper T lymphocytes (Th1) and type 2 helper T lymphocytes (Th2) responses towards Th2 bias (Larche *et al.,* 2003). Many investigators have studied the serum cytokines in asthmatic subjects (Daher *et al.,* 1995; Litonjua *et al.,* 2003; Silvestri *et al.,* 2006). In another study, the production of IL-4 and IFN-γ in phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cell cultures from atopic children was examined. The result of the study showed that highly atopic children with IgE>600U/ml produced significantly more IL-4 and less IFN-γ *in*

vitro than age-matched non-atopic controls (Tang *et al.,* 1993). Lee *et al.* (2001) investigated the serum levels of IL-4, IL-5, IL-13 and IFN-γ in asthmatics and showed that acute asthmatics had significantly increased levels of circulating IL-4, IL-5, and IL-13, although the differences were of borderline significance in serum IFN-γ when compared with control group. Smart and Kemp (2002) showed that atopic children had significantly reduced IFN-γ and increased IL-4 and IL-5 but not IL13 production to staphylococcal superantigen (SEB) stimulation when compared with non-atopic children. Bogic´ *et al.* (2004) have reported significantly higher IL-4 and IL-5 serum concentrations in asthmatic group compared to control and these were significantly higher in patients with moderate and severe asthma compared to mild asthmatics. Joseph *et al.* (2004) studied the serum level of IL-5 in asthmatics and the result of their study showed the elevated levels of IL-5 in mild and moderate persistent asthmatics compred to controls. Various studies have reported the elevated levels of Th-2 cytokines and reduced levels of Th-1 cytokines in allergic and asthmatic subjects (Cohn *et al.,* 2004; Akpinarli *et al.,* 2002; Robroeks *et al.,* 2007; Shahid *et al.,* 2002; Pukelsheim *et al.,* 2010).

1.2.14 Human Leukocyte Antigen (HLA)

The genetic loci involved in the rejection of foreign organs are known as the major histocompatibility complex (MHC), which encodes the highly polymorphic surface molecules. The human MHC is called the HLA (Human Leukocyte Antigen) system because these antigens were first identified and characterized using alloantibodies against leukocytes (Terasaki, 1990). The HLA, located on the short arm of Chromosome 6, is one of the most extensively studied regions in the human genome because of the contribution of multiple variants at this locus in autoimmune, infectious, and inflammatory diseases and in transplantation (Fernando *et al.,* 2008). The HLA system has been well known as transplantation antigens, but the primary biological role of HLA molecules is in the regulation of immune response (Bjorkman *et al.,* 1987). The importance of these MHC antigens in the immune response was first described by Baruj Benacerraf (Benacerraf, 1981). MHC molecules act as antigen-presenting structures, the particular set of MHC

molecules expressed by an individual influences the repertoire of antigens to which that individual's TH and TC cells can respond.

1.2.14.1 General Organization of the HLA system

The classical MHC encompasses approximately 3.6 megabasepairs (Mb) on 6p21.3 and is divided into three subregions: the telomeric class I, class III, and the centromeric class II regions (Figure 7). The HLA Class I genes and Class II genes each spread over approximately one third of this length. The class III region does not encode HLA molecules but contains genes for complement components (C2, C4, and factor B), 21-hydroxylase, tumor necrosis factors (TNFs), and some others (Beck and Trowsdale, 2000). Thus, the Class III region is not actually a part of the HLA complex, but is located within the HLA region, because its components are either related to the functions of HLA antigens or are under similar control mechanisms to the HLA genes (De Jong *et al*., 2003). Based on the similarity of structure and their function, HLA-I and HLA-II molecules are described below.

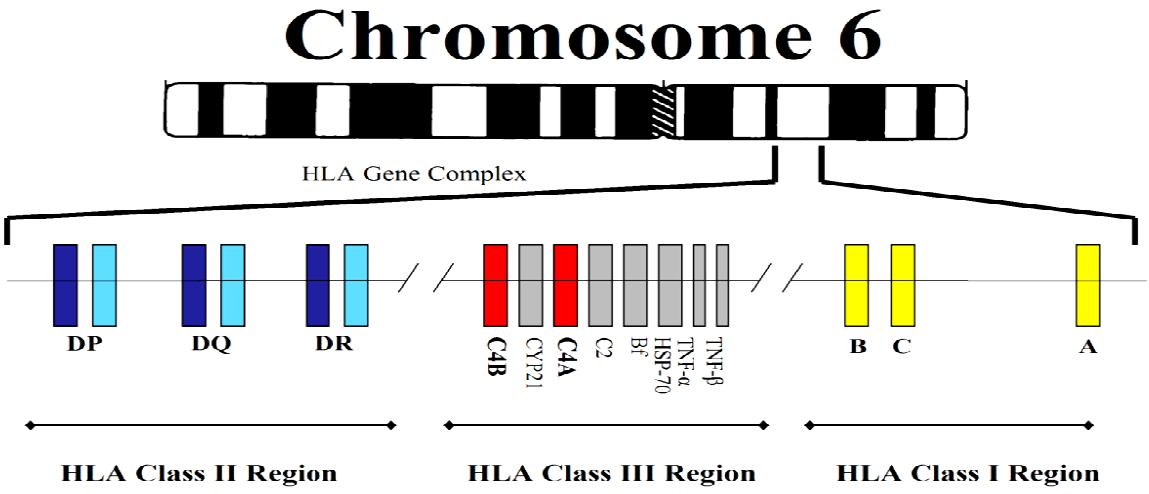

Figure 7. Genetic map of human leukocyte antigen (HLA) region. *(Adapted from:* Westover *et al.,* 2011*).*

1.2.14.2 HLA Class I molecules

Class I MHC genes encode glycoproteins expressed on the surface of nearly all nucleated cells, the major function of the class I gene products is the presentation of peptide antigens to TC cells. The class I genes code for α polypeptide chain of the class I molecule, the β chain of the class I molecule is encoded by a gene on chromosome 15, the beta 2-microglobulin gene. The α chain has five domains: two peptide-binding domains (α1 and α2), one immunoglobulin-like domain (α3), the transmembrane region, and the cytoplasmic tail (Figure 8). There are some 20 class I genes in the HLA region; three of these, HLA-A, B, and C, the so-called classic, or class Ia genes, are the main actors in the immunologic theater (Klein and Sato, 2000). Immunological studies indicate that HLA-B (which is also the most polymorphic) is the most significant HLA Class I locus, followed by HLA-A and then HLA-C. There are other HLA Class I loci (e.g. HLA-E, F, G, H, J, K and L), but most of these may not be important as loci for "peptide presenters" (Lotteau, 1992).

The HLA Class I antigens comprise a 45-kilodalton (kDa) α chain associated noncovalently with a 12-kDa β2-microglobulin molecule, which plays an important role in the structural support of the heavy chain. The HLA Class I molecule is assembled inside the cell and ultimately sits on the cell surface with a section inserted into the lipid bilayer of the cell membrane and has a short cytoplasmic tail (Figure 8). The full 3-dimensional structure of HLA Class I molecules has been determined from X-ray crystallography (Browning and McMichael, 1996). This has demonstrated that the molecule has a cleft on its outermost surface, which holds a peptide. In fact, if a cell becomes infected with a virus, the virally induced proteins within the cell are broken down into small peptides and these are then inserted into this cleft during the synthesis of HLA Class I molecules. The role of HLA Class I molecules is to take these virally enhanced peptides to the surface of the cell and by linking them to the T-Cell receptor of a cytotoxic (CD8) T cell, demonstrate the presence of the virus. The CD8+ T cell will now be "educated" and it will be able to initiate the process of killing cells which subsequently have that same viral protein/HLA Class I molecule on their surface. This role of HLA Class I molecules in identifying changed cells (e.g. virally infected) is the reason why they must be present on all cells (Roitt *et al.,* 1998).

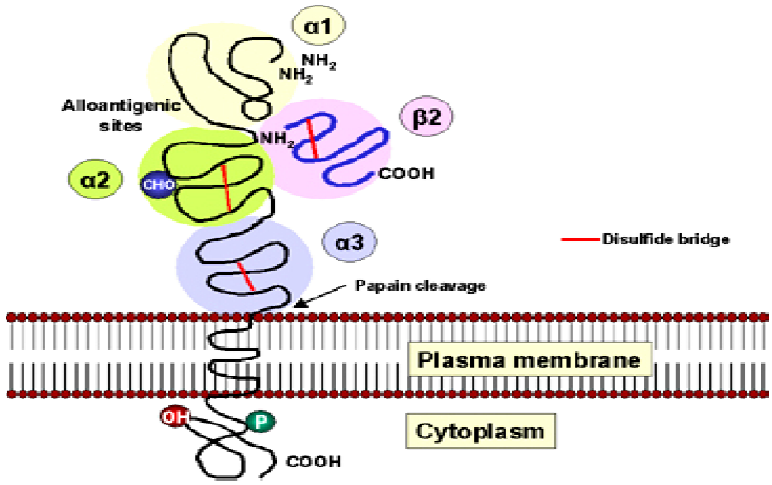

Figure 8. Structure of a class I MHC molecule showing the extracellular, transmembrane and cytosolic domains. *(Taken from: http://pathmicro.med.sc.edu/bowers/mhc.htm).*

1.2.14.3 HLA Class II Molecules

The class II genes code for the two different polypeptide chains, a 33-kDa α chain and a 28-kDa β chain, which associate by non-covalent interactions (Figure 9). The DR gene family consists of a single DRA and up to nine DRB genes (DRB1 to DRB9). The DRA gene encodes an invariable α chain and it binds various β chains encoded by the DRB genes. The DQA1 and DQB1 gene products associate to form DQ molecules, and the DPA1 and DPB1products form DP molecules (Choo, 2007). Like class Iα chains, class II MHC molecules are membrane-bound glycoproteins that contain external domains, a transmembrane segment, and a cytoplasmic anchor segment. Each chain in a class II molecule contains two external domains: α1 and α2 domains in one chain and β1 and β2 domains in the other. The membrane-distal portion of a class II molecule is composed of the α1 and β1 domains and forms the antigen-binding cleft for processed antigen (Kindt *et al.,* 2007).

Class II MHC genes encode glycoproteins expressed primarily on antigen-presenting cells (macrophages, dendritic cells, and B cells), where they present processed antigenic peptides to TH cells. Thus the “education” process, which occurs from HLA Class II presentation, involves-the helper-function of setting up a general immune reaction that will involve cytokines, cellular and humoral defense against the bacterial (or other) invasion.

This role of HLA Class II molecules in initiating a general immune response is the reason why they need only to be present on "immunologically active" cells (B lymphocytes, macrophages, etc.) and not on all tissues (Roitt *et al.,* 1998).

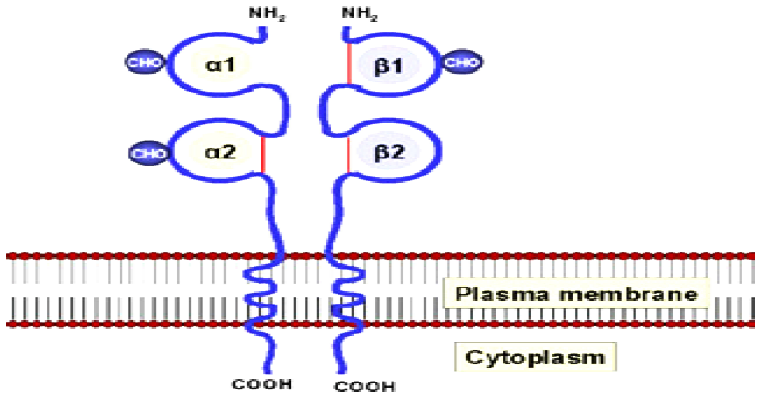

Figure 9. Structure of a class II MHC molecule showing the extracellular, transmembrane and cytosolic domains. *(Taken from: http://pathmicro.med.sc.edu/bowers/mhc.htm).*

1.2.14.4 Antigen Processing and Presentation

MHC-I and MHC-II molecules play different roles in T-cell-mediated adaptive immunity (Janeway *et al.,* 2005; Lund *et al.,* 2005). The MHC class I molecules present the peptide antigen derived from the endocytic pathway of antigen processing to cytotoxic T cells. In the endocytic pathway of antigen processing, endogenous antigens are first cleaved into peptide fragments by the proteasome, which are then generally translocated by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER). In the endoplasmic reticulum, various molecular chaperones, namely calnexin, calreticulin and tapasin, play their significant roles in the assembly of MHC-I molecule and the peptide binding to it. Finally, MHC-I molecules bind certain peptides and present them to cytotoxic T lymphocytes (CTL) stimulating cellular immunity. On the other hand, in the MHC-II pathway, exogenous antigens are first taken into the cell through endocytosis, and then degraded to peptides within endosomes and lysosomes mainly by aspartic and cysteine proteases (e.g. cathepsin). MHC-II molecules are synthesized in the ER, form complexes with invariant chain (Ii), which blocks the peptide binding cleft of MHC-II, and facilitates MHC-II entering into golgi from ER. Later MHC-II complex fuses with

endosome containing exogenous peptides. Finally, with the help of another MHC-like molecule, such as HLA-DM in humans, MHC-II can bind exogenous peptides and present them to T helper cells. Both antigen processing and presentation are important in the process of T-cell-mediated adaptive immunity, but peptide binding to MHC molecules is the most selective step (Zhang *et al.,* 2012). The antigen processing and presentation by MHC-I and MHC-II molecules are depicted in Figure 10.

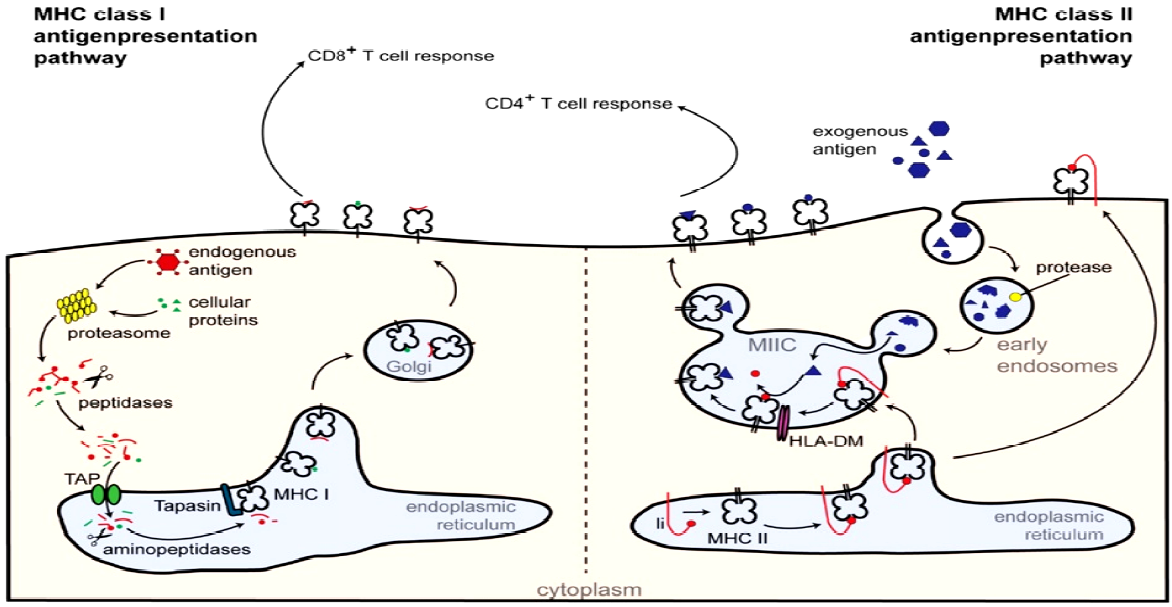

Figure 10. Antigen processing and presentation by: MHC-I molecules (left) and MHC-II molecules (right). *(Taken from*: *Neerincx et al., 2013).*

1.2.14.5 Genetics of HLA

Routine tissue typing identifies the alleles at the three HLA Class I loci (HLA-A, -B, and -C) and the three Class II loci (HLA-DR, -DP and -DQ). Thus, as each chromosome is found twice (diploid) in each individual, a normal tissue type of an individual will involve 12 HLA antigens (Sullivan and Amos, 1986). These 12 antigens are inherited co-dominantly that is to say, all 12 antigens are recognized by current typing methods and the presence of one does not affect our ability to type for the others. There are a number of

genetic characteristics of HLA antigens, they are: Polymorphism, Inheritance, Linkage disequilibrium and cross-reactivity (Browning and McMichael, 1996).

1.2.14.5.1 Polymorphism

MHC molecules are highly diverse because of an extensive range of MHC polymorphism. In fact, the IMGT/HLA database of June 2011 (Robinson *et al.,* 2011) contains over 6000 HLA alleles, which include 4946 HLA-I and 1457 HLA-II alleles. Each encoded MHC molecule binds to a distinct set of peptides, but binding preferences of most alleles have not yet been experimentally characterized, mainly because of two reasons. First, biological experiments require immense amount of time and financial cost. Second, the number of possible peptides derived from pathogens is huge, while binding peptides (binders) will be merely a tiny fraction of all those possible peptides (Assarsson *et al.,* 2007; Yewdell and Bennink, 1999).

The HLA polymorphism is not evenly spread throughout the molecule, but is clustered in the antigen–binding groove (Bjorkman and Parham, 1990; Klein and Sato, 2000). Amino acid variations in several regions change the fine shape of the groove and thus alter the peptide-binding specificity of HLA molecules (Falk *et al.,* 1991). The distribution and frequency of HLA antigens vary greatly among different ethnic groups. It has been postulated that this diversity of HLA polymorphism has evolved under unique selective pressure in different geographic regions. This could be related to the role of the HLA molecules in the presentation of prevalent infectious agents in the different areas of the world.

1.2.14.5.2 Inheritance of HLA

HLA genes are closely linked and the entire MHC is inherited as an HLA haplotype in a Mendelian fashion from each parent. The segregation of HLA haplotypes within a family can be assigned by family HLA studies. This way of presenting the HLA type is referred to as a phenotype (Thomas *et al*., 1998). This is illustrated in the figure below. Two siblings have a 25% chance of being genotypically HLA identical, a 50% chance of being HLA

haploidentical (sharing one haplotype), and a 25% chance that they share no HLA haplotypes.

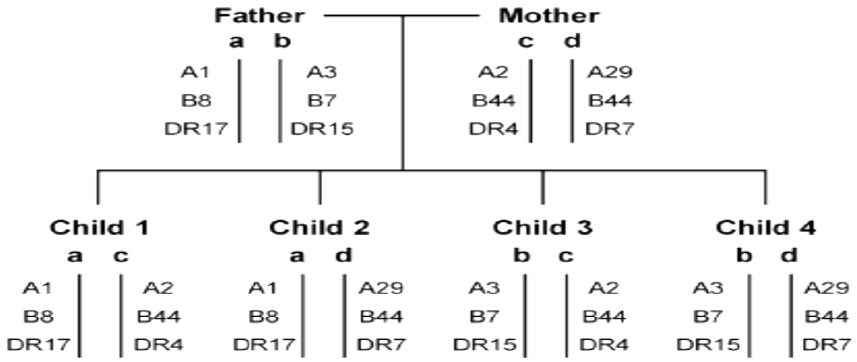

Figure 11. Mendelian inheritance of HLA haplotypes demonstrated in a family study. (*Taken from: Choo, 2007).*

1.2.14.5.3 Linkage disequilibrium

Basic Mendelian genetics states that the frequency of alleles at one locus does not influence the frequency of alleles at another locus (Law of independent segregation). However in HLA genetics this is not true. Certain HLA haplotypes are found more frequently in some populations than expected by chance. This phenomenon is called the linkage disequilibrium. The most extreme example is in Caucasians where the *HLA-A1, B8, DR3 (DRBI*O301), DQ2 (DRB1*O201)* haplotype is so conserved that even the alleles at the complement genes (Class III) can be predicted with great accuracy. Similar haplotypes are observed in selected caste groups and tribal groups of India (Tiercy *et al.,* 2002). Because of linkage disequilibrium, a certain combination of HLA Class I antigen, HLA Class II antigen and Class III products will be inherited together more frequently than would normally be expected. It is possible that these "sets" of alleles may be advantageous in an immunological sense, having a positive selective advantage.

1.2.14.5.4 Cross-reactivity

Cross-reactivity is the phenomenon whereby one antibody reacts with several different antigens, usually at one locus. The term CREG is often used to describe "Cross reacting groups" of antigens. It is useful to think in terms of CREG's when screening sera for antibodies, as most sera found are "multi-specific" and it is rare to find operationally monospecific sera. The rarity of monospecific sera means that most serological tissue typing uses sera that detects more than one specificity, and a typing is deduced by subtraction. For example, a cell may react with a serum containing antibodies to HLA-A25, A26, and A34 and be negative for pure A26 and pure A25 antisera. In this case, HLA-A34 can be assigned, even in the absence of pure HLAA34 antisera (Ferrer *et al.,* 2005).

1.2.14.6 HLA and Disease Susceptibility

Some HLA alleles occur at a much higher frequency in those suffering from certain diseases than in the general population. The diseases associated with particular MHC alleles include autoimmune disorders, certain viral diseases, disorders of the complement system, some neurologic disorders, and several different allergies. The MHC was first associated with disease in 1967 when HLA B antigens were found at increased frequency in patients with Hodgkin's lymphoma (Amiel, 1967). Since then, variation within the MHC has been found to be associated with almost every autoimmune disease, as well as several infectious and inflammatory diseases. However, because of the extensive LD that exists among alleles throughout this locus, the causal MHC variants have remained elusive for the great majority of diseases (Fernando *et al.,* 2008).

There are two general explanations for HLA and disease associations (McDevitt, 1985). Firstly, there may be a linkage disequilibrium between alleles at a particular disease associated locus and the HLA antigen associated with that disease - this is so for *HLA-A3* and Idiopathic Haemochromatosis. Another possible explanation for these associations is that the HLA antigen itself plays a role in disease, by a method similar to one of the following models: a) by being a poor presenter of a certain viral or bacterial antigen, b) by providing a binding site on the surface of the cell for a disease provoking virus or

bacterium, c) by providing a transport piece for the virus to allow it to enter the cell, d) by having such a close molecular similarity to the pathogen, that the immune system fails to recognize the pathogen as foreign and so fails to mount an immune response against it. It is most likely that all these mechanisms are involved, but to a varying extent in different diseases (Thorsby, 1997).

1.2.14.7 Association of HLA with Asthma

Asthma is a heterogeneous disease for which a strong genetic basis is firmly established. Asthma and its associated trait ‘‘atopy’’ were some of the first complex diseases for which a strong genetic basis was established (Barnes, 2001). In the early 1990s, the genome-wide linkage approach, whereby the inheritance patterns of chromosomal regions using highly polymorphic, genetic (‘‘microsatellite’’) markers evenly spaced across all chromosomes were genotyped in large samples of families, identified 10 chromosomal regions for which novel genes were subsequently identified by positional cloning i.e., *DPP10* (Allen *et al.,* 2003).

A variety of review papers describe genes associated with allergy/asthma (Peden, 2002; Ober and Hoffjan, 2006; Holloway *et al.,* 2010; Vercelli, 2008a; Vercelli, 2008b). Ober and Hoffjan (2006) listed genes associated with asthma or atopy in more than 10 studies. This study evaluated 8 of these genes (*IL4, IL13, TNF-a, HLA-DRB1, HLA-DQB1, FCER1B/MS4A2, CD14, ADAM33*) as well as 3 glutathione-s-transferase genes (*GSTM1*, *GSTP1*, and *GSTT1*) for association with asthma/allergy among urban-residing African Americans. HLA class II genes relate to non-specific modulation of inflammation. *HLA-DRB1* and *HLA-DQB1* SNPs and haplotypes have been associated with a higher risk of toluene diisocyanate-induced occupational asthma (Choi *et al.,* 2009), total serum IgE in Iranian subjects (Movahedi *et al.,* 2008), atopy in Northern Chinese (Gao *et al.,* 2003), *Dermatophagoides species*-sensitive asthma in Venuezuelan individuals (Lara-Marquez *et al.,* 1999), and asthma severity in Whites in the United States (Juhn *et al.,* 2007), suggesting a broad role for these genes in asthma pathogenesis across different ethnic groups. A study on Greek children with allergic asthma revealed that *DRB1*04* and

*DQA1*0301* might be important factors in susceptibility to asthma with sensitivity to mites (Parapanissiou *et al.,* 2005).

Numerous earlier studies have investigated the association of HLA with asthma. There are large numbers of studies which have shown the association of many different HLA class II alleles with asthma among diverse ethnic groups. Many earlier studies have also investigated the association of HLA class I alleles and/or antigens with asthma. Table 3 & 4 show the HLA class I and HLA class II alleles/haplotypes reported to be associated with asthma in different populations by various investigators, respectively.

Table 3. Association of HLA class I alleles/haplotypes with asthma as reported by various investigators

Study population	**No. of subjects**	**HLA allele/haplotype**	**Reference**
Extrinsic & intrinsic adult asthmatic patients	100 (61 atopic & 39 non-atopic)	HLA-B8 ↑	Morris *et al*., 1977
Population & family study	122 (41 intrinsic, 40 extrinsic &41 ABPA)	No association	Turton *et al*., 1979
Chinese asthmatic children	99	HLA-B*5 ↑ HLA-B*17 ↓	Huang *et al*., 1981
Korean population	-	HLA-B*08 ↑ HLA-A*03 ↓	Bondarenko *et al*., 1991
Asthmatics with ragweed Pollen allergy (Case-Control)	52 patients & 27 Controls	HLA-B*7, SC31, DR2 ↑	Blumenthal *et al*., 1992
Greek asthmatics patients	76 (35 children & 41 adults) & 400 controls	HLA-B5-B35 ↓ HLA-B8 ↑ (in adults) HLA-A10 ↑ (in children)	Apostolakis *et al*., 1996
Asian population	55 TDI exposed asthmatics, 47 asymptomatic & 95 controls	A*02-DRB1*15, A*02-DQB1*06, B*62-C*09 & A*02-DRB1*15-DQB1*06 ↑	Kim *et al*., 2006

Table 3. Continued

Study population	No. of subjects	HLA allele/haplotype	Reference
Intrinsic & allergic Asthmatics (Case-Control)	103 patients and 100 controls	HLA-B*12 ↑ A3/B7/DRw2 ↓	Morris et al., 1980
Croatian children with Allergic asthma	143 allergic asthmatic children & 163 controls	HLA-B*08 ↑	Ivković-Jureković et al., 2011

↑Increased
↓Decreased

Table 4. Association of HLA-class II alleles/haplotypes with asthma in various populations as reported by various investigators

Study population	No. of subjects	HLA allele/haplotype	Reference
Spanish Soyabean-epidemic asthmatics	78 soybean epidemic asthmatics, 67 nonepidemic asthmatics & 168 controls	DRB1*13 ↑ DRB1 *05-05, DRB1*05-06, & DRB1 *06-06↑	Soriano *et al*., 1997
Venezuelan Population	20 atopic asthmatics 64 controls (41 Non-atopic + 23 healthy)	DRB1*11 ↑ HLA-DRB1*1101 -DQA1*0501-DQB1*03031↑	Lara-Marquez *et al*., 1999
Chinese asthmatic population	98 asthmatics and 67 controls	DQA1*0101, DQA1*0601, DQB1*0303 & DQB1*0601 ↑	Guo *et al*, 2001
Caucasians with red Cedar asthma	56 asthmatics and 63 Controls	DQB1*0603 & DQB1*0302 ↑ DQB1*0501 ↓	Horne *et al*., 2000
Case-Control Hungarian mite-Sensitive asthmatics	102 asthmatics(57 mite-sensitive & 45 non-mite sensitive) & 57 controls	HLA-DR7- DQA1*0201-DQB1*0202 ↑ & HLA-DR4 - DQA1*0301-DQB1*0302 ↓	Bede *et al*., 2002
Grass allergy patients of Poland	82 atopic (40 with Asthma/rhinitis, 42 with rhinitis only & 52 nonatopic controls	DRB1*02-B5* ↑	Woszczek et al., 2002

Table 4 Continued

Study population	No. of subjects	HLA allele/haplotype	Reference
Taiwanese asthmatic Children	80 allergic asthmatics 69 non-asthmatics	HLA-DR13↑	Lin *et al*., 2002
Spanish *Artemisia vulgaris*-allergic asthmatics	213 asthmatics and 150 controls	DRB1*01 & DQB1*0501↑	Torío *et al*., 2003
Korean Aspirin-intolerant Asthmatics	76 Aspirin-intolerant, 73 Aspirin-tolerant & 91 controls	DPB1*0301↑ & DRB1*0901-DQB1*0303- DPB1*0501↑	Choi *et al*., 2004
Asian TDI-induced Asthmatics	55 TDI-induced asthmatics, 47 asymptomatic and 95 healthy controls	DRB1*15-DPB1*05↑ HLA-A*02-DRB1*15, A*02-DQB1*06, B*62- C*09 & A*02-DRB1*15-DQB1*06↑	Kim *et al*., 2006
Retrospective cohort study	340 children	HLA-DRB1*03↑	Juhn *et al*., 2006
Iranian children with allergic asthma	112 (75 males and 37 females) and 80 controls	DQB1*0603 and DQB1*0602↓	Movahedi et al., 2008
Caucasian, African-American, Hispanic children with *Alternaria*-sensitive asthma	60 moderate-severe asthmatics & 49 mild asthmatics	DQB1*03↓	Knutsen *et al*., 2010

Table 4 Continued

Study population	**No. of subjects**	**HLA allele/haplotype**	**Reference**
Retrospective cohort Study	383 children	Role of HLA DRB1*03 in asthma susceptibility independent of ancestral-haplotype-mediated linkage disequlibrium	Hanchard *et al*., 2010
Croatian children with Allergic asthma	143 asthmatics and 163 healthy controls	HLA-DRB1*03 ↑ HLA-DRB1*16 ↓	Ivković-Jureković *et al*., 2011

↑Increased ↓Decreased

1.3 OBJECTIVES OF THE STUDY

1. To estimate the prevalence of asthma in children aged between 3-12 years and to investigate the associated risk factors.

2. To determine the serum C-reactive protein (CRP) concentration in asthmatic children to understand the inflammatory process in asthma.

3. To estimate the levels of total serum IgE in asthmatic and control subjects and to investigate the relationship of various demographic and clinical characteristics with the level of total serum IgE in asthmatics.

4. To determine the serum levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines in order to investigate any alteration in Th1/Th2 balance in asthma.

5. To determine the frequency of some of the selected HLA class I and class II allelic groups in asthmatic and control groups to correlate the association, if any.

CHAPTER – 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Subjects

A total of 105 asthmatic children were included in this study. Children with asthma, who attended the Out Patient Department of Pediatrics, North Bengal Medical College & Hospital, were registered for the study. The diagnosis of asthma was made by the physician based on the medical and clinical history, physical examination, symptoms of the disease, Peak expiratory flow (PEF) estimation, chest X-ray, etc. The inclusion criteria for the participating subjects included: i. children diagnosed with asthma, ii. age group of 3 – 12 years, iii. residing in and around Siliguri, the sub-himalayan region of West Bengal, India.

A total of 110 unrelated individuals were included in the study as controls subjects. In the selection of control subjects the following criteria were taken into consideration:
i. age, sex and ethnicity matching with asthmatic subjects
ii. no history of lung disease or allergy,
iii. no airways hyperresponsiveness and no upper respiratory infections.

The study was approved by the 'Human Ethics Committee', University of North Bengal, Siliguri, West Bengal, India. The details of the study were explained to the parents/guardians of the children who participated in this study and an informed consent was obtained from each parent/guardian.

