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Variants of inflammatory mediators: alpha-1-protease inhibitors, cortisol binding globulin, interleukin-1-receptor antagonist and beta-2-adrenergic receptor genes in atopic asthmatic and non-asthmatic South Africans.

Pillay, Visva

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VARIANTS OF INFLAMMATORY MEDIATORS : ALPHA-1-PROTEASE INHIBITOR, CORTISOL BINDING GLOBULIN, INTERLEUKIN-1-RECEPTOR ANTAGONIST AND BETA-2-ADRENERGIC RECEPTOR GENES IN ATOPIC ASTHMATIC AND NON-ASTHMATIC SOUTH AFRICANS

Visva Pillay

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science.

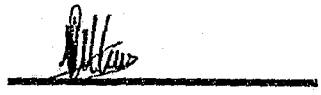
Degree awarded with distinction on 2 December 1999

Johannesburg, 1999

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

I further declare that the work presented was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand, and granted the clearance number M950219.



(Signature of candidate)

27th day of SEPTEMBER 1999

For my parents, Ganesen and Athie, in appreciation of their constant support and encouragement throughout my prolonged education.

ABSTRACT

According to current concepts asthma is primarily an inflammatory condition of the bronchi which results from the complex interactions between heterogenous genetic and environmental factors. Although the environmental allergens are fairly well known, little information concerning the genetic differences between atopic and non-atopic individuals is available. A number of candidate genes have been proposed, including genes for protease inhibitors, interleukins and the beta-2-adrenergic receptor (β_2AR). The present study was undertaken in order to determine whether molecular variation of certain atopy candidate genes (alpha-1-protease inhibitor, cortisol binding globulin, interleukin-1-receptor antagonist and β_2AR) may be associated with atopic asthma in black and white asthmatic South Africans. Alpha-1-protease inhibitor (α_1PI) has been implicated in the pathogenesis of emphysema, if it is present in one of its deficient phenotypic forms. Given that α_1PI is also an acute phase reactant in humans, it is possible that an association exists between the manifestations of asthma and α_1PI or its deficiency. This investigation looked at the various phenotypes of α_1PI in black and white asthmatic and control individuals by making use of isoelectric focusing as well as to make use of the polymerase chain reaction (PCR) which allowed for the identification of two haplotypes of the M1 phenotype of α_1PI, viz. M1 (Ala^{213}) and M1 (Val^{213}). There was a significant increase in the M1 (Ala^{213}) haplotype in the black groups as compared to the white groups. A novel finding was the identification of a new variant, the $M1E_{(JOHANNESBURG)}$. A significant difference was also found when comparing patients with severe asthma who had the rarer variants of α_1PI as compared to mild or moderate asthmatic patients with the M1M1 phenotype indicating that α_1PI plays a role in the pathogenesis of asthma.

No mutation was found in exon 2 (an amino acid substitution in this exon was shown to be responsible for abnormal CBG steroid binding activity) of the cortisol binding globulin (CBG), (as determined by sequencing analysis) in black and white asthmatic and control individuals in the present study. However, taking the fact that CBG and α_1PI share more than 40% homology of amino acid sequence, it would be advisable to continue the search for possible mutations in other exons of the gene which might act as markers for mutations in other genes that are closely linked to the CBG and which play a role in asthma.

An important role player in the control of the inflammatory process could be the IL-1 Ra since it is a powerful endogenous anti-inflammatory molecule that competitively inhibits IL-1α and IL-1β. The allelic frequency of the polymorphism in intron 2 of the IL-1 Ra gene was studied in black and white patients with asthma and control individuals. The plasma IL1 Ra concentration was also determined using a standardised Elisa kit. No significant differences in IL-1 Ra VNTR allelic frequencies were noted in the clinical groups and controls in each of the two population groups. However the 410 bp allele was increased in all black subjects as compared to all white subjects while the 240 bp allele was markedly reduced in all black subjects as compared to all white subjects. Significant

differences were observed when we compared the levels of severe patients with patients classified as having mild asthma. Significant differences were also observed when comparing moderate asthmatic children with the mild asthmatic children. Our results indicate a distinct racial difference in the IL-1 Ra gene polymorphism and although this polymorphism is unlikely to be an important determinant of overall disease susceptibility in asthma the IL-1 Ra plasma concentrations could act as a marker of disease severity in asthmatic patients.

The β_2 adrenergic receptor (β_2AR) is an important factor in the control of the inflammatory process in asthma. The gly 16 polymorphism of the β_2AR which appears to impart enhanced down regulation of receptor numbers has been found to have a higher prevalence in nocturnal white asthmatics. The allelic frequencies of the gly 16 polymorphism was studied in black and white asthmatic children control individuals. Genotyping was performed by making use of PCR and the presence of the mutation was analysed on agarose gels using ethidium bromide staining and confirmed by DNA sequencing. There was no difference in the prevalence of the gly 16 polymorphism of the β_2AR between the black and white control individuals. There was a significant increase in the frequency of the gly 16 polymorphism of the β_2AR between severe and moderate asthmatics. There was also a significant increase in the prevalence of the gly 16 polymorphism in those patients who required a long acting beta stimulant to gain symptomatic control. We did not find a difference in the prevalence of the gly 16 polymorphism between nocturnal vs. non-nocturnal asthmatics. The gly 16 polymorphism of β_2AR was predictive of more severe asthma in this study. It was also predictive for those patients who needed a long acting beta stimulant to attain symptomatic control. This polymorphism may act as a disease modifier in asthma and represents one of the many genetic variables involved in the pathogenesis of asthma.

In conclusion, the present study demonstrates the extent and complexity of genetic susceptibility to atopic asthma and it highlights the need for more refined association and functional studies that will identify additional atopy loci and their association with asthma.

Part of the work in this dissertation was the subject of conference presentations.

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LIST OF ABBREVIATIONS

A	:	adenosine
ACT	:	alpha-1-antichymotrypsin
Ala	:	alanine
α_1PI	:	alpha-1-proteinase inhibitor
AM	:	alveolar macrophage
Arg	:	arginine
Asp	:	aspartic acid
α_1AT	:	alpha-1-antitrypsin
BAL	:	bronchoalveolar fluid lavage
bis	:	N, N' -methylene-bis-acrylamide
bp	:	base pair
β_2AR	:	beta-2 adrenergic receptor
C	:	cytosine
Ca^{2+}	:	calcium ion
CBG	:	cortisol binding globulin
CD	:	cluster determinant
CF	:	cystic fibrosis
Ci	:	Curie
Cys	:	cysteine
dATP	:	2' deoxyadenosine 5' -triphosphate
dCTP	:	2' deoxycytidine 5' -triphosphate
dGTP	:	2' deoxyguanosine 5' -triphosphate
dTTP	:	2' deoxythymidine 5' -triphosphate
dd	:	double distilled
ddATP	:	2', 3' deoxyadenosine 5' -triphosphate
dCTP	:	2', 3' deoxycytidine 5' -triphosphate
dGTP	:	2', 3' deoxyguanosine 5' -triphosphate
dTTP	:	2', 3' deoxythymidine 5' -triphosphate
DMF	:	dimethyl formamide
DNA	:	deoxyribonucleic acid
dNTP	:	deoxynucleotide triphosohate
ds	:	double stranded

ECF-A	:	eosinophil chemotactic factor of anaphylaxis
ECM	:	extracellular matrix
ECP	:	eosinophil cationic protein
EDN	:	eosinophil-derived neurotoxin
EDTA	:	ethylene diaminetetra-acetic acid
EPO	:	eosinophil peroxidase
EtBr	:	ethidium bromide
eT_H	:	effector T helper cell
exo I	:	exonuclease I
Fc_eRI	:	high affinity immunoglobulin E receptor
Fc_eRII	:	low affinity immunoglobulin E receptor
Fc_eRI-β	:	beta chain of the high affinity immunoglobulin E receptor
Fc_eRIII	:	low affinity immunoglobulin G receptor
FEV_1	:	forced expiratory volume in one second
G	:	glycine
G	:	guanine
G-CSF	:	granulocyte colony-stimulating factor
GER	:	gastroeosophageal reflux
GIF	:	gastric intrinsic factor
Gly	:	glycine
Gln	:	glutamine
GM-CSF	:	granulocyte-macrophage colony-stimulating factor
HCl	:	hydrochloric acid
HDM	:	house dust mite
HETE	:	hydroxyeicosatetraenoic acid
His	:	histidine
HLA	:	human leukocyte antigen
I	:	isoleucine
IEF	:	isoelectric focusing
IFN	:	interferon
Ig	:	immunoglobulin
IL	:	interleukin
IL-1	:	interleukin-1
IL-1α	:	interleukin-1 alpha
IL-1β	:	interleukin-1 beta
IL-1Ra	:	interleukin-1 receptor antagonist
IL-1Re	:	interleukin-1 receptor
Ir	:	immune response

K^+	:	potassium ion
kbp	:	kilobase pair
KCl	:	potassium chloride
kDa	:	kilodalton
Leu	:	leucine
LPR	:	late phase response
LT	:	leukotriene
MAC	:	membrane attack complex
MBP	:	major basic protein
MC	:	mast cell
$MgCl_2$	:	magnesium chloride
MHC	:	major histocompatibility complex
MIP	:	macrophage inflammatory protein
mL	:	millilitre
mM	:	millimolar
mmol	:	millimole
mRNA	:	messenger ribonucleic acid
mT_H	:	memory T helper cell
NCF-A	:	neutrophil chemotactic factor of anaphylaxis
NE	:	neutrophil elastase
nm	:	nanomole
NO	:	nitric oxide
NO_2	:	nitrogen dioxide
NP40	:	"nonidet" P40
NSAID	:	non-steroidal anti-inflammatory drugs
O_2^-	:	superoxide anion
PAF	:	platelet activating factor
PAGE	:	polyacrylamide gel electrophoresis
PCD	:	physiological cell death
PCR	:	polymerase chain reaction
PG	:	prostaglandin
PMN	:	polymorphonuclear cell
Pro	:	proline
pT_H	:	precursor helper T cell
RFLP	:	restriction fragment length polymorphism
RNA	:	ribonucleic acid

SAP	:	shrimp alkaline phosphatase
SCF	:	stem cell factor
Ser	:	serine
SO_2	:	sulphur dioxide
SRS-A	:	slow-reacting substance of anaphylaxis
ss	:	single strand
T	:	thymine
Taq	:	*Thermus acqaticus*
TCR	:	transforming growth factor
TEMED	:	N, N, N',N' -tetramethylene diamine
TGF	:	transforming growth factor
T_H	:	helper T cell
T_m	:	melting temperature
TM	:	transmembrane domain
TNF	:	tumour necrosis factor
Tris	:	tris(hydroxymethyl) aminomethane
TX	:	thromboxane
µg	:	microgram
µL	:	microlitre
µm	:	micromolar
UV	:	ultraviolet
Val	:	valine
VNTR	:	variable number tandem repeats
VIP	:	vasoactive intestinal peptide
w/v	:	weight-to-volume

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Clinical descriptions and definitions

Initially, asthma was the name given to the disorder occurring in people with "difficult breathing," especially of an episodic kind (Howell, 1995). Although the concept of asthma as an inflammatory variety of bronchitis was firmly established by the end of the 19th century, most of the focus during the early 20th century was on "broncho-spasm" (Reed, 1995). The consequence of this was the almost universal acceptance of the American Thoracic Society's (1962) definition of asthma as reversible airway obstruction with hyper-responsiveness, but which did not include inflammation. It is only in the last ten years that the importance of inflammation has been emphasised. Despite controversy surrounding the definition, bronchial asthma is generally described as a chronic inflammatory disease in which the calibre of the airways is episodically and occasionally chronically narrowed by oedema and is unstable. This widespread narrowing of the bronchial airways, changes its severity over short periods of time either spontaneously or under treatment and there is increased responsiveness to a number of stimuli (Howell, 1995).

1.2 Categories of asthma

1.2.1 Extrinsic atopic asthma

This refers to a large group in whom asthma is due to IgE-mediated hypersensitivity reactions to inhaled antigens commonly present in the air. In some individuals the early episodes are prolonged, and masquerade initially as attacks of bronchitis. In the name used for this sort of asthma, 'extrinsic' implies that asthma is precipitated by contact with environmental antigens, and 'atopic' refers to the sort of hypersensitivity reaction that is concerned (Scadding, 1983).

1.2.2 Extrinsic non-atopic asthma

The term 'extrinsic non-atopic asthma' has been suggested to refer to patients in whom asthma can be attributed to reactions between inhaled antigens and antibodies of sorts other than the IgE type associated with atopy. Asthma attributable to hypersensitivity to a single substance is generally best specified in this way; in some contexts it may be useful to specify both the substance and the type of hypersensitivity reaction concerned (Scadding, 1983).

1.2.3 Exercise induced asthma

Increase in airways resistance of some degree after exercise can be observed in a high proportion of patients with asthma of all types. In the few in whom this gives rise to the presenting symptom, the term exercise-induced asthma can be used (Scadding,

1983).

1.2.4 Asthma associated with chronic bronchopulmonary disease

Persistent expiratory airflow limitation is a feature of a number of bronchopulmonary diseases. The commonest of these in populations with a high proportion of cigarette smokers and exposed to general air pollution is usually called chronic obstructive pulmonary (COPD) disease or chronic obstructive bronchitis. In many instances categorisation to this group can only be provisional. In a few patients, both the airways obstruction and the bronchial hypersecretion are so much improved by corticosteroid treatment that the initial diagnosis of chronic obstructive bronchitis may be replaced by one of intrinsic asthma, especially if the symptoms are not of long duration (Scadding, 1983).

1.3 Epidemiology and mortality

1.3.1 Epidemiology

Most epidemiological studies have relied upon subjective evidence for identifying subjects with asthma, the most commonly used criteria being a history of its having been diagnosed by a physician or by standard symptom questionnaires (Gregg, 1983 and Daniels *et al.*, 1996).

The incidence of asthma varies with age, but for all age groups, the incidence of asthma

has been estimated to vary between 2.65 to 4 per 1000 per year (Gergen & Weiss, 1995).

Asthma begins primarily during two periods: childhood (tends to begin before the age of 5 years) and adulthood, with a bias toward male prevalence in childhood asthma. The scenario changes during adolescence, where the female prevalence tends to equal or exceed the male prevalence. The sex difference in asthma prevalence is levelled in adulthood (Gergen & Weiss 1995). Approximately 5%-10% of children worldwide are afflicted by asthma, a third of whom do not show any symptoms of the disorder until after adolescence. On the other hand, 5-10% of people acquire asthma in adulthood and in such cases, the disorder can manifest itself at any time (Lichtenstein, 1993).

Prevalence reflects both the incidence and the duration of a disease and in the case of asthma, prevalence is highly dependent on the definition. The prevalence of asthma can be recorded in 3 ways: (a) point prevalence - asthma at the time of the survey; (b) lifetime prevalence - asthma at any time during an individual's life; or (c) period prevalence - asthma within a certain defined period, usually the last 12 months (Gergen & Weiss 1995). It is difficult to draw detailed inferences about the worldwide prevalence of asthma from various studies, however, the point prevalence of current (or active) asthma for children in industrialised countries averages around 5% with lower prevalence rates recorded in non-industrialised countries (Gergen & Weiss, 1995).

1.3.2 Epidemiological risk factors

Birth factors

The role prematurity plays in asthma has come under increased study. Mothers of premature infants have been reported to have a much higher prevalence of bronchial hyper-responsiveness than mothers of full-term infants (Gergen & Weiss, 1995). Low birth weight and prematurity, regardless of neonatal respiratory disease, has been shown to be associated with decreased flow rates and airway conductance in childhood (Gergen & Weiss, 1995).

The time of year of birth also appears to be a factor determining asthma epidemiology - if exposure to allergens in the first months of life enhances atopic sensitivity, then the logical assumption is that children born in spring would show a higher frequency of allergy than children born at other times when there are neglible levels of pollen in the atmosphere (Wilson, 1983). Other birth factors have been implicated in asthma, with a relative decrease in the prevalence of asthma reported among second-born children (Gergen & Weiss, 1995). Breast-feeding has not been shown to play a significant role in preventing asthma, in spite of the finding that breast-feeding protects against wheezing illness in the first months of life (Nesse & Williams, 1994). Prospective studies following infants through 4 and 5 years of life have found no effect of breast-feeding on development of asthma in children. This remained true whether or not there was a family history of atopy (Gergen & Weiss, 1995).

Environmental factors

Study of the factors observed to be associated with acute episodes of asthma has been a major preoccupation of epidemiologists. Smoking which is a well-recognised respiratory irritant, has been found to boost IgE levels, while suppressing IgG and IgM levels, and appears to augment sensitization to certain pollen antigens (Gerrard, 1985).