2.2 Collection of the demographic data & clinical history

The collection of demographic data and clinical history from the participating subjects was made using the questionnaire. The method of data acquisition is based on the interview of the parents and/or child. The response given to each question was recorded in the questionnaire. A sample of the questionnaire is attached in Appendix 2.

2.3 Estimation of prevalence of asthma in children between 3 to 12 years

For the estimation of the prevalence of asthma in children (3-12 years), the numbers of asthmatic and non-asthmatic children visiting the OPD were recorded in daily basis for the period of one year (May 2009 to April 2010).

2.4 Collection of Blood Samples

The clotted blood samples (4-5ml) were taken from each participant by vein puncture method in the Out Patient Department of Pediatrics, North Bengal Medical College & Hospital. The collected blood samples were brought to the Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, under appropriate conditions for further experiments.

2.4.1 Separation of serum

The blood sample was kept undisturbed for 2-3 hours at room temperature for coagulation. The blood clot was cut and centrifuged at 2000rpm for 10 minutes to separate the serum. The separated sera were stored in aliquots at -70°C until further analysis.

2.5 Determination of serum CRP level (Latex Agglutination Test)

The level of CRP was estimated in freshly separated serum samples of asthmatic subjects using the commercially available 'IMMUNOSTAT® CRP' kit (Ranbaxy Fine Chemicals Ltd., SIDCUL, Haridwar, India). The limitation of detection of the kit was <6 mg/L. The level of CRP was categorized as elevated (≥6mg/L) and normal (<6mg/L). The positive reaction with elevated CRP concentration was visible as prominent agglutination. The CRP test was performed in a total of eighty seven asthmatic subjects. The asthmatics were divided into two groups viz. ICS naïve and ICS treated based on their treatment regime.

A B C

Figure 12. Latex agglutination test for serum CRP level. A. A positive reaction where agglutination reaction is visible. B & C. Negative reactions where there is no agglutination reaction.

2.6 Determination of total serum IgE level

Total serum IgE level was measured using ELISA kits (Accu-Bind, Monobind Inc., USA). The principle of the method involves the immobilization of the biotinylated monoclonal anti-IgE antibody on the surface of a microplate well on interaction with the streptavidin coated on the well. On addition of serum containing the native antigen, antibody–antigen complex is formed. Another antibody (directed to a different epitope) labeled with an enzyme is added which results in the formation of an enzyme labeled antibody–antigen–biotinylated–antibody complex on the surface of the wells. On addition of the substrate color is formed which is measured using a microplate spectrophotometer. The concentration of the unknown samples is determined from the standard curve created using reference samples with known antigen concentration. The assay procedure was followed as per the manufacturer's instruction. The absorbance was measured at 450 nm in the ELISA plate reader (Bio-Rad). The sensitivity of the IgE AccuBind^{TM} ELISA test system was 1.0 IU/ml with the intra- and inter-assay precisions of 1.95–5.87% and 3.52–8.42%, respectively.

2.7 Determination of serum levels of IL-4 and IFN-γ

The commercially available 96 well ELISA kits (Endogen Human IL kit, Pierce Biotechnology, Inc., Rockford) were used to determine the level of IL-4 and IFN-γ in the sera of asthmatic and control subjects. The sensitivity of the kits was <2 pg/ml with inter

and intra-assay coefficient of variation of <10% in each case. The absorbance was measured at 450 nm in a microtitre plate reader (Opsys MR, Dynex Tehnologies).

2.7.1 Assay Procedure

1. 50µl of reconstituted standards and test samples were added to each well.
2. 50µl of Biotinylated Antibody Reagent was added to each well and mixed thoroughly by gently tapping the plate several times.
3. The plate was covered with an adhesive microtiter plate cover, ensuring all edges and strips are tightly sealed and the plate was incubated for two hours at room temperature, 20-25°C.
4. The adhesive plate cover was removed carefully and the plate was washed three times with wash buffer.
5. 100µl of prepared Streptavidin-HRP Solution was added to each well.
6. A new adhesive plate cover was attached, ensuring all edges and strips are tightly sealed. The plate was incubated for 30 minutes at room temperature, 20-25°C.
7. The adhesive plate cover was removed carefully, the plate contents were discarded. Then the plate was washed three times.
8. 100µl of TMB Substrate Solution was added into each well.
9. The plate was allowed to develop color reaction at room temperature in dark for 30 minutes.
10. After 30 minutes, the reaction was stopped by adding 100µl of Stop Solution to each well. The substrate reaction yielded a blue solution that turned yellow when stop solution was added.
11. The absorbance was measured on an ELISA plate reader set at 450nm within 30 minutes of stopping the reaction.

2.8 Extraction of genomic DNA

The genomic DNA was extracted from the frozen peripheral blood samples using phenol chloroform extraction method as described by Comey *et al*. (1994) with minor modifications. The procedure is described below:

1. Blood samples and all the reagents were thawed 30 minutes prior to use.
2. 500µl of blood sample was taken into a 2ml microfuge tube and equal volume of Red Cell Lysis Buffer was added. Mixed and centrifuged at 5000rpm for 5 minutes.
3. The supernatant was discarded and 1ml of 1X SSC was added. Mixed thoroughly and centrifuged at 5000rpm for 5 minutes.
4. The supernatant was discarded and 1.2ml of 1X SSC was added. Mixed and centrifuged at 5000rpm for 3 minutes.
5. The pellet was resuspended with 1.2ml of 50mM KCl and centrifuged as above.
6. The supernatant was discarded and 375µl of High Salt Buffer, 25µl 10% SDS and 12.5µl of 8mg/ml Proteinase K were added. Mixed gently and incubated at 56°C for 1 hour.
7. Equal volume of Phenol-Chlorofom was added to the PK digested suspension and spinned at 12000rpm for 20 minutes at 4 °C.
8. The DNA was precipitated in chilled absolute alcohol.
9. The DNA was rinsed with 70% ethanol, twice.
10. The DNA was dried and dissolved in 50µl TE.

2.8.1 Quantification of DNA

10µl of dissolved DNA was diluted to 1.5ml using deionized water and mixed properly. The OD was measured at wavelengths of 260nm and 280nm in a UV spectrophotometer. The DNA samples with 260/280 ratio of 1.7 and above were free of protein contaminations. The concentration of DNA was calculated using the formula:

OD at 260nm X dilution factor X 50 (1OD = 50µg of double stranded DNA).

The purity and integrity of DNA samples were also checked in 1% pre-stained agarose gel (Figure 13).

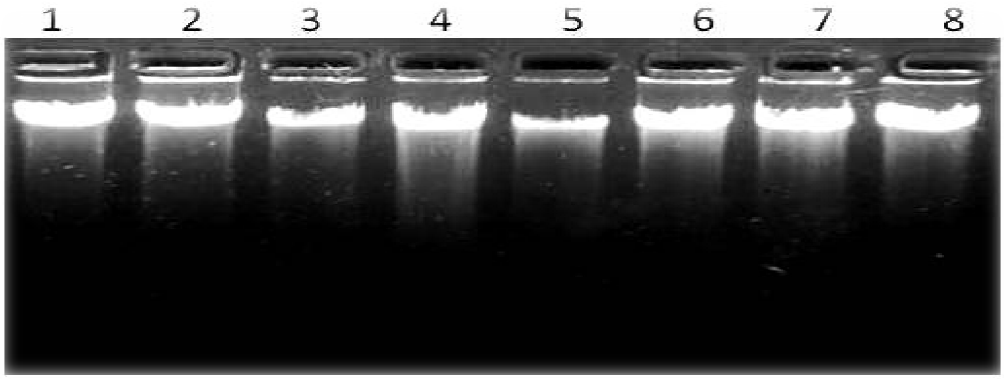

Figure 13. Electrophoregram showing DNA samples run in 1% pre-stained agarose gel after extraction.

2.9 PCR-SSP Typing of HLA alleles

Molecular typing of the selected HLA class I and class II allelic groups was performed by polymerase chain reaction using sequence-specific primers (PCR-SSP). The nucleotide sequences of the primers of HLA class I and class II allelic groups are shown in Table 5 and Table 6, respectively. The primer sequences of class I allelic groups were obtained from Bunce *et al.* (1995) and of class II allelic groups were taken from Zhu *et al*. (2007) and Sacchetti *et al*. (1997).

2.9.1 PCR amplification

A 25µl reaction mix was prepared and the reaction was carried out in a thermal cycler (MJ MiniTM Gradient Thermal Cycler, PTC-1148, Bio-Rad, Singapore). Two different internal controls (hemoglobin gene of 256bp for class I allelic groups and a fragment of human growth hormone gene1 of 439bp for class II allelic groups) were used to monitor the PCR amplification.

Table 5. Primer sequences of HLA class I allelic groups

Allelic group	Sequence	No. of bases
*HLA-A*01*	5'-GGACCAGGAGACACGGAATA-3'	20
	5'-AGG TAT CTG CGG AGC CCG-3'	18
*A*03*	5'-AGC GAC GCC GCG AGC CA-3'	17
	5'-CAC TCC ACG CAC GTG CCA-3'	18
*A*11*	5'-ACG GAA TGT GAA GGC CCA G-3'	19
	5'- CTC TCT GCT GCT CCG CCG-3'	18
*A*24*	5'-GGC CGG AGT ATT GGG ACG A-3'	19
	5'-CCT CCA GGT AGG CTC TCT G-3'	19
*A*25*	5'-TCA CAG ACT GAC CGA GAG AG-3'	20
	5'-ATG TAA TCC TTG CCG TCG TAA-3'	21
*A*26*	5'-ACT CAC AGA CTG ACC GAG C-3'	19
	5'-ATG TAA TCC TTG CCG TCG TAA-3'	21
*B*08*	5'-GAC CGG AAC ACA CAG ATC TT-3'	20
	5'-CCG CGC GCT CCA GCG TG-3'	17
*B*37*	5'-GCC GCG AGT CCG AGG AC-3'	17
	5'-CCT CCA GGT AGG CTC TGT C-3'	19
*B*44*	5'-TAC CGA GAG AAC CTG CGC-3'	18
	5'-CCA GGT ATC TGC GGA GCG-3'	18
*B*45*	5'-ACC GGG AGA CAC AGA TCT C-3'	19
	5'-CCA GGT ATC TGC GGA GCG-3'	18
*B*51*	5'-GGA GTA TTG GGA CCG GAA C-3'	19
	5'-CGT TCA GGG CGA TGT AAT CT-3'	20
*B*52*	5'-ACCGGGAGACACAGATCTC-3'	19
	5'-CGT TCA GGG GGA TGT AATCT-3'	20
Hemoglobin	5'-ATG ATG TTG ACC TTT CCA GGG-3'	21
gene (PIC-1)	5'-ATT CTG TAA CTT TTC ATC AGT TGC-3'	24

Table 6. Primer sequences of HLA class II allelic groups

Allelic group	Specific sequence	No. of bases
*HLA- DRB1*03*	5' GTTTCTTGGAGTACTCTAGGTC 3'	22
	5' TGCAGTAGTTGTCCACCCG 3'	19
*HLA-DRB1*04*	5' GTTTCTTGGAGCAGGTTAAACA 3'	22
	5' CGCTGCACTGTGAAGCTCTC 3'	20
*HLA-DRB1*12*	5' AGTACTCTACGGGTGAGTGTT 3'	21
	5' CTGTTCCAGGACTCGGCGA 3'	19
*HLA-DRB1*01*	5' TTGTGGCAGCTTAAGTTTGAAT 3'	22
	5' GCTGTTCCAGTACTCGGCAT 3'	20
*HLA-DQB1*0201*	5' GTGCGTATTGTGAGCAGAAG 3'	20
	5'GCAAGGTCGTGCGGAGCT 3'	18
*HLA-DQB1*0302*	5' GACGGAGCGCGTGCGTCT 3'	18
	5' AGTACTCGGCGTCAGGCG 3'	18
*HLA-DQB1*0603/8*	5' GGAGCGCGTGCGTCTTGTA 3'	19
	5' GCTGTTCCAGTACTCGGCAT 3'	20
*HLA-DQA1*0501*	5' AGCAGTTCTACGTGGACCTGGGG 3'	23
	5' GGTAGAGTTGGAGCGTTTAATCAGA 3'	25
Human GH gene	5' CAGTGCCTTCCCAACCATTCCCTTA 3'	25
(PIC-2)	5' ATCCACTCACGGATTTCTGTTGTGTTTC 3'	28

2.9.2 Preparation of reaction mixture

The reaction mix of 25µl was prepared in the following proportions:

Table 7. Preparation of reaction mixture for PCR

Reaction components	**Concentration**	**Reaction mixture**
MilliQ water	-	13 µl
Taq Buffer	Containing 15 mM $MgCl_2$	2.5 µl
dNTPs 100mM	10 mM	3.0 µl
Primer (Forward)	10 pm	1.5 µl
Primer (Reverse)	10 pm	1.5 µl
PIC-F	10 pm	0.6 µl
PIC-R	10 pm	0.6 µl
Taq Pol	3U/ µl	0.3 µl
Template	50 µg	2.0 µl
Volume		**25 µl**

2.9.3 Amplification procedure

A 25µl reaction mix was dispensed into PCR tubes. The tubes were spun for 1 min for mixing up of reaction components uniformly and the tubes were lodged into the thermal cycler. The touch down method was adopted for the PCR amplification. The reaction condition that was followed for PCR amplification is shown in Table 8.

Table 8. Reaction conditions followed for DNA amplification

No. of cycle	**Time**	**Temperature**
1 cycle of denaturation	3 min	94°C
5 cycles:		
Denaturation	30 s	94°C
Annealing	35 s	annealing* +2°C
Extension	40s	72°C
25 cycles:		
Denaturation	30s	94°C
Annealing	50s	annealing* -2°C
Extension	1min	72°C
1 cycle of extension	7min	72°C
Hold	Forever	12°C

** Annealing temperature varying for different alleles*

2.9.4 Amplification check by agarose gel electrophoresis

Mini gel electrophoresis apparatus (BIOTECH, India) was used for the rapid separation of amplified PCR products. The PCR products were separated on 2% agarose (Low EEO, Sisco Research Laboratories, India) gel containing 0.5µg/ml ethidium bromide (Boehringer Mannheim, Germany) in TBE buffer to check efficiency and specificity of the reaction. The step up 100bp DNA marker (Banglore Genie, India) providing even banding patterns of uniform intensity (1000bp, 900bp, 800bp, 700bp, 600bp, 500bp, 400bp, 300bp, 200bp, 100bp) was loaded in one of the wells. 10-12 µl of PCR products were mixed with loading dye (Bromophenol blue and sucrose) and loaded into the wells. The electrophoresis was carried out at 80V for 1hr to 1hr 30 minutes. Amplified PCR products of various HLA allelic groups are shown in Figure 14.

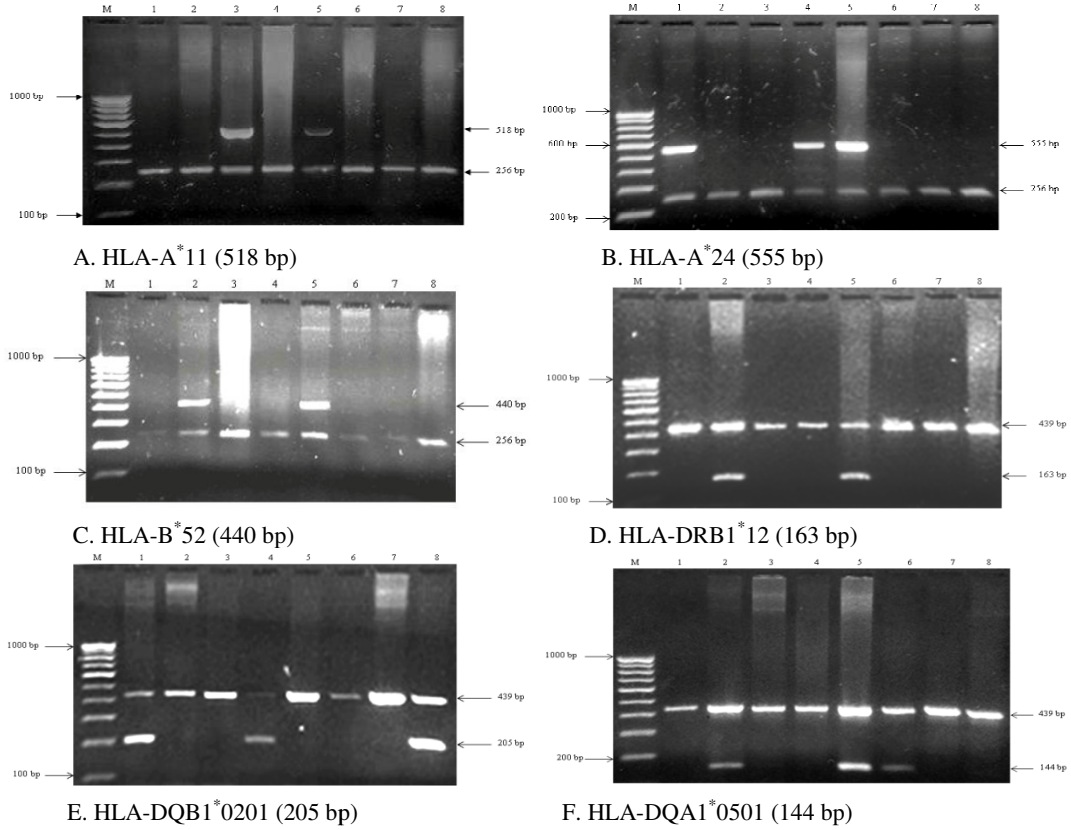

Figure 14. Electrophoregram showing amplified PCR products of various HLA class I and class II allelic groups run in 2% pre-stained agarose gel. Lane 1-8 in A, B & C: Positive internal control (256 bp) & Lane 1-8 in D, E & F: Positive internal control (439 bp). Lane M: 100 bp DNA ladder (100 bp to 1000 bp). A. Lane 3 & 5: A*11 (518bp), B. Lane 1, 4 & 5: A*24 (555bp), C. Lane 2 & 5: B*52 (440bp), D. Lane 2 & 5: DRB1*12 (163bp), E. Lane 1, 4 & 8: DQB1*0201 (205bp) and F. Lane 2, 5 & 6: DQA1*0501 (144bp).

2.9.4.1 Procedure

Agarose gels (2%) were prepared by suspending 1g dry agarose in 50ml 1X TBE buffer, then boiling the mixture until a clear solution was formed. This was allowed to cool down, 8-10µl of 0.5µg/ml ethidium bromide was added and mixed thoroughly by swirling and the content was poured in the gel cast sealed with tape on both sides. The comb was placed and it was allowed to form a rigid gel at room temperature.

2.9.4.2 Documentation and Interpretation

The gel was visualized under the UV-transilluminator (Vilber Laurmat, France) and the photographic documentation of the gel was done using Polaroid camera and analyzed using BioID Analysis software (Vilber Laurmat, France).

2.10 Statistical Analysis

Data were statistically analyzed using SPSS, version 16. Mean and standard deviation were calculated for the variables and t-test was employed for the comparisons. For the attributes, percentages were calculated and χ^2 test was used for comparisons. Figures were drawn using OriginLab 6.1. The frequencies of HLA allelic groups and HLA A-B haplotypes were calculated by direct counting (Mathews, 1984). The frequency of each allele and haplotype observed in the asthmatic group was compared to control group using χ^2 test. P-value was corrected (p_{corr}) for multiple comparisons by multiplying with the number of allelic groups. A p-value<0.05 was considered statistically significant. The odds ratio (OR) and the 95% CI for each HLA allele were calculated using the GRAPHPAD INSTAT version 3.10.

CHAPTER – 3

RESULTS AND DISCUSSION

3. RESULTS & DISCUSSION

3.1 ESTIMATION OF PREVALENCE AND ASSESSMENT OF RISK FACTORS OF ASTHMA IN CHILDREN: A HOSPITAL BASED STUDY

3.1.1 Results

In this hospital based study, we recorded a total of 6280 children aged between 3-12 years, who visited the Out Patient Department of Pediatrics, North Bengal Medical College & Hospital, Siliguri, with different illnesses during May 2009 – April 2010. Among them, 192 children were diagnosed with asthma. Therefore, the mean annual prevalence of asthma in hospital visiting children (3-12 years) during May 2009-April 2010 was observed to be 3.06%.

The demographic characteristics of the asthmatic and control subjects are presented in Table 9 and the association between various risk factors and asthma is shown in Table 10. Analyses of the various risk factors showed that 33 (33%) asthmatic children and 17 (15.5%) control subjects were having the family history of asthma and/or atopy. This difference was statistically significant ($p<0.01^{**}$). Therefore, the family history of asthma and/or atopy was found to be significantly associated with asthma in children. Other factors viz. exclusive breastfeeding up to 6 months, overcrowding and cooking mode did not show any association with asthma in children.

Table 9. Characteristics of asthmatic and control subjects

	Asthmatics	Controls
No. of subjects (n)	100	110
Age (years): Mean±SD	7.26±2.64	7.15±2.52
Gender		
Male	55	59
Female	45	51

Table 10. Association of risk factors with asthma

Risk factors	Asthmatics (N=100)	Controls (N=110)	$\chi 2$	p-value
EBF upto 6 months				
Given: n (%)	75 (75)	92 (83.64)		
Not given: n (%)	25 (25)	18 (16.36)	2.40	0.121
Family history of asthma/atopy				
Yes: n (%)	33 (33)	17 (15.45)		
No: n (%)	67 (67)	93 (84.55)	8.89	0.003**
Overcrowding at home				
Yes	39 (39)	31 (28.18)		
No	61 (61)	79 (71.82)	2.76	0.097
Cooking mode				
With smoke	78 (78)	91 (82.73)		
Smokeless	22 (22)	19 (17.27)	0.75	0.388

**Significant at p<0.005

EBF, Exclusive breastfeeding

3.1.2 Discussion

In our hospital based study, the mean annual prevalence of asthma in children was found to be 3.06%. Various school survey, community based studies and hospital based studies have shown diverse prevalence of asthma in children (Awasthi *et al.*, 2004; Jain *et al.*, 2010). Singh *et al.* (2002) showed 2.6% prevalence of asthma in pediatric population of the age group 1-15 years residing in five villages of Dehlon block of Ludhiana. In a recent study on school children (12-15 years) of rural Puducherry, Ganesh *et al.* (2012) reported the overall prevalence of bronchial asthma to be 8.7%. They found comparatively higher

rate of prevalence in 12-13 year age group (11.4%) compared to 14-15 year age group (7.1%). Boys had a higher prevalence of asthma (10.1%) compared to girls (7.1%). They further reported that family history of bronchial asthma (OR=6.64), presence of hey in the house (OR=9.79), exercise as aggravating factor (OR=4.63) and 2nd birth order (OR=0.06) were independently associated with bronchial asthma. In a cross sectional study, the prevalence of childhood asthma in Pune city was found to be 6.7% (7% amongst 6-7-year olds and 6.3% amongst 13-14 year olds. They further observed that Asthma was more common amongst boys (8.1%) than girls (4.9%) and more frequent in students studying in private schools (7.3%) than in those studying in public schools (5.8%). Risk factors such as family history of atopy, caesarian delivery, use of biomass fuel for cooking, absence of separate kitchen, absence of exclusive breastfeeding during the first 6 months of life, preterm birth, snoring, dampness at home, male sex, and parental smoking were significantly associated with asthma (Cheraghi *et al.,* 2012).

Although some recent reports suggest the prevalence of asthma to be declining but no overall global declining trend in the prevalence of asthma was shown in a recent review of epidemiological studies conducted to examine international trends in asthma prevalence in children and adults for the period 1990-2008 (Anandan *et al.,* 2010). The genetic predisposition, family history of asthma, is considered to be an important risk factor for the development of asthma. The finding of our study is consistent with various other studies which have well documented strong association of family history of asthma and asthma development. Vishwanathan *et al.* (1966) have observed the family history of asthma in 42% of asthmatic subjects but in only 10% nonasthmatics. Similarly, Ninan *et al.* (1995) observed parental history of asthma in 42% patients with polysymptomatic asthma as compared to 13% in asymptomatic children.

Analyses of data showed that there was no significant association of asthma with exclusive breast feeding, hygiene condition around house, overcrowding and cooking mode. These observations are consistent with previous studies. The findings of Gergen *et al.* (1988) showed a non-significant association between overcrowding and asthma. Schenker *et al.*

(1983) and Chhabra *et al.* (1998) have reported no significant association between the prevalence of wheeze and asthma with the type of fuel used in kitchen.

The importance of breastfeeding to childhood asthma is a controversial issue. Several investigators have claimed that breastfeeding is highly protective against asthma (Saarinen *et al.*, 1979; Hide and Guyer, 1981; Raisler *et al.*, 1999), while many other studies failed to show any significant association (Taylor *et al.,* 1983; Cogwell *et al.*, 1987; Zeiger *et al.*, 1989; Lucas *et al.,* 1990; Gustafsson *et al.,* 1992). On the other hand, few studies have suggested that breastfeeding is associated with increased risk for asthma (Martin *et al*., 1981; Savilahti *et al.,* 1987; Takemura *et al.*, 2001). In fact, most of these studies were based on a relatively small sample population and had poor adjustment of the confounding factors.

The present study was a preliminary epidemiological study carried out in the North Bengal region. There are indeed certain limitations of this study. We could not involve the general pediatric population of specific age group in this study as it was restricted only to the hospital visiting children. Future study based on school survey, using the standard questionnaire designed particularly for the epidemiological study, may be conducted to further strengthen this study.

In conclusion, the mean annual prevalence of childhood asthma was estimated to be 3.06% in our hospital based study conducted at North Bengal Medical College & Hospital, Siliguri. Results of our study also indicate that the family history of asthma/atopy is associated with asthma in children suggesting the genetic predisposition to be an important etiology for the development of asthma.

3.2 DETERMINATION OF SERUM LEVEL OF C-REACTIVE PROTEIN

3.2.1 Results

A total of eighty-seven asthmatic children were recruited for the study. They were divided into two groups viz. inhaled corticosteroid (ICS)-naïve and ICS-treated on the basis of their treatment status. ICS-naïve group was comprised of 15 subjects and ICS-treated group was comprised of 72 subjects. A comparison of demographic characteristics and CRP levels between ICS-naïve and ICS-treated groups is shown in Table 11. The elevated serum CRP level was detected in 13 ICS-naïve individuals (86.7%) and in only 3 ICS-treated individuals (4.2%), shown in Figure 15. The difference was statistically significant (χ^2=50.23, p<0.001). No significant difference was observed in other parameters between the two groups. The result revealed that the CRP concentration in serum of the ICS-naïve asthmatics is significantly elevated compared to ICS-treated asthmatics reflecting the presence of systemic inflammation in the ICS-naïve asthmatics.

Table 11. Characteristics of ICS-naïve and ICS-treated asthmatic groups

Characteristics	**ICS-naïve (n=15)**	**ICS-treated (n=72)**	**χ^2/t test**	**p value**
Age (yrs); Mean ± SD	7.11 ± 2.14	7.08 ± 2.64	t = - 0.30, df = 85	0.96
Gender; M/F (%)	9/6 (60/40)	40/32 (55.6/44.4)	χ2 = 0.10, df=1	0.752
FH; yes/no (%)	6/9 (40/60)	25/47 (34.7/65.3)	χ2 = 0.151, df=1	0.698
EBF; done/not done (%)	12/3 (80/20)	56/16 (77.8/22.2)	χ2 = 0.36, df=1	0.85
CRP level; elevated/nornal (%)	13/2 (86.7/13.3)	3/69 (4.2/95.8)	χ2 = 50.23, df=1	< 0.001

FH, Family history of asthma/atopy; EBF, Exclusive breastfeeding

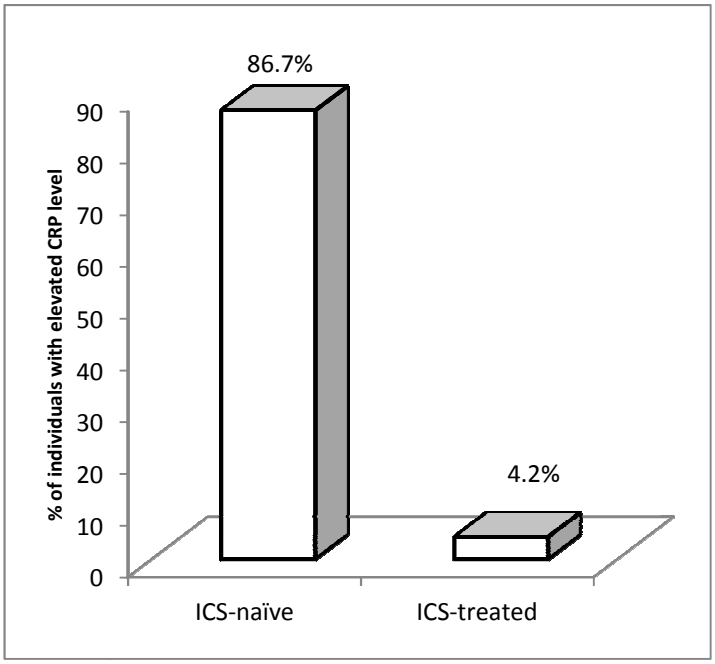

Figure 15. Comparison of serum CRP levels between ICS-naïve and ICS-treated groups of asthmatic children.

3.2.2 Discussion

The finding of the present study revealed the elevated and normal level of serum CRP in ICS-naïve and ICS-inhaling asthmatic children respectively. It is consistent with the findings of several other studies. Takemura *et al.* (2006) reported significantly increased levels of serum hs-CRP in steroid-naïve patients compared with controls, but not in patients on inhaled corticosteroid. Further, among steroid-naïve patients, serum hs-CRP levels negatively correlated with indices of pulmonary function and positively with sputum eosinophil count. Similarly, in another study, it was reported that plasma CRP levels were significantly reduced in corticosteroid-naïve asthmatic patients treated with inhaled corticosteroid for 3 months (Kasayama *et al.,* 2009). Therefore, it is provable that the ICS, which has well characterized anti-inflammatory properties, might have reduced serum CRP concentration to the normal state among the ICS-inhaling asthmatic children.

Bronchial hyperresponsiveness (BHR) is a crucial attribute of asthma. It is due to chronic asthmatic inflammation and may reflect the level of the inflammatory process (Nogami *et*

al., 2003). In an elegant work on a population-based study, Kony *et al*. (2004) revealed an association between a higher frequency of BHR and higher CRP levels in study participants, which could reflect local inflammation within the bronchi. Fujita *et al.* (2007) reported that increased hs-CRP levels may be associated with allergic inflammation, particularly eosinophilic inflammation, and the degree of airway obstruction in asthmatic patients. Thus, these findings suggest that asthmatic inflammation results in the elevation of CRP concentration. However, the exact role of the CRP in asthmatic inflammation is not clearly understood.

Though CRP is structurally distinct from the immunoglobulins, it shares some functional similarities with them. These include precipitation (Tillet and Francis, 1930), opsonization (Ganrot and Kindmark, 1969), capsular swelling (Hedlund, 1961) and agglutination (Patterson and Higginbotham, 1965). Two major biological activities of CRP have been well defined: first, it is able to bind several biological substrates that are distributed widely in nature (Gotschlich *et al.,* 1982), and second, it has significant activation capabilities, in particular to activate the complement system (Kaplan and Volanakis, 1974) and to bind to and modulate the function of phagocytic leukocytes (Wood, 1951; Kindmark, 1971). These effects support the concept that this serum protein may have a potentially central role in the host defense mechanisms.

The present study was carried out to monitor the serum CRP level in asthmatic children. It was observed that the ICS-naïve status of the asthmatic children was associated with the elevated serum CRP concentration. The finding of the study suggests that serum CRP may be used as a marker for assessing the degree of inflammation in asthma. It was further observed that serum CRP concentration was reduced in the ICS-inhaling asthmatic children. The ICS, which has potent anti-inflammatory properties, might have reduced the CRP concentration to the normal level in ICS-inhaling asthmatic children.

3.3 DETERMINATION OF TOTAL SERUM IMMUNOGLOBULIN E

3.3.1 Results

Total serum IgE was determined in 70 asthmatic and 70 control subjects. Characteristics of the asthmatic and control subjects are shown in Table 12. The results showed that the asthmatic subjects had a significantly elevated level of total serum IgE compared to the control subjects (269.21 ± 150.97IU/ml versus 146.89 ± 77.32IU/ml; $p<0.001^{***}$; Table 13 and Figure 16). It was further observed that out of 70, 50 (71.4%) asthmatics had total serum IgE >150 IU/ml. The associations of various demographic and clinical characteristics of the asthmatic subjects with the elevated level of total serum IgE were investigated (Table 14). No association of gender, family history of asthma/atopy, exclusive breastfeeding up to 6 months, and residential set up of the asthmatics with the elevated level of total serum IgE was found. The result showed the significant associations of age group, raised eosinophil count and exposure to cigarette smoke with the elevated level of total serum IgE in asthmatics. The data of IL-4 and total serum IgE of 44 asthmatic subjects were further analyzed to find out the correlation between them. The result showed the significant positive correlation ($r = 0.56$, $p<0.001^{***}$) between these two parameters (Figure 17).

Table 12. Demographic characteristics of asthmatic and control subjects

Characteristics	**Asthmatic subjects (N=70)**	**Control subjects (N=70)**
Age (years): Mean ±SD	6.93±2.63	7.02±2.29
Gender: M/F (%)	37/33 (52.86/47.14)	39/31 (55.71/44.29)
Height (cm): Mean±SD	116.03±17.16	119.59±13.28
Weight (kg): Mean±SD	18.74±6.01	19.80±5.57

3.3.2 Discussion

In the present study, out of 70 subjects 50 (71.43%) asthmatics had the elevated level of total serum IgE (>150IU/ml) suggesting that the majority of asthmatics had an allergic etiology. The asthmatic group had a significantly higher level of total serum IgE when compared to controls (269.21±150.97IU/ml versus 146.89±77.32IU/ml, $p<0.001^{***}$). Numerous earlier studies have shown the involvement of IgE in the allergic etiology of asthma and an elevation in its level in serum of the asthmatic subjects (Borish et al., 2005; Sharma *et al.,* 2006; Afshari *et al*. 2007; Sandeep *et al.*, 2010; Al-Quraishi, 2013). The finding of the elevated total serum IgE in asthmatics of the present study is in accordance with the well known fact that IgE plays a central role in the pathophysiology of asthma and other allergic diseases. Therefore, determination of total serum IgE level may be considered as a useful parameter to find out the involvement of allergic component in asthma.

Table 13. Comparison of total serum IgE levels between asthmatic and control groups

Variable	**Asthmatics (n=70)**	**Controls (n=70)**	**t-test**	**p-value**
IgE (IU/ml)	269.21 ± 150.97	146.89 ± 77.32	t = 6.034 df = 138	$p<0.001^{***}$

Data shown as mean±SD

When the association between elevated level of total serum IgE and various characteristics of asthmatic subjects was investigated, it was found that higher age group, raised eosinophil count and exposure to cigarette smoke showed a significant association with elevated level of total serum IgE (Table 14). These findings of the present study are consistent with the findings of various earlier studies. In a study on general population of Tuscon, Arizona, Cline and Burrows (1989) reported that serum IgE concentrations tend to peak around the age of 8-12 years and then decline gradually throughout adult life. Similarly, Sherrill *et al.* (1990) showed that total serum IgE levels track markedly with age during childhood. Subjects having high levels of serum IgE at the age of 9 months had still higher levels of serum IgE than their peers at the age of 6 and 11 years. Therefore, the early allergic sensitization and high production of IgE in early life implicates the pattern of

development of the immune system in early years as an important determinant of a predisposition to asthma in childhood (Peat *et al.,* 1990; Martinez *et al.,* 1995).

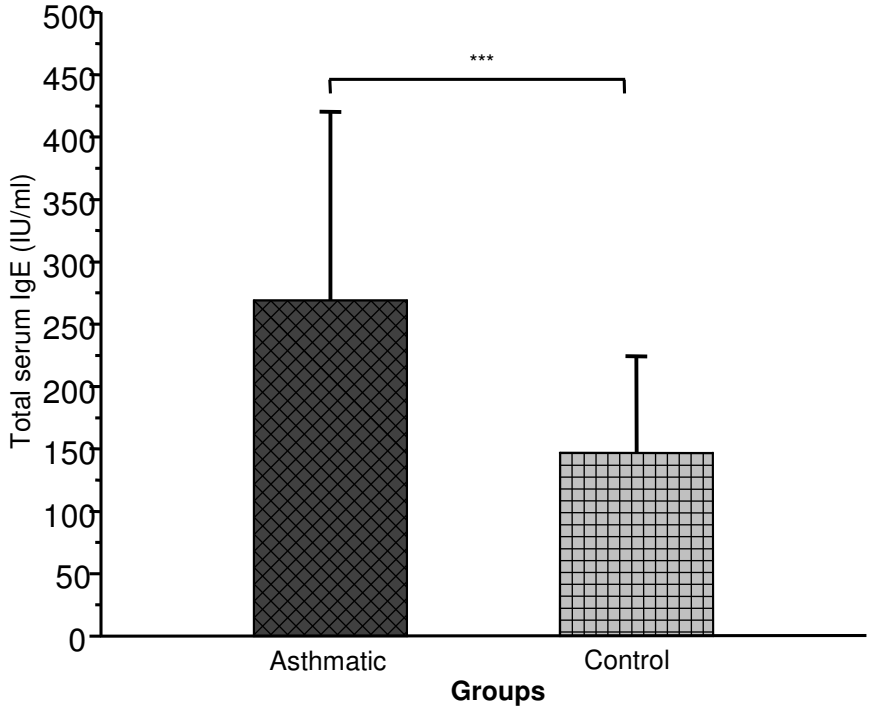

Figure 16. Levels of total serum IgE in asthmatics and controls. Asthmatic group had significantly elevated level of total serum IgE than the control group ($p<0.001^{***}$).

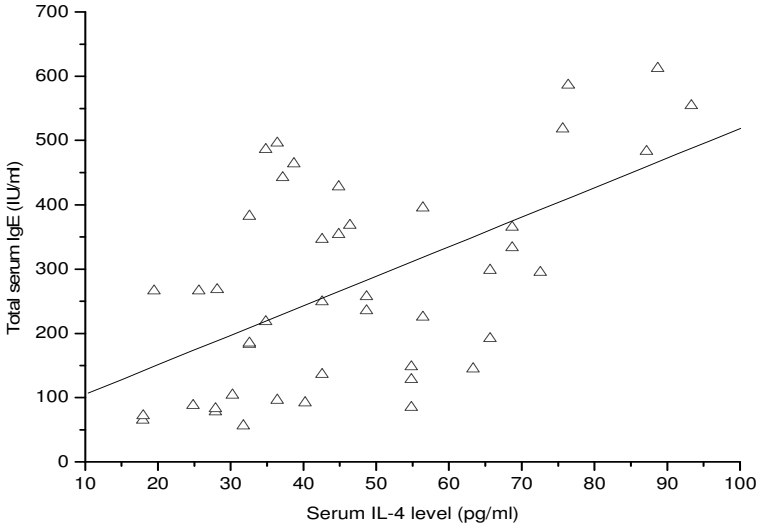

Fig. 17. Correlation between serum levels of IL-4 and total IgE among 44 asthmatic subjects. The correlation coefficient was 0.56 and was statistically significant ($p<0.001^{***}$).

Table 14. Association of demographic and clinical characteristics of asthmatics with the elevated level of total serum IgE (>150IU/ml)

Characteristics	**Total no. of asthmatics (N=70)**	**Total serum IgE >150IU/ml (N=50) n (%)**	χ^2	***p* value**
Age Group				
3-7 years	40	24 (60.0)		
8-12 years	30	26 (86.7)	5.973	0.015*
Gender				
Male	37	29 (78.4)		
Female	33	21 (63.6)	1.857	0.173
Eosinophil Count				
Raised	45	37 (82.2)		
Normal	25	13 (52.0)	7.193	0.007**
FHA				
Yes	22	19 (86.4)		
No	48	31 (64.6)	3.507	0.061
EBF up to 6 months				
Given	52	37 (71.2)		
Not given	18	13 (72.2)	0.007	0.931
Exposure to cigarette smoke				
Exposed	24	21 (87.5)		
Not exposed	46	29 (63.0)	4.622	0.032*
Residential set up				
Rural	56	41 (73.2)		
Urban	14	09 (64.3)	0.438	0.508

Abbreviations: FHA, family history of asthma/atopy; EBF, exclusive breastfeeding up to 6 months; ECS, exposure to cigarette/tobacco smoke
*Significant at p<0.05, **Significant at p<0.01

Kartasamita *et al.* (1994) showed the higher values of IgE and eosinophil count in children with asthma and a significant correlation between IgE level and eosinophil count. Satwani *et al.* (2009) showed eosinophilia along with raised serum IgE levels to be a significant allergic marker. Peripheral blood eosinophil counting has important clinical implication in

order to demonstrate the allergic etiology of the disease, to monitor its clinical course and to address the choice of therapy (Mesinga *et al.,* 1994). The significant association of exposure to cigarette smoke and elevated level of total serum IgE in asthmatic children observed in our study is in concordance with the finding of Strachan and Cook who showed the potential role of passive smoking on IgE in a study conducted in children (Strachan and Cook, 1998). Sapigni *et al*. (1998) found that peak IgE concentration at 8-14 years in a general population (8-73 years). They also found that passive smoking was significantly related to increased IgE values.

Further, it was also observed that there was a significant correlation between total IgE and IL-4 in sera of 44 asthmatic subjects. This finding is consistent with the finding of Afshari *et al*. (2007) who reported considerably higher levels of serum IgE and IL-4 in asthmatics than in nonasthmatic controls. IL-4 is one of the two cytokines known to cause switching in B-cells, a prerequisite for elevated IgE synthesis (Del Prete *et al.,* 1988). Kaminuma *et al.* (1999) observed that IL-4 participates in the mediation of local tissue inflammatory infiltration in patients with asthma and also activates eosinophils and aggravates asthma symptoms. Further, IL-4 down-regulates Fas expression on T cells and induces Bcl-2 expression to inhibit apoptosis and promote proliferation (Vella *et al.,* 1997).

In conclusion, the elevated level of total serum IgE may demonstrate the allergic etiology of asthma in the subjects studied. The data of the present study supports the significant association of higher age, raised eosinophil count and exposure to cigarette smoke with the elevated level of total serum IgE in asthmatics.

3.4 DETERMINATION OF SERUM LEVEL OF IL-4 AND IFN-γ

3.4.1 Results

A total of forty-eight asthmatic subjects, divided into two groups viz. steroid-naïve (n=18) and steroid-treated (n=30) and 32 control subjects were included in the study. The characteristics of the subjects are summarized in Table 15. Among the various demographic and clinical characteristics, the eosinophil count was found to be significantly higher in asthmatic subjects than in the controls ($p<0.05$). The demographic and clinical characteristics such as total lymphocyte count, eosinophil count, etc. did not show significant correlation with serum levels of IL-4 and IFN-γ.

Table 15. Demographic and clinical characteristics of asthmatic and control subjects

Characteristics	**Asthmatics (N=48)**	**Controls (N=32)**	**p-value**
Gender			
Male: n (%)	25 (52.08)	18 (56.25)	
Female: n (%)	23 (47.92)	14 (43.75)	0.134
Age (yrs): Mean±SD	6.74 ± 2.7	6.35 ± 2	0.428
Age of onset			
Up to 4 yrs: n (%)	34 (70.83)		
5-8 yrs: n (%)	11 (22.92)		
9-12 yrs: n (%)	3 (6.25)		
TC (/mm^3): Mean±SD	9448±1831.16	9891.67±1756.25	0.506
Eosinophil Count (/mm^3): Mean±SD	848±741.2	380.42±138.43	0.04*
Treatment status			
Steroid-naïve: n (%)	18 (37.5)		
On steroid treatment: n (%)	30 (62.5)		
Family history of asthma/atopy			
Yes: n (%)	22 (46)	8 (25)	
No: n (%)	26 (54)	24 (75)	0.06

*$p<0.05$

Serum levels of IL-4 and IFN-γ in steroid-naïve group, steroid-treated group and the control group are presented in Figure 18 and Figure 19, respectively. Comparisons of serum IL-4 levels between steroid-naïve asthmatics and controls, steroid-treated asthmatics and controls and steroid-naïve and steroid-treated asthmatics are made in Table 16, Table 17 & Table 18, respectively. Similarly, comparisons of serum IFN-γ levels between steroid-naïve asthmatics and controls and steroid-treated asthmatics and controls are made in Table 19 & Table 20, respectively. The steroid-naïve group had significantly higher serum level of IL-4 than the control group (52.25 ± 21.91 versus 32.81 ± 16.28 pg/ml; $p < 0.001^{***}$). It was observed that serum level of IL-4 was lower in steroid-treated group but not statistically significant when compared with steroid-naive group (40.80 ± 17.77 versus 52.25 ± 21.91 pg/ml; p = 0.054, NS). In contrast, serum level of IFN-γ was significantly lower in both steroid-naïve group and steroid-treated group compared to the control group (21.62 ± 9.91 versus 30.79 ± 14.28; $p = 0.02^{*}$ and 23.03 ± 10.54 versus 30.79 ± 14.28 pg/ml; $p=0.019^{*}$), respectively.

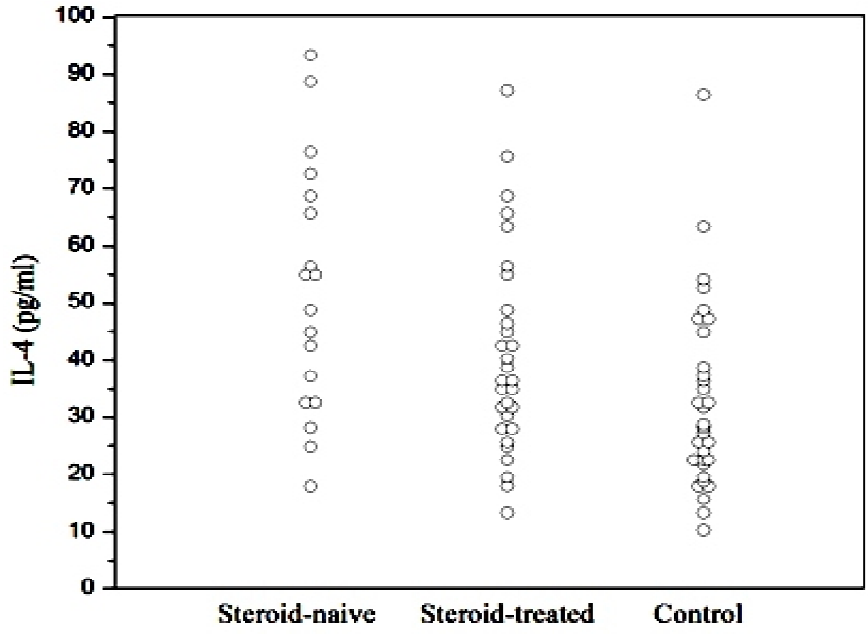

Figure 18. Serum levels of IL-4 (pg/ml) in steroid-naïve, steroid-treated and control group.

Table 16. Comparison of serum IL-4 levels between steroid-naïve asthmatic and control groups

Variable	Steroid-naïve Asthmatics (n=18)	Controls (n=32)	t-test	p-value
IL-4 (pg/ml)	52.25 ± 21.91	32.81 ± 16.28	t = 3.57 df = 48	$p<0.001^{***}$

Data shown as mean±SD

Table 17. Comparison of serum IL-4 levels between steroid-treated asthmatic and control groups

Variable	Steroid-treated Asthmatics (n=30)	Controls (n=32)	t-test	p-value
IL-4 (pg/ml)	40.80 ± 17.77	32.81 ± 16.28	t = 1.85 df = 60	p = 0.0697 (NS)

Data shown as mean±SD

Table 18. Comparison of serum IL-4 levels between steroid-naïve and steroid-treated groups of asthmatic subjects

Variable	Steroid-naïve (n=18)	Steroid-treated (n=30)	t-test	p-value
IL-4 (pg/ml)	52.25 ± 21.91	40.80 ± 17.77	t = 1.98 df = 46	p = 0.054 (NS)

Data shown as mean±SD

3.4.2 Discussion

The findings of higher serum level of IL-4 in steroid-naïve group and lower serum level of IFN-γ in both steroid-naïve and steroid-treated groups of asthmatic children are consistent with the findings of various other studies. Numerous studies have estimated and analyzed the serum levels of cytokines in asthma (Matsumoto *et al.*, 1991; Daher *et al.,* 1995; Lee *et al.,* 2001; Litonjua *et al.,* 2003; Silvestri *et al.*, 2006). Bogic´ *et al*. (2004) have reported significantly higher IL-4 and IL-5 serum concentrations in asthmatic group compared to control and these were significantly higher in patients with moderate and severe asthma compared to mild asthmatics. Shahid *et al.* (2002) have shown an increased concentration

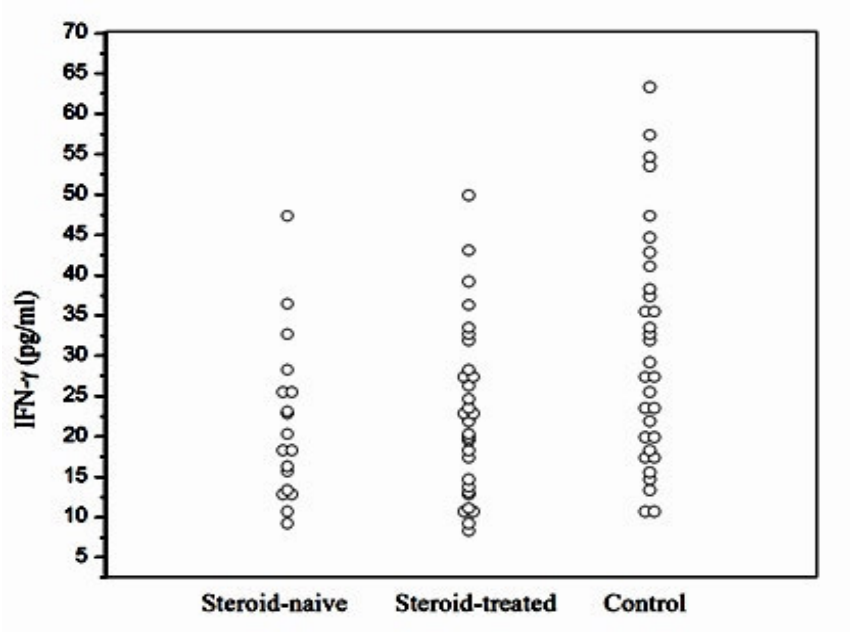

Figure 19. Serum levels of IFN-γ (pg/ml) in steroid-naïve, steroid-treated and control group.

Table 19. Comparison of serum IFN-γ levels between steroid-naïve asthmatic and control groups

Variable	**Steroid-naïve Asthmatics (n=18)**	**Controls (n=32)**	**t-test**	**p-value**
IFN-γ (pg/ml)	21.62 ± 9.91	30.79 ± 14.28	t = -2.41 df = 48	p = 0.20*

Data shown as mean±SD

Table 20. Comparison of serum IFN-γ levels between steroid-treated asthmatic and control groups

Variable	**Steroid-treated Asthmatics (n=30)**	**Controls (n=32)**	**t-test**	**p-value**
IFN-γ (pg/ml)	23.03 ± 10.54	30.79 ± 14.28	t = -2.42 df = 60	p = 0.019*

Data shown as mean±SD

of exhaled IL-4 in steroid-naïve group of asthmatic children and a decreased concentration of exhaled IFN-γ in both steroid-naive and steroid-treated groups compared to control subjects. Further, they have also reported that the exhaled IL-4 level was significantly lower in asthmatic children who were on steroid treatment. In a study, it was revealed that the expression of T-bet mRNA and the level of IFN-γ were lower, but the level of serum IL-4 was higher in asthma patients compared to healthy subjects. With the Astragalus membranaceus intervention, the level of IFN-γ and the expression of T-bet mRNA were increased and the level of IL-4 was decreased in the peripheral blood mononuclear cells (PBMCs) supernatant (Wang *et al.,* 2006). Similarly, Al-Daghri *et al.* (2014) reported significantly increased IL-4 mRNA expression in children with asthma compared to healthy control group. They also reported significantly higher serum IL-4 and poly-aromatic hydrocarbons (PAHs) concentration and significantly lower IFN-γ in children with asthma. Several other studies have also reported that allergic and asthmatic subjects are more likely to have elevated levels of Th-2 cytokines and reduced levels of Th-1 cytokines (Daher *et al.,* 1995; Walker *et al.,* 1994; Cohn *et al.,* 2004; Romagnani, 1994; Sanchez-Guerrero *et al.*, 1997; Akpinarli *et al.*, 2002; Robroeks *et al.,* 2007).

It is evident that IL-4 demonstrates a broad range of biological activities. It is a key cytokine implicated in the pathogenesis of allergic responses and at the same time it can also down-regulate acute inflammatory changes (Chung and Barnes, 1999). IL-4 has also got additional effects on asthma pathogenesis which include stimulation of mucus producing cells and fibroblasts leading to airway remodeling (Dabbagh *et al.,* 1999; Trautmann *et al.*, 1998; Doucet *et al.,* 1998). It has also been confirmed that the crucial role of IL-4 lies in its effect on Th-2 development, rather than on the induction of IgE synthesis and subsequent mast cell degranulation (Coyle *et al.,* 1995). In contrary, IFN-γ is a potent inhibitor of IgE synthesis (Vercelli *et al.,* 1990). Thus, this imbalance in the serum levels of IL-4 and IFN-γ is predicted to drive the asthma pathogenesis.

In the present study, it was observed that serum level of IL-4 was reduced in response to the treatment with steroid in asthmatic children. This finding is consistent with the earlier studies which suggest that steroids inhibit both IL-4 and IFN-γ synthesis but the inhibitory

action on IFN-γ is less marked (Ninan *et al.,* 1995). It appears that the steroid treatment down-regulates the IL-4 concentration in sera of the asthmatic subjects.

Although Th-1/Th-2 paradigm provided a simplistic model for initially describing involvement of T cells in asthma but still it does not fully support the complexities of this disease. Moreover, IFN-γ possesses a number of proinflammatory activities including the up-regulation of ICAM-1 (Marguet *et al*., 2000) and the receptor for TNF-α (Ruggiero *et al.,* 1986), it is likely that under certain circumstances IFN-γ may exert its proinflammatory activities and potentiate the inflammatory response in children with asthma. Therefore, it appears that some Th-1 and Th-2 cytokines are indeed elevated in asthma phenotypes of children. However, their effects in childhood asthma are largely unknown. In fact, there is an urgent need for complete understanding of T cell cytokine responses in childhood asthma. Moreover, it is crucial to understand the disease process for unraveling such complexities.

To conclude, the findings of our study support the hypothesis of Th1/Th2 cytokine imbalance and suggest that serum level of IL-4 may be elevated in concert with decreased level of IFN-γ in asthma. Determination of serum levels of IL-4 and IFN-γ may be useful for understanding and monitoring the inflammatory response in asthma.

3.5 PCR-SSP TYPING OF HLA CLASS I & CLASS II ALLELIC GROUPS

3.5.1 Results

The characteristics of asthmatic and control subjects are summarized in Table 21. Electrophoregrams of various PCR amplified HLA allelic groups are presented in Figure 20. The frequencies of HLA alleles in asthmatic and control groups are compared in Table 22. The comparison of the frequencies of HLA-A, HLA-B and HLA class II allelic groups between asthmatic and control groups are also made in Figure 21, Figure 22 & Figure 23, respectively. Analysis of HLA alleles showed the higher frequencies of *A*01* (15.71% vs. 10.45%), *A*03* (17.62% vs. 14.09%), *A*24* (16.67% vs. 13.64%), *A*25* (10.48% vs. 9.09%), *A*26* (12.86% vs. 8.64%), *B*08* (21.90% vs. 18.64%), *B*44* (5.71% vs. 3.64%), *B*45* (7.14% vs. 5.00%), *B*37* (19.05% vs. 15.45%), *DRB1*01* (6.67% vs. 3.18%), *DRB1*03* (11.43% vs. 3.64%), *DRB1*12* (8.57% vs. 5.45%), *DQB1*0201* (18.57% vs. 14.09%), *DQB1*0302* (14.29% vs. 10%), and *DQA1*0501* (14.76 vs. 10.91%) and lower frequencies of *A*11* (8,57% vs. 10%), *B*51* (6.19% vs. 8.18%), *B*52* (4.29% vs. 5.91%), *DRB1*04 (15.24% vs. 18.18%) and DQB1*0603 (2.86% vs. 4.55%)* in asthmatic group than in controls. Among these, *HLA-DRB1*03* allelic group was significantly associated with asthma (OR=3.78, 95%CI=1.61-8.85, p=0.0025, p_{corr}<0.05). None of the HLA class I allelic groups showed a significant association with asthma. Although the *HLA-DRB1*01* allelic group was not significantly associated with asthma, the odds ratio for this allele was greater than 2 (OR = 2.26, 95% CI = 0.88 – 5.85, p=0.136).

The frequencies of HLA allelic groups in two groups of asthmatic subjects with high total serum IgE level (tIgE>150IU/ml) and low total serum IgE level (tIgE<150IU/ml) are presented in Table 23. The frequencies of HLA-A allelic groups, HLA-B allelic groups and HLA class II allelic groups in two groups of asthmatic subjects viz. tIgE>150IU/ml and tIgE<150IU/ml are presented in Figure 24, Figure 25 & Figure 26, respectively. Comparisons of the frequencies of HLA allelic groups between two groups of asthmatic subjects revealed higher frequencies of *HLA-A*01, A*24, A*25, B*51, B*52, B*37, DRB1*01, DRB1*03, DQB1*0302, DQB1*0603/8* and *DQA1*0501* and lower frequencies of *HLA-A*03, A*26, B*08, DRB1*12* and *DQB1*0201* in tIgE>150IU/ml than

tIgE<150IU/ml group. Rests of the alleles were observed in nearly equal frequencies in both the groups. None of these alleles showed a significant association with the elevated level of total serum IgE in asthmatic subjects.

Table 21. Characteristics of asthmatic and control subjects

	Asthmatics	**Controls**
No. of subjects	105	110
Males	57	58
Females	48	52
Age (years): Mean ± SD	7.33±2.62	7.71±2.74
Age of onset		
Before 5 years	85	-
After 5 years	20	-
Height (cm): Mean ± SD	116.35±13.28	117.63±13.03
Weight (Kg): Mean ± SD	19.19±5.94	20.95±6.28
Study groups		
Bengali	78	69
Bihari	12	15
Nepali	7	15
Others	8	11

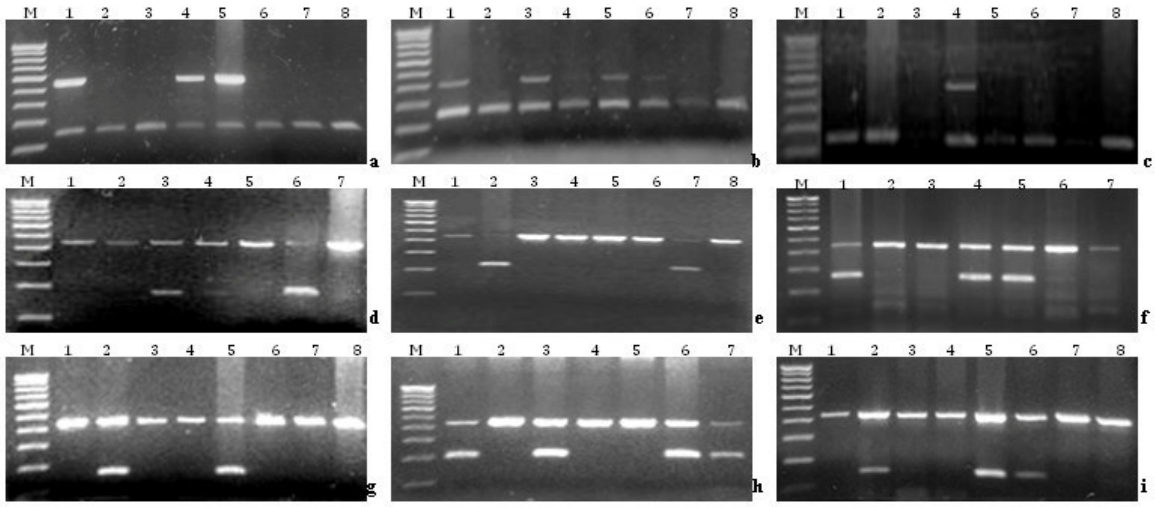

Figure 20. Electrophoregram showing various HLA class I and class II allelic groups run in 2% agarose gel after PCR amplification. Lane M: 100bp DNA ladder (100-1000bp). Positive internal controls, 256bp (a-c) and 439bp (d-i). a. Lane 1, 4 & 5: *HLA-A*24* (555bp). b. Lane 1, 3, 5 & 6: *A*25* (398bp). c. Lane 4: *B*44* (575bp). d. Lane 3 & 6: *DRB1*01* (168bp). e. Lane 2 & 7: *DRB1*03* (222bp). f. Lane 1, 4 & 5: *DRB1*04* (262bp). g. Lane 2 & 5: *DRB1*12* (163bp). h. Lane 1, 3, 6 & 7: *DQB1*0201* (205bp). i. Lane 2, 5 & 6: *DQA1*0501* (144bp).