Smoking increases airway resistance in normal individuals and in patients with respiratory diseases, it causes hypertrophy of bronchial mucous glands and also inhibits bronchial ciliary motion, all of which can be harmful in patients with abnormal bronchial function caused by allergic asthma (Joubert *et al.*, 1988). Infants and young children whose parents smoke tend to have more problems with bronchial hyper-responsiveness, lower respiratory tract infections and asthma, than children with non-smoking parents, especially in the first year of life (Joubert *et al.*, 1988). Maternal smoking has been shown to be more important to the development of allergic asthma than paternal smoking due to the greater exposure of the child to maternal than paternal smoke (Gergen & Weiss, 1995). Tobacco smoke increases the severity of asthma (Murray & Morrison, 1988; Gergen & Weiss, 1995), males have been reported to be more "sensitive" to environmental tobacco smoke than females. This may be mediated through increased bronchial reactivity in "smoke-sensitive" asthmatics (Gergen & Weiss, 1995).

Asthma has been reported more frequently among children in urban areas as compared to rural areas. Individuals who have moved from rural to urban areas (in the same country) have developed allergies (Gerrard, 1985). However, data from south

Australia suggest that among 5-16 year old children, asthma may be more prevalent in rural areas (Gergen & Weiss, 1995). Home dampness and the presence of moulds in the home have been reported to be associated with an increased prevalence of respiratory symptoms, including asthma and wheeze (Wilson, 1983; Nesse & Williams, 1994). Such an association has been reported, but has been attributed to the dust mite content of damp rooms (Wilson, 1983). It has also been suggested that modern airtight houses, with wall-to-wall carpeting provide excellent breeding sites for house dust mites and therefore provoke allergies (Nesse & Williams, 1994). It has however, also been suggested that this association may in part be explained by a tendency of parents from homes with visible moulds to over-report symptoms (Gergen & Weiss, 1995).

The role of air pollution in asthma is still unclear. However, various atmospheric air pollutants are known to exacerbate rhinitis and asthma. Recent years have seen a dramatic increase in asthma morbidity and mortality in a number of countries, and air pollution has been implicated as a plausible causative agent (Antò & Sunyer, 1995). Asthma has been reported to be increased in polluted areas as compared to non-polluted areas. In Los Angeles, increased asthma attacks have been associated with high oxidant and particulate pollution (Gergen & Weiss, 1995). Also in Los Angeles, increases in asthma emergency room visits and hospitalizations have been associated with the levels of nitric oxide, coefficient of haze, and hydrocarbons, in addition to Santa Ana wind conditions and airborne allergen counts (Gergen & Weiss, 1995). Sulfur dioxide has been shown to cause bronchoconstriction and asthma-like symptoms, even at low levels, with chronic exposure. Nitrogen dioxide, associated with gas stoves, has been implicated as a cause of respiratory disease and as having an

inconsistent effect on pulmonary function (Neas *et al.*, 1991). Ozone has been shown to increase bronchial reactivity and airborne acidity has been reported to be associated with daily symptoms among moderate to severe asthmatics (Gergen & Weiss, 1995). Other "natural" products if handled carelessly, can cause pollution problems; a report from Barcelona, Spain linked an epidemic of asthma caused by soybean dust being blown into nearby neighbourhoods from unloading ships (Antò *et al.*, 1989). The complex mixture of atmospheric pollutants could induce larger inflammatory and functional changes than reported, and as a result the effect of air pollution on allergic asthma could be of a much greater magnitude than that seen in laboratory studies (Antò & Sunyer, 1995).

The children of migrants have been reported to acquire the prevalence of the asthma in the area to which the parents move (Gergen & Weiss, 1995). One expects that ethnic individuals, who have lived in a particular environment for a number of generations, would adapt to that environment and might become allergic when such groups move to new and strange environments where they encounter new allergens (Gerrard, 1985). This phenomenon has been observed in Filipinos who were atopic in their homeland but who have developed eczema and asthma on moving to California, while Polynesians have developed asthma when they have migrated from their isolated islands to New Zealand (Gerrard, 1985). Tokelauan children living in New Zealand developed asthma at a higher rate than Tokelauan children living in the Tokelau Islands (Gergen & Weiss, 1995).

Race and socioeconomic conditions

The effects of socioeconomic status on the prevalence of asthma are not clear, however asthma has been reported to vary by racial groups across the world and it appears that poverty plays a central role in these differences (Lichtenstein, 1993; Gergen & Weiss, 1995; Arrighi, 1995). In the US, black persons, at any age, have been reported to have a higher prevalence of asthma than whites. Puerto Rican children living in the New York city area have been reported to have some of the highest rates of asthma in the US. The rate of hospitalizations and mortality is also higher among black persons than white persons (Gergen & Weiss, 1995; Arrighi, 1995). In New York city, asthma morbidity and mortality have been reported to cluster in poor neighbourhoods with a predominant minority population (Gergen & Weiss, 1995). Similar increases in asthma morbidity and mortality among groups living in poverty are found in other parts of the world. In New Zealand, Maoris and Pacific island Polynesians have higher rates of hospitalizations and mortality. The higher rates of hospitalizations among the Polynesians appear to be due to different drug management at discharge from hospitals and in the community, with their being less likely to be on prophylactic therapy (Gergen & Weiss, 1995).

A formal epidemiological study is yet to be undertaken in South Africa (where several different cultures and a wide range of socioeconomic standards are represented) to ascertain whether allergic diseases and asthma are more or less common in black South Africans than in other races. Official South African death certification data suggest that the asthma mortality rate is much higher in black South Africans than in white

South Africans (Benatar & Ainslie, 1986). It would appear that poor access to health care facilities, economic factors (including the cost of medication) and a general lack of compliance in the use of prophylactic therapy, contribute to the high asthma mortality rate in black South Africans (Walls & Ordman, 1983; Benatar & Ainslie, 1986; Joubert *et al.*, 1988). It has been noted previously that asthma among black children in Durban is apparently rare (Walls & Ordman, 1983), which is believed to be due to the high incidence of parasitic infections. Whether this scenario is applicable to the whole of the black South African population is uncertain, as it has been found that the prevalence of allergic diseases and parasitic infections (especially *Ascaris lumbricoides*, which has a potent IgE-stimulatory effect that causes a parasite-specific immune response that has been shown to correlate with enhanced tendency to asthma) is very high (Walls & Ordman, 1983; Joubert *et al.*, 1988). Racial/ethnic differences in lung size have also been proposed to explain some of these differences. Mexican-Americans have been reported to have higher expiratory flow rates than non-Hispanics and black persons to have lower rates than white persons or Mexican-Americans (Gergen & Weiss, 1995).

Season and altitude

Seasonal variations in morbidity and mortality of asthma have been reported. Changes in temperature, pollen levels and fungal spore counts have been reported to be associated with asthma emergency room visits (Gergen & Weiss, 1995). The Western Cape in South Africa, with its temperate climate, prevailing winds, high rainfall and rich variety of flora is a natural endemic area for allergic asthma (Joubert

et al., 1988). Asthma is reported to be less prevalent at higher altitudes; a decreased concentration of house dust mites is hypothesized to be responsible for this decrease (Gergen & Weiss, 1995). Aggravation of asthma symptoms in winter has been described in patients sensitive to house dust mites (Walls & Ordman, 1983). Asthma hospitalization has also been associated with rainfall, low barometric pressure, and counts of coloured basidiospores and green algae (Gergen & Weiss, 1995).

Infection

Viral infections are well recognised to be factors precipitating asthma attacks. Viral infections have been reported to enhance the release of inflammatory mediators from mast cells and basophils in the lung. Asthmatics have also been reported to have more viral infections (Gergen & Weiss, 1995). A study by Potter *et al.* (1984) showed that upper and lower respiratory tract viral infections were the major cause of severe asthma attacks in children in the Cape Peninsula. Viral infections of the lower respiratory tract cause wheezing (Stark & Graziano, 1995).

Viral infections may facilitate specific allergic sensitization by altering the function of lung mast cells and they may reduce β-receptor function, thereby inducing resistance to drug therapy. They may also stimulate cholinergic and non-adrenergic, non-cholinergic bronchoconstriction or they may alter the control of the immune response, lymphocyte proliferation and interferon production, all of which could induce the predominant pathophysiology of acute airway injury and inflammation that accompanies lower airway viral infections (Figure 1.1) (Potter *et al.*, 1984; Stark &

Graziano, 1995).

Bacterial infections do not seem to be important triggers of asthma, however sinusitis, regardless of etiology, is felt to make asthma difficult to control until it is adequately treated. Croup and lower respiratory tract infections, such as bronchiolitis and pneumonia, have been reported to be associated with later development of asthma (Gergen & Weiss, 1995).

Viral infections have also been implicated in the development of allergenic sensitization, however other groups have not confirmed this finding. Sensitization to the viral agent has been shown to play a role wheezing illness, as production of viral-specific IgE has been found to be associated with the clinical manifestation of wheezy illness for both respiratory syncytial virus and para-influenza viruses (Gergen & Weiss, 1995).

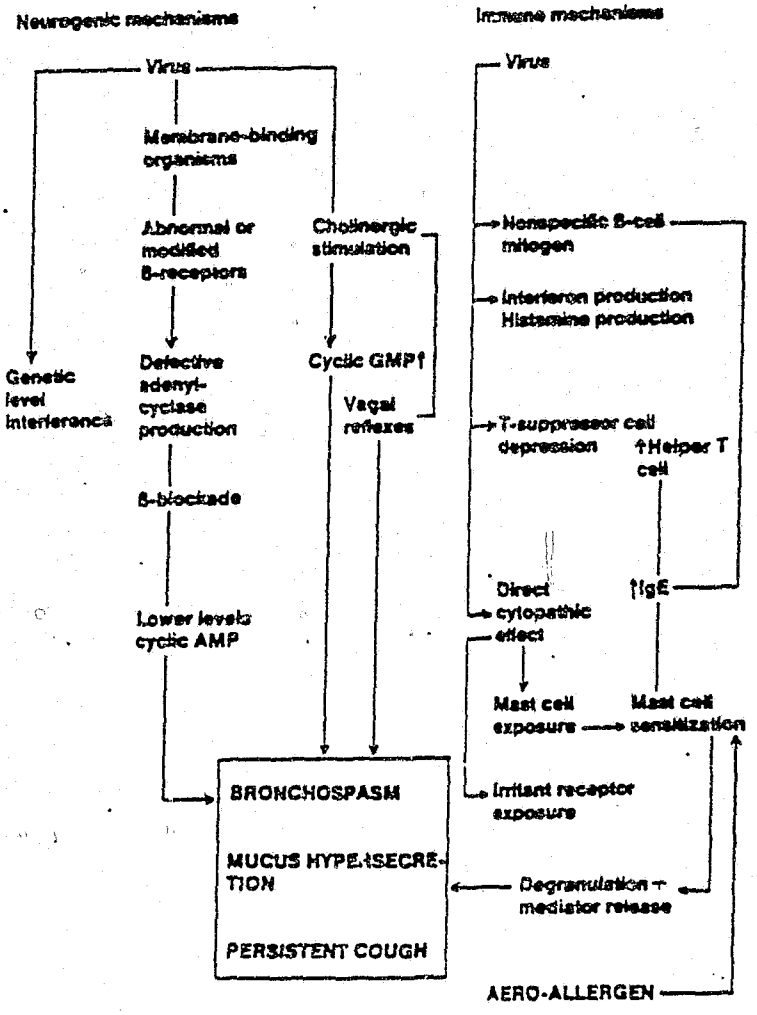


Figure 1.1 Several animal models emphasise the importance of the immune system and of neural innervation of airway smooth muscle tone in determining the outcome of respiratory viral infections. The interaction of several factors, including environmental (e.g. exposure to cigarette smoke) , genetic and developmental (e.g. the status of the humoral and cell-mediated immune responses) is necessary for viral-induced airway obstruction and bronchial hyper-responsiveness. These interacting factors predispose an individual to "wheezing" following viral infections of the lower respiratory tract, which trigger subsequent events that induce the pathophysiology of acute airway inflammation (from Potter *et al.*, 1984; Stark & Graziano, 1995).

1.3.3 Mortality

Asthma deaths were very rare in the first half of the century, but since that time, the patterns of asthma mortality have become considerably more complex. There were epidemics of asthma deaths in the 1960's, and again in New Zealand in the 1970's (Pearce *et al.*, 1995). These have been superimposed on a more gradual underlying increase in asthma mortality in many countries, which commenced in the 1940's and has been particularly marked in the 1980's (Pearce *et al.*, 1995).

Asthma mortality rates in the United States doubled from 1978-1987, and this increase in asthma mortality has been accompanied by a concomitant increase in asthma morbidity (Arrighi, 1995). Data suggest that the South African asthma mortality rate is greater than that of the UK (Benatar & Ainslie, 1986). Almost all comparative studies of asthma mortality have been confined to the 5-34 age group, because the diagnosis of asthma mortality is more firmly established in this group (Pearce *et al.*, 1995). The gradual increase in asthma mortality during 1940-1960 was thought to be due to a number of different factors, including changes in environmental risk factors such as chemical pollutants, environmental aeroallergens, and foods and preservatives. Alternatively, the gradual changes in mortality could be due to changes in the management of asthma (Pearce *et al.*, 1995).

Studies found that the epidemic in England and Wales was real and was not due to changes in death certification, disease classification or diagnostic practice. They also concluded that it was unlikely to be due to a sudden increase in asthma prevalence, but

was rather due to an increase in case fatality due to new methods of treatment (Pearce *et al.*, 1995). It was concluded that the excess deaths followed the introduction of pressurised β-agonist aerosols in 1961 (Arrighi, 1995; Crane *et al.*, 1995; Pearce *et al.*, 1995). It was noted that with the use of these β-agonist aerosols, relief of symptoms could enable a patient to tolerate worsening hypoxia and to unduly delay seeking medical help (the "delay" hypothesis) (Pearce *et al.*, 1995). It was also argued that both chronic and acute side effects could occur and that direct toxicity could occur in certain circumstances due to the over use of isoprenaline which is a non-selective β-agonist (the "toxicity" hypothesis) (Crane *et al.*, 1995).

A number of studies revealed that the toxicity hypothesis and the delay hypothesis were not incompatible; in fact, they were complementary in that acute toxicity was most likely to occur in the presence of delay (Pearce *et al.*, 1995). Despite mounting evidence, the aerosol/toxicity hypothesis was continued to be regarded with scepticism particularly in the US and Germany where there had been considerable sales of the β-agonist aerosol without any mortality epidemics. This apparent contradiction was explained by studies which indicated that a high-dose formulation of isoprenaline (isoprenaline forte) which contained five times the dose per administration of other isoprenaline aerosols had only been licenced in eight countries, six of which (England, Wales, Ireland, Scotland, Australia, New Zealand and Norway) had mortality rates that coincided with the early introduction of the drug as well as high sales volume of the drug (Pearce *et al.*, 1995). Overall, there was a strong positive correlation internationally between the asthma mortality rate and isoprenaline forte sales in the eight countries in which the drug was licenced, whereas no mortality epidemics

occurred in countries in which isoprenaline forte was not licenced, such as Sweden, Canada, West Germany and the US (Pearce *et al.*, 1995).

Until recently, epidemiological studies of asthma mortality had largely concentrated on the causes of the mortality epidemics which have only occurred with isoprenaline forte and fenoterol, which are high-dose, poorly selective, full agonists, with relatively greater cardiac side effects (Crane *et al.*, 1995). No other β-agonists have been associated with such epidemics. In recent years, attention has also focussed on the possible causes of the gradual increase in mortality which appears to have occurred in a number of countries during the 1980's. Although there is still some debate as to whether this increase is occurring, there does appear to have been a gradual increase in mortality in many countries since the 1980's (Barnes, 1993). The causes of this increase are difficult to determine, because the changes have been so gradual and could conceivably be due to changes in diagnostic practice, however it is also possible that the gradual increases in asthma mortality could be due to a class effect of β-agonists or to changes in the environmental causes of asthma (Pearce *et al.*, 1995). It is important to resolve the question whether excessive use of inhaled β-agonists may worsen asthma and contribute to the worldwide increased asthma mortality rate, as the number of prescriptions for these drugs is increasing worldwide at a much faster rate than the number of prescriptions for other anti-asthma drugs (Barnes, 1993).

1.3.4 Mortality risk factors

Class effect of β-agonists

In recent years, attention has increasingly focussed on the possibility that the increase in mortality could be due to a class effect of β-agonists. In particular, concern has been raised by several clinical trials which have suggested that regular use of β-agonists could lead to worsening asthma (Pearce *et al.*, 1995). The implications are that β-agonists or bronchodilators in general can cause deterioration in lung function and increases in asthma severity when used regularly. However, there has been considerable debate as to whether this phenomenon is specific to fenoterol or whether it is a class effect of β-agonists, and as to the extent to which it is dose-dependent (Burrows & Lebowitz, 1992). The findings of various studies are incompatible with each other, since, in most developed countries it is difficult to investigate a class effect of β-agonists using epidemiological studies, because virtually all asthmatics use these drugs, and there is no appropriate comparison group. Epidemiological methods are relatively robust when making comparisons within the class of β-agonists, but randomised controlled trials are more appropriate when attempting to identify a class effect of β-agonists (Pearce *et al.*, 1995).