Table 22. Frequencies of HLA class I and class II allelic groups in asthmatic and control subjects

HLA allelic group	**Asthmatics (N=105) Freq. % (n)**	**Controls (N=110) Freq. % (n)**	χ^2	**p**	**OR (95% CI)**
*A*01*	15.71 (33)	10.45 (23)	2.564	0.109	1.73 [0.94-3.21]
*A*03*	17.62 (37)	14.09 (31)	0.932	0.334	1.39 [0.78-2.47]
*A*11*	8.57 (18)	10.00 (22)	0.132	0.717	0.83 [0.42-1.65]
*A*24*	16.67 (35)	13.64 (30)	0.670	0.413	1.33 [0.74-2.39]
*A*25*	10.48 (22)	9.09 (20)	0.116	0.734	1.19 [0.61-2.34]
*A*26*	12.86 (27)	8.64 (19)	1.802	0.179	1.66 [0.86-3.21]
*B*08*	21.90 (46)	18.64 (41)	0.701	0.403	1.31 [0.76-2.26]
*B*44*	5.71 (12)	3.64 (8)	0.662	0.416	1.65 [0.64-4.20]
*B*45*	7.14 (15)	5.00 (11)	0.569	0.451	1.50 [0.65-3.44]
*B*51*	6.19 (13)	8.18 (18)	0.406	0.524	0.72 [0.33-1.56]
*B*52*	4.29 (9)	5.91 (13)	0.314	0.575	0.70 [0.29-1.71]
*B*37*	19.05 (40)	15.45 (34)	0.931	0.335	1.38 [0.78-2.42]
*DRB1**					
01	6.67 (14)	3.18 (7)	2.223	0.136	2.26 [0.88-5.85]
03	11.43 (24)	3.64 (8)	9.106	0.0025*#	3.78 [1.61-8.85]
04	15.24 (32)	18.18 (40)	0.593	0.441	0.77 [0.43-1.35]
12	8.57 (18)	5.45(12)	1.258	0.262	1.69 [0.77-3.71]
*DQB1**					
0201	18.57 (39)	14.09 (31)	1.578	0.209	1.51 [0.85-2.67]
0302	14.29 (30)	10.00 (22)	1.710	0.191	1.60 [0.85-3.01]
0603/8	2.86 (6)	4.55 (10)	0.467	0.495	0.61 [0.21-1.73]
*DQA1**					
0501	14.76 (31)	10.91 (24)	1.295	0.255	1.50 [0.81-2.78]

OR, Odds ratio; CI, confidence interval

% = (n/2N) x 100

* p corrected <0.05

Fisher exact= 0.0018

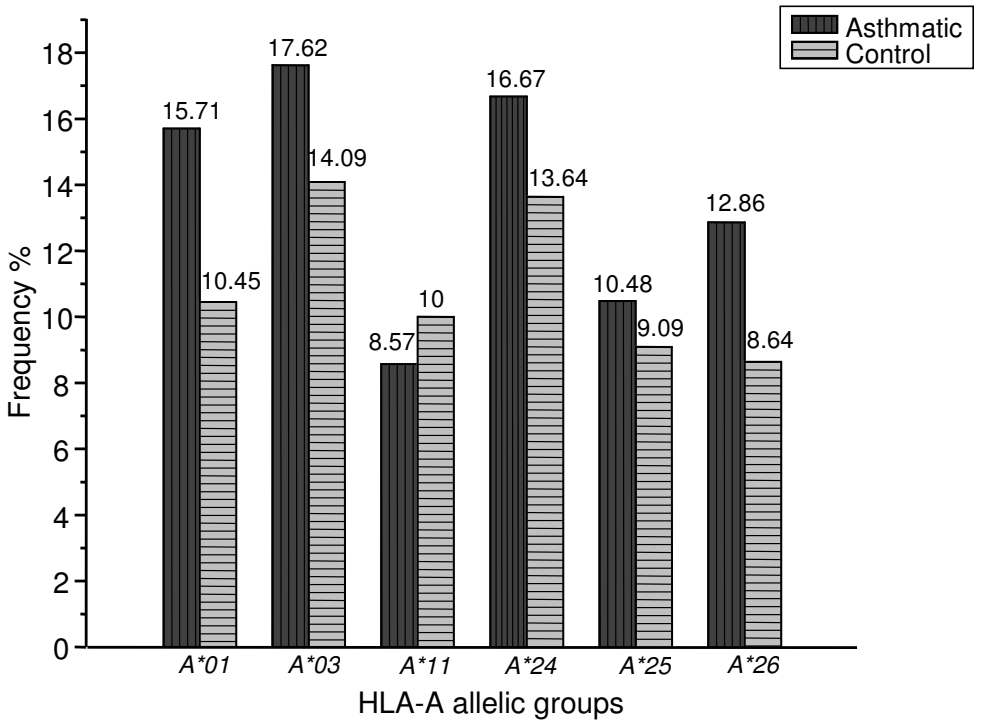

Figure 21. Comparison of Frequencies of HLA-A allelic groups between asthmatic and control groups.

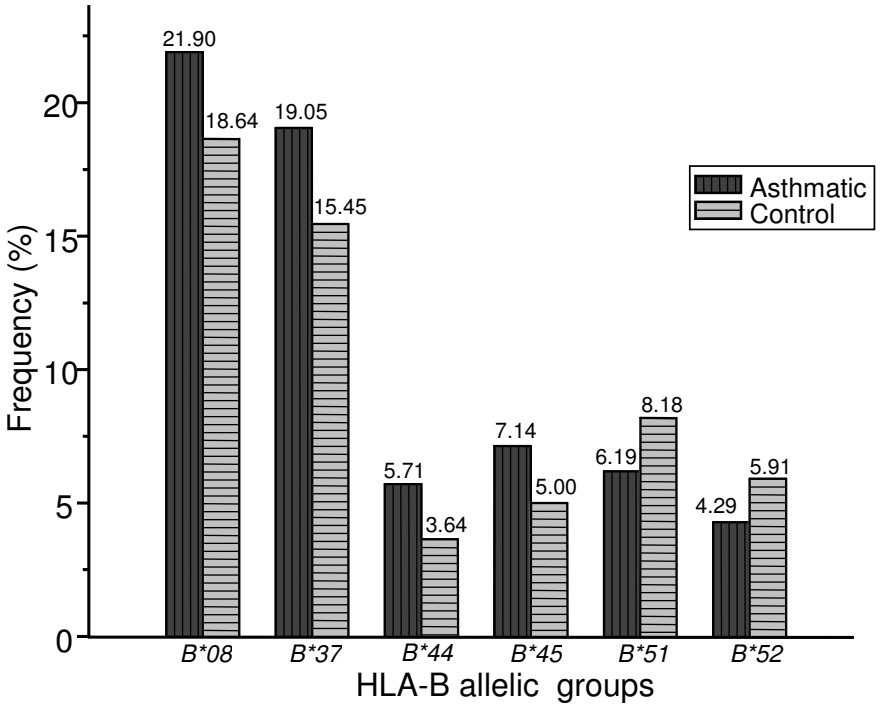

Figure 22. Comparison of Frequencies of HLA-B allelic groups between asthmatic and control groups.

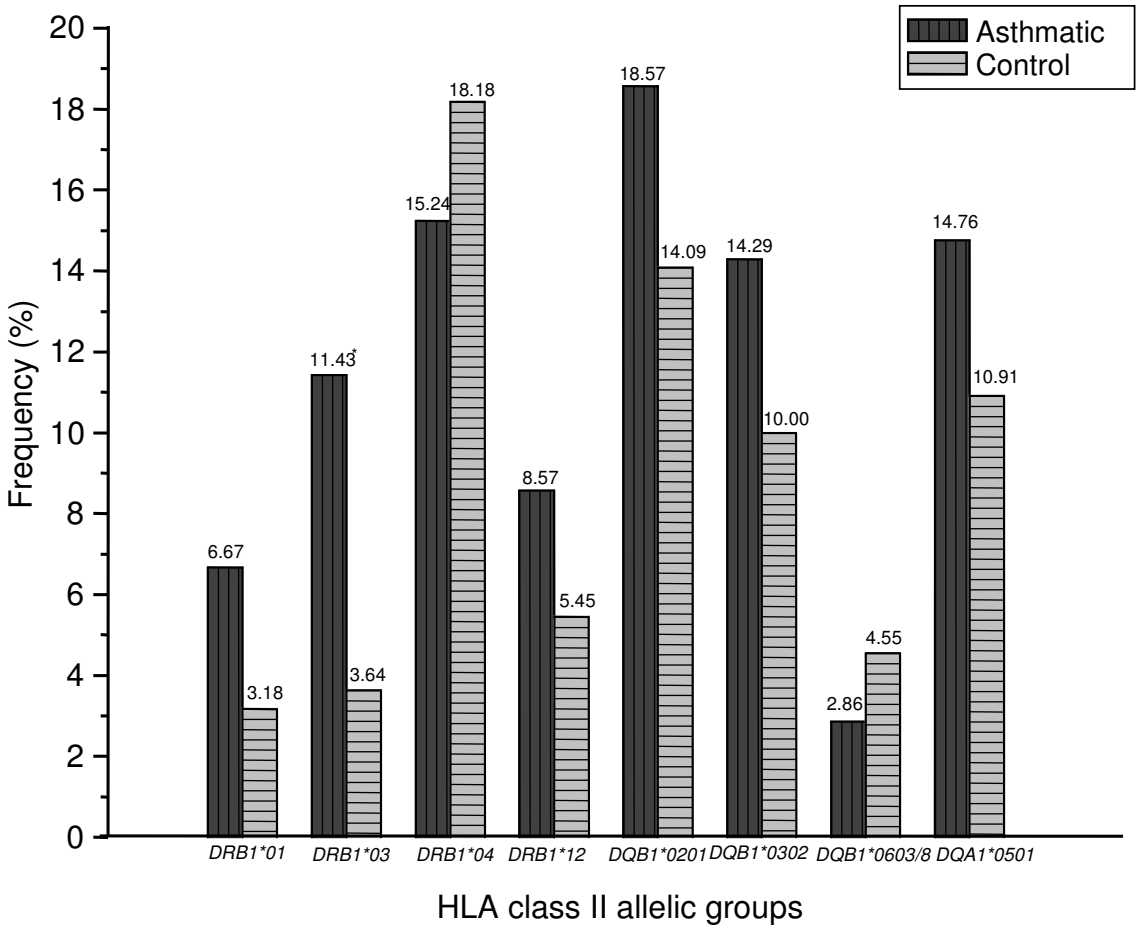

Figure 23. Comparison of Frequencies of HLA class II allelic groups between asthmatic and control groups.

Distribution of significant HLA A-B haplotypes in asthmatic and control groups is shown in Table 24. The result of the haplotype analysis revealed significantly higher frequencies of *A*01-B*37* (6.19% vs. 1.82%, OR=3.74, p=0.018*), *A*24-B*08* (7.14% vs. 2.73%, OR=2.89, p= 0.029*) and *A*26-B*37* (5.71% vs. 1.36%, OR=4.60, p=0.012*) haplotypes and lower frequencies of *A*11-B*44* (0.48% vs. 3.18%, OR = 0.14, p=0.036*) and *A*25-B*52* (0.95% vs. 4.09%, OR = 0.22, p = 0.037*) haplotypes in asthmatic subjects than in controls. Since only few selected HLA class II allelic groups were studied, thus detailed haplotype analysis of class I and class II loci were not undertaken.

Table 23. Frequencies of HLA allelic groups in two groups of asthmatic subjects viz. tIgE>150IU/ml and tIgE<150IU/ml

HLA allelic group	**tIgE>150IU/ml (N=50) Freq. % (n)**	**tIgE<150IU/ml (N=20) Freq. % (n)**	χ^2	**p**	**OR [95% CI]**
*A*01*	18.0 (18)	12.5 (5)	0.364	0.546	1.69 [0.53-5.41]
*A*03*	14.0 (14)	17.5 (7)	0.083	0.773	0.72 [0.24-2.18]
*A*11*	9.0 (9)	10.0 (4)	0.021	0.884	0.88 [0.24-3.26]
*A*24*	17.0 (17)	12.5 (5)	0.201	0.654	1.55 [0.48-4.98]
*A*25*	12.0 (12)	7.5 (3)	0.256	0.612	1.79 [0.45-7.17]
*A*26*	13.0 (13)	15.0 (6)	0.002	0.966	0.82 [0.26-2.58]
*B*08*	20.0 (20)	22.5 (9)	0.013	0.908	0.81 [0.29-2.32]
*B*44*	6.0 (6)	5.0 (2)	0.032	0.859	1.23 [0.23-6.66]
*B*45*	8.0 (8)	7.5 (3)	0.067	0.795	1.08 [0.26-4.56]
*B*51*	7.0 (7)	5.0 (2)	0.003	0.955	1.47 [0.28-7.74]
*B*52*	5.0 (5)	2.5 (1)	0.041	0.840	0.23 [2.11-19.30]
*B*37*	19.0 (19)	15.0 (6)	0.126	0.723	1.43 [0.47-4.36]
*DRB1**					
01	9.0 (9)	5.0 (2)	0.218	0.640	1.98 [0.39-10.08]
03	11.0 (11)	7.5 (3)	0.109	0.741	1.60 [0.39-6.47]
04	14.0 (14)	15.0 (6)	0.016	0.900	0.91 [0.29-2.83]
12	8.0 (8)	12.5 (5)	0.286	0.593	0.57 [0.16-2.02]
*DQB1**					
0201	17.0 (17)	20.0 (8)	0.039	0.844	0.77 [0.27-2.25]
0302	16.0 (16)	10.0 (4)	0.506	0.477	1.88 [0.54-6.55]
0603/8	3.0 (3)	0 (0)	0.218	0.641	Undefined
*DQA1**					
0501	15.0 (15)	10.0 (4)	0.305	0.581	1.71 [0.49-5.99]

tIgE, total serum immunoglobulin E; OR, Odds ratio; CI, confidence interval

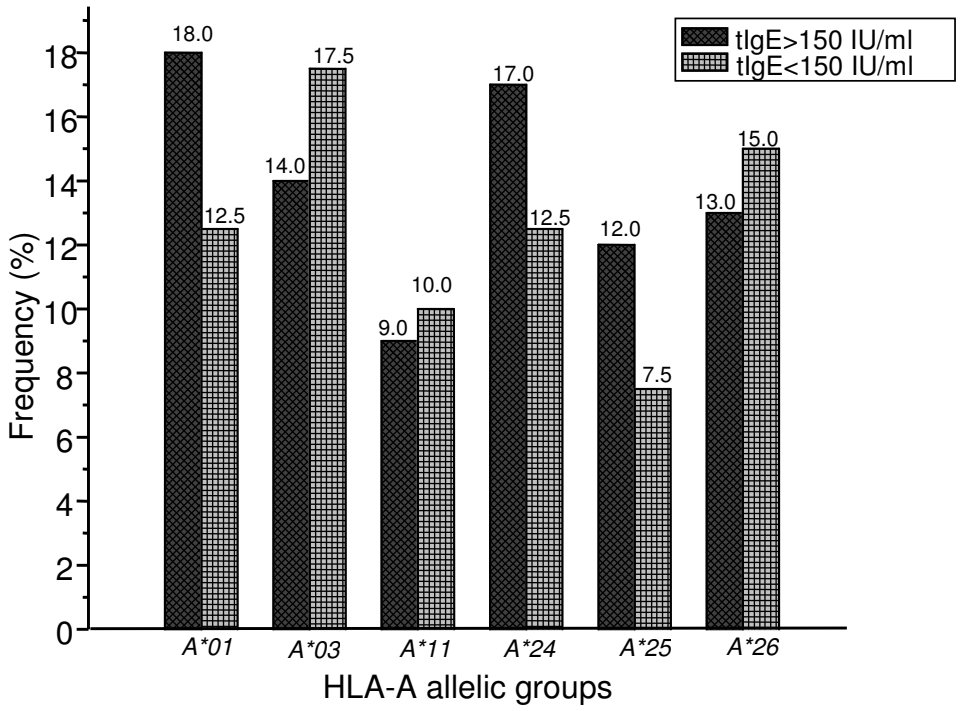

Figure 24. Comparison of frequencies of HLA-A allelic groups in two groups of asthmatic subjects viz. tIgE>150IU/ml and tIgE<150IU/ml.

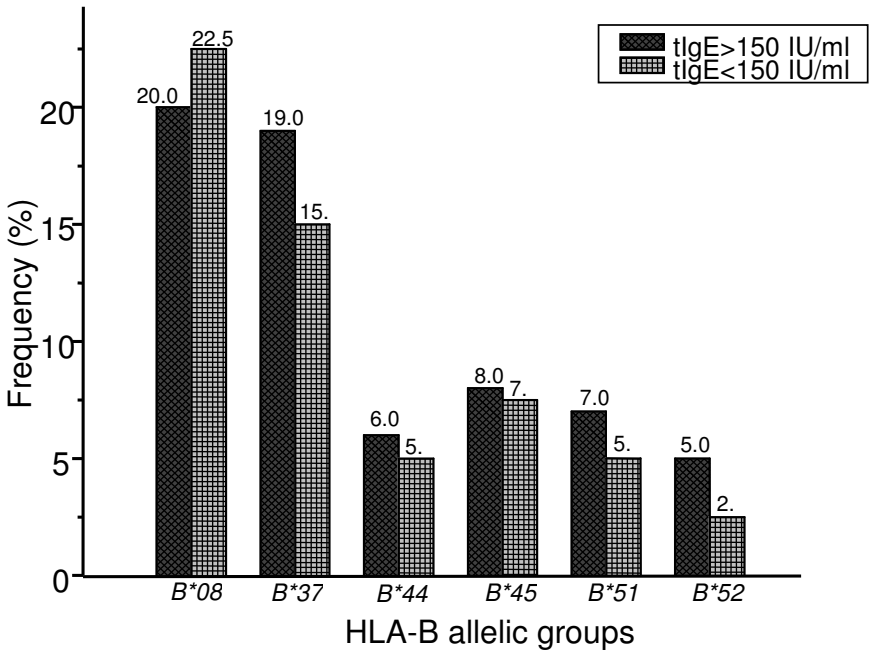

Figure 25. Comparison of frequencies of HLA-B allelic groups in two groups of asthmatic subjects viz. tIgE>150IU/ml and tIgE<150IU/ml.

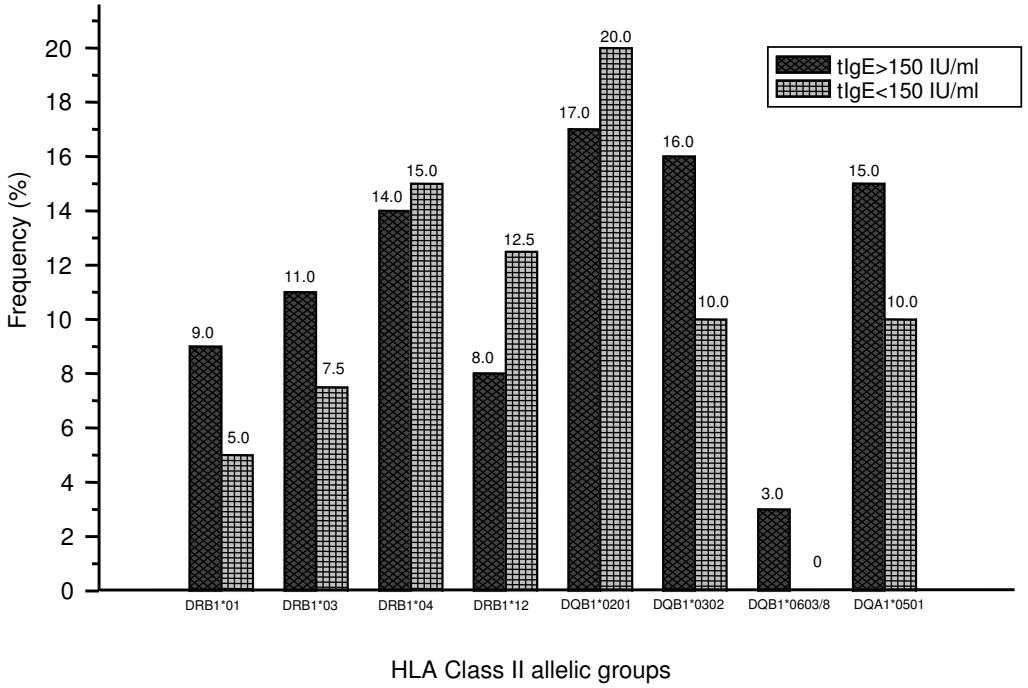

Figure 26. Comparison of frequencies of HLA class II allelic groups in two groups of asthmatic subjects viz. tIgE>150IU/ml and tIgE<150IU/ml.

Table 24. Distribution of significant HLA A-B haplotypes in asthmatic and control subjects

Haplotype	**Asthmatics (N=105) Freq. % (n)**	**Controls (N=110) Freq. % (n)**	**$\chi 2$**	**p**	**OR [95% CI]**
*A*01-B*37*	6.19 (13)	1.82 (4)	5.64	0.018	3.74 [1.18-11.88]
*A*24-B*08*	7.14 (15)	2.73 (6)	4.75	0.029	2.89 [1.08-7.76]
*A*26-B*37*	5.71 (12)	1.36 (3)	6.27	0.012	4.60 [1.26-16.81]
*A*11-B*44*	0.48 (1)	3.18 (7)	4.39	0.036	0.14 [0.02-1.17]
*A*25-B*52*	0.95 (2)	4.09 (9)	4.36	0.037	0.22 [0.05-1.03]

Freq. % = n/2NX100
OR, Odds ratio; CI, Confidence interval

3.5.2 Discussion

In the present study, it was observed that the frequency of *HLA-DRB1*03* was significantly higher in asthmatic subjects than in controls (OR=3.78, 95%CI=1.61-8.85,

p=0.0025, p_{corr}<0.05). The association of *HLA-DRB1*03* with childhood asthma, in the present study, is consistent with the findings of various earlier studies. A study conducted by Ivković-Jureković *et al*. (2011) in Crotian asthmatic children, showed a significantly higher frequency of *HLA-DRB1*03* specificity among the asthmatic patients with total serum IgE≥400KU/L. In another study, Juhn *et al.* (2007) reported that *HLA-DRB1*03* allele was the most significantly associated with an increased risk of asthma (HR: 1.5, 95% CI: 1.0-2.4, p=0.050). Similarly, Hanchard *et al.* (2010) suggested the role of *HLA-DRB1*03* in asthma susceptibility independent of ancestral-haplotype-mediated linkage disequilibrium. In addition, it has been reported that *HLA-DRB1*03* allele plays an important role in determining the eosinophilic airway inflammation, a Th2 mediated inflammation (Rajagopalan *et al.,* 2006). The frequency of *HLA-DRB1*01* was higher in asthmatic subjects than in controls, though the difference was not significant (6.67% vs. 3.18% respectively; OR = 2.26, 95% CI = 0.88 – 5.85, p=0.136). Analysis of HLA alleles in association with the elevated level of total serum IgE showed that none of the HLA allelic groups was found to be associated with the elevated level of total serum IgE in asthmatic subjects. Many studies have reported the positive significant association of *HLA-DRB1*01* with the elevated level of total serum IgE in asthmatics and/or allergic patients. Torío *et al.* (2003) showed a significant association of *DRB1*01* with the elevated level of total serum IgE in the Spanish *Artemisia* sensitive asthmatics. Woszczek *et al.* (2002) reported the significantly higher total serum IgE levels in allergic patients with *HLA-DRB1*01* compared to patients without these allele. Similarly, Ulbrecht *et al.* (1997) reported a weak association of *HLA-DRB1*01* with specific IgE-positive cases compared to negative controls.

Asthma and its associated trait ‘‘atopy’’ were some of the first complex diseases for which a strong genetic basis was established (Barnes, 2001). HLA class II antigens play a key role in antigen presentation to CD4+ T-lymphocytes and therefore influence the specificity of the immune response. HLA genes have been implicated in triggering an allergen-specific IgE response. The amino acid constituents of the specific epitopes of allergens have been identified and specific HLA-DR and DQ gene products have been shown to present these epitopes (Verhoef *et al.,* 1993). HLA-DR alleles are found to be associated with the development of specific IgE response to seasonal as well as perennial allergens

(Marsh *et al.,* 1982; O'Hehir *et al.,* 1990). Murray (1998) suggested the potential role of HLA genes in determining the Th1 vs Th2 immune response through the interaction between T-cell receptor, peptide, and MHC molecules. Blumenthal *et al.* (1992) have suggested a different role of HLA gene polymorphism. They showed that in pollen allergy, the asthma phenotype may be associated with MHC-extended haplotype (*HLA-B7/SC31/DR2*).

Several studies have reported the appearance of various susceptible and protective HLA haplotypes in different clinical forms of asthma. Kim *et al.* (2006) reported the significant association of many two loci and three loci HLA haplotypes with isocyanate-induced occupational asthma in an Asian population. These include *A*02-B*62, A*02-DRB1*15, A*02-DQB1*06, B*62-C*09, A*02-DRB1*15-DQB1*06, DRB1*15-DPB1*05, DRB1*09-DPB1*05* and *DRB1*09-DQB1*0303-DPB1*05*. Lara-Marquez *et al.* (1999) reported a significant association between the *HLA-DRB1*1101-DQA1*0501-DQB1*0301* haplotype and *Dermatophagoides spp*.-sensitive asthma in Venezuelan population. Similarly, Choi *et al.* (2004) reported a higher frequency of *HLA-DRB1*0901-DQB1*0303-DPB1*0501* haplotype in aspirin intolerant asthmatic patients than in aspirin tolerant asthmatic patients. *HLA-DRB1*0901-DPB1*0201* and *DQB1*0303-DPB1*0201* haplotypes were also shown to be significantly associated with aspirin intolerant asthma (AIA vs. NC, $p<0.0005$, $p_c<0.05$). Further, the frequency of *DQB1*02-DPB1*0301* haplotype was significantly higher in patients with aspirin intolerant asthma than in those with aspirin tolerant asthma and control subjects. Bede *et al.* (2002) reported an increased frequency of HLA-*DR7-DQA1*0201-DQB1*0202* haplotype in mite sensitive asthmatic children compared to non-atopic controls and non-mite sensitive asthmatic controls and a decreased frequency of *HLA-DR4-DQA1*0301-DQB1*0302* haplotype among mite sensitive asthmatic patients compared to non-atopic controls.

Although it has been known that HLA is associated with asthma and/or related phenotypes but its exact role in disease pathogenesis is still not clearly understood. Probably, HLA alleles act in association with other genetic loci responsible for the regulation of total IgE. It has been shown that genetic loci of chromosome 11q and 5q are strongly associated with

high total IgE levels (Cookson *et al.,* 1989; Marsh *et al.,* 1994). Therefore, the regulation of the total IgE response is a complicated process involving several genetic loci.

In the present study, we failed to establish the association of HLA alleles with high level of total serum IgE in asthmatic subjects. It could be due to the limitations of our study. These include: relatively small number of subjects in two groups of asthmatics viz. total IgE<150IU/ml and total IgE>150IU/ml. Further, we selected only few alleles which have been reported to be associated with pediatric asthma in diverse ethnic populations. Therefore, further study in large cohort of asthmatic subjects of Siliguri and adjoining areas taking as many HLA alleles as possible is needed to support the present findings.

In conclusion, the present finding suggests the significant association of *HLA-DRB1*03* with asthma in the pediatric population of Siliguri region of West Bengal, India. Therefore, *HLA-DRB1*03* allele may be implicated in the susceptibility to asthma in the pediatric population of this region.

CHAPTER – 4

COMPREHENSIVE DISCUSSION

4. COMPREHENSIVE DISCUSSION

Asthma is a chronic inflammatory disorder of the airways, in which many cells and cellular elements play a role, in particular mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible persons, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, especially at night and in the early morning hours (NHLBI, 1997). The inflammation results in airflow obstruction, bronchial hyperresponsiveness to a variety of stimuli, and mucus hypersecretion. Irreversible structural remodeling may then occur in some patients, contributing to persistent abnormalities in lung function. Asthma usually begins in childhood. Determinants commonly associated with persistent childhood asthma include viral respiratory infections, history of allergy, family history of allergy, and atopy. Asthma exacerbations commonly result from respiratory viral infections, especially rhinovirus infection (Johnston *et al.,* 1995). Childhood asthma is frequently associated with atopy, which involves a genetic susceptibility to produce IgE in response to common environmental allergens, particularly house dust mites, pet dander, and fungi (Koren, 1997). Many airborne allergens can cause asthma, but these airborne allergens are most commonly associated with asthma onset. Atopy is probably the strongest predisposing factor in the development of asthma.

The etiology of asthma is complex and multifactorial. It involves the interaction between genetic factors and environmental stimuli. The strong familial clustering of asthma has encouraged an increasing volume of research into the genetic predisposition to disease. Although identification of all asthma genes is incomplete, genetic findings are already changing the prevailing view of asthma pathogenesis. Candidate-gene and linkage studies followed by positional cloning have already provided a large number of genes accountable for the susceptibility to asthma (Vercelli, 2008a). In addition, many genome-wide association studies (GWASs) published in recent decade have identified several genetic loci to be associated with asthma and/or its related phenotypes in different populations (Mathias *et al.,* 2010; Moffatt *et al.,* 2010; Li *et al.,* 2010).

In asthma, the development of immune response depends on a repertoire of cytokines produced by numerous cells, including CD4+ helper T cells. These lymphocytes can be

divided into two subsets, T helper type 1 (Th-1) and T helper type 2 (Th-2), based on their cytokine profiles (Romagnani, 1992). Effector Th1 cells are involved in delayed-type hypersensitivity through their production of IFN-γ and IL-2, whereas Th2 cells secrete IL-4, IL-5, IL-9 and IL-13, and promote antibody-mediated humoral immune responses (Brown and Ennis, 2005). It has been suggested that an alteration in cytokine milieu, with excess Th-2 products (IL-4, IL-5, and IL-13) in concert with decreased Th-1 products (IFN-γ and IL-2), is predicted to drive the asthma phenotype (Castro *et al.,* 2000). A hypersensitivity reaction initiated by immunologic mechanisms mediated by IgE antibodies occurs in allergic asthma. IgE plays a central role in the initiation and propagation of the inflammatory cascade and thus the allergic response (Buhl, 2005). Indeed, recent studies reveal that IgE, through its high affinity IgE receptor (FcεRI), is a critical regulator of Th2 responses (Peng, 2009). There are clear parallels between human allergic disease and the "Th-1/Th-2 paradigm" originally described in rodents (Romagnani, 1991; Mosmann and Sad, 1996). A substantial body of evidence implicates Th-2 type cytokines (IL-4, IL-5, IL-9, and IL-13) in the development and expression of allergy and airway inflammation (Chang *et al.,* 1996; Gabrielsson *et al.,* 1997; Kimura *et al.,* 2000; Jenmalm *et al.,* 2001).

Asthma and allergic diseases remain the most common diseases in the modern world, making it essential to determine the causal pathways and underlying mechanisms, with the hope that this may lead to more definitive treatment and prevention strategies. A more complete understanding of the processes involved in local and systemic immune development is a crucial part of this process. Therefore, the objectives of the study were to estimate the prevalence of asthma in children aged between 3-12 years and to investigate the associated risk factors, to determine the serum C-reactive protein (CRP) concentration in asthmatic children to understand the inflammatory process(es) in asthma and to study the effect of corticosteroid on serum CRP level, to estimate the levels of total serum IgE in asthmatic and control subjects and to investigate the relationship of various demographic and clinical characteristics with the level of total serum IgE in asthmatics, to determine the serum levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines in order to investigate the alteration

in Th1/Th2 balance in asthma, if any and to determine the frequency of some of the selected HLA class I and class II allelic groups in asthmatic and control groups.

4.1 Assessment of prevalence and associated risk factors of asthma

Asthma and other allergic disorders are common health problems especially in children. A high proportion of infants with early symptoms of atopic dermatitis and food allergy frequently develop more persistent, recurrent airway pathology. Asthma has a negative impact on quality of life. It impairs the child's social interaction and academic achievement (von Mutius, 2000). The burden of asthma and/or allergic disorders has been steadily rising in Western countries, with almost 40% of these populations showing evidence of allergic sensitization. There is now mounting concern that billions of people will be affected as developing countries begin to show the same trends (Lewis, 1998).

The prevalence of asthma has been reported to increase in many places around the world during the last decades (Manning *et al.*, 2007). Many factors have been reported that contribute to this increase. These include genetic factors as well as environmental factors such as lifestyle, infections and diet. In our preliminary hospital based study, the mean annual prevalence of asthma in children, aged 3 to 12 years, was observed to be 3.06%. The assessment of the risk factors revealed the association of family history of asthma/atopy with asthma.

Rising prevalence and morbidity of childhood asthma and allergic diseases have been observed globally (Pearce *et al.,* 2007; Eder *et al.,* 2006). Although some recent reports suggest the declining trend of prevalence of asthma but no overall global declining trend in the prevalence of asthma was shown in a recent review of epidemiological studies conducted to examine the international trends in asthma prevalence in children and adults for the period 1990-2008 (Anandan *et al.,* 2010). Many factors have been reported that contribute to the increased prevalence of asthma which include genetic factors as well as environmental factors such as lifestyle, infections and diet (Kiadeh *et al.,* 2013). Numerous epidemiologic studies have reported the varied rates of asthma prevalence in Indian children (Singh *et al.,* 2002; Awasthi *et al.,* 2004; Jain *et al.,* 2010; Ganesh *et al.,* 2012;

Cheraghi *et al.,* 2012). The variations in the prevalence of asthma in children as reported by various authors in Indian children could be due to regional variation, differences in diagnostic criteria, and most importantly the sample size.

Family history of asthma/atopy was found to be associated with development of asthma (χ^2 =8.89, p=0.003**). Previous studies from different parts of the world have also reported a strong association between family history of atopy and asthma with reported prevalence of asthma (Jenkins *et al.,* 1993; Christie *et al.,* 1999; Lee *et al.,* 2003; Rönmark *et al.,* 1999). The finding of the association between family history of asthma/atopy and development of asthma in children in our study highlights the fact that family history of asthma/atopy is indeed an important risk factor for asthma.

4.2 Serum C-reactive protein level

C-reactive protein was discovered in humans in 1930 as a serum component that binds the C polysaccharide of Streptococcus pneumoniae (hence CRP). Structurally, CRP is usually composed of five identical subunits (hence pentraxin), each of 23 kDa in mass, which are linked noncovalently to form a disc-like pentagonal ring (Anderson, 2006). It has long been used clinically to evaluate the presence and degree of inflammation because CRP blood levels increase as much as 1,000-fold within 24 hours after the onset of inflammation (Kushner *et al*., 1981). It has been demonstrated that CRP levels are associated with age, sex, race (African-American), body mass index (BMI), smoking, serumlipids, blood pressure, presence of diabetes mellitus, 2-h post-challenge glucose, frequency of exercise, and cardio-respiratory fitness (Folsom *et al*., 2002; Hashimoto *et al*., 2004).

The serum CRP concentration was determined in asthmatic children with and without ICS treatment in order to understand if serum CRP concentration could be taken as a marker for asthmatic inflammation. The result showed that the CRP concentration was significantly elevated in the serum of ICS-naïve group of asthmatic children compared to ICS-inhaling asthmatic children ($p < 0.001$). Recent publications suggest that CRP could be taken into

consideration as a simple, cheap and reliable marker for monitoring asthmatic inflammation (Kony *et al*., 2004; Olafsdottir *et al*., 2005).

The CRP is predominantly synthesized in the Liver in response to inflammation and tissue damage. Monocytes, lymphocytes and neutrophils are also able to produce CRP (Baumann and Gauldie, 1994). CRP is regulated by pro-inflammatory cytokines, with a recognized important role in the pathophysiology of asthma, primarily the TNF-α, NF-kappa B, IL-6 and IL-1β (Voleti and Agrawal, 2005). Although its function is still unclear, the CRP may serve as a general scavenger protein and play an important role to recognize bacteria and damaged human cells and to mediate their elimination through opsonization, phagocytosis, and cell-mediated cytotoxicity. The CRP can also activate the classical complement cascade by binding directly to the complement fragment C1q (Pepys and Hirshfield, 2003). A correlation of peripheral blood CRP levels with severity, extent, and progression of inflammatory pathologies has been well established. Recent publications suggested that hs-CRP has a contributing role in the pathogenesis of disease in addition to its being merely an inflammatory marker (Devaraj *et al.,* 2005).

Many studies have focused on the possible role of inhaled steroids in the attenuation of CRP levels in asthma and chronic obstructive pulmonary disease (COPD). Pinto-Plata *et al*. (2006) have reported that CRP levels were lower in COPD patients treated with inhaled steroids. Similarly, Karthikeyan *et al.* (2014) have reported significantly higher CRP levels in steroid naïve asthmatics compared to control subjects. While the serum CRP levels in steroid inhaling asthmatics were comparable with control group.

4.3 Total serum IgE level

Total serum immunoglobulin E (IgE) is known to be elevated in various allergic disorders such as allergic asthma, allergic rhinitis (AR), eczema, atopic dermatitis, bronchial hyper-responsiveness, and sometimes forms the basis of allergic diseases (Yunginger, 1988). A number of epidemiological studies have shown a strong association between total serum

IgE levels, skin test reactivity to aeroallergens, and asthma phenotype (Friedhoff and Marsh, 1993; Sherrill *et al.,* 1999). It has also been shown that noncognate production of IgE (i.e. production of IgE that is not driven by one or few specific allergens) is a significant inherited risk factor for the development of asthma (Suayer *et al.,* 1996).

The level of total serum IgE was measured in asthmatic and control subjects using the ELISA kits (AccuBind, Monobind Inc., USA).The mean total serum IgE level was significantly higher in asthmatic subjects compared to that of the control subjects (269.21 ± 150.97IU/ml versus 146.89 ± 77.32IU/ml; $p<0.001^{***}$). Increased serum IgE levels in asthma may be due to increases in IgE-dependent processes and cellular components of the immune system. The secretion of IgE by lymphocytes defines the allergic state of an individual. The cellular events associated with IgE-dependent processes are very much important in asthma (Tracey *et al.,* 1995). Higher IgE levels indicate some types of inherent susceptibility and/or presence of a disease process involving airway inflammation (Chowdary *et al.,* 2003; Sherril *et al*., 1995).

Analyses of the demographic and clinical characteristics in association with total serum IgE revealed that higher age group (8-12 years), raised eosinophil count and exposure to cigarette smoke were significantly associated with the elevated level of total serum IgE in asthmatic subjects. In a study, a significant relation between age and IgE levels was reported in the allergic patients (Sharma *et al.,* 2002). In this study, the authors have observed the highest mean IgE levels in 8-12 years age group (642IU/ml). Similarly, in an American population, it was reported that in asthmatic patients, IgE increases until the age of 9 years and after reaching a peak, the IgE levels decrease in the teenage years (Grundbacher and Massie, 1985). According to Halonen *et al.* (1982), a significant relationship exists between serum IgE levels and eosinophilia in populations presumed to be free of parasites where IgE levels presumably provide a better clue to atopy than do skin tests. Various earlier studies have also identified exposure to cigarette smoke (passive smoking) as an important risk factors for the elevated level of total serum IgE in asthmatics (Satwani *et al*., 2009; Kartasamita *et al.,* 1994). The mechanism of modulation of IgE levels by tobacco smoke is not well understood (Sherrill *et al.,* 1994, Sapigni *et al*., 1998). There could be indirect and direct actions of tobacco smoke on IgE levels (Villar and

Holgate, 1995). An indirect action could increase the likelihood of developing sensitivities to inhaled allergens. In fact, smoke increases permeability in the lungs possibly facilitating and enhancing penetration of allergens. Tobacco smoke could have a direct action on IgE levels through immune system cellular regulation changing the function of T lymphocytes (Holt, 1987). Th2 lymphocytes regulate IgE production. Thus, newborns with smoking parents have higher cord IgE levels, regardless of parental atopy, than newborns born to non-smokers (Magnusson, 1986).

Elevated serum levels of specific IgE towards common environmental allergens are a key component in the pathogenesis of allergic asthma. IgE antibodies cause chronic airway inflammation through effector cells such as mast cells, basophils, etc., activated via high-affinity (FcεRI) or low-affinity (FcεRII) IgE receptors. IgE has been viewed as a target for immunological drug development in asthma. Despite an increase in the availability of drugs for asthma, a number of strategies aimed at inhibiting the proinflammatory action of IgE have been developed in recent years (D'Amato *et al*., 2014).

4.4 Serum levels of IL-4 and IFN-γ

Asthma is characterized by chronic inflammation in the airways and the presence of a predominance of CD4+ T-helper 2 (Th2) cells that secrete IL-4, IL-5, and IL-13 cytokines (Ober, 2005). Th2 cells contribute to the immunopathogenesis of asthma by recruiting eosinophils and mast cells to the airways (Ober, 2005; Romagnani, 1994; de Vries *et al.*, 2000) and by inducing B-cells to produce immunoglobulin E antibodies (Kuo *et al.*, 2001). Allergic and asthmatic subjects are more likely to have elevated levels of the Th2 cytokines and reduced levels of the Th1 cytokines (IFN-γ and TNF-β).

Serum levels of IL-4 and IFN-γ were determined among 48 asthmatic children (18 steroid-naïve and 30 steroid-treated) and 32 control subjects using Enzyme linked immunosorbent assay (ELISA) method. It was observed that serum level of IL-4 was significantly higher in steroid-naïve group as compared to control subjects but it was lower in steroid-treated group. In contrast, serum levels of IFN-γ were significantly lower in both steroid-naïve as

well as steroid-treated groups of asthmatic children compared to control subjects. It has been suggested that an alteration in cytokine milieu, with excess Th-2 products (IL-4, IL-5, and IL-13) in concert with decreased Th-1 products (IFN-γ and IL-2), is predicted to drive the asthma phenotype (Castro *et al.,* 2000). Elevated levels of IL-4, an essential cofactor for IgE production, and IL-5, responsible for the final differentiation, activation and recruitment of eosinophils (Kay, 1991), have been found in serum of patients with asthma (Matsumoto *et al.,* 1991; Hashimoto *et al.,* 1993; Matsumoto *et al.,* 1994; Tang *et al.,* 1995). On the other hand, IFN-γ is thought to protect against the development of asthma by regulating Th-2 cytokine production, although a mixed Th-1/Th-2 pattern has also been reported (Heaton *et al.,* 2005). In a recent study, Figueiredo *et al.* (2012) have shown that non-atopic asthma was associated with IFN-γ and elevated monocytes in blood and suggested that IFN-γ and monocytes might play a role in immunopathology of non-atopic asthma in Latin American children.

One of the first studies measuring cytokine concentrations in children with allergic disease, revealed a significant increase in the level of IL-4 in serum from atopic asthmatics compared to controls, which correlated with IgE (Matsumoto *et al.,* 1991). Other subsequent studies in serum and blood supported the importance of IL-4 in childhood asthma (Akcakaya *et al.,* 1994; Daher *et al.,* 1995; Krogulska *et al.,* 2009). IL-4 demonstrates a broad range of biological activities. It is a main cytokine involved in the pathogenesis of allergic responses and at the same time it can also down-regulate acute inflammatory changes (Chung and Barnes, 1999). IL-4 has also got additional effects on asthma pathogenesis which include stimulation of mucus producing cells and fibroblasts leading to airway remodeling (Dabbagh *et al.,* 1999; Trautmann *et al.,* 1998; Doucet *et al.,* 1998). It has also been confirmed that the crucial role of IL-4 lies in its effect on Th-2 development, rather than on the induction of IgE synthesis and subsequent mast cell degranulation (Coyle *et al.,* 1995). Several other invasive studies involving bronchoalveolar lavage (BAL) fluid and lung biopsies have confirmed that a Th-2-like mediated immune response is seen in asthma (Robinson *et al.,* 1992; Umetsu *et al.,* 1997; Walker *et al.,* 1992). Gemou-Engesaeth *et al.* (1997) and Krouwels *et al.* (1996) have reported the imbalance in the production of IL-4 and IFN-γ in children with atopic asthma, and corticosteroids appear to correct it.

4.5 Typing of HLA allelic groups

Allergic asthma is considered a multifactor disease, the possibility of increasing understanding of the mechanisms by which inherited factors influence disease has stimulated the study of human leukocyte antigen (HLA). HLA is controlled by genes within the major histocompatibility complex which is associated with other aspects of immune response, some complement components, and susceptibility to certain diseases (Svejgaard *et al*., 1975). The products of major histocompatibility complex play a fundamental role in regulating immune responses since they encode the molecules that represent the linkage elements between environmental allergens and the immune system. HLA genes have been implicated in the development of asthma and atopy, but the importance of associations between HLA genes and asthma remains unclear. Different HLA genes may represent factors conferring risk or protection for the development of allergic diseases. It is assumed that HLA genes as genetic markers have influence on the atopy and asthma as well as on sensitization against specific inhalant allergens.

According to the published data, it seems that individual antigens do not have a significant influence on the intensity of specific IgE immunological response. It is more probable that environmental factors or other loci (e.g. genes for T-cell receptor or TNF-α) are important in determining the individual sensitization to a specific allergen (Li *et al.,* 1995). The class I genes of MHC may have important effects on atopic responses, but these have not yet been adequately investigated. Similarly, the class III complement genes contain polymorphisms which may be of relevance to inflammatory or immune diseases. Polymorphism in the HLA class II molecules may lead to allelic forms that are more effective in binding allergenic peptides (i.e. epitopes) on the membranes of antigen-presenting cells, thus leading to allelic disequilibrium of HLA class II alleles among sensitized individuals (Howell and Holgate, 1995; Tomlinson and Bodmer, 1995). The most replicated and possibly the strongest association between the HLA system and allergic disease is that between increased IgE production in response to the ragweed *Artemisia artemisiifolia* pollen allergens Amb aV, Amb tV, AmbpV, and Amb aVI in individuals expressing the *HLA-DR5* allele (Marsh *et al.,* 1987).

In the present study, we studied the selected HLA class I and class II allelic groups which have been reported to be associated with childhood asthma in various populations. The result of our study did not show the significant association of HLA class I allelic groups with asthma. The HLA A-B haplotype analysis revealed the higher frequencies of *A*01-B*37*, *A*24-B*08*, *A*26-B*37* haplotypes and lower frequencies of *A*11-B*44* and *A*25-B*52* haplotypes in asthmatic subjects than in controls. As only few selected class II allelic groups were included in the study, haplotypes of HLA class I and class II loci were not undertaken. Several studies have investigated the association of HLA class I alleles with the asthma. The work of Thorsby *et al*. (1997) has been cited showing an increased frequency of *A1/B8* in asthmatic children, but the figures were not significant. Morris *et al.* (1980) reported that *HLA- B*12* (B44 and B45) was increased in the allergic asthmatics compared to controls (46% *vs.* 29%) and it is suggested that *B*12* is associated with the ability to produce the IgE antibodies. *A3/B7/DRw2* (which are in linkage disequilibrium) all show a decreased frequency in intrinsic asthmatic patients compared to controls (24%, 12% and 9% *vs.* 32%, 26% and 24% respectively). Besides, *HLA-B*8* and *DRw3*, which showed a moderate increase in frequency in all three groups of asthmatics, were found in five of seven patients with low atopy but persisting antibodies to *A. fumigates.* Wang *et al.* (1988) reported the much higher frequencies of *HLA-A*9*, *-A*10*, *-BW*61* and *-BW*62* and much lower frequency of *HLA-A*03* in the asthmatic subjects than in the normal controls. However, after the p-values were corrected, the significant difference only existed in *HLA-Bw61*.

Among class II allelic groups, *HLA-DRB1*03* was found to be associated with childhood asthma (OR=3.78, 95% CI=1.61-8.85, p=0.0025, $p_{corr} < 0.05$). Further analysis of HLA allelic groups in asthmatics with high and low total serum IgE levels did not show the significant association. Various studies have reported the association of HLA class II alleles and haplotypes with asthma in different populations. In a study, *HLA-DQA1*0104* and *-DQB1*0201* were reported to be positively associated while *HLA-DQA1*0301* and *-DQB1*0301* alleles were negatively associated with asthma (Gao *et al*., 2003). Another study in Greek children with allergic asthma revealed that *DRB1*04* and *DQA1*0301* might be important factors in susceptibility to asthma with sensitivity to mites (Parapanissiou *et al*., 2005). Horne *et al.* (2000) have shown that *HLA-DRB1*0401-*

*DQB1*0302* haplotype as the most susceptible haplotype in development of asthma due to red cedar. While the presence of the *DRB1*0101-DQB1*0501* haplotype appeared to confer protection. Similarly, Wosczek *et al.* (2002) showed a significant association between *HLA-DRB1*02, B5** haplotype and asthma phenotype in patients with grass-pollen allergy. They also showed the association between *HLA-DRB1*01* alleles and higher total serum IgE levels in the patients with grass pollen allergy. HLA class II genes relate to non-specific modulation of inflammation. *HLA-DRB1* and *DQB1* SNPs and haplotypes have been associated with a higher risk of toluene diisocyanate-induced occupational asthma (Choi *et al.,* 2009), total serum IgE in Iranian subjects (Movahedi *et al.,* 2008), atopy in Northern Chinese (Gao *et al.,* 2003), *Dermatophagoides spp*.-sensitive asthma in Venuezuelan individuals (Lara-Marquez *et al.,* 1999), and asthma severity in whites in the United States (Juhn *et al.,* 2007), suggesting a broad role for these genes in asthma pathogenesis across ethnic groups.

Among genetic factors contributing to the development of atopic diseases, HLA genes have been implicated in triggering an allergen specific IgE response. HLA-DR alleles were found to be associated with the development of specific IgE reactions to seasonal (Marsh *et al.,* 1982) and perennial allergens (O'Hehir *et al.,* 1990) and to some drugs (Kowalski *et al.,* 1998). A different role for HLA gene polymorphisms has been suggested by a study of Blumenthal *et al.* (1992), who found that in pollen allergy the asthma phenotype may be associated with MHC extended haplotype *HLA-B7/SC31/DR2* and patients with rhinitis alone have increased frequency of *HLA-B8/SC01/DR3* haplotype. It has been reported that a potent pro-inflammatory cytokine TNF-α gene polymorphism, which is in linkage disequilibrium with the HLA loci, may affect cytokine generation and also the severity of the disease (Brinkman *et al.,* 1997). Interestingly, an association of extended TNF-α haplotype *LTα Ncol*1/TNF-308*2/HLA-DRB1*02* and asthma has also been reported (Moffatt *et al.,* 1999), suggesting that it is the combination of different polymorphic loci localized to chromosome 6 (particular extended haplotype) that influences asthma phenotype.

CHAPTER – 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Although the propensity for allergy can manifest at any age, allergic diseases frequently develop in the first years of life, suggesting that very early events play an important role in initiating these disease processes. Decades of research have provided a detailed knowledge of the immunological processes that underlie the acute allergic immune response. Surprisingly, little is known about how or why these inflammatory processes are initiated and why an increasing number of individuals are affected. A better knowledge of early initiating events is critical in both understanding disease pathogenesis and planning prevention strategies. The objectives of the present study were to estimate the prevalence of asthma in children aged between 3-12 years and to investigate the associated risk factors, to assess the systemic inflammation by estimating serum CRP levels, to estimate the levels of total serum IgE in asthmatic children, to determine the serum levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines in order to investigate the alteration in Th1/Th2 balance in asthma, if any and to determine the frequency of some of the selected HLA class I and class II allelic groups in asthmatic and control groups.

Assessment of prevalence and associated risk factors of asthma

Epidemiological study provides an assessment of disease frequency and burden of pediatric asthma. In addition, it allows researchers to explore associations of risk factors for childhood asthma and the study of disease progression as well as the effect of therapeutic interventions. Asthma is a major global health problem, characterized as a chronic disease affecting a major proportion of pediatric population. The prevalence of asthma has been reported to increase in many places around the world during the last decades. Many factors have been reported that contribute to this increase. Increased prevalence of asthma is multifactorial in etiology.

In this hospital based study, the prevalence of asthma and the association of risk factors among the pediatric population in the age group of 3-12 years were investigated. Children who visited the Out-Patient Department of Pediatrics, North Bengal Medical College and

Hospital, from May 2009 to April 2010, were registered for the study. Asthma was diagnosed by the physician. The relevant data were collected using the questionnaire.

The prevalence of asthma among children in the age group between 3-12 years was 3.06%. The assessment of risk factors showed that the family history of asthma was significantly associated with asthma in children (33% vs. 15.45% in asthmatic and control subjects respectively). The present finding of the prevalence of childhood asthma in and around Siliguri seems to be similar to the prevalence rates in other rural areas of the country as reported by various studies. Results of our study also indicated that asthma is associated with the family history of asthma/atopy suggesting that genetic predisposition may be an important etiology for the development of asthma.

Serum level of CRP

Asthma is characterized by airway hyperresponsiveness and inflammation, in which various cells (such as eosinophils, neutrophils, macrophages and T-lymhocytes), cytokines and mediators play a role. Beside local inflammation, systemic inflammation is also present in asthma. C-reactive protein (CRP) is an acute-phase reactant secreted by hepatocytes in response to circulating inflammatory cytokines. It has long been used clinically to evaluate the presence and degree of inflammation because CRP blood levels increase as much as 1,000-fold within 24 hours after the onset of inflammation. Therefore, serum CRP concentration was determined in inhaled corticosteroid (ICS)-naïve and ICS-inhaling asthmatic children to understand the inflammatory process(es) in asthma.

Serum level of CRP was studied among 87 asthmatic children (15 ICS-naïve and 72 ICS-inhaling). Freshly separated serum samples were used for the test. Commercially available CRP kit 'IMMUNOSTAT' (Ranbaxy Fine Chemicals Ltd., HP, India) was used for the detection of CRP level in the serum sample. The limitation of detection of the test was less than 6 mg/L. Further, CRP was treated as a categorical variable: elevated (≥6 mg/L) and normal (<6 mg/L).

The result of the study revealed that the elevated serum CRP concentration was detected in 13 (86.7%) ICS-naïve children and in only 3 (4.2%) ICS-inhaling children. The CRP concentration was significantly elevated in the serum of ICS-naïve asthmatic subjects ($p<0.001^{***}$). This study suggests that the asthmatic inflammation is associated with the elevation of serum CRP concentration and the ICS, which has the anti-inflammatory properties, might have played a role in reducing the CRP concentration to normal level in the ICS-inhaling children.

Total serum immunoglobulin E

IgE has been shown to be a major contributing factor for the development of bronchial hyperresponsiveness in asthma. An elevation in serum IgE level contributes to asthma and is considered a potent predictor of the development of asthma. The objectives of the present study were to estimate the levels of total serum IgE in asthmatic and control subjects and to investigate the relationship of various demographic and clinical characteristics with the total serum IgE level in asthmatics.

The levels of total serum IgE were measured in asthmatic and control subjects using the ELISA kits (AccuBind, Monobind Inc., USA). The relevant demographic and clinical data were obtained using the questionnaire. The results showed significantly elevated level of total serum IgE in asthmatic children compared to the controls subjects (269.21 ± 150.97 and 146.89 ± 77.32 IU/ml, respectively, $p<0.001^{***}$). The levels of total IgE and IL-4 in sera of 44 asthmatic children showed a significant positive correlation ($r=0.56$, $p<0.001^{***}$). In the present study, the higher age group, exposure to cigarette smoke, and the raised eosinophil count showed the significant association with the elevated level of total serum IgE in asthmatic children. The present findings suggest the allergic etiology of asthma in the subjects studied. Further, it also reveals the significant association of higher age, exposure to cigarette smoke and raised eosinophil count with the elevated level of total serum IgE in asthmatics.

Serum Levels of IL-4 and IFN-γ

Asthma is a chronic disease of the lung characterized by shortness of breath, wheeze, cough, reduced airflow on expiration, and airway hyperreactivity to non-specific bronchoconstrictors. Recent evidence suggests that asthma is not a single disease, but consists of several subtypes, including allergic and steroid-resistant asthma. Allergic asthma is mediated by the Th-2 cytokines. It has been suggested that an alteration in cytokine milieu, with excess Th-2 products (IL-4, IL-5, and IL-13) in concert with decreased Th-1 products (IFN-γ and TNF-β), is predicted to drive the asthma phenotype. Elevated levels of IL-4, an essential cofactor for IgE production, and IL-5, responsible for the final differentiation, activation and recruitment of eosinophils, have been found in serum of patients with asthma. On the other hand, IFN-γ is thought to protect against the development of asthma by regulating Th-2 cytokine production, although a mixed Th-1/Th-2 pattern has also been reported.

Serum levels of IL-4 and IFN-γ were determined among 48 asthmatic children (18 steroid-naïve and 30 steroid-treated) and 32 control subjects using Enzyme Linked Immunosorbent Assay (ELISA) kits with the objectives of comparing the serum levels of IL-4 and IFN-γ between the asthmatic and control subjects, investigating any alteration in Th-1/Th-2 balance, and analyzing whether there is any deviation in the levels of cytokines with corticosteroid treatment in asthmatic subjects.

Serum level of IL-4 was significantly higher in steroid-naïve group of asthmatic children as compared to control group (52.25 ± 21.91 versus 32.81 ±16.28 pg/ml; $p< 0.001^{***}$) while it was lower in steroid-treated group of asthmatic children but not statistically significant when compared with steroid-naïve group (40.80 ± 17.77 versus 52.25 ± 21.91 pg/ml; p = 0.054, NS). In contrast, serum level of IFN-γ was significantly lower in both steroid-naive and steroid-treated groups of asthmatic children compared to control group (21.62 ± 9.91 versus 30.79 ± 14.28; $p = 0.02^{*}$ and 23.03 ± 10.54 versus 30.79 ± 14.28 pg/ml; $p = 0.019^{*}$), respectively. The results of our study suggest that serum level of IL-4 may be elevated in concert with decreased level of IFN-γ in asthma. Determination of serum levels of IL-4 and IFN-γ may be a useful tool for understanding the disease processes in asthma.

Association of HLA with asthma

Asthma is a heterogeneous disease for which a strong genetic basis is firmly established. It is a complex disorder influenced by gene-environment interaction. HLA genes have been shown to be consistently associated with asthma and its related phenotypes in various populations. The aim of the present study was to determine the frequency of the selected HLA class I and class II allelic groups in asthmatic and control subjects.

Frequencies of HLA alleles were determined among 105 asthmatic and 110 control subjects using PCR-SSP method. The allele and two loci haplotype frequencies were estimated by direct counting. Frequency of each HLA allele and/or haplotype was compared between asthmatic group and control group using χ^2 test. P-value was corrected by multiplying with the number of the allelic groups studied. Odds ratio (OR) and 95% CI for each allele were calculated using GRAPHPAD INSTAT version 3.10.

The result of our study did not show the significant association of HLA class I allelic groups with asthma. Among class II allelic groups, the frequency of *HLA-DRB1*03* was significantly higher in asthmatic children than in controls (11.43% vs. 3.64%, OR=3.78, 95% CI=1.61 – 8.85, p=0.0025, p_{corr}<0.05). Further analysis of HLA allelic groups in two groups of asthmatic subjects with high and low total serum IgE levels did not show the significant association. Therefore, *HLA-DRB1*03* may be implicated in the susceptibility to asthma in the pediatric population.

On the basis of our observation, the following concluding remarks may be drawn:

1. The prevalence of childhood asthma observed in our hospital based study is comparable to the prevalence rates reported from other rural areas of India, although we need to estimate the actual prevalence among the general pediatric population taking into consideration the school going children of different age groups.

2. The family history of asthma/allergy was found to be associated with asthma suggesting that genetic predisposition may be an important etiology for the development of asthma in children.

3. Serum CRP level was found to be elevated in ICS-naïve asthmatics reflecting the ongoing systemic inflammation. While serum CRP level in ICS-treated asthmatics was normal and this could be due to the anti-inflammatory action of the ICS. Therefore, CRP may be considered as a marker of inflammation in asthma.

4. The increased level of IL-4 and decreased level of IFN-γ in serum of asthmatic children suggest the Th-2 mediated pathogenesis supporting the hypothesis of Th1/Th2 imbalance in asthma.

5. Significantly higher level of total serum IgE in asthmatic children may indicate the allergic etiology of asthma. Further, the study showed the association of higher age group, exposure to cigarette smoke, and raised eosinophil count with the high titer of total serum IgE in asthmatic children.

6. The present preliminary finding suggests the possible association of *HLA-DRB1*03* with asthma in children. Further study in a large cohort of asthmatic subjects needs to be done to strengthen the present finding.

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INDEX

Questionnaire

PULMONOLOGY CLINIC

DEPT. OF PAEDIATRIC MEDICINE
NORTH BENGAL MEDICAL COLLEGE, SILIGURI

GENERAL INFORMATION:

- Name _____
- Father's/Mother's name _____
- Address _____
- Phone _____
- Date of Birth _____
- Sex _____
- Caste/ Ethnic group _____

Registration No. - _____

Date: _____

DATE:

	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY 10
COUGH/ BREATHLESSNESS	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E
BRONCHODIALATOR USE	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E

	DAY 11	DAY 12	DAY 13	DAY 14	DAY 15	DAY 16	DAY 17	DAY 18	DAY 19	DAY 20
COUGH/ BREATHLESSNESS	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E
BRONCHODIALATOR USE	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E

	DAY 21	DAY 22	DAY 23	DAY 24	DAY 25	DAY 26	DAY 27	DAY 28	DAY 29	DAY 30	DAY 31
COUGH/ BREATHLESSNESS	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E
BRONCHODIALATOR USE	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E	M E

PRE: POST:

NAME: _____

AGE:_____ SEX:_____ HEIGHT:_____ WT:_____

Immunization History

Vaccines not taken:

DPT	*OPV*	*MEASELS*	*TYPHOID*	*VARICELLA*	*HIB*	*INFLUENZA*	*HEP-A*	*MMR*

Exclusive Breastfeeding: ☐

When symptoms first started?

☐ < 1yr	☐ 1-3 yrs
☐ 4-6 yrs	☐ 7-12 yrs

How it started and progressed?

Family History: Parents / Siblings

ASTHMA	ECZEMA	ALLERGIC RHINITIS	URTICARIA	TB	OTHERS

Significant Past History

KNOWING COUGH

1. Is it associated with fever? Yes / No

2. Is it associated with running nose initially? Yes / No

3. When the cough is worst? Day / Night

4. Any early morning cough? Yes / No

5. Does sleep get disturbed with cough / breathlessness? Yes / No

6. What increases cough?

7. Is cough associated with vomiting? Yes / No

8. Any relation with exercise? Yes / No

9. Any relation with crying, laughing, depression or other emotions? Yes / No

10. What relieves symptoms?

11. Can you hear wheeze? Yes / No

12. History of hospitalization for cough / breathlessness. Yes / No

13. History of any inhalers used / prescribed. Yes / No

14. Seasonality. Yes / No

ASSOCIATED CO-MORBIDITIES

- ALLERGIC RHINITIS
- EAR DISCHARGE
- FEATURES OF SINUSITIS
- FEATURES OF GERD
- ALLERGIC CONJUNCTIVITIS

HABITAT:

SANITATION:

BATH + TOILET …….. (in the house / Community)

WASHING ……..(Laundry / Local arrangement)

HYGIENIC CONDITION AROUND HOUSE (Good / Fair / Poor)

NUTRITION …….

GENERAL:

OVERCROWDING …… (Yes / No)

COOKING MODE ……..(Smokeless / With smoke)

TRIGGERS:

PET	
PLANTS	
SMOKE	
CIG. SMOKE	
MOSQUITO REPELLANT	
INSECTICIDE	
BODY SPRAY	
PERFUMES	
ROOM FRESHNER	
TOYS	
CARPET	
FOOD	
CHANGE IN CLIMATE	
EXERCISE	
OTHERS	

ANY COMMENT:

PEAK FLOW METER READING:

VISIT	1ST	2ND	3RD	4TH	5TH	6TH	7TH	8TH	9TH
PEF									

TC, DC: ……

Mx TEST: ……..

IgA + IgE LEVEL: ……

CD4 COUNT: ……

GRADATION & TREATMENT

STEPS	SYMPTOMS DAY / NIGHT	FEV1 / PEF VARIABLE	SPECIFIC NOTE
MILD INTERMITTENT			
MILD PERSISTENT			
MODERATE PERSISTENT			
SEVERE PERSISTENT			

DRUG HISTORY

ORAL		**INHALED**	
NAME	DURATION	NAME	DURATION

RESULTS AFTER STARTING MEDICATION IF ANY:

☐ IMPROVED ☐ NO CHANGE ☐ DETERIORATED

ANY SPECIFIC RESPONSE TO MEDICATION:

PEFR	**WT:**	**HEIGHT:**	**DATE:**
PRE:		POST:	

ADVICE (medication)

NAME	**DURATION**

Devices

Technique:	**Duration:**	**Maintenance:**

OTHER ADVICE:

APPENDIX - 2

CHEMICALS, REAGENTS & KITS

1. 20 X SSC (pH 8.0)

NaCl - 175.3g

Na-Citrate - 88.2g

Dissolve in 1000ml distilled water and adjust pH to 8.0 with NaOH.