Changes in environmental causes of asthma

The recent gradual increases in asthma mortality could be due to changes in environmental causes of asthma. There are no studies which link such causes directly

to asthma mortality trends, however there is some evidence that asthma prevalence is increasing, and there are a number of studies, which suggest that changes in environmental causes of asthma could lead to an increase in asthma prevalence and morbidity, and thus indirectly, to increasing asthma mortality (Pearce *et al.*, 1995). These include: (a) early exposure, especially during infancy, to house dust mite allergens (Pearce *et al.*, 1995); (b) the introduction of blankets and changes in sleeping habits in developed countries, which is believed to increase exposure to indoor inhaled allergens (Gerrard, 1985); (c) an increase in noxious air pollutants in urban air (Antó & Sunyer, 1995); (d) migration from developing to industrialized countries (Pearce *et al.*, 1995); and (e) modern conveniences, such as wall-to-wall carpeting and an increase in indoor humidity, as a result of newer energy-efficient homes, both of which favour breeding of house dust mites (Nesse & Williams, 1994). It should be stressed that the evidence concerning increased exposure to various inhaled allergens primarily relates to asthma prevalence and morbidity, and does not directly relate to asthma mortality. Nevertheless, it is certainly plausible that such allergens may be indirectly causing asthma deaths, and thus contributing to the gradual rise in asthma mortality.

Asthma severity

Various studies have examined the association between markers of asthma severity and risk of asthma death in adults. These have involved three markers of chronic asthma severity: (1) a hospital admission during the previous 12 months; (2) prescription of 3 or more categories of asthma drugs; and (3) prescription of oral corticosteroids. All three markers of chronic asthma severity are associated with an increased risk of

subsequent death (Pearce *et al.*, 1995).

Characteristics of the asthmatic

Several studies have examined characteristics of the asthmatic which may be associated with an increased risk of death. It has been noted that the risk of asthma death in adults is associated with psychosocial problems and other psychological characteristics (e.g. depression and the denial of asthma symptoms) of the patient, as well as the underlying severity of the asthma (Arrighi, 1995; Pearce *et al.*, 1995). Strunk *et al.* (1985) have reached similar conclusions in a small study in children, which found that various psychosocial factors were associated with an increased risk of asthma death, including conflicts between the patient's parents and hospital staff regarding medical management, depressive symptoms, and disregard of asthma symptoms.

1.4 The genetics of atopy and asthma

Studies of monozygotic and dizygotic twins raised apart show that genetic factors are important in the etiology of atopic asthma and other allergic diseases. There is little doubt that there is a major hereditary contribution to the etiology of asthma and allergic diseases. However, the inheritance of asthma and allergy does not follow classical Mendelian patterns that are characteristic of single-gene disorders (Cookson, 1994; Sandford *et al.*, 1996). The inheritance pattern of asthma demonstrates that it is a "complex genetic disorder". A similar complex inheritance is seen in disorders such as hypertension, atherosclerosis, diabetes mellitus and arthritis. All of these

diseases show a clear hereditary pattern, but the mode of inheritance cannot be simply classified as autosomal dominant, recessive, or sex-linked (Sandford *et al.*, 1996).

Another important feature that distinguishes these complex genetic disorders from single-gene disorders is their prevalence. Atopy in the Western world has a prevalence of 40-50% (Daniels *et al.*, 1996), with asthma occurring in 4-8% of the population, and allergic rhinitis reported in 25% of some populations, as compared to the most frequent Mendelian disorder affecting the lungs, cystic fibrosis (CF), which occurs once in every 2 000 live Caucasian births, which is approximately 100 times less frequent than asthma (Sandford *et al.*, 1996). Possible reasons that these conditions are not inherited in a simple Mendelian fashion include the following: (a) there may be a number of genes that predispose people to develop these complex traits - either more than one gene in the same individual (polygenic inheritance) or different combinations of genes in different individuals (genetic heterogeneity); and (b) environmental factors may be necessary for expression of the disease phenotype (Sandford *et al.*, 1996).

1.4.1 Candidate genes for atopy and asthma

Figure 1.2 illustrates the functional roles of the various candidate genes that have been implicated in the pathogenesis of allergy and asthma.

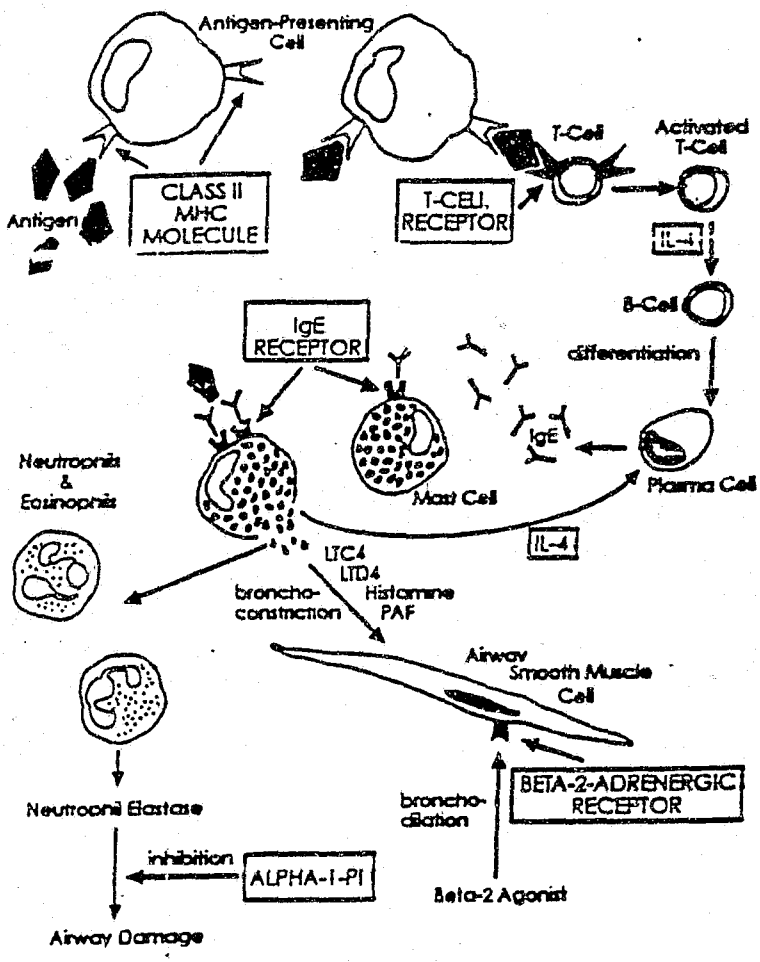


Figure 1.2 Functional roles of various candidate genes that may be involved in the pathogenesis of atopy and asthma. The candidate genes are shown in the boxes: (1) specific MHC/HLA class II antigens allow specific inhaled allergens to be presented more effectively to T lymphocytes by monocytes and other antigen presenting cells. (2) Specific T cell receptor (TCR) types allow more effective T cell responses to particular combinations of MHC/HLA II antigen-allergen complexes. (3) More active, or increased production of IL-4 results in enhanced IgE synthesis. (4) Enhanced binding and signalling by high affinity IgE receptors on mast cells, basophils and other effector cells cause an exaggerated response to allergen-IgE complexes, causing the release of more inflammatory mediators and more IL-4, which in turn further boosts IgE levels by a positive feedback loop. (5) The allergic inflammatory response to mediators and cytokines is increased because of the decreased antiproteolytic activity of alpha-1-protease inhibitor (α_1PI) and other antiproteases, and/or because of a disruption of the protease-antiprotease balance. (6) The smooth muscle response to contractile agonists released from effector cells is enhanced due to defective or downregulated β_2-adrenergic receptors (from Sandford *et al.*, 1996).

Linkage to Chromosome 11q13 and the High-Affinity Immunoglobulin E Receptor

Chromosome 11q13 was implicated in the pathogenesis of atopy during a random genome search by Cookson *et al.* (1989), although further evidence exists for non-linkage in many large families (Cookson, 1994). The initial study caused much controversy over its broad definition of atopy and it's failure to replicate linkage in other populations (Hizawa *et al.*, 1992; Lympany *et al.*, 1992). A sib-pair analysis on 743 subjects conducted to reassess the evidence for linkage showed robust evidence for linkage of atopy to chromosome 11q13. This linkage was independent of the definition of atopy and was only observed through the female line (maternal inheritance) (Cookson, 1994). A suggested mechanism for this was the influence of the maternal immune system on the foetus or neonate (Sandford *et al.*, 1996) or imprinting of the paternal copies of the gene (Hopkin, 1995). Imprinting is a process by which a particular gene is differentially activated or silenced (probably by the demethylation or methylation of CPG islands on the gene) depending on the sex of the parent from whom it was inherited from (Razin & Cedar, 1994; Sandford *et al.*, 1996). It was suggested that the atopy gene was only expressed when inherited from the mother, indicating that immunological interactions between mother and child, rather than genomic imprinting, might be responsible for the maternal effect on the inheritance of the atopy gene on chromosome 11q13 (Daniels *et al.*, 1996).

In subsequent studies, investigators have used a maternal inheritance model for the linkage analysis. Shirakawa and colleagues (1996) found linkage to an atopy gene on chromosome 11q13 in a Japanese population. All these observations support the notion that atopic asthma is indeed a polygenic disorder.

The most likely position of the atopy gene was found to coincide with that of the high affinity receptor for IgE (FcεRIβ) (Sandford *et al.*, 1993). Linkage studies of the FcεRIβ locus have generated conflicting results and it has been suggested that FcεRIβ (or another gene nearby) may predispose patients to bronchial hyper-responsiveness, rather than to atopy (Van Herwerden *et al.*, 1995). A variant of the FcεRI receptor (Leu 181) was detected that segregated with atopy when maternally inherited, a finding which supported the hypothesis that FcεRIβ was the gene responsible for the linkage in 11q13. A possible model for the action of this mutation is that it increases the signal transduction activity of the receptor. This change would also increase the level of activation of mast cells which release interleukin 4 (IL-4), which would, in turn, stimulate the synthesis of higher levels of IgE (Sandford *et al.*, 1996). The study by Shirakawa and co-workers (1996), also showed the absence of association between atopy and gastric intrinsic factor (GIF) and CD20 (the 5' and 3' flanking genes of FcεRIβ, respectively), further supporting the candidacy of FcεRIβ as the atopy gene on chromosome 11q13. Another two studies have also shown positive associations between atopy phenotypes and polymorphisms in the 11q13 region; one within the FcεRIβ gene (Shirakawa *et al.* 1995) and one with a marker from the 11q13 region (Hizawa *et al.*, 1995).

Linkage to Chromosome 5q and the Cytokine Gene Cluster

The cluster of cytokine genes on chromosome 5q31 has made this region of the genome attractive with respect to containing an atopy gene. Recently, a linkage of total serum IgE levels to the IL-4 gene in this region has been reported in 11 large Amish families (Marsh, *et al.*, 1994).

In the presence of linkage , siblings who share alleles from the marker would be expected to have similar IgE levels, and those not sharing these alleles would be expected to have a larger difference in their IgE levels. The sib-pair analysis demonstrated that the strongest linkage evidence for linkage was found at the IL-4 gene locus (Marsh *et al.*, 1994; Sandford, *et al.*, 1996).

Another study found that evidence for linkage of total serum IgE levels was significantly higher when only subjects with relatively low IgE levels and without specific IgE responsiveness were included in the analysis (Postma, *et al.*, 1995; Sandford, *et al.*, 1996). This study suggests that specific IgE responsiveness is a confounding factor in the analysis of the genetics of total serum IgE and it also showed that a trait for total serum IgE is co-inherited with a trait for bronchial hyper-responsiveness (Postma, *et al.*, 1995). A second group has published data showing linkage to chromosome 5q with high levels of IgE assumed to be recessive (Meyers, *et al.*, 1994). Further segregation analyses showed evidence for a second major locus unlinked to chromosome 5q (Xu *et al.*, 1995).

The same group has also shown linkage of markers in this region with a phenotype based on bronchial hyper-responsiveness (Postma *et al.*, 1995). In these studies the region with the best evidence for linkage was not found to be around the IL-4 locus but in an adjacent region around the β_2-adrenergic receptor gene. This finding makes the β_2-adrenergic receptor gene a candidate for the bronchial hyper-responsiveness linkage, because variants in this gene have been associated with hyper-responsiveness (Postma *et al.*, 1995; Sandford *et al.*, 1996).

None of the studies had sufficient power to produce an accurate localisation of the disease gene, and there are many possible candidates in the region, including IL-4, IL-5, IL-13, interferon regulatory factor-1 and granulocyte-macrophage colony stimulating factor (GM-CSF), (Sandford *et al.*, 1996). It is also possible that more than one of these loci may be involved in the pathogenesis of atopy. Further progress with linkage analysis will therefore be difficult, but association studies may be able to define the important loci (Sandford *et al.*, 1996). It has been suggested that a polymorphism within the IL-4 gene regulatory region may be a candidate for this linkage (Boyer *et al.*, 1995).

Linkage to chromosome 14q and the T Cell Antigen Receptor (TCR)

Linkage has been demonstrated between the α region of the TCR locus and specific IgE responses (Moffatt *et al.*, 1994). The evidence for linkage was assessed using 312 sib-pairs. The linkage pattern suggested a recessive genetic effect that was detected in two separate population samples. The results of this study showed that a gene or

genes in the TCR-α region may modify specific IgE responses in atopic individuals (Moffatt *et al.*, 1994). The region also contains the δ chain TCR genes, which are found within the α locus; therefore, they are also candidates for linkage (Sandford *et al.*, 1996). Although this result has not been replicated independently, it suggests that polymorphisms within the TCR genes may limit an individual's ability to respond to specific antigens (Sandford *et al.*, 1996).

Human Leukocyte Antigen (HLA)/Major Histocompatibility Complex (MHC)

In efforts to further understand the role of the immune response in asthma, both population and family studies have examined relationships between HLA type, asthma and atopy. Comparisons between studies are complicated by differences in the ethnic group studied, the phenotypic definitions and the HLA typing methods (Sandford *et al.*, 1996).

The HLA class II antigens (designated DP, DQ and DR) play a key role in antigen presentation and therefore influence the specificity of the immune response. Many associations between class II alleles and IgE hyper-responsiveness to specific allergens have been investigated. In general, significant associations are found only with highly purified simple allergens and not with more complex ones (Sandford *et al.*, 1996). For example, the complex allergens of house dust mite (*Der p* and *Der f*) have not been associated with a specific HLA type, however specific epitopes of *Der p* have been identified at the amino acid level and have been shown to be present by specific HLA-DR and DQ gene products (Sandford *et al.*, 1996).

In contrast, in a recent study by Young and colleagues (1994) that tested the responses of 431 subjects to six aeroallergens, found that there was very little evidence to suggest an important role for HLA in atopy.

Few studies have linked HLA antigens directly with allergic asthma, some of which suggest a HLA-linked recessive gene controlling IgE responses to specific allergens. Disease associations with HLA were first studied with class I antigens (designated A, B and C). In some cases, the original class I HLA associations could have been the result of linkage disequilibrium with a class II allele or an unrelated gene outside the HLA region (Sanford *et al.*, 1996). This phenomenon may explain some of the conflicting results of atopy associations with the class I antigens.

A family study of class I haplotypes in house dust mite allergy reported an HLA-linked recessive pattern of inheritance for nasal allergy and specific IgE levels to house dust mite. In particular, HLA-B 8 (which is in linkage disequilibrium with DR 3) has a possible role in the pathogenesis of atopy. HLA-B 8 is well known for its association with autoimmune diseases and because both autoimmune diseases and atopy result from hyperimmune responses, B 8 may be a marker for decreased immunoglobulin suppression (Sandford *et al.*, 1996). This suggestion received further support from the recent finding of linkage of a region on chromosome 6 (near the class I antigens) to eosinophil counts (Daniels *et al.*, 1996).

After 25 years of HLA typing atopic and asthmatic subjects, there are now powerful allergy models with which to further study the molecular basis of the human immune

responses. HLA loci have been identified as candidate genes for the pathogenesis of atopy and asthma, primarily due to the role of class II antigens in antigen presentation and T cell restriction. The presence of a particular HLA allele may be necessary but not sufficient for hyper-responsiveness to an epitope. Future studies may implicate an HLA locus in combination with one or more other candidate genes (in particular the TCR loci) (Sandford *et al.*, 1996). Such studies will be needed if HLA typing is to be powerful enough to predict individuals at risk for atopy or asthma.

β_2-adrenergic receptor

The possible involvement of the β_2-adrenergic receptor in bronchial asthma has been a long-standing hypothesis (Szentivanyi, 1968). With this in mind, studies were conducted to determine whether any variants of the gene could be detected and whether any of them could be associated with asthma. The result was the discovery of nine different point mutations, four of which caused amino-acid substitutions. None of these mutations were more prevalent in asthma patients than in normal control subjects (Reihsaus *et al.*, 1993; Barnes *et al.*, 1995; Turki *et al.*, 1995). However, in the asthma group one mutation (substitution of glycine for arginine at position 16) correlated with more severe asthma as indicated by the use of corticosteroids and immunization therapy. This study suggests that mutations in the β_2-adrenergic receptor gene do not play a primary role in the pathogenesis of asthma but may modulate the severity of symptoms in affected individuals (Barnes *et al.*, 1995; Turki *et al.*, 1995). These observations could explain the decreased β_2-adrenergic responsiveness observed in the airway smooth muscle from fatal asthma, a finding that is not seen in airway

tissue obtained during surgical resection from less severe asthmatics (Sandford *et al.*, 1996).