2. HSB (High Salt Buffer) pH 7.6

10mM Tris-HCl - 605.7mg

10mM KCl - 372.8mg

10mM MgCl2 - 1.02g

0.4 M NaCl - 11.69 g

2mM EDTA - 404.7g

Dissolve in 1000 ml distilled water and adjust pH to 7.6.

3. 10% SDS

Dissolve 1g SDS in 10ml distilled water.

4. 50mM KCl

3.728g of KCl in 1000ml distilled water.

5. 4M NaCl

116.9g NaCl in 500ml distilled water.

6. 4M NaOH

Dissolve 50g of NaOH in 500ml distilled water. Filter the solution through Whatman no. 3 filter paper.

7. Tris hydrochloride (Tris-HCl), Mol. Wt. 157.6 (Himedia)

8. Tris NH_4Cl

TRIS - 20.6g/L dH_2O

NH_4Cl - 0.83g/100ml dH_2O

9. RCLB (Red Cell Lysis Buffer)

NH4Cl	-	4.15 g
0.1M Tris HCL	-	50mL

Make up to 500mL with distilled water and adjust pH to7.5 ±0.2.

10. Proteinase-K Solution (8mg/ml)

Dissolve 8mg Proteinase-K to 1ml distilled water.

11. Phenol Chloroform (4:1)

4 parts Phenol + 1 part Chloroform. The pH of phenol should be adjusted to 8.5-9.0 adding Tris-HCl.

12. Deoxyribonucleotide Triphosphate (dNTPs) set: Bangalore Genei, India

The deoxyribonucleotide triphosphates are the monomers of DNA polymer consisting of dATP, dCTP, dGTP, dCTP. The dNTPs are used at saturating concentration in PCR amplification of DNA.

13. PCR Buffer with $MgCl_2$ (Bangalore Genei, India)

The PCR buffer is optimized for use in PCR experiments. Generally, the PCR buffer is supplied along with Taq Polymerase by the commercial companies.

14. Ethidium Bromide (Gibco BRL, USA): 0.5µg/ml TBE Buffer)

15. Gel Loading Dye/ Solution

0.05% Bromophenol blue	-	50 mg
4.0% Sucrose	-	20 g
0.1 M EDTA	-	1.46 g
0.5% SDS	-	250 mg

Dissolve EDTA in 25ml distilled water by adjusting the pH to 8.0 with 5N NaOH and add bromophenol blue. Once dissolved add sucrose and finally SDS. Adjust the final volume to 50 ml and stir at 80°C to make the solution viscous. 1 volume of gel loading solution is optimal to 1-4 volumes of sample. Bromophenol blue serves as the tracking dye while

sucrose adds density and facilitates sample loading. EDTA is included to terminate the action of intrinsic DNAase activity. SDS helps to dissociate DNA-protein complexes which can otherwise interfere the electrophoresis.

16. Taq DNA Polymerase (Bangalore Genei, India)

17. Genomic DNA (100ng -50µg)

18. 10 X TBE Buffer

0.9 M TRIS	-	109.06 g
0.02 M EDTA	-	7.44 g
0.9 M Boric Acid	-	55.647 g

Dissolve in 1000ml distilled water and store at 4°C. Prepare1X as working buffer.

19. TE Buffer/Solution

1mM TRIS	-	121.16 mg
0.1 mM EDTA	-	37.224 mg

Dissolve in 950ml distilled water and adjust pH to 7.5. Adjust the final volume to 1000ml adding distilled water.

20. Phosphate Buffered Saline (PBS), pH 7.2 (Himedia, India)

HUMAN IL-4 ELISA KIT (Pierce Biotechnology, Inc., Rockford)

INSTRUCTIONS

Human IL-4 ELISA Kit

Thermo SCIENTIFIC

EH2IL4 EH2IL45

1358.2

Number	Description
EH2IL4	**Human Interleukin-4 (IL-4) ELISA Kit,** sufficient reagents for 96 determinations
	Kit contents:
	Anti-Human IL-4 Precoated 96-well Strip Plate, 1 each
	Lyophilized Recombinant Human IL-4 Standard, 2 vials
	Standard Diluent, 14 ml
	Biotinylated Antibody Reagent, 8 ml
	30X Wash Buffer, 50 ml
	Streptavidin-HRP Concentrate, 75 µl
	Streptavidin-HRP Dilution Buffer, 13 ml
	TMB Substrate, 12 ml
	Stop Solution, 13 ml, contains 0.16 M sulfuric acid
	Adhesive Plate Covers, 4 each
EH2IL45	**Human Interleukin-4 ELISA Kit,** sufficient reagents for 5 × 96 determinations
	Kit contents:
	Anti-Human IL-4 Precoated 96-well Strip Plate, 5 each
	Lyophilized Recombinant Human IL-4 Standards, 5 vials
	Standard Diluent, 75 ml
	Biotinylated Antibody Reagent, 35 ml
	Streptavidin-HRP Concentrate, 250 µl
	Streptavidin-HRP Dilution Buffer, 70 ml
	TMB Substrate, 5 × 13 ml
	30X Wash Buffer, 200 ml
	Stop Solution, 55 ml, contains 0.16 M sulfuric acid
	Adhesive Plate Covers, 30 each

For Research Use Only. Not for use in diagnostic procedures.

Storage: For maximum stability, store in a non-defrosting -20ºC freezer and refer to the expiration date for frozen storage on the label. Alternatively, store at 2-8ºC and refer to the expiration date for refrigerated storage. Once thawed, store at 4ºC until the expiration date for refrigerated storage. Kit is shipped on dry ice.

Introduction

This Thermo Scientific ELISA Kit is for measuring human IL-4 in serum, plasma, urine and culture supernatants.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermo.com/pierce

HUMAN IFN-γ ELISA KIT (Pierce Biotechnology, Inc., Rockford)

INSTRUCTIONS

Thermo SCIENTIFIC

Human IFNγ ELISA Kit

EHIFNG EHIFNG2 EHIFNG5

1278.7

Number	Description
EHIFNG	**Human Interferon gamma (IFNγ) ELISA,** sufficient reagents for 96 determinations
EHIFNG2	**Human Interferon gamma (IFNγ) ELISA,** sufficient reagents for 2 × 96 determinations
EHIFNG5	**Human Interferon gamma ELISA,** sufficient reagents for 5 × 96 determinations

Kit Contents	EHIFNG	EHIFNG2	EHIFNG5
Anti-human IFNγ Precoated 96-well Strip Plate	1 each	2 each	5 each
Lyophilized Recombinant Human IFNγ Standard	2 vials	4 vials	5 vials
Standard Diluent, contains 0.1% sodium azide	12mL	2 × 12mL	75mL
30X Wash Buffer	50mL	2 × 50mL	200mL
Biotinylated Antibody Reagent, contains 0.1% sodium azide	8mL	2 × 8mL	35mL
Streptavidin-HRP Concentrate	75µL	2 × 75µL	250µL
Streptavidin-HRP Dilution Buffer	14mL	2 × 14mL	70mL
TMB Substrate	13mL	2 × 13mL	5 × 13mL
Stop Solution, contains 0.16M sulfuric acid	13mL	2 × 13mL	55mL
Adhesive plate covers	6 each	12 each	30 each

For research use only. Not for use in diagnostic procedures.

Storage: For maximum stability, store in a non-defrosting -20ºC freezer and refer to the expiration date for frozen storage on the label. Alternatively, store at 2-8ºC and refer to the expiration date for refrigerated storage. Once thawed, store at 4ºC until the expiration date for refrigerated storage. Kit is shipped on dry ice.

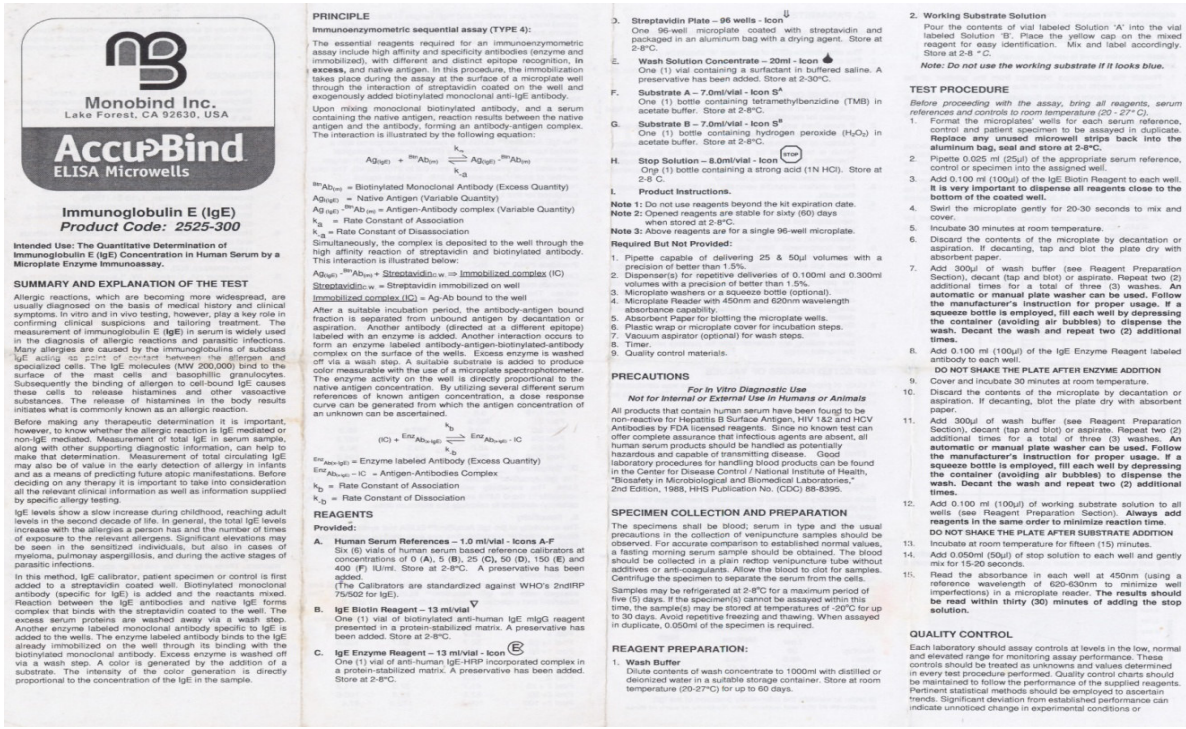
Table of Contents

Introduction

The Thermo Scientific Human Interferon gamma (IFNγ) ELISA Kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of IFNγ in serum, plasma, urine and culture supernatant.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

IgE ELISA KIT (Accu-Bind, Monobind Inc., USA)



Monobind Inc.
Lake Forest, CA 92630, USA

Accu-Bind
ELISA Microwells

Immunoglobulin E (IgE)
Product Code: 2525-300

Intended Use: The Quantitative Determination of Immunoglobulin E (IgE) Concentration in Human Serum by a Microplate Enzyme Immunoassay.

SUMMARY AND EXPLANATION OF THE TEST

Allergic reactions, which are becoming more widespread, are usually diagnosed on the basis of medical history and clinical symptoms. In vitro and in vivo testing, however, play a key role in confirming clinical suspicions and tailoring treatment. The measurement of immunoglobulin E (IgE) in serum is widely used in the diagnosis of allergic reactions and parasitic infections. Many allergies are caused by the immunoglobulins of subclass IgE acting as point of contact between the allergen and specialized cells. The IgE molecules (MW 200,000) bind to the surface of the mast cells and basophilic granulocytes. Subsequently the binding of allergen to cell-bound IgE causes these cells to release histamines and other vasoactive substances. The release of histamines in the body results initiates what is commonly known as an allergic reaction.

Before making any therapeutic determination it is important, however, to know whether the allergic reaction is IgE mediated or non-IgE mediated. Measurement of total IgE in serum sample, along with other supporting diagnostic information, can help to make that determination. Measurement of total circulating IgE may also be of value in the early detection of allergy in infants and as a means of predicting future atopic manifestations. Before deciding on any therapy it is important to take into consideration all the relevant clinical information as well as information supplied by specific allergy testing.

IgE levels show a slow increase during childhood, reaching adult levels in the second decade of life. In general, the total IgE levels increase with the allergies a person has and the number of times of exposure to the relevant allergens. Significant elevations may be seen in the sensitized individuals, but also in cases of myeloma, pulmonay aspergillosis, and during the active stages of parasitic infections.

In this method, IgE calibrator, patient specimen or control is first added to a streptavidin coated well. Biotinylated monoclonal antibody (specific for IgE) is added and the reactants mixed. Reaction between the IgE antibodies and native IgE forms complex that binds with the streptavidin coated to the well. The excess serum proteins are washed away via a wash step. Another enzyme labeled monoclonal antibody specific to IgE is added to the wells. The enzyme labeled antibody binds to the IgE already immobilized on the well through its binding with the biotinylated monoclonal antibody. Excess enzyme is washed off via a wash step. A color is generated by the addition of a substrate. The intensity of the color generation is directly proportional to the concentration of the IgE in the sample.

PRINCIPLE

Immunoenzymometric sequential assay (TYPE 4):

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme and immobilized), with different and distinct epitope recognition, in **excess,** and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-IgE antibody.

Upon mixing monoclonal biotinylated antibody, and a serum containing the native antigen, reaction results between the native antigen and the antibody, forming an antibody-antigen complex. The interaction is illustrated by the following equation:

$$Ag_{(IgE)} + {}^{Bio}Ab_{(m)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} Ag_{(IgE)} - {}^{Bio}Ab_{(m)}$$

$^{Bio}Ab_{(m)}$ = Biotinylated Monoclonal Antibody (Excess Quantity)
$Ag_{(IgE)}$ = Native Antigen (Variable Quantity)
$Ag_{(IgE)} - {}^{Bio}Ab_{(m)}$ = Antigen-Antibody complex (Variable Quantity)
k_a = Rate Constant of Association
k_{-a} = Rate Constant of Disassociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$Ag_{(IgE)} - {}^{Bio}Ab_{(m)} + Streptavidin_{(C.W.)} \Rightarrow$ Immobilized complex (IC)

$Streptavidin_{(C.W.)}$ = Streptavidin immobilized on well
Immobilized complex (IC) = Ag-Ab bound to the well

After a suitable incubation period, the antibody-antigen bound fraction is separated from unbound antigen by decantation or aspiration. Another antibody (directed at a different epitope) labeled with an enzyme is added. Another interaction occurs to form an enzyme labeled antibody-antigen-biotinylated-antibody complex on the surface of the wells. Excess enzyme is washed off via a wash step. A suitable substrate is added to produce color measurable with the use of a microplate spectrophotometer. The enzyme activity on the well is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

$$(IC) + {}^{Enz}Ab_{(IgE)} \underset{k_b}{\overset{k_b}{\rightleftharpoons}} {}^{Enz}Ab_{(m)} - IC$$

$^{Enz}Ab_{(IgE)}$ = Enzyme labeled Antibody (Excess Quantity)
$^{Enz}Ab_{(m)} - IC$ = Antigen-Antibodies Complex
k_b = Rate Constant of Association
k_{-b} = Rate Constant of Dissociation

REAGENTS

Provided:

A. **Human Serum References – 1.0 ml/vial - Icons A-F**
Six (6) vials of human serum based reference calibrators at concentrations of 0 **(A)**, 5 **(B)**, 25 **(C)**, 50 **(D)**, 150 **(E)** and 400 **(F)** IU/ml. Store at 2-8°C. A preservative has been added.
(The Calibrators are standardized against WHO's 2ndIRP 75/502 for IgE).

B. **IgE Biotin Reagent – 13 ml/vial**
One (1) vial of biotinylated anti-human IgE mIgG reagent presented in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

C. **IgE Enzyme Reagent – 13 ml/vial - Icon**
One (1) vial of anti-human IgE-HRP incorporated complex in a protein-stabilized matrix. A preservative has been added. Store at 2-8°C.

D. **Streptavidin Plate – 96 wells - Icon**
One 96-well microplate coated with streptavidin and packaged in an aluminum bag with a drying agent. Store at 2-8°C.

E. **Wash Solution Concentrate – 20ml - Icon**
One (1) vial containing a surfactant in buffered saline. A preservative has been added. Store at 2-30°C.

F. **Substrate A – 7.0ml/vial - Icon S^A**
One (1) bottle containing tetramethylbenzidine (TMB) in acetate buffer. Store at 2-8°C.

G. **Substrate B – 7.0ml/vial - Icon S^B**
One (1) bottle containing hydrogen peroxide (H_2O_2) in acetate buffer. Store at 2-8°C.

H. **Stop Solution – 8.0ml/vial - Icon** (STOP)
One (1) bottle containing a strong acid (1N HCl). Store at 2-8 C.

I. **Product Instructions.**

Note 1: Do not use reagents beyond the kit expiration date.
Note 2: Opened reagents are stable for sixty (60) days when stored at 2-8°C.
Note 3: Above reagents are for a single 96-well microplate.

Required But Not Provided:

1. Pipette capable of delivering 25 & 50µl volumes with a precision of better than 1.5%.
2. Dispenser(s) for repetitive deliveries of 0.100ml and 0.300ml volumes with a precision of better than 1.5%.
3. Microplate washers or a squeeze bottle (optional).
4. Microplate Reader with 450nm and 620nm wavelength absorbance capability.
5. Absorbent Paper for blotting the microplate wells.
6. Plastic wrap or microplate cover for incubation steps.
7. Vacuum aspirator (optional) for wash steps.
8. Timer.
9. Quality control materials.

PRECAUTIONS

For In Vitro Diagnostic Use
Not for Internal or External Use In Humans or Animals

All products that contain human serum have been foung to be non-reactive for Hepatitis B Surface Antigen, HIV 1&2 and HCV Antibodies by FDA licensed reagents. Since no known test can offer complete assurance that infectious agents are absent, all human serum products should be handled as potentially hazardous and capable of transmitting disease. Good laboratory procedures for handling blood products can be found in the Center for Disease Control / National Institute of Health, "Biosafety in Microbiological and Biomedical Laboratories," 2nd Edition, 1988, HHS Publication No. (CDC) 88-8395.

SPECIMEN COLLECTION AND PREPARATION

The specimens shall be blood; serum in type and the usual precautions in the collection of venipuncture samples should be observed. For accurate comparison to established normal values, a fasting morning serum sample should be obtained. The blood should be collected in a plain redtop venipuncture tube without additives or anti-coagulants. Allow the blood to clot for samples. Centrifuge the specimen to separate the serum from the cells.

Samples may be refrigerated at 2-8°C for a maximum period of five (5) days. If the specimen(s) cannot be assayed within this time, the sample(s) may be stored at temperatures of -20°C for up to 30 days. Avoid repetitive freezing and thawing. When assayed in duplicate, 0.050ml of the specimen is required.

REAGENT PREPARATION:

1. **Wash Buffer**
Dilute contents of wash concentrate to 1000ml with distilled or deionized water in a suitable storage container. Store at room temperature (20-27°C) for up to 60 days.

2. **Working Substrate Solution**
Pour the contents of vial labeled Solution 'A' into the vial labeled Solution 'B'. Place the yellow cap on the mixed reagent for easy identification. Mix and label accordingly. Store at 2-8 °C.
Note: Do not use the working substrate if it looks blue.

TEST PROCEDURE

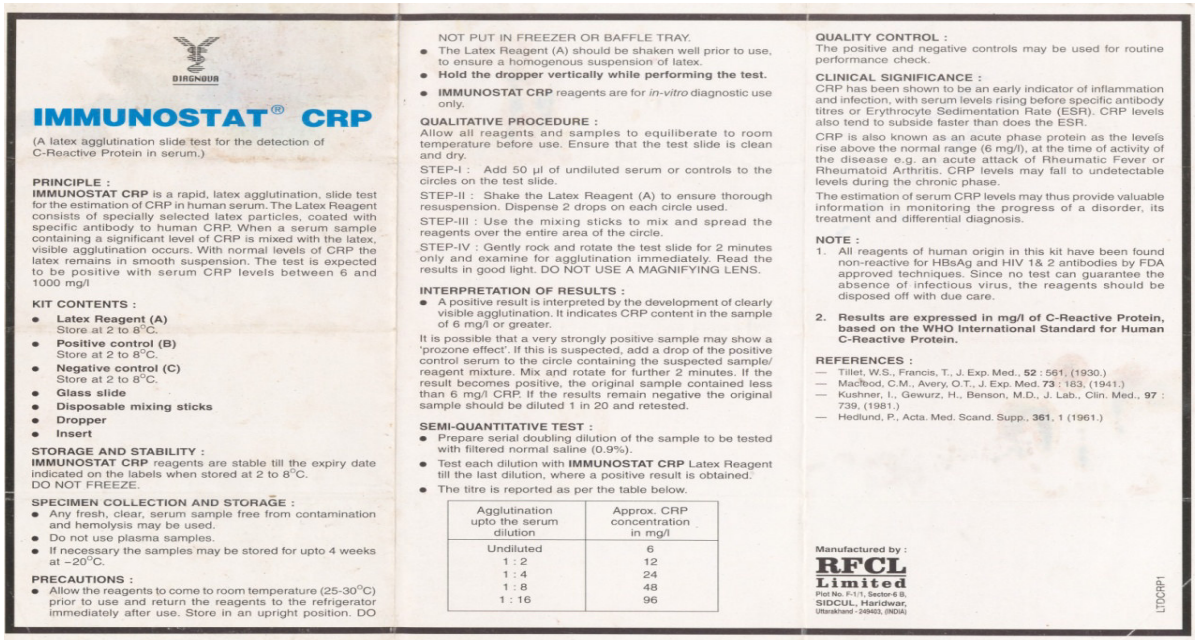
Before proceeding with the assay, bring all reagents, serum references and controls to room temperature (20 - 27° C).

1. Format the microplates' wells for each serum reference, control and patient specimen to be assayed in duplicate. **Replace any unused microwell strips back into the aluminum bag, seal and store at 2-8°C.**
2. Pipette 0.025 ml (25µl) of the appropriate serum reference, control or specimen into the assigned well.
3. Add 0.100 ml (100µl) of the IgE Biotin Reagent to each well. **It is very important to dispense all reagents close to the bottom of the coated well.**
4. Swirl the microplate gently for 20-30 seconds to mix and cover.
5. Incubate 30 minutes at room temperature.
6. Discard the contents of the microplate by decantation or aspiration. If decanting, tap and blot the plate dry with absorbent paper.
7. Add 300µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
8. Add 0.100 ml (100µl) of the IgE Enzyme Reagent labeled antibody to each well.
DO NOT SHAKE THE PLATE AFTER ENZYME ADDITION
9. Cover and incubate 30 minutes at room temperature.
10. Discard the contents of the microplate by decantation or aspiration. If decanting, blot the plate dry with absorbent paper.
11. Add 300µl of wash buffer (see Reagent Preparation Section), decant (tap and blot) or aspirate. Repeat two (2) additional times for a total of three (3) washes. **An automatic or manual plate washer can be used. Follow the manufacturer's instruction for proper usage. If a squeeze bottle is employed, fill each well by depressing the container (avoiding air bubbles) to dispense the wash. Decant the wash and repeat two (2) additional times.**
12. Add 0.100 ml (100µl) of working substrate solution to all wells (see Reagent Preparation Section). **Always add reagents in the same order to minimize reaction time.**
DO NOT SHAKE THE PLATE AFTER SUBSTRATE ADDITION
13. Incubate at room temperature for fifteen (15) minutes.
14. Add 0.050ml (50µl) of stop solution to each well and gently mix for 15-20 seconds.
15. Read the absorbance in each well at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. **The results should be read within thirty (30) minutes of adding the stop solution.**

QUALITY CONTROL

Each laboratory should assay controls at levels in the low, normal and elevated range for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. Significant deviation from established performance can indicate unnoticed change in experimental conditions or

CRP KIT (IMMUNOSTAT®, RANBAXY FINE CHEMICALS LTD.)



DIAGNOVA

IMMUNOSTAT® CRP

(A latex agglutination slide test for the detection of C-Reactive Protein in serum.)

PRINCIPLE :
IMMUNOSTAT CRP is a rapid, latex agglutination, slide test for the estimation of CRP in human serum. The Latex Reagent consists of specially selected latex particles, coated with specific antibody to human CRP. When a serum sample containing a significant level of CRP is mixed with the latex, visible agglutination occurs. With normal levels of CRP the latex remains in smooth suspension. The test is expected to be positive with serum CRP levels between 6 and 1000 mg/l

KIT CONTENTS :
- **Latex Reagent (A)** Store at 2 to 8°C.
- **Positive control (B)** Store at 2 to 8°C.
- **Negative control (C)** Store at 2 to 8°C.
- **Glass slide**
- **Disposable mixing sticks**
- **Dropper**
- **Insert**

STORAGE AND STABILITY :
IMMUNOSTAT CRP reagents are stable till the expiry date indicated on the labels when stored at 2 to 8°C. DO NOT FREEZE.

SPECIMEN COLLECTION AND STORAGE :
- Any fresh, clear, serum sample free from contamination and hemolysis may be used.
- Do not use plasma samples.
- If necessary the samples may be stored for upto 4 weeks at –20°C.

PRECAUTIONS :
- Allow the reagents to come to room temperature (25-30°C) prior to use and return the reagents to the refrigerator immediately after use. Store in an upright position. DO NOT PUT IN FREEZER OR BAFFLE TRAY.
- The Latex Reagent (A) should be shaken well prior to use, to ensure a homogenous suspension of latex.
- **Hold the dropper vertically while performing the test.**
- **IMMUNOSTAT CRP** reagents are for *in-vitro* diagnostic use only.

QUALITATIVE PROCEDURE :
Allow all reagents and samples to equiliberate to room temperature before use. Ensure that the test slide is clean and dry.

STEP-I : Add 50 µl of undiluted serum or controls to the circles on the test slide.

STEP-II : Shake the Latex Reagent (A) to ensure thorough resuspension. Dispense 2 drops on each circle used.

STEP-III : Use the mixing sticks to mix and spread the reagents over the entire area of the circle.

STEP-IV : Gently rock and rotate the test slide for 2 minutes only and examine for agglutination immediately. Read the results in good light. DO NOT USE A MAGNIFYING LENS.

INTERPRETATION OF RESULTS :
- A positive result is interpreted by the development of clearly visible agglutination. It indicates CRP content in the sample of 6 mg/l or greater.

It is possible that a very strongly positive sample may show a 'prozone effect'. If this is suspected, add a drop of the positive control serum to the circle containing the suspected sample/ reagent mixture. Mix and rotate for further 2 minutes. If the result becomes positive, the original sample contained less than 6 mg/l CRP. If the results remain negative the original sample should be diluted 1 in 20 and retested.

SEMI-QUANTITATIVE TEST :
- Prepare serial doubling dilution of the sample to be tested with filtered normal saline (0.9%).
- Test each dilution with **IMMUNOSTAT CRP** Latex Reagent till the last dilution, where a positive result is obtained.
- The titre is reported as per the table below.

Agglutination upto the serum dilution	Approx. CRP concentration in mg/l
Undiluted	6
1 : 2	12
1 : 4	24
1 : 8	48
1 : 16	96

QUALITY CONTROL :
The positive and negative controls may be used for routine performance check.

CLINICAL SIGNIFICANCE :
CRP has been shown to be an early indicator of inflammation and infection, with serum levels rising before specific antibody titres or Erythrocyte Sedimentation Rate (ESR). CRP levels also tend to subside faster than does the ESR.

CRP is also known as an acute phase protein as the levels rise above the normal range (6 mg/l), at the time of activity of the disease e.g. an acute attack of Rheumatic Fever or Rheumatoid Arthritis. CRP levels may fall to undetectable levels during the chronic phase.

The estimation of serum CRP levels may thus provide valuable information in monitoring the progress of a disorder, its treatment and differential diagnosis.

NOTE :
1. All reagents of human origin in this kit have been found non-reactive for HBsAg and HIV 1& 2 antibodies by FDA approved techniques. Since no test can guarantee the absence of infectious virus, the reagents should be disposed off with due care.
2. **Results are expressed in mg/l of C-Reactive Protein, based on the WHO International Standard for Human C-Reactive Protein.**

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Manufactured by :
RFCL Limited
Plot No. F-1/1, Sector-6 B,
SIDCUL, Haridwar,
Uttarakhand - 249403, (INDIA)

LTDCRP1

APPENDIX - 3

PUBLICATIONS

1. **Lama, M.**, Chatterjee, M., and Chaudhuri, T.K. (2014). A study of the association of childhood asthma with HLA alleles in the population of Siliguri, West Bengal, India. Tissue Antigens *84*(3), 316-320.

2. **Lama, M.**, Chatterjee, M., and Chaudhuri, T.K. (2013). Total Serum Immunoglobulin E in Children with Asthma. Ind. J. Clin. Biochem. *28*, 197-200.

3. **Lama, M.**, Chatterjee, M., Nayak, C.R., and Chaudhuri, T.K. (2011). Increased interleukin-4 and decreased interferon-γ levels in serum of children with asthma. Cytokine *55*(3), 335-338.

4. **Lama, M.**, Chatterjee, M., and Chaudhuri, T.K. (2011). Epidemiology of Childhood Asthma: A Hospital Based Study. The Child and Newborn *15*, 41-44.

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BRIEF COMMUNICATION

A study of the association of childhood asthma with HLA alleles in the population of Siliguri, West Bengal, India

M. Lama[1,2], M. Chatterjee[3] & T. K. Chaudhuri[1]

1 Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri, West Bengal, India
2 Department of Zoology, The University of Burdwan, Golapbag, West Bengal, India
3 Department of Pediatrics, North Bengal Medical College & Hospital, Siliguri, India

Key words
allelic group; asthma; children; *DRB1*03*; human leukocyte antigen

Correspondence
Cellular Immunology Laboratory
Department of Zoology
University of North Bengal
Rajarammohanpur
Siliguri 734013
West Bengal
India
Tel: +91 353 2582124
Fax: +91 353 2699001
e-mail: dr_tkc_nbu@rediffmail.com

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Abstract

Asthma is a heterogeneous disease for which a strong genetic basis is firmly established. It is a complex disorder influenced by gene–environment interaction. Human leukocyte antigen (HLA) genes have been shown to be consistently associated with asthma and its related phenotypes in various populations. The aim of this study was to determine the frequency of the selected HLA classes I and II allelic groups in asthmatic and control groups. HLA typing was performed using polymerase chain reaction-sequence-specific typing (PCR-SSP) method. The allele frequency was estimated by direct counting. Frequency of each HLA allelic group was compared between asthmatic group and control group using χ^2 test. *P*-value was corrected by multiplying with the number of the allelic groups studied. Odds ratio (OR) and its corresponding 95% confidence interval (CI) for each allelic group were calculated using GRAPHPAD INSTAT 3.10. The results of this study showed a significantly higher frequency of *HLA-DRB1*03* in asthmatics than in controls (11.43% *vs* 3.64%, OR = 3.78, 95% CI = 1.61–8.85, $P = 0.0025$, $P_{corr} < 0.05$). Analysis of HLA alleles in low and high total serum immunoglobulin E (IgE) level in asthmatics revealed no significant association. *HLA-DRB1*03* may be implicated in the susceptibility to asthma in the pediatric population.

Asthma is a common chronic inflammatory disease of the conducting airways which undergo distinct structural and functional changes, leading to non-specific bronchial hyper-responsiveness (BHR) and airflow obstruction that fluctuates over time (1). It is a complex disease determined by many genes and molecular mechanisms. Indeed, the genetic factors contribute to disease susceptibility but the manifestation of the disease is modulated by environmental exposures and the interactions between both of these components. Candidate-gene and linkage studies followed by positional cloning have already provided a large number of genes accountable for the susceptibility to asthma (2). In addition, many genome-wide association studies (GWASs) published in recent decade have identified several genetic loci to be associated with asthma and/or its related phenotypes in different populations (3–5).