Alpha-1-Proteinase Inhibitor (α_1PI)

The gene for α_1PI has been known to play a role in the pathogenesis of chronic obstructive lung disease (which includes asthma) and may result from a disturbed balance between extracellular matrix (ECM) proteases and their inhibitors (Walls, 1995), resulting in the destruction of alveolar walls (Cox, 1995). Studies have provided evidence for the involvement of this important proteinase inhibitor in bronchial asthma (Gaillard *et al.*, 1994; Cox, 1995). There are two main alleles associated with α_1PI deficiency, denoted Z and S. A study of 151 children with severe asthma found similar frequencies of the Z allele in the asthma group compared to the control group. However, the children with steroid-dependent asthma contained more Z heterozygotes than the nonsteroid-dependent asthmatics and normal control subjects. This suggests a link between the Z allele and asthma severity (Sandford *et al.*, 1996). There have been other reports concerning asthma and the α_1PI gene. One of the most common alleles at the α_1PI (locus) is known as M_2 and one report has identified an increased prevalence of M_2 in asthmatics (Gaillard *et al.*, 1992). The association of bronchial hyper-responsiveness and S heterozygosity has been detected in a group of asthmatic families (Townley *et al.*, 1990). In addition, there have been reports of an association between asthma and another antiprotease deficiency; that is low levels of α_1-antichymotrypsin. However, the genetic mechanisms have not yet been elucidated (Sandford *et al.*, 1996).

Other candidate genes

There have been studies suggesting that atopy, asthma and bronchial hyper-responsiveness are associated with heterozygosity for cystic fibrosis (CF) of the pancreas (Gerrard, 1985). It was found that heterozygosity for the most common CF allele (ΔF508) may protect against asthma. It was suggested that this heterozygote advantage could account for the high prevalence of the allele in the Caucasian population, however further studies have not been able to find an association between CF and atopic asthma (Sandford *et al.*, 1996).

Similarly, a possible connection between asthma and familial Mediterranean fever (FMF) has recently been investigated. The high rate of heterozygosity for the FMF gene in some populations suggests that there may be a heterozygote advantage for FMF, however the results of this study was inconclusive (Sandford *et al.*, 1996).

A plausible candidate for corticosteroid resistance in asthmatic subjects is the glucocorticoid receptor gene. A missense mutation in this gene had previously been found to be responsible for familial glucocorticoid resistance (Hurley *et al.*, 1991). In one study the investigators analysed six corticosteroid-resistant and six corticosteroid-responsive asthmatics and compared the data to the wild type gene sequence. As no mutations were found in any of the subjects, this suggested that the cause of corticosteroid resistance in these asthmatics does not lie in the structure of this gene (Sandford *et al.*, 1996).

The results of a recent study have provided evidence that the gene for interferon-γ (INF-γ) may be involved in the production of the atopic state. INF-γ is a good candidate gene for atopy, since it acts as an antagonist to IL-4 and downregulates the production of IgE from B cells (Tang *et al.*, 1994). The authors found that low cord blood INF-γ levels were predictive of atopic symptoms at age 12 months, suggesting that reduction of INF-γ secretion is a cause rather than a result of the atopic state (Tang, *et al*, 1994).

There are complete genome searches underway that systematically attempt to discover all the genes involved in susceptibility to atopy and asthma. The results of these searches will identify additional candidate genes from those known to be part of the allergic pathway. It is also likely that previously unknown genes will be identified. This search should lead to considerable advances in our knowledge of the mechanisms that lead to asthma and other allergic diseases (Sandford *et al.*, 1996).

1.5 Mechanisms of airway inflammation in asthma

Until recently, asthma has been viewed primarily as a respiratory disease of acute airway obstruction. Thus, research and therapeutic attention focussed principally upon mechanisms leading to acute bronchospasm. Consequently, first-line therapy consisted of bronchodilators to regulate airway smooth muscle contraction. The contribution of other components of airway obstruction, as well as the potential benefit of anti-asthma therapy directed towards control of these abnormalities, has been largely neglected. However, the approach and focus in asthma therapy and concepts of

pathogenesis have begun to change considerably.

There is currently a general concensus that patients with asthma have inflammatory changes in the airway wall, with increased numbers of activated mast cells, eosinophils, macrophages and T-lymphocytes. In addition, there is commonly epithelial shedding. These changes have been observed in bronchial biopsies of even the mildest of asthmatic patients and have been observed in patients with asthma of different clinical types (Saetta *et al.*, 1992).

It is now becoming clear that cytokines released from inflammatory and structural cells in the airway may play a major role in co-ordinating and perpetuating chronic inflammation. Identification of the cytokines involved in asthma and their effects on cell function is now an important focus for research (see Figure 1.3). Cytokines produce chronic changes in cell responsiveness by influencing the transcription of certain genes that may result in the altered expression of receptors, enzymes and regulatory proteins (Muegge & Durum, 1990).

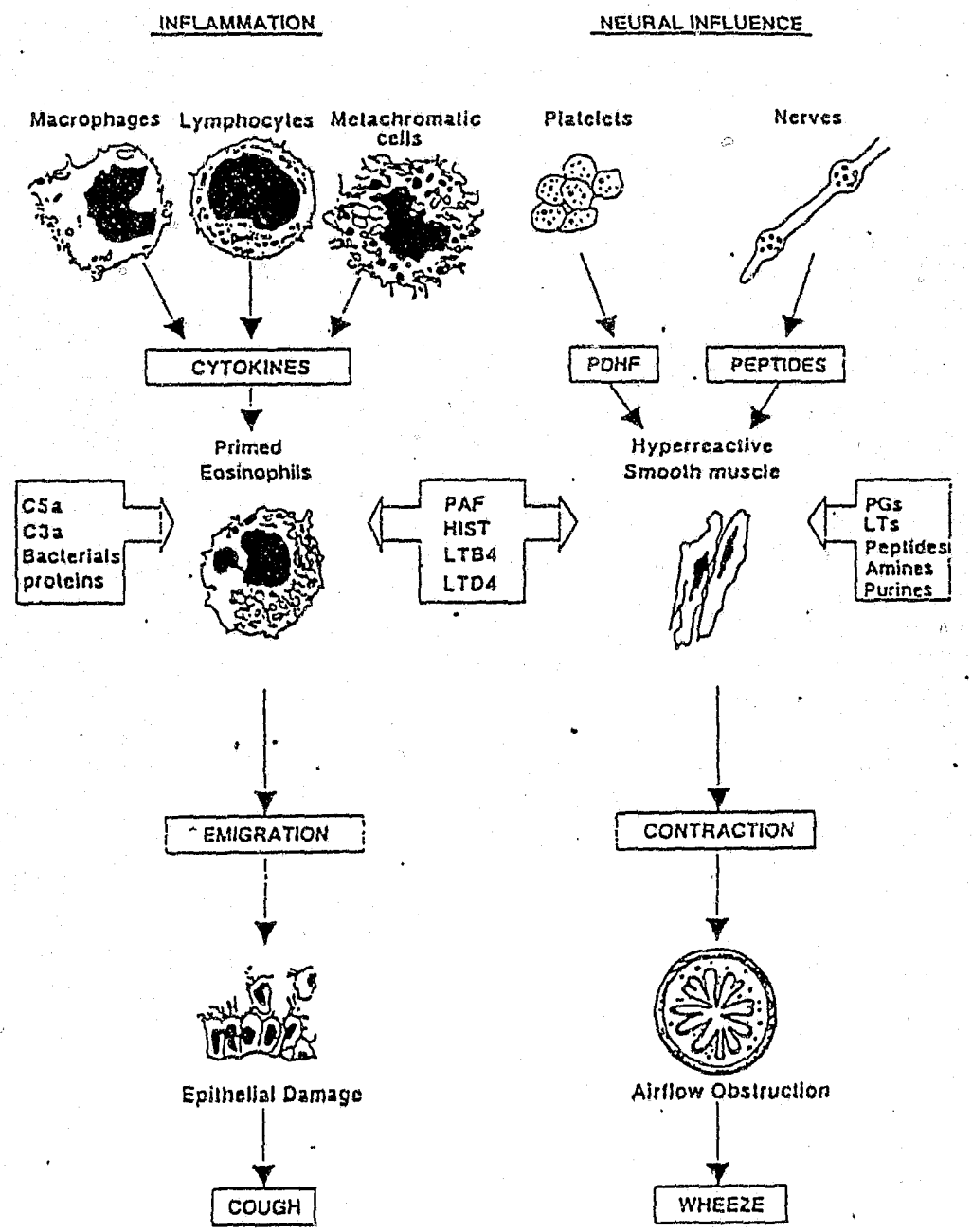


Figure 1.3 Schematic representation of airway hyperreactivity and inflammation in asthma, emphasising a parallel rather than sequential interrelationship (from Chapman *et al.*, 1993).

Elucidating the mechanisms that are involved in the development and perpetuation of the inflammatory and obstruction of the airways that is characteristic of asthma is crucial. The following brief review of the cells participating in allergic airway reactions focuses on the cellular and mediator mechanisms that are believed to be essential for the asthmatic diathesis.

1.5.1 Mast cells

Mast cells (MCs) are high affinity IgE receptor bearing cells that are present in the airway epithelium which appear to be activated in allergic and inflammatory responses. Mast cell mediators such as histamine and tryptase are present in bronchoalveolar fluid. Mast cells may be activated by an inhaled allergen via an IgE dependent mechanism (which involves the cross-linking by allergen, and internalisation of IgE receptors), (Nilsson & Schwartz, 1995). Mast cells possess high-affinity receptors which bind IgE. Cross linkage of cell-bound IgE by binding of allergen results in a rise in intracellular calcium concentration and activation of protein kinase C, leading to phosphorylation of a granule membrane protein. Mast cell degranulation releases histamines and tryptase (Marom, 1991). This IgE-mediated mechanism initiates arachadonic acid metabolism to generate leukotrienes and prostaglandins, which in conjunction with histamine, are undoubtedly involved in the bronchospastic response of their airway smooth muscle contractile properties (Drazen & Austen, 1981). The MCs generation and release of chemotactic factors then sets the stage for recruitment of other inflammatory cells to the airways, and the development of the late asthmatic response (LAR) (see Figure 1.4).

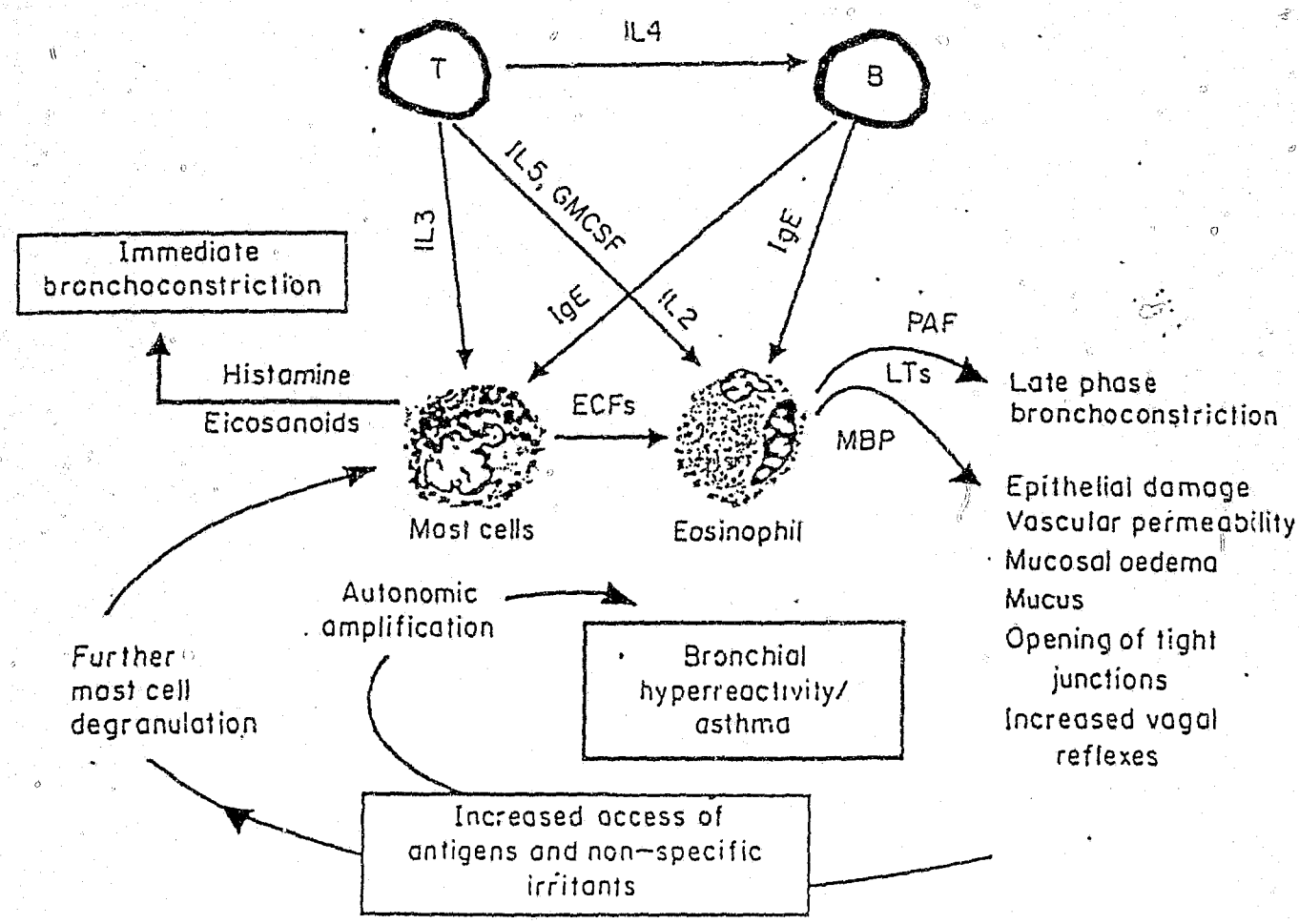


Figure 1.4 Mechanisms involved in the development of airway inflammation and bronchial hypereactivity in asthma (from Richards *et al.*, 1992).

It has been reported that murine MCs synthesise a variety of cytokines upon IgE receptor aggregation and internalisation, and although data on the cytokine production by human MCs are limited, it is possible that MCs are pivotal in recruiting and priming inflammatory cells in sensitised individuals (Arm & Lee, 1992). *In vitro* studies have demonstrated that several different cytokines are produced by MCs, including interleukins (IL)-3, 4, 5 and 6, interferon (INF)-γ and tumor necrosis factor (TNF)-α (Valent, 1994). Both IL-3 and stem cell factor (SCF), a unique activation factor for human MCs, are the major cytokines involved in MC differentiation (Valent, 1994). Furthermore, emerging data suggest a similar MC cytokine profile in human (Valent, 1994). The ability of MCs to produce such a plethora of cytokines provides them with great potential for interactions with other cell types to modulate homeostatic, allergic, inflammatory and host defence functions. Furthermore, an autocrine role for these cytokines on cell function, growth and differentiation should be considered (Nilsson & Schwartz, 1995).

1.5.2 Basophils

Basophils have long been considered the circulating counterpart of MCs. Their exact function remained elusive for many years, however, recent studies have shed some light on the contribution of basophils to various illnesses. The blood basophil count increases during the pollen season, suggesting that basophilopoiesis may be influenced by environmental factors, such as allergens (Alam & Grant, 1995).

The late-phase allergic response (LPR) induced by antigen challenge in highly allergic patients seems closely related to the naturally occurring reaction in chronic allergic disorders. The number of metachromatic cells increases at the site of LPR.

Since both basophils and mast cells release similar mediators, the exact contribution of each cell type proved difficult to determine (Alam & Grant, 1995). However, it was discovered that the profile of mast-cell/basophil-derived mediators released in the early and late phase allergic reaction is not identical. Histamine is detectable in both phases. Prostaglandin D_2 (PGD_2), a major mediator produced by mast cells, but not by basophils, is detectable in the early but not the late phase allergic reaction (Naclerio *et al.*, 1985).

Metachromatic granules of basophils contain a large number of pharmacologically active mediators that are released upon basophil activation. Mediators are typically classified as preformed and newly generated. The preformed mediators are histamine, neutrophil chemotactic factor, N-α-ρ-tosyl-L-arginine methyl ester hydrochloride (TAME) esterase and kallikrein, while the leukotrienes LTC_4, LTD_4 and LTE_4 and platelet activating factor (PAF), are the most important lipid mediators that are generated almost immediately after allergen challenge (Busse *et al.*, 1994; Alam & Grant, 1995).

It has been well established that viral infection of the airways causes exacerbation of asthma. Although the mechanism of such an exacerbation may be complex, incubation of basophils with viruses results in enhanced mediator release and chemotaxis

(Chonmaitree *et al.*, 1991). Thus, the heightened mediator release from basophils may exacerbate the symptoms of asthma.

1.5.3 Eosinophils

Of the effector cells in asthma, the eosinophil has possibly the most important and potentially pivotal role in generating airway inflammation. The biology of the eosinophil is an indication of this cell's potential relevance to asthma (Weller, 1991; Howell, 1995). In 1975, Horn and coworkers examined the usefulness of total eosinophil counts in the diagnosis and management of steroid dependent asthma. They found that eosinophil counts in a cohort of asthma patients paralleled disease severity. Total eosinophil counts were inversely related to pulmonary function and a rising eosinophil count was found to be associated with increasing bronchial obstruction as reflected by changes in specific conductance, forced expiratory volume in 1 second (FEV_1), and maximum mid-expiratory flow rate.

Recent data indicate that eosinophils themselves can elaborate proinflammatory cytokines in allergic reactions. Initial studies demonstrated that human eosinophils express and release transforming growth factor β (TGF-β) and interleukin-1 (IL-1), (DelPozo *et al.*, 1990; Wong *et al.*, 1990). Although the relevance of TGF-β to allergic inflammation is unclear, IL-1 has the capacity to induce adhesion-molecule expression on endothelial cells and is itself an eosinophil activator. Recent studies have suggested that eosinophils have the capacity to synthesize IL-3, GM-CSF and IL-5 (Desreumaux *et al.*, 1992; Kita *et al.*, 1991; Moqbel *et al.*, 1991).

Thus, although the T lymphocyte is considered to be a major source of these cytokines, it is possible that eosinophils themselves also contribute to the overall cytokine production in allergic airway inflammation. This raises the possibility of an autocrine mechanism whereby stimulated eosinophils may both release and respond to cytokines, such as IL-1, IL-3, IL-5 and GM-CSF. Thus there is the potential for a self-perpetuating cycle, with continuous eosinophil infiltration and activation, and consequently chronic inflammation (Björnsdottir *et al.*, 1995).

Besides autocrine generation of cytokines, such as IL-3 and GM-CSF, the eosinophil can synthesize and secrete lipid mediators, such as LTC_4, LTD_4, LTE_4 and PAF, which can further mediate bronchoconstriction and vascular permeability. Macrophage processing of antigen results in release of cytokines, such as IL-1 and INF-γ, which activate endothelium by up regulating adhesion-molecule expression (ICAM-1, VCAM-1 and ELAM-1). This leads to transendothelial and intestitial migration of the eosinophil (Björnsdottir *et al.*, 1995).

En route to the airway, eosinophil priming occurs and is governed by multiple factors, including endothelial adhesion, migration through lung matrix, trans-epithelial movement and exposure to proinflammatory mediators and activators. The generation of IL-4 by Th2 lymphocytes can selectively induce endothelial expression of VCAM-1 and play a pivotal role in isotype switching from IgG to IgE. Thus, sensitized tissue mast cells bind plasma-cell-derived IgE and release inflammatory mediators into the airway, resulting in the immediate asthmatic response (Björnsdottir *et al.*, 1995).

The interplay of these mediators and cytokines induces migration of the eosinophils from the circulation to the bronchial tissue. On arrival in the airway, the eosinophil is again acted upon by cytokines, generated by lymphocytes, mast cells and the eosinophil itself, thus further priming the cell and enhancing its capacity as a proinflammatory cell, through release of preformed granule mediators in *de novo*-generated lipid mediators and reactive oxygen species, contributing to LAR (Björnsdottir *et al.*, 1995). It is clear that eosinophils are a major component of the inflammatory response observed in asthma and that products derived from eosinophils are capable of producing the physiologic and histopathologic findings compatible with asthma. However, the factors which initiate the phenomenon of eosinophilic inflammation have not been completely defined. Although allergen exposure can be implicated in some patients, a specific inciting or perpetuating agent cannot be identified in all subjects. The recent observations indicating a dependence on the presence of functional T lymphocytes to produce LAR eosinophilic inflammation of the airways, suggest that a product derived from T lymphocytes will be the "activation" factor for eosinophils. However, further in-depth studies of eosinophil-T-lymphocyte interaction will be required (Björnsdottir *et al.*, 1995).

1.5.4 Neutrophils

The evidence that neutrophils play a critical role in bronchial asthma is controversial. Neutrophils appear to be normal resident cells of the larger airway. Similar numbers of neutrophils have been found in bronchial wash and in bronchial biopsies in asthmatic subjects, in subjects with chronic bronchitis and in control healthy subjects (Kirby *et*

al., 1987; Fabbri and Ciaccia, 1992; Saetta *et al.*, 1992). The fact that the number of cells is no different does not exclude the possibility that cells may have a different degree of activation. For instance, the plasma chemotactic activity for neutrophils is increased and peripheral-blood neutrophils show markers of activation during active asthma, after exercise-induced asthma and during early and late asthmatic reactions induced by allergens and toluene diisocyanate (TDI) (Sastre *et al.*, 1990).

Interestingly, the percentage increase in plasma neutrophil chemotactic activity correlates with the percentage decline of FEV_1 of the early asthmatic reaction (Sastré *et al.*, 1990). In humans, bronchoalveolar neutrophilia occurs before the onset and during the first few hours of the allergen-induced late asthmatic response and the increase of neutrophils is much larger than the increase of the number of airway eosinophils, the neutrophil increase usually precedes the eosinophil increase, the airway neutrophilia and the increased plasma chemotactic activity and activation of circulating cells are all prevented by anti-inflammatory steroids and cromolyn, which attenuate spontaneous allergen-precipitated asthma attacks, thereby suggesting as association between asthma attacks and the migration and activation of neutrophils in airways. Circulating neutrophil-generated superoxide anion (O_2-) is increased in young asthmatics and correlates with the degree of histamine-induced bronchial hyperesponsiveness (Fabbri *et al.*, 1995).

Even stronger evidence for the role of neutrophils in asthma comes from several studies which have demonstrated that these cells have the ability to synthesize and release immunoregulatory cytokines, which include TNF-α, IL-1β, and the IL-1 receptor

antagonist (IL-1Ra), IL-8, transforming growth factor (TGF)-β1 and IL-6, although the evidence for the release of the latter is contradictory. All these cytokines (excluding IL-1ra), as well as granulocyte colony-stimulating factor (G-CSF) and GM-CSF significantly prolong neutrophil survival (Cassatella, 1995).

In addition, the neutrophil is a potential source of a wide variety of mediators, including potent lipid mediators such as prostaglandins, thromboxanes, leukotrienes B_4 and PAF, which may then contribute to airway responses and/or exacerbation of the inflammatory response. From lavage studies, it is known that there is a neutrophilia present before, during and after late (but not early) asthmatic reactions induced by allergens (Metzger *et al.*, 1987).

Although there is substantial evidence that neutrophils may be involved in asthma attacks and asthma exacerbations, and although neutrophil products can indeed alter airway function, the role of these cells in causing the pathologic and physiologic features of asthma remains to be established.

1.5.5 T lymphocytes

It has long been recognised that $CD4^+$ T lymphocytes, after activation by specific antigen , have the ability to orchestrate the influx of granulocytes and other cells into chronic inflammatory reactions in the presence or absence of antibodies ("cell-mediated" immunity). It has more recently become clear that this control is exerted through the release of lymphokines, which influence the differentiation, life span,

accumulation and activation of specific granulocytes. It is likely, therefore, that $CD4^+$ T lymphocytes play a role in all inflammatory responses which are antigen-driven, including those which characterise asthma (Corrigan & Kay, 1995).

$CD4^+$ T cells are divided into T_{H1} and T_{H2} subtypes, depending on their cytokine producing profile: T_{H1} cells produce IL-2, TNF-β and IFN-γ, mediate classical cell mediated immune responses to delayed hypersensitivity and are well suited to induce enhanced antimicrobial activity in macrophages (Figure 1.5) (Paul & Seder, 1994; Busse *et al.*, 1995).

T_{H2} clones produce IL-3, IL-4, IL-5, IL-6, IL-10 and possibly IL-9, all of which regulate B lymphocyte differentiation into antibody-secreting cells, and which modulate allergic responses (Figure 1.5) (Paul & Seder, 1994; Busse *et al.*, 1995). It has been suggested that a selective imbalance, or inappropriate activation of T_{H1} or T_{H2} clones is crucial to the pathogenesis of allergic and chronic inflammatory disorders (Figure 1.6) (Peltz, 1991). The discovery of a functional dichotomy of activated $CD4^+$ T lymphocytes, which have the propensity either to mediate delayed type hypersensitivity (DTH) reactions and suppress IgE synthesis (T_{H1}) or to mediate allergic and asthmatic inflammation and promote IgE synthesis (T_{H2}), is likely to have a profound impact on our understanding of the pathogenesis of asthmatic bronchial inflammation and inappropriate IgE synthesis (Corrigan & Kay, 1995). Of importance when assessing the role of T cells in asthma, is the fact that these cells are able to generate a memory response, which partly explains why patients have recurring symptoms of asthma upon exposure to appropriate stimuli (Peltz, 1991; Busse *et al.*, 1995).

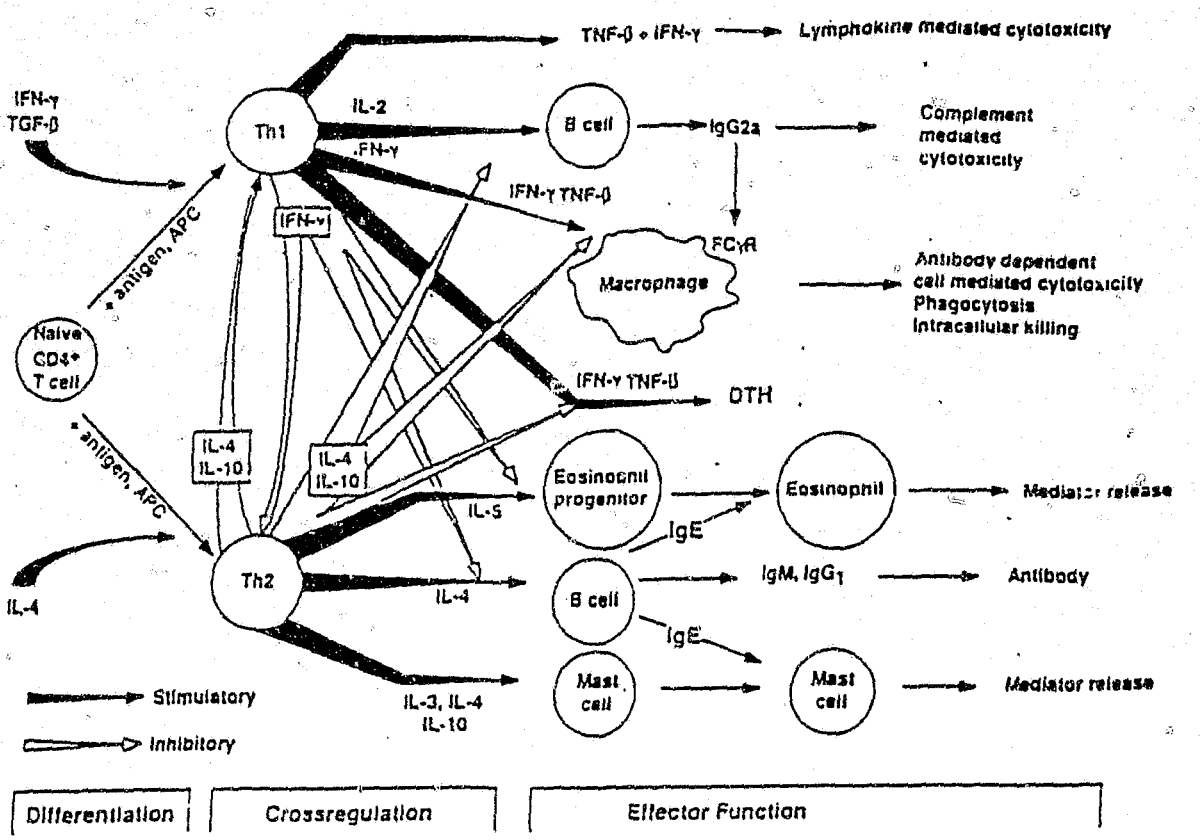


Figure 1.5 Regulatory interactions between $CD4^+$ T cell subsets (from Busse *et al.*, 1995)

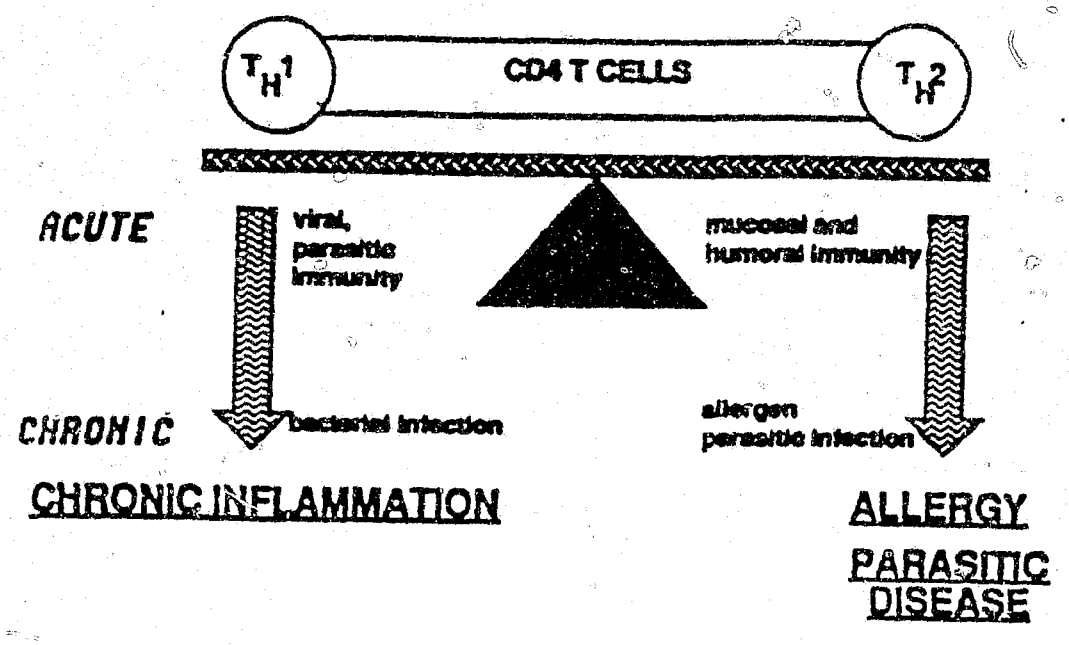


Figure 1.6 Schematic diagram indicating the development of chronic inflammation and allergy as a result of selective activation of $CD4^+$ T cell subsets. Protective immunity results from a balanced or appropriate activation of $CD4^+$ T cell subsets, while immune-mediated disease results from excessive or biassed activation of a particular subset (from Peltz, 1991).

Not much is known about the nature of the activating antigen(s) in asthma. In cases where a particular provoking antigen can be implicated (e.g. in experimental allergen challenge and occupational asthma), it should be possible to test whether or not antigen-specific T lymphocytes can be linked to disease pathogenesis by determining the cloning frequency of allergen-specific T lymphocytes retrieved from bronchial biopsies or BAL fluid. In other cases (e.g. asthma not associated with atopy), the task is much more difficult, since there is no clue as to the nature of the provoking antigen(s). In such cases, the antigens might originate from an external source (aeroallergens or viruses); alternatively, it is possible that $CD4^+$ T lymphocytes in asthmatics may inappropriately recognise antigens within the bronchial mucosa; in other words asthma may be an "autoimmune" disease (Corrigan & Kay, 1995).

One indirect approach to this problem might be to examine the usage of antigen receptor Vβ genes by T lymphocytes in the bronchial mucosa of 'intrinsic' asthmatics. Limited usage of Vβ genes would suggest that the T lymphocytes are responding to a single antigen (although it is unlikely that the particular antigen could then be identified) (Corrigan & Kay, 1995). This approach has been used to demonstrate, for example, that the T lymphocyte response in a subset of patients with Crohn's disease was relatively oligoclonal, suggesting a response to a single (unknown) antigen (Postnett *et al.*, 1990).

It seems clear that a better understanding of the antigen specificity and functional capacity of $CD4^+$ T lymphocytes in the asthmatic bronchial mucosa will play a large part in our further understanding of the pathogenesis of the disease.

1.5.6 Macrophages and monocytes

Alveolar macrophages (AM) are the most prevalent cells in the airways of normal subjects and symptomatic or asymptomatic asthmatics. Although eosinophils are clearly most characteristic of asthma and correlate with its severity (Bousquet *et al.*, 1990; Walker *et al.*, 19991), the critical processes which control eosinophil influx is not known. It has been proposed that AM subserve this role in asthma (Fuller, 1989).