The major histocompatibility complex (MHC), designated as human leukocyte antigen (HLA) in human, genes map on chromosome 6p21 play an important role in the regulation of the immune system (6). Many studies have documented that 6p21 region is strongly linked to atopic phenotype and asthma and it is considered a major locus influencing allergic diseases (7–9). Further, numerous studies have investigated the association of HLA alleles and/or antigens with asthma. Some of the earlier studies have reported the association of various HLA class I alleles/antigens (10, 11), while large number of studies have investigated the association of HLA class II alleles/haplotypes with asthma in different populations. Choi et al. reported a strong association between *HLA-DPB1*0301* and aspirin-intolerant asthma in Korean population (12). In an another study, Movahedi et al. reported the association of HLA class II alleles with asthma and high total IgE levels in Iranian children (13). Lara Marquez et al. have reported the association of HLA class II alleles and haplotypes with *Dermatophagoides species* sensitive asthmatics in Venezuelan population (14). Similarly, Guo et al. also reported the association of HLA-DQ genes with asthma and positive specific IgE response to *Dermatophagoides species* in Chinese population (15). In a study on Korean population, Kim et al. have reported the association of HLA haplotype with isocyanate-induced occupational asthma (16). Another study on Greek children with allergic asthma revealed the association of *HLA-DRB1*04*

and *DQA1*0301* with susceptibility to mites sensitive asthma (17).

Siliguri is located in the foothills of the Himalayas. It is a city in the Indian state of West Bengal and known as the gateway to North East India, Bhutan, Nepal and Bangladesh. The adjoining areas of Siliguri are comprised of tea gardens and villages. People of diverse communities are the inhabitants of this region. The majority of population belongs to Bengali community while other minority communities include Nepali, Marwari, Bihari and native tribal community (http://en.wikipedia.org/wiki/Siliguri. Accessed on 16.1.2014). There is a paucity of data particularly on HLA association with asthma in pediatric population of this region. Therefore, the purpose of this study was to determine the frequencies of some of the selected HLA classes I and II allelic groups in asthmatic and control subjects.

A total of 105 unrelated asthmatic children, aged 3–12 years, were recruited in this study from the Out Patient Department of Pediatrics, North Bengal Medical College & Hospital, Siliguri, West Bengal, India. Asthma was diagnosed by a physician based on the physical examination, clinical symptoms, response to bronchodilators, etc. Age and sex matched 110 unrelated children of the same ethnic background were included as controls. The criteria for the selection of control subjects included: no history of allergy, airway hyper-responsiveness, upper respiratory tract infections and lung disease. The study was approved by the Institutional Ethics Committee, University of North Bengal. The written informed consent was obtained from the parents/guardians for their children to participate in the study. The blood samples were collected under appropriate conditions by vein puncture method and the demographic and clinical characteristics of the participating subjects were collected using the questionnaire.

Genomic DNA was extracted from the venous blood using Phenol-Chloroform method. Molecular typing of the selected HLA classes I and II allelic groups was performed by polymerase chain reaction using sequence-specific primers (PCR-SSP). We studied the allelic groups which have been reported previously to be associated with childhood asthma in various populations (10, 11, 13, 17–25). The primer sequences of HLA class I allelic groups were taken from Bunce et al. (26) and of class II allelic groups were obtained from Zhu et al. (27) and Sacchetti et al. (28). The primers were synthesized and supplied by Integrated DNA Technologies (IDT).

A 25 µl reaction mix was prepared and the reaction was carried out in a thermal cycler (MJ Mini^{TM} Gradient Thermal Cycler, PTC-1148, Bio-Rad, Singapore). Touchdown PCR was adopted for the amplification of the DNA with the following reaction conditions: Initial denaturation at 94°C for 3 min followed by five cycles of 94°C denaturation for 30 s, 2°C higher annealing temperature (varying for different alleles) for 35 s and 72°C extension for 40 s, and 25 cycles of 94°C denaturation for 30 s, 2°C lower annealing temperature for 50 s and 72°C extension for 1 min. A final extension at 72°C for 7 min was performed. In order to avoid technical error in amplification, two different internal controls were used. An internal control (hemoglobin gene) of 256 bp was used for typing of class I allelic groups and another internal control (a fragment of human growth hormone gene1) of 439 bp was used for class II allelic groups. The PCR products were electrophoresed in 2% prestained agarose gel. We also analyzed the association of HLA allelic groups with the elevated level of total serum immunoglobulin E (IgE) in asthmatic subjects. Available data of total serum IgE of 70 asthmatic subjects of our earlier study were used for this purpose (29).

Table 1 Characteristics of asthmatic and control subjects

	Asthmatics	Controls
No. of subjects	105	110
Males	57	58
Females	48	52
Age (years): mean ± SD	7.33 ± 2.62	7.71 ± 2.74
Age of onset		
Before 5 years	85	
After 5 years	20	
Height (cm): mean ± SD	116.35 ± 13.28	117.63 ± 13.03
Weight (kg): mean ± SD	19.19 ± 5.94	20.95 ± 6.28
Study groups		
Bengali	78	69
Bihari	12	15
Nepali	7	15
Others	8	11

SD, standard deviation.

The frequencies of the HLA allelic groups were determined by direct counting. The frequency of each allelic group observed in the asthmatic group was compared to control group using χ^2 test. *P*-values were corrected (P_{corr}) by multiplying with the number of allelic groups studied. A *P*-value <0.05 was considered to be statistically significant. The odds ratio (OR) with its corresponding 95% CI for each HLA allelic group was calculated using the GRAPH PAD IN STAT version 3.10.

The characteristics of asthmatic and control subjects are summarized in Table 1. The result showed the higher frequencies of *A*01* (15.71% *vs* 10.45%), *A*03* (17.62% *vs* 14.09%), *A*24* (16.67% *vs* 13.64%), *A*26* (12.86% *vs* 8.64%), *B*08* (21.90% *vs* 18.64%), *B*37* (19.05% *vs* 15.45%), *DRB1*01* (6.67% *vs* 3.18%), *DRB1*03* (11.43% *vs* 3.64%), *DQB1*0201* (18.57% *vs* 14.09%), *DQB1*0302* (14.29% *vs* 10.00%) and *DQA1*0501* (14.76% *vs* 10.91%) and the lower frequencies of *A*11* (8.57% *vs* 10.00%), *B*51* (6.19% *vs* 8.18%), *B*52* (4.29% *vs* 5.91%), *DRB1*04* (15.24% *vs* 18.18%) and *DQB1*0603/8* (2.86% *vs* 4.55%) in asthmatic group than in controls, respectively (Table 2). Among these, the frequency of *HLA-DRB1*03* was significantly higher in asthmatics than in controls (11.43% *vs* 3.64%, OR = 3.78, 95% CI = 1.61–8.85, $P = 0.0025$, $P_c < 0.05$) while the frequencies of rest of the allelic groups did not show significant difference between the two groups. When the frequencies of *HLA-DRB1*03* were compared between asthmatic

Table 2 Frequencies of HLA class I and class II allelic groups in asthmatic and control subjects[a]

HLA allelic group	Asthmatics ($N = 105$) Freq. % (n)	Controls ($N = 110$) Freq. % (n)	χ^2	P	OR (95% CI)
*A*01*	15.71 (33)	10.45 (23)	2.564	0.109	1.73 [0.94–3.21]
*A*03*	17.62 (37)	14.09 (31)	0.932	0.334	1.39 [0.78–2.47]
*A*11*	8.57 (18)	10.00 (22)	0.132	0.717	0.83 [0.42–1.65]
*A*24*	16.67 (35)	13.64 (30)	0.670	0.413	1.33 [0.74–2.39]
*A*25*	10.48 (22)	9.09 (20)	0.116	0.734	1.19 [0.61–2.34]
*A*26*	12.86 (27)	8.64 (19)	1.802	0.179	1.66 [0.86–3.21]
*B*08*	21.90 (46)	18.64 (41)	0.701	0.403	1.31 [0.76–2.26]
*B*44*	5.71 (12)	3.64 (8)	0.662	0.416	1.65 [0.64–4.20]
*B*45*	7.14 (15)	5.00 (11)	0.569	0.451	1.50 [0.65–3.44]
*B*51*	6.19 (13)	8.18 (18)	0.406	0.524	0.72 [0.33–1.56]
*B*52*	4.29 (9)	5.91 (13)	0.314	0.575	0.70 [0.29–1.71]
*B*37*	19.05 (40)	15.45 (34)	0.931	0.335	1.38 [0.78–2.42]
*DRB1**					
01	6.67 (14)	3.18 (7)	2.223	0.136	2.26 [0.88–5.85]
03	11.43 (24)	3.64 (8)	9.106	0.0025[b,c]	3.78 [1.61–8.85]
04	15.24 (32)	18.18 (40)	0.593	0.441	0.77 [0.43–1.35]
12	8.57 (18)	5.45(12)	1.258	0.262	1.69 [0.77–3.71]
*DQB1**					
0201	18.57 (39)	14.09 (31)	1.578	0.209	1.51 [0.85–2.67]
0302	14.29 (30)	10.00 (22)	1.710	0.191	1.60 [0.85–3.01]
0603/8	2.86 (6)	4.55 (10)	0.467	0.495	0.61 [0.21–1.73]
*DQA1**					
0501	14.76 (31)	10.91 (24)	1.295	0.255	1.50 [0.81–2.78]

CI, confidence interval; HLA, human leukocyte antigen; OR, odds ratio.

[a] $\% = (n/2N) \times 100$.

[b] P corrected <0.05.

[c] Fisher exact $= 0.0018$.

Bengali and control Bengali groups to exclude the possibility of significant influence of population admixture, it was found significantly higher frequency of *HLA-DRB1*03* in asthmatic Bengali group than in control Bengali group (13.16% *vs* 3.62% respectively; OR = 4.41, 95% CI = 1.56−12.52, $P = 0.006^{**}$). Although the *HLA-DRB1*01* allelic group was not significantly associated with asthma, the OR for this allele was greater than 2 (OR = 2.26, 95% CI = 0.88−5.85, $P = 0.136$). Further analysis of the frequencies of HLA allelic groups in two groups of asthmatic subjects viz. tIgE > 150 IU/ml and tIgE < 150 IU/ml showed the higher frequencies of *A*01* (18.0% *vs* 12.5%), *A*24* (17.0% *vs* 12.5%), *A*25* (12.0% *vs* 7.5%), *B*52* (5.0% *vs* 2.5%), *B*37* (19.0% *vs* 15.0%), *DRB1*01* (9.0% *vs* 5.0%), *DRB1*03* (11.0% *vs* 7.5%), *DQB1*0302* (16% *vs* 10.0%) and *DQA1*0501* (15.0% *vs* 10.0%), and the lower frequencies of *A*03* (14% *vs* 17.5%), *A*26* (13% *vs* 15%), *B*08* (20.0% *vs* 22.5%), *DRB1*12* (8.0% *vs* 12.5%) and *DQB1*0201* (17.0% *vs* 20.0%), respectively. None of these showed the significant difference between the two groups.

In this study, it was observed that the frequency of *HLA-DRB1*03* was significantly higher in asthmatic subjects than in controls (OR = 3.78, 95% CI = 1.61–8.85, $P = 0.0025$, $P_c < 0.05$). The association of *HLA-DRB1*03* with childhood asthma, in this study, is consistent with the findings of various earlier studies. A study conducted by Ivković-Jureković et al. in Croatian asthmatic children, showed a significantly higher frequency of *HLA-DRB1*03* specificity among the asthmatic patients with total serum IgE ≥ 400 kU/l (30). In another study, Juhn et al. reported that *HLA-DRB1*03* allele was the most significantly associated with an increased risk of asthma (HR: 1.5, 95% CI: 1.0–2.4, $P = 0.050$) (23). Similarly, Hanchard et al. suggested the role of *HLA-DRB1*03* in asthma susceptibility independent of ancestral-haplotype-mediated linkage disequilibrium (31). In addition, Rajagopalan et al. have reported that *HLA-DRB1*03* allele plays an important role in determining the eosinophilic airway inflammation, a Th2 mediated inflammation (32).

The frequency of *HLA-DRB1*01* was higher in asthmatic subjects than in controls, although the difference was not significant (6.67% *vs* 3.18%, respectively; OR = 2.26, 95% CI = 0.88–5.85, $P = 0.136$). Analysis of HLA alleles in association with the elevated level of total serum IgE showed that none of the HLA alleles was found to be associated with the elevated level of total serum IgE in asthmatic subjects. Many studies have reported the positive significant association of *HLA-DRB1*01* with the elevated level of total serum IgE in asthmatics and/or allergic patients. Torio et al. showed a significant association of *DRB1*01* with the elevated level of total serum IgE in the Spanish Artemisia sensitive asthmatics (24). Woszczek et al. reported the significantly higher total serum IgE levels in allergic patients with HLA-*DRB1*01* compared to patients without these allele (25). Similarly,

Ulbrecht et al. reported a weak association of *HLA-DRB1*01* with specific IgE-positive cases compared to negative controls (33).

Asthma and its associated trait 'atopy' were some of the first complex diseases for which a strong genetic basis was established (34). HLA class II antigens play a key role in antigen presentation to $CD4^+$ T-lymphocytes and therefore influence the specificity of the immune response. HLA genes have been implicated in triggering an allergen-specific IgE response. The amino acid constituents of the specific epitopes of allergens have been identified and specific HLA-DR and DQ gene products have been shown to present these epitopes (35). HLA-DR alleles are found to be associated with the development of specific IgE response to seasonal as well as perennial allergens (36, 37). Murray suggested the potential role of HLA genes in determining the Th1 *vs* Th2 immune response through the interaction between T-cell receptor, peptide and MHC molecules (38). Blumenthal et al. have suggested a different role of HLA gene polymorphism. They showed that in pollen allergy, the asthma phenotype may be associated with extended haplotype HLA-B7/SC31/DR2 (39).

Although it has been known that HLA is associated with asthma and/or related phenotypes but its exact role in disease pathogenesis is still not clearly understood. Probably, HLA alleles act in association with other genetic loci responsible for the regulation of total IgE. It has been shown that genetic loci of chromosome 11q and 5q are strongly associated with high total IgE levels (40, 41). Therefore, the regulation of the total IgE response is a complicated process involving several genetic loci.

In this study, we failed to establish the association of HLA alleles with high level of total serum IgE in asthmatic subjects. It could be because of the limitations of our study which include: relatively small number of subjects in total IgE < 150 IU/ml group of asthmatics. Further, we studied only limited number of allelic groups which have been reported previously to be associated with pediatric asthma in different populations. Therefore, further study in large cohort of asthmatic subjects of this region taking into account as many HLA allelic groups as possible is needed to support the present finding.

In conclusion, the present preliminary finding suggests the possible association of *HLA-DRB1*03* allelic group with asthma in the pediatric population of Siliguri region of West Bengal, India. Therefore, *HLA-DRB1*03* allelic group may be implicated in the susceptibility to childhood asthma.

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Conflicts of interest

The authors have declared no conflicting interests.

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SHORT COMMUNICATION

Total Serum Immunoglobulin E in Children with Asthma

M. Lama · M. Chatterjee · T. K. Chaudhuri

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Abstract Immunoglobulin (Ig) E has been shown to be a major contributing factor for the development of bronchial hyperresponsiveness in asthma. An elevation in serum IgE levels contributes to asthma and is considered a potent predictor of the development of asthma. The objectives of the present study were to estimate the levels of total serum IgE in asthmatic and healthy control subjects and to investigate the relationship of various demographic and clinical characteristics with the total serum IgE level in asthmatics. We measured the levels of total serum IgE using the ELISA kits (AccuBind, Monobind Inc., USA). The relevant demographic and clinical data were obtained using the questionnaire. The results showed that asthmatic children had significantly elevated level of total serum IgE compared to that of the healthy controls. The levels of total IgE and IL-4 in sera of 44 asthmatic children showed a significant positive correlation. Total serum IgE >150 IU/mL was found to be significantly associated with the age, exposure to cigarette smoke, and raised eosinophil count in asthmatic children. In conclusion, the elevated level of total serum IgE may demonstrate the allergic etiology of asthma in the subjects studied.

Keywords Immunoglobulin E · Asthma · Children · Eosinophil · Interleukin-4

M. Lama · T. K. Chaudhuri (✉)
Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Rajarammohanpur, Siliguri 734 013, West Bengal, India
e-mail: dr_tkc_nbu@rediffmail.com

M. Lama
Department of Zoology, The University of Burdwan, Golapbag 713 104, West Bengal, India

M. Chatterjee
Department of Pediatrics, North Bengal Medical College & Hospital, Sushrutnagar, Siliguri, India

M. Chatterjee
Department of Pediatrics, NRS Medical College & Hospital, Kolkata, India

Introduction

Asthma is the most common chronic disorder in childhood, characterized by reversible airway obstruction, bronchial hyperresponsiveness (BHR) and atopy [1]. Total IgE level estimation provides evidence in support of atopy. Atopy is a nearly universal finding in children with asthma which is described as a tendency to produce excess amount of immunoglobulin (Ig) E antibodies when exposed to allergens [2]. Patients with asthma tend to have increase airway reactivity to a variety of stimuli such as allergens, irritants, exercise, cold air, and viruses [3]. The concentration of IgE in serum is age dependent and normally remains at levels less than 10 IU/mL in most infants during the first year of life [4].

Various population studies have shown an association between the prevalence of asthma/BHR and the total serum IgE levels, independent of specific reactivity to common allergens or symptoms of allergy [5, 6]. Burrows and his colleagues found a close correlation between serum IgE levels and the self-reported asthma [2].

The objectives of the present study were to estimate the levels of total serum IgE in asthmatic and healthy control subjects and to investigate the relationship of various demographic and clinical characteristics with the total serum IgE level in asthmatics.

Materials and Methods

Subjects and Collection of Blood Samples

In the present study, a total of 140 (70 asthmatic and 70 control) subjects were included. Children of the age group 3–12 years with asthma but free of other ailments such as parasitic infection, etc., diagnosed by the physicians were registered for the study. Each patient was thoroughly examined by the physician and the proforma was filled accordingly. Age and sex matched healthy subjects with no history of respiratory disorder, other atopic signs and symptoms, helminths or parasitic infection, were considered as control subjects. The registration of participants, data collection, and blood sample collection were done in the Out-Patient Department of Pediatrics, North Bengal Medical College & Hospital, Siliguri, West Bengal, India. All the laboratory investigations were performed in the Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri, West Bengal, India. The blood samples were collected by vein puncture method at appropriate conditions. Sera were separated and stored in aliquots at −70 °C until analysis.

Measurement of Total Serum IgE

Total serum IgE level was measured using Immunoenzymometric sequential assay (Type 4), ELISA kits (AccuBind, Monobind Inc., USA). The principle of the method involves the immobilization of the biotinylated monoclonal anti-IgE antibody on the surface of a microplate well on interaction with the streptavidin coated on the well. On addition of serum containing the native antigen, antibody–antigen complex is formed. Another antibody (directed to a different epitope) labeled with an enzyme is added which results in the formation of an enzyme labeled antibody–antigen–biotinylated–antibody complex on the surface of the wells. On addition of the substrate color is formed which is measured using a microplate spectrophotometer. The concentration of the unknown samples is determined from the standard curve created using reference samples with known antigen concentration.

The assay procedure was followed as per the manufacturer's instruction. The absorbance was measured at 450 nm in the ELISA plate reader (Bio rad). The sensitivity of the IgE AccuBind™ ELISA test system was 1.0 IU/mL with the intra- and inter-assay precisions of 1.95–5.87 % and 3.52–8.42 %, respectively.

Determination of Serum Level of Interleukin-4

In our previous study, we investigated serum levels of IL-4 and IFN-γ in 48 asthmatic and 32 control subjects [7]. A total of 44 asthmatics whose sera were used for both IL-4 and IgE estimation were considered for determining the correlation between IL-4 and total serum IgE.

Ethics

This study was approved by the "Institutional Human Ethics Committee", University of North Bengal, Siliguri, West Bengal, India. The written informed consent was obtained from the guardians/parents for their children to participate in this study.

Statistical Analysis

The data were compiled and tabulated in MS Excel 2007. Statistical analyses were done by the statistical computer software SPSS 16.0. First, means and SDs were calculated for the variables and *t* tests were applied for the comparison of means. For attributes, the percentages were calculated and χ^2 test was used for the comparison. Pearson's Chi-square test was used for analyzing the correlation between the total serum IgE and IL-4 in asthmatic subjects. A p value of <0.05 was considered to be statistically significant. The scatter diagram was plotted using the OriginLab v8.5.

Results and Discussion

The demographic and biochemical profile of asthmatics and controls are presented in Table 1. Table 2 shows the relationship of demographic and clinical characteristics of asthmatic subjects with the elevated level of total serum IgE. The results showed no significant associations of gender, family history of asthma/atopy, exclusive breast-feeding up to 6 months and residential set up with the elevated level of total serum IgE. The higher age group, exposure to cigarette smoke and the raised eosinophil count showed the significant associations with the elevated levels of total serum IgE in asthmatics. Further, there was a significant positive correlation ($r = 0.56$, $p < 0.001$***) between the total serum IgE and IL-4 in 44 asthmatic children (Fig. 1).

It was observed that out of 70 asthmatics, 50 (71.43 %) subjects had total serum IgE > 150 IU/mL. The mean total serum IgE level was significantly higher in asthmatic subjects compared to that of the control subjects, 269.21 ± 150.97 IU/mL versus 146.89 ± 77.32 IU/mL; $p < 0.001$*** (Fig. 2).

In the present study, the higher age group, exposure to cigarette smoke, and the raised eosinophil count showed the significant association with the elevated level of total serum IgE in asthmatic children. These findings are

Table 1 Demographic and biochemical profile of asthmatic and control subjects

	Asthmatic subjects ($n = 70$)	Control subjects ($n = 70$)		p value
Age (years)	6.93 ± 2.63	7.02 ± 2.29	$t = -0.211$	0.833
Sex				
Male/female (%)	37/33 (52.86/47.14)	39/31 (55.71/44.29)	$\chi^2 = 0.115$	0.734
Height (cm)	116.03 ± 17.16	119.59 ± 13.28	$t = -1.372$	0.172
Weight (kg)	18.74 ± 6.01	19.80 ± 5.57	$t = -1.079$	0.268
Study community				
Bengali/non-Bengali	54/16 (77.14/22.86)	46/24 (65.71/34.29)	$\chi^2 = 2.24$	0.134
Level of total serum IgE (IU/mL)	269.21 ± 150.97	146.89 ± 77.32	$t = -6.03$	<0.001***

*** Significant at $p < 0.001$

Table 2 Relationship of demographic and clinical characteristics of asthmatic children with elevated level of total serum IgE (>150 IU/mL)

Characteristics	Total no. of asthmatic subjects ($n = 70$)	Total serum IgE, >150 IU/mL ($n = 50$) (%)	χ^2	p value
Age group				
3–7 years	40	24 (60.0)	5.973	0.015*
8–12 years	30	26 (86.7)		
Sex				
Male	37	29 (78.4)	1.857	0.173
Female	33	21 (63.6)		
Eosinophil count				
Raised	45	37 (82.2)	7.193	0.007**
Normal	25	13 (52.0)		
FHA				
Yes	22	19 (86.4)	3.507	0.061
No	48	31 (64.6)		
EBF up to 6 months				
Given	52	37 (71.2)	0.007	0.931
Not given	18	13 (72.2)		
Exposure to cigarette smoke				
Exposed	24	21 (87.5)	4.622	0.032*
Not exposed	46	29 (63.0)		
Residential set up				
Rural	56	41 (73.2)	0.438	0.508
Urban	14	09 (64.3)		

FHA family history of asthma/atopy; *EBF* exclusive breastfeeding

* Significant at $p < 0.05$, ** Significant at $p < 0.01$

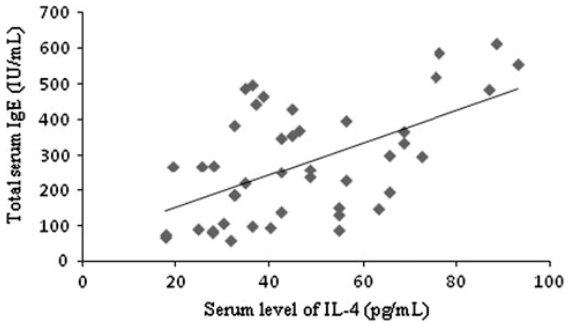

Fig. 1 Correlation between serum levels of IL-4 and total IgE in 44 asthmatic subjects. The correlation coefficient was 0.56 and was statistically significant ($p < 0.001$***)

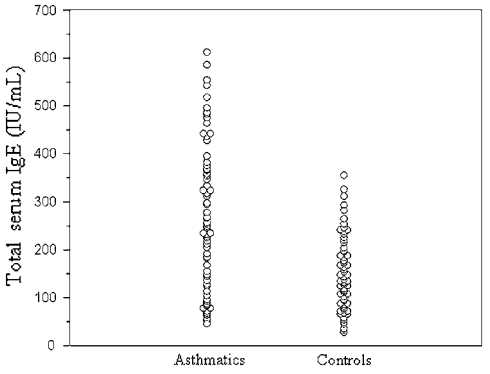

Fig. 2 Comparison of total serum IgE levels (IU/mL) between asthmatic and control subjects

consistent with the findings of several earlier studies. Cline et al. [8] reported the higher total serum IgE levels in the age group of 8–14 years. Similarly, Strachan and Cook [9] showed the potential role of passive smoking on IgE in a study conducted in children. Satwani et al. [10] showed eosinophilia along with raised serum IgE levels to be a significant allergic marker. Peripheral blood eosinophil counting has tremendously important clinical implication in order to demonstrate the allergic etiology of the disease, to monitor its clinical course and to address the choice of therapy [11].

The major finding of the present study confirmed that 71.43 % of the asthmatic subjects had total serum IgE levels >150 IU/mL. The mean total serum IgE level in asthmatic group was 269.21 ± 150.97 and 146.89 ± 77.32 IU/mL in control group. The difference was

statistically significant ($p < 0.001$***). Several studies have reported the elevated levels of total serum IgE in asthmatics [12, 13]. Therefore, it is in accordance with the well known fact that IgE plays a central role in the pathophysiology of allergic disorder such as asthma.

In the present study, it was also observed that there was a significant correlation between total IgE and IL-4 in sera of 44 asthmatic subjects. This finding is consistent with the finding of Afshari et al. [14] who reported considerably higher levels of serum IgE and IL-4 in asthmatics than non-asthmatic controls. IL-4 is one of the two cytokines known to cause switching in B-cells, a prerequisite for elevated IgE synthesis [15].

This study is a preliminary investigation and it has of course certain limitations. Further study investigating the prevalent allergens and the specific IgE estimation is warranted to strengthen the present study.

In conclusion, the elevated level of total serum IgE may demonstrate the allergic etiology of asthma in the subjects studied. Further, it also reveals the significant association of higher age, exposure to cigarette smoke and raised eosinophil count with the elevated level of total serum IgE in asthmatics.

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Short Communication

Increased interleukin-4 and decreased interferon-γ levels in serum of children with asthma

Manoj Lama [a], Mridula Chatterjee [b], C.R. Nayak [c], Tapas Kumar Chaudhuri [a,*]

[a] Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Rajarammohanpur, Siliguri 734 013, West Bengal, India
[b] Department of Pediatrics, North Bengal Medical College and Hospital, Siliguri, West Bengal, India
[c] Computer Centre, University of North Bengal, Siliguri 734 013, West Bengal, India

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ABSTRACT

Background and purpose: Immune and inflammatory responses, mediated by cytokines, play important roles in the pathophysiology of asthma. These responses are associated with over expression of T helper (Th)-2 cytokine, particularly interleukin (IL)-4 and IL-5, and decreased expression of Th-1 cytokine, IL-2 and IFN-γ. We hypothesized that there would be an imbalance in the levels of circulating IL-4 and IFN-γ in the asthmatic subjects.
Method: We investigated serum levels of IL-4 and IFN-γ among eighty children (18 steroid-naïve, 30 steroid-treated children with asthma and 32 healthy controls) using commercially available ELISA kits.
Results: Serum level of IL-4 was significantly higher in steroid-naïve group of asthmatic children compared to the healthy control subjects and was lower in steroid-treated group though the level was statistically not significant. In contrast, serum levels of IFN-γ were significantly lower in both steroid-naïve and steroid-treated groups of asthmatic children compared to healthy control subjects.
Conclusion: The results of our study suggest that serum level of IL-4 may be elevated in concert with decreased level of IFN-γ in asthma. Determination of serum levels of IL-4 and IFN-γ may be a useful tool for understanding the disease processes in asthma.

1. Introduction

Asthma is the most common chronic disease of childhood and the leading cause of childhood morbidity as measured by school absences, emergency department visits, and hospitalizations [1]. The asthma phenotype is characterized by a T helper (Th)-2 mediated inflammatory response involving alteration in the fine balance between Th-1 and Th-2 responses towards Th-2 bias and a complex interaction between a wide network of inflammatory and structural cells and the inflammatory mediators which they release [2].

In asthma, the development of immune response depends on a repertoire of cytokines produced by numerous cells, including $CD4^+$ helper T cells. These lymphocytes can be divided into two subsets, T helper type 1 (Th-1) and T helper type 2 (Th-2), based on their cytokine profiles [3]. Effector Th1 cells are involved in delayed-type hypersensitivity through their production of IFN-γ and IL-2, whereas Th2 cells secrete IL-4, IL-9, IL-10 and IL-13, and promote antibody-mediated humoral immune responses [4]. It has been suggested that an alteration in cytokine milieu, with excess Th-2 products (IL-4, IL-5, and IL-13) in concert with decreased Th-1 products (IFN-γ and IL-2), is predicted to drive the asthma phenotype [5]. Elevated levels of IL-4, an essential cofactor for immunoglobulin E (IgE) production, and IL-5, responsible for the final differentiation, activation and recruitment of eosinophils [6], have been found in serum of patients with asthma [7–10]. On the other hand, IFN-γ is thought to protect against the development of asthma by regulating Th-2 cytokine production, although a mixed Th-1/Th-2 pattern has been reported [11].

One of the first studies measuring cytokine concentrations in children with allergic disease, revealed a significant increase in the level of IL-4 in serum from atopic asthmatics compared to controls, which correlated with IgE [7]. Other subsequent studies in serum and blood supported the importance of IL-4 in childhood asthma [12–14]. The differences in IL-4 levels are likely to be dependent on disease severity, since analysis of children with mild/moderate asthma revealed no differences in IL-4 concentrations compared to normal controls [15]. Several other invasive studies involving bronchoalveolar lavage (BAL) fluid and lung biopsies have confirmed that a Th-2-like mediated immune response is seen in asthma [16–18]. Gemou-Engesaeth et al. and Krouwels et al. [19,20] have reported the imbalance in the production of

Abbreviations: IL, interleukin; IFN, interferon.

* Corresponding author. Fax: +91 353 2699001.
E-mail address: dr_tkc_nbu@rediffmail.com (T.K. Chaudhuri).

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IL-4 and IFN-γ in children with atopic asthma, and corticosteroids appear to correct it.

Although there are various studies showing cytokine profile of asthmatic subjects, mostly employing either bronchealveolar lavage (BAL) fluid or the exhaled breath condensate, the data on the circulating levels of cytokines in the asthmatic subjects are limited. Moreover, it has been suggested that asthma being the most heterogeneous airway disease, it may also demonstrate the systemic pattern of the disease outside the respiratory tract. Therefore, the aims of the present study were: (1) to characterize Th-1 cytokine (IFN-γ) and Th-2 cytokine (IL-4) profiles in children with asthma and healthy control, (2) to investigate any alteration in Th-1/Th-2 balance, and (3) to analyze whether there is any deviation in the levels of cytokines with corticosteroid treatment in asthmatic subjects.