Macrophages may play a pivotal role in asthma and allergic disease by directing the recruitment and activation of inflammatory cells, by generating factors which directly promote bronchospasm and tissue injury, and by production and release of cytokines which modulate and control the airway inflammatory response. The evidence that AM contribute to airway inflammation can be grouped into several broad areas: (1) AM are the most numerous cells of both the conducting airways and the distal airspace in health, in quiescent asthma, and in clinically active asthma (Calhoun & Jarjour, 1995); (2) AM bear low-affinity surface receptors for IgE ($Fc_{\varepsilon}RII$, CD23) and can be activated for proinflammatory functions by binding of antigen to IgE linked to those receptors; (3) is the capability of AM to produce a broad range of mediators, cytokines, and inflammogens. which are plausibly or experimentally linked to asthma and allergic responses, and (4) AM from asthmatics and other allergic subjects are functionally and phenotypically distinct from normal macrophages and from those in other pulmonary diseases (Calhoun & Jarjour, 1995).

AM and mononuclear cells express $Fc_{\varepsilon}RII$ (CD23). The proportion of monocytes and macrophages expressing CD23 is increased in allergic patients compared to normal individuals and is further enhanced following airway antigen challenge (Borish *et al.*, 1991). Macrophages and monocytes are also activated by antigen in an IgE-dependent manner to transcribe and secrete cytokines (IL-1β) and TNF-α (Borish *et al.*, 1991), produce superoxide anion, release β-glucuronidase and other lysosomal enzymes and augment cytotoxicity (Calhoun & Jarjour, 1995).

Upon activation, AM and mononuclear cells release a plethora of compounds which have been implicated in or linked to asthma. These factors can be broadly grouped as: (1) chemotactic factors such as LTB_4, IL-8, complement fragments and a neutrophil chemotactin distinct from IL-8, all of which can serve to recruit granulocytes; (2) cytokines and other cell-activating factors which includes IL-1 β, TNF-α, IL-6 and GM-CSF; (3) smooth muscle and mucous gland activators, including LTC_4, PGD_2 (smooth muscle spasmogen), endothelins 1 and 3 (regulate smooth muscle tone), LTB_4 and IL-1β (promote dose-dependent mucous glycoprotein secretion); and (4) direct inflammogens which include β-glucuronidase, neutral proteases and several lysosomal enzymes, which, as mentioned previously, are released following IgE receptor cross-linking, and all of which are believed to be instrumental in amplifying the inflammatory response (Arm & Lee, 1992; Calhoun & Jarjour, 1995; Drazen *et al.*, 1995).

1.5.7 Platelets

Platelets release a variety of autocoids, which are capable of inducing certain aspects of the inflammatory response. These include platelet activating factor 4 (PF-4) which is a chemoattractant for neutrophils, monocytes, and eosinophils; various lipoxygenase metabolites of arachadonic-acid metabolism; and cationic proteins (which induce vascular permeability and neutrophil accumulation), as well as a variety of mediators acting on airway or vascular smooth muscle (Page, 1995). An increased number of circulating platelet aggregates has been reported to occur in asthmatic subjects. Platelet activation has been shown to accompany both allergen and exercise-induced bronchoconstriction, and nocturnal asthma. Platelet activation within the systemic circulation is often associated with trapping of platelets within the microvasculature of the lung which has been observed in a number of lung tissue biopsies from asthmatic subjects undergoing allergen-induced late-onset airway obstruction (Page, 1995). Platelets have also been observed on the epithelial surface of symptomatic, but not asymptomatic subjects, in histologic sections of lung tissue obtained from asthmatic subjects (Jeffrey *et al.*, 1989). There is certainly overwhelming evidence that the platelet is more than an innocent bystander, and the precise contribution of this cell type to the pathogenesis of asthma requires further studies.

1.5.8 Epithelial damage

The epithelial lining of airways is a complex structure with several roles in airway function: (1) protection and regulation of airways from the external environment; (2) regulation of fluid and ion transport across the airway and into the airway lumen; (3) potential modulation of airway calibre and airway smooth-muscle tone; (4) potential modulation of inflammatory cells and mediator secretions (White & Leff, 1995). Perturbation of these functions may contribute substantially to airway inflammation, oedema formation, mucous plugging, and bronchoconstriction in the late asthmatic state (White & Leff, 1995).

Several factors are believed to play a role in the epithelial damage seen in asthmatic airways. These factors include: (1) the cytotoxic effects of eosinophil-derived products such as major basic protein (MBP), which has been found at sites of epithelial loss in postmortem studies of status asthmaticus patients; (2) active oxygen radicals, neutral proteases and the effects of subepithelial oedema; (3) respiratory tract viral infections which are known to cause large-scale epithelial damage in the airways of non-asthmatic individuals, and which are known to exacerbate asthma in children and in adults; and (4) physiological death (PCD), probably via apoptosis, which facilitates the detachment of groups of cells from the basement membrane (Holloway *et al.*, 1995; White & Leff, 1995).

Although the bronchial epithelium basement membrane is of normal thickness in asthmatics, there is a dense deposition of collagen fibrils beneath the basement

membrane, which could contribute further to chronic damage within the epithelium (Arm & Lee, 1992; White & Leff, 1995). In addition, damaged epithelium could initiate cytokine signals, thereby altering host defence responses to signals for normal repair and restoration, all of which is likely to contribute to the severity of airway obstruction and bronchial hyperreactivity in asthma (Holloway *et al.*, 1995; White & Leff, 1995).

1.5.9 Eicosanoids

Eicosanoids is a term that was derived from *eicosa* (20 carbon atoms), *-ene* (indicates that double bonds are present in the compounds), and *-oid* (indicates structural similarity to one another) (Kleeberger & Freed, 1995). Leukotrienes, prostaglandins and thromboxanes are important mediators of bronchial asthma. They are potent pro-inflammatory and spasmogenic compounds and they are present in asthmatic airways at rest and during acute asthma attacks (Arm & Lee, 1992). Figure 1.7 shows the arachadonic acid cascade that results in the metabolism of leukotrienes (LTs), prostaglandins (PGs) and thromboxanes (TXs).

Leukotrienes

The leukotrienes are biologically active fatty acids derived from the oxidative metabolism of arachadonic acid. They consist of the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4, and the non-cysteinyl leukotriene LTB_4 (Drazen, 1995). The formation of the cysteinyl leukotrienes results from five distinct processes: membrane

bound arachadonic acid is cleaved from cell membranes, probably by cytosolic phospholipase A_2; the cleaved arachadonic acid interacts with 5-lipoxygenase which adds molecular oxygen to the arachadonic acid to form the unstable intermediate 5-hydroxy-eicosatetraenoic acid (5-HETE), or is converted to the unstable intermediate epoxide LTA_4; LTA_4 is then converted to LTB_4 by an epoxide hydrolase or by glutathione-S-transferase to LTC_4; LTC_4 is cleaved by γ-glutamyl-transpeptidase to LTD_4 and further to LTE_4 by a variety of dipeptidase (Figure 1.7) (Arm & Lee, 1992; Drazen *et al.*, 1995).

Prostaglandins and Thromboxanes

Arachadonic acid is released to the cell via hydrolysis of phospholipids by phospholipase A_2, phospholipase C, and/or diglyceride lipase. The prostaglandins and thromboxanes are generated from arachadonic acid through the cycloxygenase pathway (Kleeberger & Freed, 1995). Cycloxygenase (prostaglandin endoperoxide synthetase), located in endoplasmic reticulum and nuclear membranes, catalyses the oxygenation of arachadonic acid to prostaglandin endoperoxide-prostaglandin G_2 (PGG_2), which is subsequently reduced to PGH_2 by peroxidase activity. The PGH_2 compound then becomes a substrate for the terminal enzymes to produce thromboxane (TX) A_2 or to prostaglandins PGD_2, $PGF_{2\alpha}$, PGE_2 and PGI_2 (Figure 1.7) (Arm & Lee, 1992).

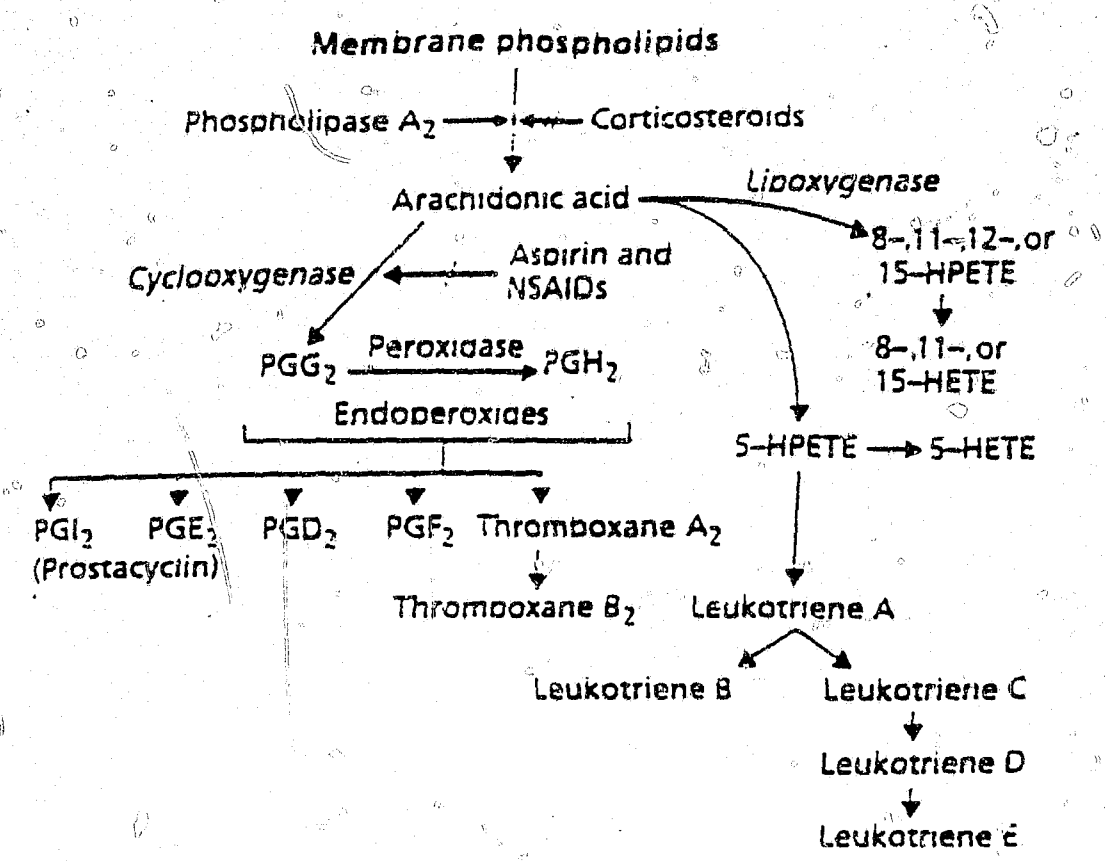


Figure 1.7 The metabolism of arachadonic acid by 5-lipoxygenase and cyclooxygenase pathways to leukotrienes (LT), prostaglandins (PG) and thromboxanes (TX); aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase, while corticosteroids inhibit phospholipase A_2 (from Bush & Ashbury, 1995).

1.6 Nitric oxide

A potential neurotransmitter of the non-adrenergic inhibitory nervous system is nitric oxide (NO). NO is formed from L-arginine by the removal of the terminal guanidonitrogen groups. The process is catalysed by a cytosolic, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme, tentatively labelled NO synthase (NOS) (Pueringer & Casale, 1995). NOS exists in two main isoforms: constitutive NOS (cNOS), which acts locally, to form a small amount of NO and mediates endothelium-dependent vasodilation and non-adrenergic, non-cholinergic neural responses, and inducible NOS (iNOS) which is induced by cytokines such as TNF-α, IL-1β and INF-γ (Barnes & Liew, 1995).

NO might play a role in the eosinophilic inflammation that is characteristic of asthma, and high concentrations of the gas may contribute to epithelial damage and shedding in asthmatic airways (Liggett *et al.*, 1995). It has also been suggested that the inappropriate activation of NO synthesis by airway epithelium might augment and perpetuate asthmatic inflammation by inhibiting T_{H1} IFN-γ production (Barnes & Liew, 1995). The consequence of this would be an increase in IL-4 and IL-5 production by T_{H2} cells, which would upregulate IgE synthesis (IL-4) and recruit eosinophils into the airways (IL-5) (Figure 1.8) (Hamid *et al.*, 1993; Barnes & Liew, 1995; Liggett *et al.*, 1995).

Treatment of smooth muscle preparations with a scavenger of superoxide, superoxide dismutase, potentiates NO-induced smooth-muscle relaxation. NO appears to contribute a portion of the bronchodilating activity of the non-adrenergic nervous system. Indeed, NO has been shown to be a potent bronchodilator in isolated guinea-pig tracheal smooth muscle. Moreover, there is at least some evidence to suggest that, in humans, NO may be the predominant non-adrenergic bronchodilator. Although the mechanisms by which NO mediate airway smooth-muscle relaxation is unknown, NO does lead to the production of cAMP (Pueringer & Casale, 1995).

With their known bronchodilating and anti-inflammatory effects, the putative neurotransmitters of the non-adrenergic system are potentially protective against some of the manifestations of asthma. Therefore, a reduced functional amount of NO could potentiate the symptoms of asthma. Effective therapy directed at altering non-adrenergic inhibitory nervous system activity has not yet been developed, however with the recognition of NO as a potent bronchodilator, efforts at developing a potential exogenous nondegradable source of NO may lead to new therapeutic strategies (Pueringer & Casale, 1995).

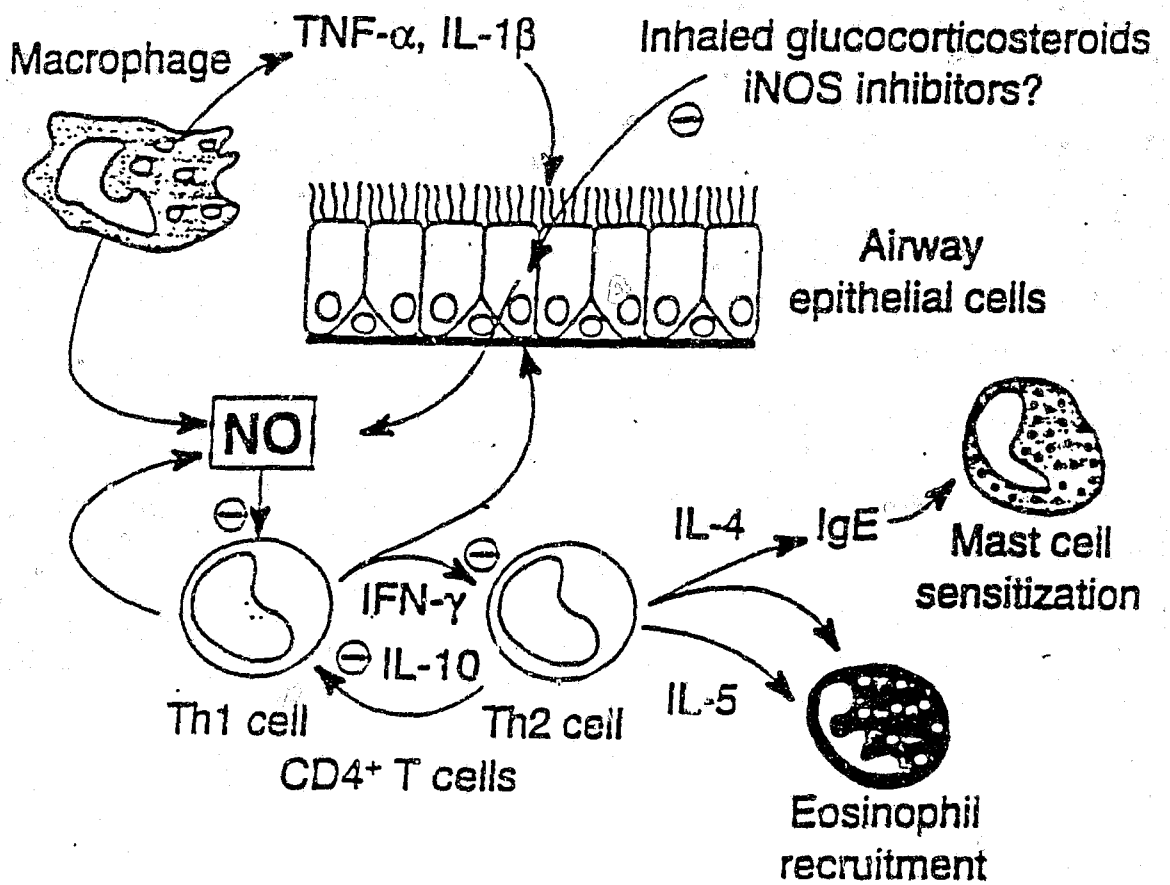


Figure 1.8 The role of nitric oxide (NO) in asthmatic inflammation. NO produced by epithelial cells and macrophages in asthma inhibits T_{H1} cells, allowing the activation of T_{H2} cells, which results in the release of IL-4 and IL-5. These cytokines stimulate IgE synthesis and eosinophil invasion of airways. Inhaled glucocorticoids might inhibit TNF-α, IL-1β and IFN-γ-induced iNOS expression by epithelial cells and, thereby, reduce airway inflammation (from Barnes & Liew, 1995).

1.7 Asthma in children

1.7.1 Epidemiology and natural history

Although asthma in children is a condition which every paediatrician and general family practitioner encounters frequently, there are a number of important facts and equally important misunderstandings about childhood asthma which require clarification. The importance of asthma in children both to the individual child, the family and the community cannot be overemphasised. In most developed societies asthma is a common cause of chronic ill health. In one study in young children it was found that some two thirds of all drugs prescribed were for the treatment of wheezing and 7% of all children had missed school (for a median period of 7 days in a year) because of wheezing (Godfrey, 1997).

Asthma is a leading cause of morbidity among children throughout the world. In the US, asthma accounts for 2.2 million paediatrician visits per year and 28 million restricted activity days. In various surveys, the incidence of asthma measured at 10 to 15 year intervals showed that in the 8-13 age range there had been an approximate doubling in asthma in the past 10 years (Eggleston & Szefler, 1995; Godfrey, 1997). Risk factors for both morbidity and mortality trends include race, urbanisation and poverty. Both mortality and hospitalisations are 3 times higher in black than in white persons. However, when poverty is accounted for, hospitalisation rate differences between black and white children almost disappear. Racial differences in asthma morbidity may be related to differences in access to care or a cultural over-reliance on

crisis-oriented care through emergency rooms (Eggleston & Szefler, 1995).

The median age of onset of asthma is 4 years; > 20% of children develop symptoms within the first year of life. Risk factors for early onset include atopy. This risk factor is clearly genetic, in that parental history is almost as strong a risk factor as an elevated level of IgE in cord blood. The association with parental smoking is much less clear, and most studies have shown a weak or insignificant association with either the onset or severity of asthma. Other obvious risk factors for early onset include neonatal lung disease, reduced lung volumes in otherwise healthy infants and respiratory infections, especially with the respiratory syncytial virus (RSV). Forty to 50% of infants with RSV broncholitis develop chronic asthma (Eggleston & Szefler, 1995).

In 60% of cases that begin in childhood, asthma resolves by young adult life. In general those who resolve have less severe, intermittent asthma, do not have multiple positive skin prick tests to inhalant allergens and do not have persistent wheezing. Some studies indicate that heavy exposure to pollution, allergens or cigarette smoke may decrease the likelihood of resolution (Eggleston & Szefler, 1995).

1.7.2 Pathophysiology

Two environmental processes affect the course of asthma. Firstly, those factors that initiate inflammation and secondly, those that act on inflamed airways to precipitate immediate obstructive responses.

Initiators of inflammation

Respiratory viral infections are more obvious initiators of inflammation in children than in adults. This may in part be because the infant host is immunologically naive and therefore more susceptible to severe infections with common respiratory viruses and to unusual illness patterns. RSV, which cause "colds" in older children and adults is a typical example. In infants, RSV causes a significant febrile illness with a high frequency of pneumonia and bronchiolitis (Eggleston & Szefler, 1995).

Allergen exposure is capable of altering asthma symptoms and increasing bronchial hyper-responsiveness. In children, this applies not only to aeroallergens, such as house dust mite (Sporik *et al.*, 1990), but also to food allergens.

Exposure to passive cigarette smoking has been associated more clearly with the expression of allergic sensitivity and with asthmatic symptoms in children. The association is especially strong when the child is exposed to maternal smoke (Eggleston & Szefler, 1995).

Acute precipitants

Most asthmatics have acute obstructive responses to various precipitants, including viral respiratory infections, allergens, exercise, irritants, drugs, and weather changes. The association between respiratory virus infections and significant wheezing episodes in asthmatic patients is much clearer in children, where 60% of such infections are accompanied by wheezing, than in adults where 20% of such infections are accompanied by wheezing (Eggleston & Szefler, 1995). When children are together in groups such as schools and day care centres, they are exposed to respiratory viral infection more frequently. In addition, day care settings may expose them to higher concentrations of allergens or cigarette smoke that may also contribute to respiratory disease. In other cases, children's response to a precipitant may not be different from that of adults, but the relative importance of a precipitant may be different. For example, childhood is a much more physical time of life, therefore, exercise-induced asthma is both more commonly reported and will interfere with normal functioning to a greater extent (Eggleston & Szefler, 1995).

Asthma in children is probably due to inheritance of a permissive gene or genes, which, if exposed to an appropriate environmental inducing agent, allows the child to develop the specific type of bronchial hyperreactivity associated with asthma. It appears that two thirds of children with asthma will lose their symptoms entirely and remain symptom free until middle adult life. It is possible that the earlier use of corticosteroids therapy in children with moderate to severe perennial asthma may shorten the course of the disease and improve the long-term prognosis (Godfrey, 1997).

1.8 New treatments for asthma

There are currently only five classes of anti-asthmatic drugs in widespread use: (a) β-agonists which remain the cornerstone of anti-asthmatic drugs (relax airway smooth muscle; inhibit mast cell mediator release and results in bronchodilation); (b) anticholinergics which are used particularly in acute severe asthma and may be of some benefit in nocturnal asthma (inhibits acetylcholine); (c) methylxanthines which inhibit phosphodiesterase and decrease bronchial hypersensitivity; (d) disodium cromoglycate (DSCG) has been used in the treatment of asthma for the past 20 years and the related drug nedocromil sodium has been introduced more recently (acts as a mast cell stabiliser, inhibit activation of effector cells and mediator release, inhibit chemotaxis and has an anti-inflammatory action); and (e) corticosteroids, which are recognised as fundamental in the treatment of all but the mildest of asthmatics, are now being used in an increasing number of children and at higher doses, particularly in adults (inhibit phospholipase A_2, leukotrienes and prostaglandins, redirect protein synthesis) (Barnes *et al.*, 1995).

Increased understanding of the pathophysiology of asthma has led to new targets for drug development, and in many cases compounds that interfere with these pathways have been developed. These new drugs include:

1. Potassium-channel activators
2. Calcium-channel blockers
3. Drugs affecting asthmatic mediators
4. Adhesion blockers

1.8.1 Potassium-channel activators

Potassium (K^+) channels are present on smooth muscle, and activation of K^+ channels leads to muscle relaxation. K^+ channels are present on some inflammatory cells, so K^+ channel activators might have additional anti-inflammatory properties (Barnes *et al.*, 1995). The K^+ channel activators ability to reverse bronchial hyper-responsiveness is believed to depend on hyperpolarisation of vagal neurones and therefore presynaptic inhibition (Morley, 1993). For K^+ channels activators to be of clinical use, the cardiovascular side effects need to be overcome. Unless the current concerns about β_2-agonists become more problematic, it seems unlikely that K^+ channel activators will supersede them (Barnes *et al.*, 1995).

1.8.2 Calcium-channel blockers

Alterations in calcium flux are important in activating airway smooth muscle and a number of inflammatory cells. Calcium antagonists affect voltage-dependent calcium channels. It now seems likely that receptor-operated calcium channels and intracellular mobilisation of calcium are of greater importance in asthma as they offer the prospect of being both bronchodilators and anti-inflammatory agents (Barnes *et al.*, 1995).

1.8.3 Drugs affecting asthmatic mediators

Several pharmaceutical companies are developing new drugs that interfere with the activity of mediators made by mast cells, basophils, eosinophils and several other

inflammatory cells (Lichtenstein, 1993). These include PAF antagonists, drugs that block the functioning of leukotrienes, cyclooxygenase inhibitors, which block the production of prostaglandins, and thromboxanes and bradykinin antagonists, which all help to alleviate cough and chest tightness (Morley, 1993; Barnes *et al.*, 1995).

1.8.4 Adhesion blockers

Adhesion of mast cells, basophils, eosinophils, T lymphocytes and several other inflammatory mediator cells to the extracellular matrix (ECM) causes important regulatory changes in these cells and therefore plays an important role in the inflammatory cascade (Hamaway *et al.*, 1994). This attachment is mediated by specialised cell-surface receptors/adhesion molecules, and results in the activation of a cascade of intracellular signalling events that induce cellular responses and regulate the responses of these cells to other stimuli (Hamaway *et al.*, 1994). As these adhesion molecules facilitate the migration of cells from blood to sites of inflammation in tissue, they are therefore a recently identified target which may be amenable to manipulation by drugs. These drugs have not yet entered clinical trials for the treatment of asthma, but preliminary studies in primates of an anti-intercellular adhesion molecule I monoclonal antibody showed activity against eosinophil migration after antigen challenge, but no effect on chronic bronchial inflammation (Lichtenstein, 1993; Barnes *et al.*, 1995). It has often been claimed that so many mediators and cells are present in the asthmatic response that a drug acting exclusively against one element is unlikely to be effective. As more novel therapies become available, it can be seen that this gloomy prospect is not true. The new approaches to treating asthma will lead to a

decreased role for older drugs and teach us more about the pathophysiology of the condition. It is also salutory to remember that it takes many years before the importance of a drug is recognised, with inhaled steroids taking 15 years from their introduction to the recognition of their central role in asthma therapy (Barnes *et al.*, 1995).

1.9 Objectives of the present investigation

According to current concepts asthma is primarily an inflammatory condition of the bronchi which results from the complex interactions between heterogenous genetic and environmental factors. Although the environmental allergens are fairly well known, little information concerning the genetic differences between atopic and non-atopic individuals is available. The present study was undertaken in order to determine whether molecular variation of certain candidate genes may play a role in the development of asthma in black and white South Africans. The present study was undertaken in order to: (a) confirm reported frequencies of alpha-1-protease inhibitor variants in black and white asthmatic patients and control individuals in South Africa; (b) assess the frequency of the interleukin-1 receptor antagonist (IL-1 Ra), β_2 adrenergic receptor and cortisol binding globulin (CBG) variants in black and white asthmatic patients and control individuals in South Africa; (c) look for associations between alpha-1-protease inhibitor, IL-1ra, β_2 adrenergic receptor and CBG; (d) make comparisons of the above parameters between population groups; and (e) investigate the expression of IL-1ra in black and white asthmatic patients and control individuals in South Africa.

CHAPTER 2

THE ROLE OF ALPHA-1-PROTEINASE INHIBITOR IN ASTHMA

2.1 Introduction

The current understanding of the relationship of the plasma protein α_1-PI and human disease evolved from the discovery by Laurell & Erikson (1963) of a marked reduction of the α_1-globulin peak on plasma protein electrophoresis associated with the development of emphysema. Soon afterwards, it was recognised that the deficiency was due to a deficiency of one protein. This protein was a serine protease inhibitor, capable of inhibiting proteolytic enzymes with serine at their active site (Travis & Salversen, 1983). Since trypsin was the first serine protease evaluated as a target for this protease inhibitor, the inhibitor was termed alpha-1-antitrypsin (α_1-AT), and the hereditary disease called α_1-AT deficiency.

α_1-AT plays a central role as a protease inhibitor in controlling tissue degradation. As a major protease inhibitor in human plasma, α_1-AT can complex with a broad spectrum of proteases, including elastase, trypsin, chymotrypsin, thrombin and bacterial proteases. The most important inhibitor action is that against leukocyte elastase, a protease that degrades the elastin of the alveolar walls as well as other structural proteins of a variety of tissues.

2.1.1 Structure of α_1-AT

α_1-AT is a glycoprotein consisting of a single polypeptide chain of 394 residues and a carbohydrate content of 12%; the resulting molecular mass is 52kDa. The small size of the protein allows it to diffuse through interstitial body fluids and into tissues such as the lung. The locus (PI locus) for α_1-AT is on chromosome 14, at 14q32.1, close to the locus for the protease inhibitor α_1-antichymotrypsin, and in a cluster of sequence-related genes that includes cortisol binding globulin (CBG) and protein C-inhibitor. The gene is 12.2 kb long and contains six introns. α_1-AT produced in hepatocytes has a 1.4 kb mRNA transcript, while macrophages have longer RNA transcript, beginning in exons 5' to the first exon for hepatocyte α_1-AT (Cox, 1995).

α_1-AT, has been crystallized in its active form (Elliott *et al.*, 1996; Elliott *et al.*, 1998 and Ryu *et al.*, 1996) and it crystallizes after proteolytic cleavage at the reactive site (Loebermann *et al.*, 1984). Analysis of the crystal structure indicates that the single polypeptide chain is organised into well-defined secondary structural elements : three β sheets and eight α helixes. α_1-AT contains one cysteine residue, as indicated by both protein and DNA analysis. No disulfide bridge is present in the protein, although the thiol group can form a disulfide bond with other proteins, such as the IgA heavy chain and the thiol group of immunoglobulin κ light chain (Cox, 1995).

α_1-AT shows considerable genetic variability, having more than 70 genetic variants (PI types), many of which have been sequenced. Most variants are associated with quantitatively and qualitatively normal α_1-AT. Further variation can be revealed at the

DNA level where a number of restriction enzymes reveal polymorphisms.

α_1-AT is modified during its passage through the endoplasmic reticulum. Some of this modification is reflected in the micro-heterogeneity observed in acid starch gel and agarose electrophoresis and in polyacrylamide isoelectric focussing, as is typical for glycoproteins near their isoelectric point. Eight bands were originally noted, numbered from 1 (anodal) to 8 (cathodal); bands 4 and 6 contain 40 and 35 percent, respectively, of the total α_1-AT and have isoelectric points of 4.52 and 4.59 (Cox, 1995). Much of the heterogeneity is due to differences in the type of carbohydrate side chain.. Three carbohydrate side chains per molecule are attached at asparagine residues 46, 83, and 247. The carbohydrate chains may be biantennary or triantennary, terminating in two or three N-acetylneuraminic acid residues. The electrophoretic mobility of α_1-AT is sequentially shifted cathodally by incubation with neuraminidase as N-acetylneuraminic acid residues are removed.

The gene coding for α_1-AT, 12.2kb in length, includes a 1434-bp coding region. The gene contains six introns; exons 1A through 1C, the 5' portion of exons 2, and the 3' portion of exon 5 are non-coding regions (Figure 2.1). The largest intron (between exons 1C and 2), is 5.3kb in length and contains a 143-amino acid open reading frame, an *Alu* sequence, and a pseudo-transcription-initiation region. The open reading frame does not appear to be an actual protein-coding region (Cox, 1995).

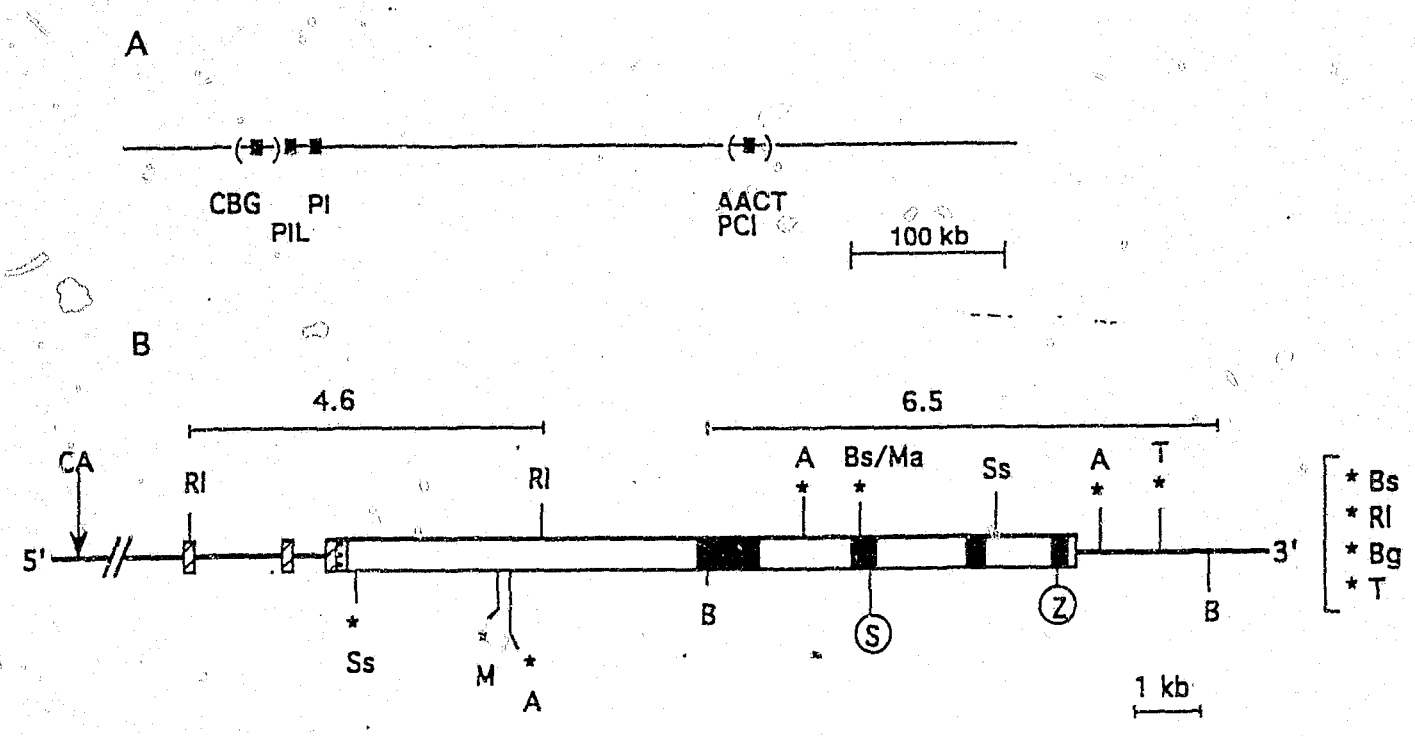


Figure 2.1: (A) PI and other loci in the serpin superfamily cluster on chromosome 14, as determined by pulsed field gel electrophoresis. PIL = PI-like, CBG =corticosteroid binding globulin, PCI = protein C inhibitor, AACT = α_1-antichymotrypsin. (B) α_1-AT gene and flanking regions. Coding regions are solid rectangles, introns are open rectangles, and untranslated regions are dotted rectangles. Cross-hatched regions are exons of macrophage DNA. Asterisks indicate site of polymorphisms for the following restriction enzymes (those in the square brackets at the right indicate polymorphisms in the 3' homologous region) : A = *Avu* II; B = *Bam* HI; Bg = *Bgl* II; BS = *Bst* EII; M = *Msp* I; Ma = *Mae* III; T = *Taq* I; RI = *Eco* RI; Ss = *Sst* I; S and Z circled = site of mutations in PI*S and PI*Z, respectively. Genomic probes 4.6 and 6.5, are indicated. The arrow marks the position of the CA repeat (from Cox, 1995).