2. Materials and methods

2.1. Subjects

This study included asthmatic as well as healthy children in the age group of 3–12 years. Children with asthma were recruited from the pediatric Outpatient Department of North Bengal Medical College and Hospital, Siliguri, West Bengal, India. Healthy age-matched control subjects were selected on the basis of having no history of lung disease or allergy, no airways hyperresponsiveness and no upper respiratory infections. We obtained approval for the study from the 'Human Ethics Committee', University of North Bengal, Siliguri, West Bengal, India. The study was explained in detail to the parents/guardians of the children who participated in this study and an informed consent was obtained from each parent/guardian.

2.2. Study design

Physical examination of each child was performed by the physician. Demographic data and other clinical characteristics of the study subjects were collected using the questionnaires. Weight and height were recorded for every child. Asthma was diagnosed by the physician on the basis of medical history, physical examination, chest X-ray, and peak expiratory flow rate (PEFR). The entire asthmatic subjects were divided into two groups: asthmatic children who were not under steroid treatment were included in steroid-naïve group and those under steroid treatment were included in the steroid-treated group.

2.3. Sample size and blood collection

A total of 48 asthmatic subjects (18 under steroid-naïve group and 30 under steroid-treated group) and 32 healthy control subjects were included in this study. All the participating subjects were from Indian origin residing in and around Siliguri, West Bengal, India. The majority of the children were Bengali. The blood samples from the healthy controls and asthmatic subjects were collected between 11 am and 2 pm by veinpuncture method at appropriate conditions. Serum was acquired after coagulation of the blood for 1–2 h at room temperature. The supernatant was centrifuged for 10 min at 2000*g*. The serum thus acquired was then aliquoted and stored at −70 °C until analysis.

2.4. Determination of serum levels of cytokines

Serum levels of IL-4 and IFN-γ were determined by Enzyme Linked Immunosorbent Assay (ELISA) method. Commercially available 96 well ELISA kits (Endogen Human IL kit, Pierce Biotechnology, Inc., Rockford) were used to measure serum levels of IL-4 and IFN- γ. The sensitivity for both IL-4 and IFN-γ was <2 pg/ml with inter and intra-assay coefficient of variation of <10% in each case. Absorbance was measured by a microtitre plate reader (Opsys MR, Dynex Tehnologies) at 450 nm. Each assay was carried out by the same investigator in the Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Siliguri, India.

2.5. Data analysis

The data collected were statistically analyzed by the statistical computer software SPSS, version 15.0. A p value of less than 0.05 was considered to be statistically significant. Mean and standard deviation were calculated for the variables and t-test was employed for the comparisons. For the attributes, percentages were calculated first and then χ^2 test was used for comparisons. The figures were drawn with the help of OriginLab 6.1.

3. Results

Forty-eight asthmatic children, 18 steroid-naïve and 30 steroid-treated, and 32 healthy children participated in this study. The demographic and clinical characteristics of the study groups are presented in Table 1. Eosinophil count in the asthmatic subjects was significantly higher as compared to control subjects ($p < 0.05$). The age, gender and other clinical characteristics did not differ significantly between the two groups. The demographic and clinical characteristics such as family history of asthma, total lymphocyte count, eosinophil count, etc. did not show significant correlation with serum levels of IL-4 and IFN-γ.

Serum levels of IL-4 and IFN-γ in three groups, viz. steroid-naïve, steroid-treated children with asthma and healthy control subjects, are summarized in Figs. 1 and 2, respectively. Serum IL-4 level was significantly higher in steroid-naïve group of asthmatic children as compared to healthy control group (52.25 ± 21.91 versus 32.81 ± 16.28 pg/ml; $p < 0.001^{***}$) and it was lower in steroid-treated group but not statistically significant when compared with steroid-naïve group (40.80 ± 17.77 versus 52.25 ± 21.91 pg/ml; $p = 0.054$, NS). In contrast, serum level of IFN-γ was significantly lower in both steroid-naïve and steroid-treated groups of

Table 1
Demographic and clinical characteristics of asthmatic and healthy control subjects.

	Asthmatic subjects Mean ± SD/[%]	Control subjects Mean ± SD/[%]	p value
Age (years)	6.74 ± 2.70	6.35 ± 2.00	0.428
Age of onset			
Up to 4 years	34 [70.83%]		
5–8 years	11 [22.92%]		
9–12 years	03 [6.25%]		
Gender			
Male/female	25/23 [52.08%/47.92%]	18/14 [56.25%/43.75%]	0.134
Treatment status			
Steroid-naïve	18 [37.5%]		
On steroid treatment	30 [62.5%]		
Total Leukocyte Count (/mm^3)	9448 ± 1831.16	9891.67 ± 1756.25	0.506
Eosinophil Count (/mm^3)	848 ± 741.20	380.42 ± 138.43	0.04*
Family history of asthma/atopy			
Yes/no	22/26 [45.83%/54.17%]	8/24 [25%/75%]	0.06

* $p < 0.05$.

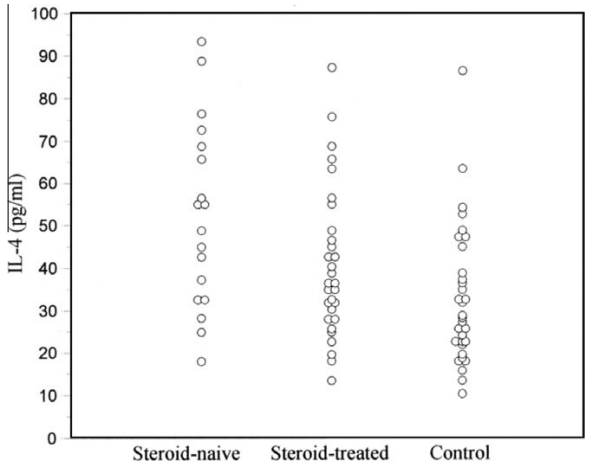

Fig. 1. Comparison of serum levels of IL-4 (pg/ml) among three groups viz. steroid-naïve, steroid-treated subjects with asthma and healthy control.

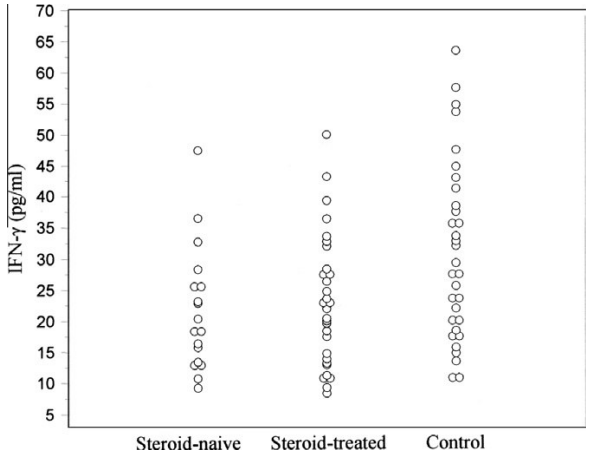

Fig. 2. Comparison of serum levels of IFN-γ (pg/ml) among three groups viz. steroid-naïve, steroid-treated subjects with asthma and healthy control.

asthmatic children compared to healthy control subjects (21.62 ± 9.91 versus 30.79 ± 14.28; $p = 0.02^*$ and 23.03 ± 10.54 versus 30.79 ± 14.28 pg/ml; $p = 0.019^*$), respectively.

4. Discussion

In the present study, serum levels of IL-4 and IFN-γ were well above their detection levels in both the asthmatic as well as control subjects. The mean IL-4 level was significantly higher in steroid-naïve group as compared to control subjects but in steroid-treated group it was lower, though statistically not significant, as compared to steroid-naïve group. In contrast, levels of IFN-γ were significantly lower in both steroid-naïve as well as steroid-treated groups of children with asthma compared to control subjects.

The findings of higher and lower serum levels of IL-4 and IFN-γ respectively, in our study, are consistent with various other findings. A number of authors have studied serum cytokines in asthmatic subjects [7,13,21–23]. Bogić et al. [24] have reported significantly higher IL-4 and IL-5 serum concentrations in asthmatic group compared to control and these were significantly higher in patients with moderate and severe asthma compared to mild asthmatics. Shahid et al. [25] have shown an increased concentration of exhaled IL-4 in steroid-naïve group of asthmatic children and a decreased concentration of exhaled IFN-γ in both steroid-naïve and steroid-treated groups compared to control subjects. Further, they have also reported that the exhaled IL-4 level was significantly lower in asthmatic children who were on steroid treatment. In a study, it was revealed that the expression of T-bet mRNA and the level of IFN-γ were lower, but the level of serum IL-4 was higher in asthma patients compared to healthy subjects. With the *Astragalus membranaceus* intervention, the level of IFN-γ and the expression of T-bet mRNA were increased and the level of IL-4 was decreased in the peripheral blood mononuclear cells (PBMCs) supernatant [26]. Several other studies have also reported that allergic and asthmatic subjects are more likely to have elevated levels of Th-2 cytokines and reduced levels of Th-1 cytokines [13,27–32].

In fact, IL-4 demonstrates a broad range of biological activities. It is a main cytokine involved in the pathogenesis of allergic responses and at the same time it can also down-regulate acute inflammatory changes [33]. IL-4 has also got additional effects on asthma pathogenesis which include stimulation of mucus producing cells and fibroblasts leading to airway remodeling [34–36]. It has also been confirmed that the crucial role of IL-4 lies in its effect on Th-2 development, rather than on the induction of IgE synthesis and subsequent mast cell degranulation [37]. On the other hand IFN-γ is a potent inhibitor of IgE synthesis [38]. Thus, this imbalance in the serum levels of IL-4 and IFN-γ is predicted to drive the asthma pathogenesis.

The finding of our study also revealed the association of steroid treatment with the reduction of IL-4 level in serum of asthmatic subjects. Serum level of IL-4 was lower in steroid-treated asthmatic subjects, although not significant as compared to steroid-naïve asthmatic subjects (40.80 ± 17.77 versus 52.25 ± 21.91 pg/ml; $p = 0.054$, NS). The previous findings suggest that steroids inhibit both IL-4 and IFN-γ synthesis but the inhibitory action on IFN-γ is less marked [39]. Therefore, our finding of lower level of IL-4 in steroid-treated asthmatic subjects supports the earlier findings and suggests that steroid treatment down-regulates the level of IL-4 in asthma.

Although Th-1/Th-2 paradigm provided a simplistic model for initially describing involvement of T cells in asthma but still it does not fully support the complexities of this disease. Moreover, IFN-γ possesses a number of proinflammatory activities including the up-regulation of ICAM-1 [40] and the receptor for TNF-α [41], it is likely, that under certain circumstances IFN-γ may exert its pro-inflammatory activities and potentiate the inflammatory response in children with asthma. Therefore, it appears that some Th-1 and Th-2 cytokines are indeed elevated in asthma phenotypes of children. However, their effects in childhood asthma are largely unknown. In fact, there is an urgent need for complete understanding of T cell cytokine responses in childhood asthma. Moreover, it is crucial to understand the disease process for unraveling such complexities.

To conclude, the findings of our study support the hypothesis of Th1/Th2 cytokine imbalance and suggest that serum level of IL-4 may be elevated in concert with decreased level of IFN-γ, in asthma. Determination of serum levels of IL-4 and IFN-γ may be useful for understanding and monitoring the inflammatory response in asthma.

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ORIGINAL ARTICLE

Epidemiology of Childhood Asthma: A Hospital Based Study

Manoj Lama *, Mridula Chatterjee **, Tapas Kumar Chaudhuri *

** Cellular Immunology Laboratory, Dept. of Zoology, University of North Bengal, Siliguri*

*** Dept. of Pediatrics, North Bengal Medical College & Hospital, Sushrutnagar, Siliguri*

ABSTRACT

a) Introduction: *Asthma is a major global health problem, characterized as a chronic disease affecting a major proportion of pediatric population. Increased prevalence of asthma is multifactorial in etiology.* ***b) Aims and Objectives:*** *The prevalence of asthma among the pediatric population in the age group 3-12 years and its associated risk factors have been investigated in a hospital based study carried out from May 2009-April 2010 in the Outpatient Department of Pediatrics (OPD), North Bengal Medical College & Hospital, Siliguri.* ***c) Methodology:*** *Children who visited the Out-Patient Department of Pediatrics, North Bengal Medical College and Hospital, from May 2009 to April 2010, were registered for the study. Asthma was diagnosed by the physician. The relevant data were collected using the questionnaire.* ***d) Result and Analysis:*** *In this hospital-based study, the mean prevalence of asthma among children in the age group between 3 and 12 years was found to be 3.06%. It was noted that 33 (33%) asthmatic children and 17 (15.45%) control subjects had the family history of asthma. This difference was statistically significant.* ***e) Conclusion:*** *The prevalence rate of childhood asthma in and around Siliguri seems to be roughly equal to the prevalence rates prevailing in other rural areas of the country as reported by various studies. Results of our study also indicated that asthma is associated with the family history of asthma/atopy suggesting that genetic predisposition may be an important etiology for the development of asthma.*

KEY WORDS: *Asthma, Children, Prevalence*

INTRODUCTION

Pediatric asthma is a major global health concern. It is now one of the most common chronic diseases affecting an estimated 300 million people worldwide[1]. Asthma has also increased the number of preventable hospital visits and admissions. Apart from being the leading cause of hospitalization for children, it is one of the most important chronic conditions causing elementary school absenteeism[2,3]. The increased prevalence of asthma is multifactorial in etiology. The pathogenesis has not been clearly elucidated, but various factors such as economic development, exposure to tobacco smoke, exposure to air pollution, infection, climate, diet, obesity, antibiotic use, and exposure to allergens are known to be associated with childhood asthma[4].

In Indian scenario, large numbers of studies have reported the varying rates of asthma prevalence in pediatric population. A study has reported a wide variation (4-19%) in the prevalence of asthma in school-going children from different geographic areas in India[5]. A recent investigation by Jain et al.[6] in a cross sectional community based study on rural Indian children showed the prevalence of bronchial asthma to be 10.3%. Another study by Awasthi et al.[7] on school-going children in Lucknow

Corresponding Author: *Dr Tapas Kumar Chaudhuri*
E-mail: *dr_tkc_nbu@rediffmail.com*

showed the prevalence of asthma to be 2.3%in age group of 6-7 years and 3.3%in age group of 13-14 years. The prevalence of childhood asthma in this region is still not known.

AIMS AND OBJECTIVE OF THE STUDY

To determine the prevalence of childhood asthma and to study the risk factors associated with the development of asthma.

METHODOLOGY

This study was carried out for one year from May 2009 to April 2010 on the pediatric population in the age group of 3- to 12-years. The North Bengal Medical College & Hospital is a main hospital in this region. The children residing in rural as well as urban areas in and around Siliguri who visited the Out-Patient Dept. of Pediatrics, North Bengal Medical College & Hospital, were registered in this study. Most of the children were from the rural areas (villages and tea gardens).

Asthma was diagnosed by a physician based on the medical history, physical examination, clinical history, family history of atopy and asthma, etc. For the study of prevalence of asthma, total number of children in the age group 3-12 years who were registered in the OPD was recorded. For the study of associated risk factors of asthma, data were collected using the questionnaires from 100 asthmatic and 110 control subjects. Age, sex, height and weight were also recorded. For each case to participate in the study we obtained informed consent from the parent and/or guardian of the patient.

Data Analysis

Data obtained from the study were statistically analyzed with the statistical software SPSS (version 15.0). For variables, first the means were calculated and t-test was employed for comparing the equality of means. For attributes, the percentages were calculated and χ^2 test was used for the comparisons. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

A total of 6280 children were registered in one year in the age group of 3-12 years out of which 192 were diagnosed with asthma. The monthly prevalence of pediatric asthma for one year duration from May 2009-April 2010 is shown in fig. 1. The mean prevalence was observed to be 3.06%.

The age and sex distributions of asthmatic and control subjects are presented in table I and associated risk factors of asthma are presented in table II. In this study, it was noted that 25 (25%) asthmatic children and 18 (16.36%) non-asthmatic children were not breast fed up to 6

Table I. *Age and sex distribution of asthmatic and control subjects*

	Age (yrs)		Sex	
	Mean	**SD**	**Male**	**Female**
Asthmatic subjects	7.26	2.64	55 [55%]	45 [45%]
Control subjects	7.15	2.52	59 [53.64]	51 [46.36]
	0.752 NS		0.843 NS	

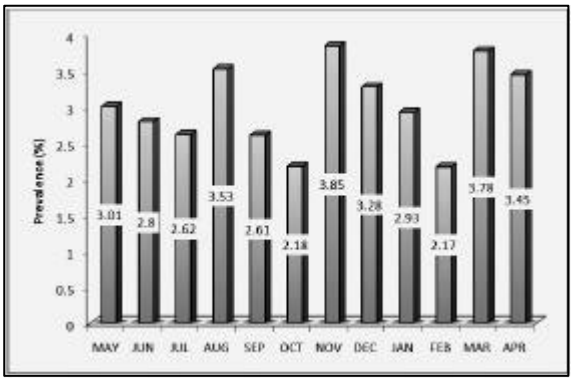

Fig. 1. *Prevalence of pediatric asthma for one year duration (from May 2009 - April 2010)*

Table II. *Associated risk factors of asthma as noted in asthmatic and control subjects*

Risk factors	Type of risk Factors	Asthmatic subjects (n=100)	Control subjects (n=110)	p-value
EBF up to 6 months	Done	75 [75%]	92 [83.64%]	0.1214 NS
	Not done	25 [25%]	18 [16.36%]	
Family history of asthma/ atopy	Yes	33 [33%]	17 [15.45%]	0.0029 S**
	No	67 [67%]	93 [84.55%]	
Overcrowding	Yes	39 [39%]	31 [28.18%]	0.0967 NS
	No	61 [61%]	79 [71.82%]	
Cooking mode	With smoke	78 [78%]	91 [82.73%]	0.388 NS
	Smokeless	22 [22%]	19 [17.27%]	

S=Significant, NS=Non significant, Abbreviation: EBF, Exclusive breastfeeding, **Significant at $p < 0.01$

months. This difference was not statistically significant. The family history of asthma/ atopy was recorded in 33 (33%) asthmatic children and in 17 (15.45%) control subjects. This difference was found to be statistically significant ($p<0.01$**). The rest of the factors, hygienic condition around house, overcrowding and cooking mode did not differ significantly between asthmatic and control subjects.

DISCUSSION

In this hospital based study, the prevalence of pediatric asthma was recorded to be the highest during the month of November then followed by March, August and April. The lowest prevalence rate was observed during the months of February and October. This variation in prevalence rate may be due to seasonal variation, climatic factors, exposure to certain environmental factors, cold allergy, etc. The annual mean prevalence was found to be 3.06%. Various school survey, community based studies and hospital based study have shown diverse rates of asthma prevalence [5-7]. Singh et al.[8] in their study on the pediatric population in the age group of 1-15 years residing in five villages of Dehlon block of Ludhiana showed the prevalence of asthma to be 2.6%.

Although some recent reports suggest the prevalence of asthma to be declining but no overall global declining trend in the prevalence of asthma was shown in a recent review of epidemiological studies to examine international trends in asthma prevalence in children and adults for the period 1990-2008[9].

The genetic predisposition (family history of asthma) is considered to be an important risk factor for the development of asthma. The finding of our study is consistent with various other studies which have well documented strong association of family history of asthma and asthma development. Vishwanathan et al.[10] have observed the family history of asthma in 42% of asthmatic subjects but in only 10% non-asthmatics. Similarly, Ninan et al.[11] observed parental history of asthma in 42% patients with polysymptomatic asthma as compared to 13% in asymptomatic children ($p<0.001$).

The present study showed no significant association of asthma with exclusive breast feeding, hygiene condition around house, overcrowding and cooking mode (with smoke/ smokeless). These observations are consistent with previous studies. The findings of Gergen et al.[12] showed non-significant association between overcrowding and asthma. Schenker et al.[13] and Chhabra et al.[14] have reported no significant association between the prevalence of wheeze and asthma with the type of fuel used in kitchen. The importance of breast feeding to childhood

asthma is a controversial issue. However, in a study by Oddy et al.15 it was reported that the exclusive breast feeding >4 months was a significant protective factor for wheezing LRI, current asthma and atopy, following multivariate adjustment.

This is a preliminary epidemiological study carried out in the North Bengal region. There are indeed certain limitations of this study. We could not involve the general pediatric population of specific age group in this study as it was restricted only to the hospital visiting children. Future study based on school survey using the standard questionnaire (ISSAC questionnaire) designed particularly for the epidemiological study may be warranted to further support this study.

CONCLUSION

The present hospital-based study shows the mean annual prevalence of childhood asthma to be 3.06% in and around Siliguri, West Bengal. Results of our study also indicate that the family history of asthma/atopy is associated with asthma in children suggesting the genetic predisposition to be an important etiology for the development of asthma.

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ELEVATED SERUM C-REACTIVE PROTEIN CONCENTRATION IN INHALED CORTICOSTEROID-NAÏVE CHILDREN WITH ASTHMA

MANOJ LAMA, MRIDULA CHATTERJEE[a], CHITTA R. NAYAK[b] and TAPAS KUMAR CHAUDHURI[*]

Cellular Immunology Laboratory, Department of Zoology, University of North Bengal, Rajarammohunpur, SILIGURI – 734013 (W.B.) INDIA
[a]Department of Pediatrics, North Bengal Medical College & Hospital, SUSHRUTNAGAR – 734 012, Dist. Darjeeling, (W.B.) INDIA
[b]Computer Centre, University of North Bengal, 734 013 Dist. Darjeeling, (W.B.) INDIA

ABSTRACT

Objective-Serum C-reactive protein (CRP) concentration was determined in inhaled corticosteroid (ICS)-naïve and ICS-inhaling asthmatic children to understand the inflammatory process (es) in asthma. The latex agglutination test was performed for determining the serum CRP concentration among 87 asthmatic children. The limitation of detection of the test was less than 6 mg/L. Further, CRP was treated as a categorical variable: elevated (≥ 6 mg/L) and normal (< 6 mg/L). Among 87 asthmatic children, 15 children were ICS-naïve and 72 were ICS-inhaling. The elevated serum CRP concentration was detected in 13 (86.7%) ICS naïve-children and in only 3 (4.2%) ICS-inhaling children. The CRP concentration was significantly elevated in the serum of ICS-naïve sub-group of asthmatic children ($p < 0.001$). This study suggests that the asthmatic inflammation is associated with the elevation of serum CRP concentration and the ICS, which has the anti-inflammatory properties, might have played a role in reducing the CRP concentration in the ICS-inhaling children.

Key words: Asthma, C-reactive protein, Inflammation, Inhaled corticosteroid.

INTRODUCTION

Asthma is the most common chronic disease in childhood. It is responsible for significant social, economic and psychological impact on the family. Acute asthma leads to disturbed sleep, restriction in day to day activities and school absenteeism. Risk factors associated with development of asthma include: family history of asthma and atopic diseases,

[*]Author for correspondence; Ph.: (M) 9434377127 (O) +91-353-2699124; Fax: +91353 2581546; E-mail: dr_tkc_nbu@rediffmail.com

bronchiolitis during infancy, sensitization to allergens during childhood and passive smoking[1].

Asthma is characterized by airway hyperresponsiveness and inflammation, in which various cells (such as eosinophils, neutropnils, macrophages and T-lymhocytes) cytokines and mediators play a role. Beside local inflammation, systemic inflammation is also present in asthma, as shown by the plasma fibrinogen and serum amyloid A[2].

C-Reactive protein (CRP), the best-studied major acute phase protein in humans, was initially described in 1930 by Tillet and Francis Jr.[3] as the serum factor responsible for the precipitation of acute phase sera with the C-substance (C-polysaccharide,CPS) of pneumococcal cell walls. It is produced by the liver at a higher concentration, when the organism is challenged by a significant inflammatory stimulus, such as endotoxins from the membranes of Gram-negative bacteria inhaled into the bronchial tree of asthmatic patients[4]. A population based study has shown associations of increased levels of serum CRP with a high frequency of airway hyperresponsiveness and low forced expiratory volume in one second (FEV1) among subjects without heart disease[5], suggesting that systemic inflammation may be associated with respiratory impairment. Another epidemiological study showed that elevated levels of hs-CRP correlate significantly with respiratory symptoms and with prevalence of non-allergic asthma[6]. Recent publications suggest that CRP could be taken into consideration as a simple, cheap and reliable marker for monitoring asthmatic inflammation[5-7].

In the present study, we therefore, determined the serum CRP concentration in asthmatic children with and without ICS treatment in order to understand if serum CRP concentration could be taken as a marker for asthmatic inflammation. The relationships of the elevated serum CRP concentration to the other demographic variables were then investigated.

EXPERIMENTAL

Study subjects

The study included children (3-12 years) with asthma, who attended the out-patient department of North Bengal Medical College & Hospital, Siliguri, West Bengal. Children were diagnosed to have asthma according to the clinical criteria (history, physical examination, chest X-rays and peak flow expiratory rate (PEFR). A total of 87 asthmatic children (49 males and 38 females, age = 7.09 ± 2.55 years) were registered in the study from February 2009 – March 2010. After obtaining the informed consents from the

parents/guardians of the patients, blood samples of 2-3 mL were obtained in appropriate conditions by vein-puncture method.

Ethics

This study was approved by the Institutional Human Ethics Committee, University of North Bengal.

CRP test

The blood sample was allowed to coagulate at room temperature for 3-4 hours. Blood clot was cut and centrifuged at 2000Xg for 10 minutes to separate the serum. Freshly separated serum samples were used for the C-reactive protein test. Commercially available CRP kit 'IMMUNOSTAT' (Ranbaxy Fine Chemicals Ltd., HP, India) was used for the detection of CRP concentration in the serum. The limitation of detection of the test was < 6 mg/L. We treated CRP as a categorical variable, elevated (≥ 6 mg/L) and normal (≤ 6 mg/L). A positive result indicating the elevated serum CRP concentration was interpreted by the development of a clearly visible agglutination. It indicated CRP content of 6 mg/L and above in the test samples. Serum samples that showed no visible agglutination were considered to have normal CRP concentration.

Statistical analysis

Data were statistically analyzed with statistical software version 15.0 (SPSS package). Firstly, we calculated the mean, standard deviation and the frequency tables. To compare the two sub-groups (ICS-naïve and ICS-inhaling), the Student's t-test and the chi-square test were used. Chi-square test was employed for the homogeneity testing, t-test for equality of means of independent populations and paired t-test for the equality of means for the dependent variables. The p-values of less than 0.05 were considered as significant.

RESULTS AND DISCUSSIONS

Among 87 asthmatic children, 15 children were ICS-naïve and 72 were ICS-inhaling. The elevated serum CRP concentration was detected in 13 (86.7%) ICS-naïve children and in only 3 (4.2%) ICS-inhaling children.

The demographic data and the ICS status of patients (ICS-naïve and ICS-inhaling) of the entire group and the two sub-groups of children with asthma are shown in Table 1. Age, sex distribution, exclusive breast feeding up to 6 months and the family history of asthma/atopy did not differ between the two sub-groups. The result showed that the CRP

concentration was significantly elevated in the serum of ICS-naïve sub-group of asthmatic children ($p < 0.001$).

Table 1: Demographic data and CRP concentration (elevated and normal) in the entire group and two sub-groups of patients (ICS-naïve and ICS-inhaling)

Demographic data and serum CRP concentration	Age (Years) Mean ± SD	Sex M/F	Exclusive breast feeding (up to 6 months) Yes/No	Family history of asthma (atopy) Yes/No	Elevated CRP/ Normal CRP
Entire group (N = 87)	7 .09 ± 2.55	49/38 (56.3% /43.7%)	68/19 (78.2% /21.8%)	31/56 (35.6% /64.4%)	16/71 (18.4% /81.6%)
ICS-naïve sub-group (N = 15)	7.11 ± 2.14	9/6 (60% /40%)	12/3 (80% /20%)	6/9 (40% /60%)	13/2 (86.7% /13.3%)
ICS-inhaling sub-group (N = 72)	7.08 ± 2.64	40/32 (55.6% /44.4%)	56/16 (77.8% /22.2%)	25/47 (34.7% /65.3%)	3/69 (4.2% /95.8%)
Statistical	t = - 0.30 df = 85 p = 0.96 NS	$\chi^2 = 0.10$ df = 1 p = 0.752 NS	$\chi^2 = 0.36$ df = 1 p = 0.850 NS	$\chi^2 = 0.151$ df =1 p = 0.698 NS	$\chi^2 = 50.23$ df = 1 $p < 0.001$ S***

NS: Not Significant
S: Significant
*significant at $p < 0.05$
**significant at $p < 0.01$
***significant at $p < 0.001$

In the present study, It was observed that the serum CRP concentration was elevated in the ICS-naïve children with asthma while ICS-inhaling children showed to have normal serum CRP concentration. Our finding is similar to the finding of Takemura M et al.[8] They reported that serum hs-CRP levels were significantly increased in steroid-naïve patients compared with controls, but not in patients on inhaled corticosteroid. Further, among steroid-naïve patients, serum hs-CRP levels negatively correlated significantly with indices of pulmonary function and positively with sputum eosinophil count. In another study by Kasayama et al.,[9] it was revealed that the plasma CRP levels were significantly reduced in corticosteroid-naïve asthmatic patients treated with inhaled corticosteroid for 3 months. In this respect, it is likely that in the present study, the ICS, which has well characterized anti-inflammatory properties, might have reduced serum CRP concentration to the normal state among the ICS-inhaling group of asthmatic children.

Bronchial hyperresponsiveness (BHR) is a crucial attribute of asthma. It is due to chronic asthmatic inflammation and may reflect the level of the inflammatory process[10]. An elegant work on a population-based study, Kony et al.,[5] revealed an association between a higher frequency of BHR and and higher CRP levels in study participants, which could reflect local inflammation within the bronchi. Fujita M et al.,[11] reported that increased hs-CRP levels may be associated with allergic inflammation, particularly eosinophilic inflammation, and the degree of airway obstruction in asthmatic patients. In another study by Szalai et al.[12], it has been suggested that an increase in CRP concentration may accompany the acute phase of allergic inflammation. This phenomenon may occur as a secondary reaction connected with the ability of CRP to stimulate the expression of anti-inflammatory cytokine-10, which down-regulates the activity of the Th_2 lymphocyte population. Thus, these findings suggest that asthmatic inflammation results in the elevation of CRP concentration. However, the exact role of the CRP in asthmatic inflammation is not clearly understood.

Although CRP is structurally distinct from the immunoglobulins, it shares with them the ability to initiate several biological functions including precipitation[3], opsonization[13], capsular swelling[14] and agglutination[15]. Two major biological activities of CRP have been well defined: first, it is able to bind several biological substrates that are distributed widely in nature[16]. Second, it has significant activation capabilities, in particular to activate the complement system[17] and to bind to and modulate the function of phagocytic leukocytes[18,19]. These effects support the concept that this serum protein may have a potentially central role in the host defense mechanisms.

To our knowledge, this is the first such attempt to investigate the interrelationship between serum CRP concentration and asthma in this region. The present study has certain limitations: we did not consider the severity score of the disease in our study. Moreover, the sample size in the present study is also small; study in large sample size will be needed to strengthen the present study further.

CONCLUSION

In conclusion, it was observed that the ICS-naïve status of the asthmatic children was associated with the elevated serum CRP concentration, which suggests that the serum concentration of CRP can be a marker, reflecting the degree of inflammation in asthma. It was further observed that the serum CRP concentration was reduced in the ICS-inhaling asthmatic children. The ICS, which has potent anti-inflammatory properties, might have reduced the CRP concentration to the normal state.

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