DNA and protein sequencing studies have indicated homology not only between some of the protease inhibitors, but also between inhibitors and other plasma proteins, as well as with chicken ovalbumin. Human α_1-AT and α_1-antichymotrypsin share 56% homology in their coding nucleotide sequences and 42% homology in their amino acids. Human α_1-AT and protein C inhibitor share 42% amino acid identity. An unexpectedly high degree of homology has been observed between α_1-AT and two non-inhibitor human plasma proteins: thyroxine-binding globulin, located on the X chromosome (58%) (Flink *et al*, 1986) and corticosteroid-binding globulin, located on chromosome 14 (53%) (Underhill & Hammond, 1989). There is 28% homology between the amino acid sequences of antithrombin III and α_1-AT (Kurachi *et al.*, 1981), and 27% homology between C1 inhibitor and α_1-AT (Tosi *et al.*, 1986); the position and number of introns is very different however, probably indicating a relatively ancient divergence from a common ancestral gene several hundred million years ago.

2.1.2 Physiology of α_1-AT

Alpha-1 antitrypsin inhibits a broad spectrum of serine proteases. Because of its efficiency of inhibition, broad substrate specificity and ready access to tissues, α_1-AT plays an important role in defending tissues from proteolysis. α_1-AT inhibits most serine proteases tested to date, including pancreatic and neutrophil elastase, neutrophil cathepsin G, pancreatic trypsin and chymotrypsin, collagenase from skin and synovia, acrosin, kallikrein, urokinase and renin (Cox, 1995). Since α_1-AT increases during the acute-phase response and is also a trypsin inhibitor, and since trypsin inhibitors are

known to have antibacterial activity, α_1-AT may play some role in resistance to infection. The association of α_1-AT deficiency with a spectrum of inflammatory diseases suggests that α_1-AT is important in the inflammatory response.

The two major sites of α_1-AT gene expression are hepatocytes and the mononuclear phagocyte family of cells (Permutter *et al.*, 1985). On average, hepatocytes contain approximately two-hundred fold more α_1-AT mRNA transcripts per cell than do mononuclear phagocytes, including blood monocytes and alveolar macrophages, suggesting that the liver is the major site of α_1-AT biosynthesis (Perlino *et al.*, 1987).

The path of α_1-AT biosynthesis is typical of a secretory glycoprotein (Figure 2.2). The α_1-AT is translated on ribosomes bound to the endoplasmic reticulum (ER). As the α_1-AT polypeptide is synthesised, it proceeds through the membrane of the ER to the lumen, a process thought to be directed by the 24 amino acid N-terminal signal peptide on the newly synthesised α_1-AT molecule. Within the cisterna of the rough ER, the three carbohydrate side chains are added as the protein begins to fold into its three-dimensional configuration. Like other N-linked oligosaccharides, the carbohydrate side chains of α_1-AT are initially added in the form of a branched oligosaccharide, containing nine mannose residues, among other components. Once linked to the α_1-AT molecule, the so-called "high mannose" side chains are trimmed and the protein is translocated to the Golgi apparatus, where the side chain processing of the oligosaccharides is completed, to form the final "complex" type of side chains found on mature α_1-AT (Pfeffer & Rothman, 1987).

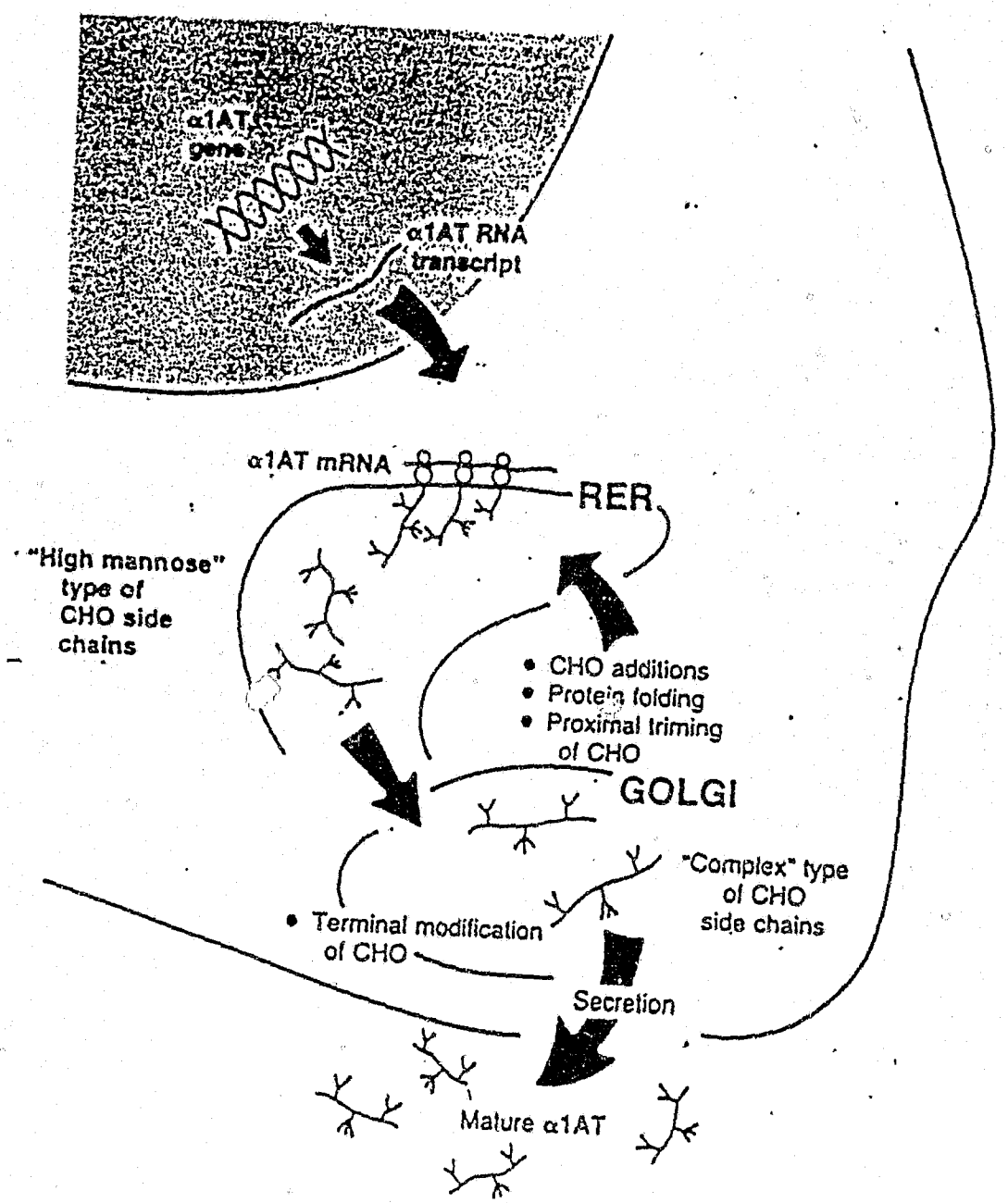


Figure 2.2: Processes involved in the normal biosynthesis of α_1-AT. In the nucleus, the mRNA precursor is transcribed from the gene. After splicing and translocation, the α_1-AT is translated on the rough ER. The newly synthesised polypeptide chain is secreted into the cisterna of the rough ER, the signal peptide is cleaved, the carbohydrate side chains are added as the protein folds. The initial "high mannose" carbohydrate side chains undergo proximal trimming, and the protein is then translocated to the Golgi apparatus. Within the Golgi, terminal modification of the carbohydrate side chains yields a mature 55 kDa protein (from Brantly *et al.*, 1988)

2.1.3 Genetic Variation

In 1974, isoelectric focussing in polyacrylamide gel was first introduced for resolution of PI variants. According to the nomenclature guidelines, with subsequent modifications according to general guidelines for human gene nomenclature, alleles at the PI locus are designated PI*M, PI*S *etc.* Phenotypes are designated as PI MZ, PI M (or MM if confirmed in family studies). Genotype is indicated as PI*M/PI*Z *etc* (Cox, 1995). The PI (protease inhibitor) variants, initially identified primarily by the method of acid starch gel electrophoresis, were named in order of their mobility: F (fast), M (medium), S (slow), and Z (the most cathodal). There are many inherited variants of α_1-AT (Figure 2.3). PI*M, which can be further classified into subtypes, is the most common allele in all populations. The PI*S allele reaches polymorphic frequencies in many populations, as does the PI*Z allele, which produces a deficiency of α_1-AT. In addition to common variants of α_1-AT, more than 60 rare variants of α_1-AT have been identified.

The amino acid sequence has been identified for a number of types of α_1-AT, either directly or by DNA sequence analysis. An alanine-valine substitution at amino acid position 213, noted by amino acid sequencing was further identified by DNA studies of both Z and M1. The alanine substitution is found in about 34% of PI*M1 alleles and differentiates two subtypes of M1 : M1(Ala^{213}) and M1(Val^{213}). The PI*Z allele has the Ala^{213} substitution; however , PI types M2, M3, and S all have valine at position 213 (Nukiwa *et al.*, 1987).

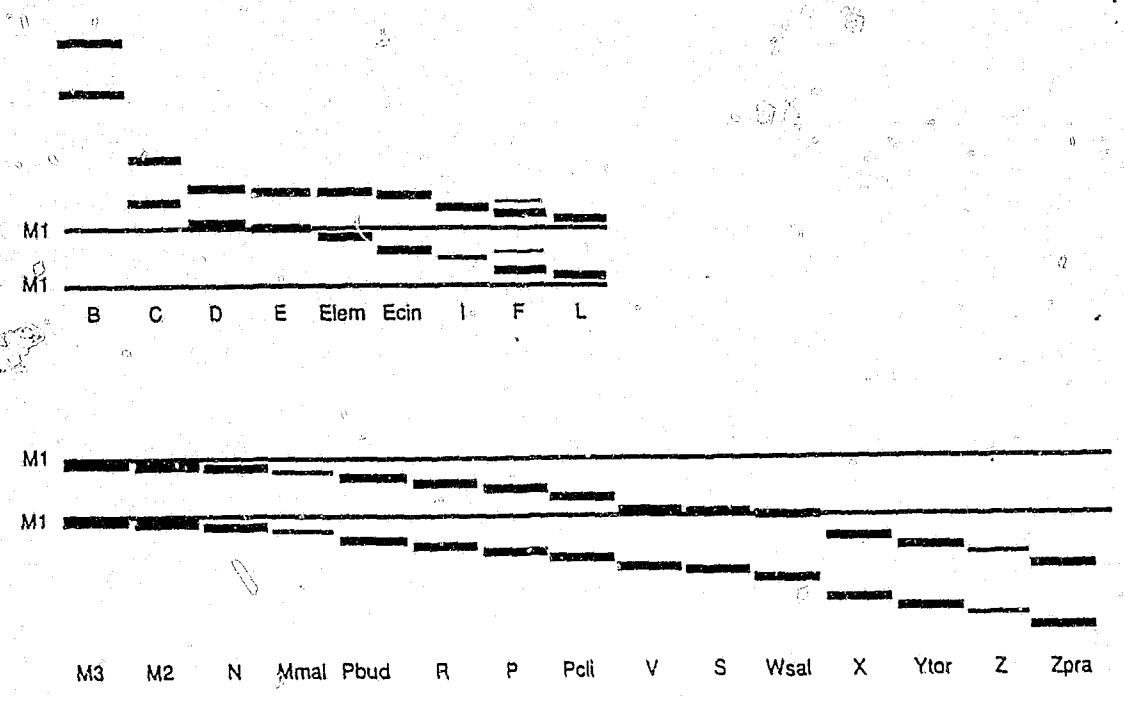


Figure 2.3: Diagram of selected anodal (top row) and cathodal (bottom row) PI variants as revealed by isoelectric focussing in polyacrylamide gels. The positions of the two major bands of M1 are indicated by the solid lines. Anode at top. (From Cox, 1995)

The distribution of the PI alleles has now been determined for many populations. In all populations, PI*M is the most common allele. PI*M1 is distributed in all populations with the highest frequency in all populations studied, with PI*M2 the next most frequent, while PI*M3 is found to be relatively uncommon. The additional subtype allele PI*M4 has been described in several populations at a frequency of 0.002 to 0.050 (Cox, 1995). In many studies, no differentiation was made between PI types M3 and M4, since these alleles are not easily separated by isoelectric focussing. The PI*S allele is rare or absent in black and oriental populations, but show the highest frequency in Spain and Portugal, a lower frequency in France, and low generally in other parts of Europe. The frequency of PI*Z is highest in Scandinavian countries, and the allele is present throughout white populations including those of the Middle East, and is absent from oriental and black populations, except in those populations known to have a white admixture, as in the United States (Cox, 1995).

2.1.4 α_1-Antitrypsin Deficiency

The most common of the deficiency alleles is PI*Z, and most individuals with α_1-AT deficiency are of PI type ZZ. The estimated frequency of the PI*Z allele in North American white populations is 0.0122, corresponding to a frequency of PI ZZ homozygotes of 1 in 6 700 (Cox, 1995).

The Z variant differs from the M_1 (Ala^{213}) by the substitution of lysine for glutamic acid at position 342. This mutation reduces the stability of the molecule in its monomeric form and predisposes to a novel protein-protein interaction between the reactive centre

loop of one molecule and the A β sheet of a second (Lomas *et al.*, 1992). This "loop-sheet" polymerisation interferes with the secretion of the molecules from the hepatocytes and macrophages and results in decreased plasma levels of 10%-15% of normal.

The S variant differs from the M_1 (Val^{213}) by the substitution of valine for glutamic acid at the 264 position (Brantly *et al.*, 1988). This mutation also leads to the production of polymers (Elliott *et al.*, 1996). This effect reduces plasma α_1-AT concentrations to 60% of normal.

The S and Z variants have been associated with emphysema (Brantly *et al.*, 1988), liver disease (Brantly *et al.*, 1988), panniculitis (Pinto *et al.*, 1993) and asthma (Colp *et al.*, 1993). Elliott *et al.* (1998) provided the first demonstration of Z α_1-AT polymers in the lungs of patients with emphysema. The inactivated α_1-AT is unable to play any role in the anti-proteinase screen, and this serves to exacerbate lung disease associated with plasma defiency of the Z mutation of α_1-AT (Elliott *et al.*, 1998).

However, several studies suggest that individuals with certain α_1-AT phenotypes may be predisposed to developing asthma. Gaillard *et al.* (1992) reported an increased prevalence of the non-deficient M2M2 α_1-AT phenotype in white asthmatics in South Africa, while an increased prevalence of the Z allele of α_1-AT was found to be associated with severe asthma in Swedish children (Cox, 1994; Walls, 1995). Interestingly, a heterozygote advantage has been suggested to account for the high incidence of the S and Z α_1-AT alleles in the European population. These alleles have

been implicated in protection against pulmonary tuberculosis (Carrell, 1984).

It has also been shown that contrary to current thinking, common mutations of α_1-AT that are associated with mild to moderate deficiency of the protein predict a subgroup of cystic fibrosis patients with less severe pulmonary disease (Mahadeva *et al.*, 1998).

2.1.5 Amplification of DNA: The Polymerase Chain Reaction

The development of the polymerase chain reaction (PCR) technique for nucleic acid amplification has had a major impact on many diverse areas of both basic and clinical research. Since its inception (Saiki *et al.*, 1985), reports on a wide variety of applications for PCR have received much attention in scientific and medical literature. This technology has been shown to have a vast application to the diagnosis of human disease including such diverse areas as infectious disease, genetic disorders and cancer (Arends & Bird, 1992; Gunther *et al.*, 1993).

Basic Principle of PCR

PCR is a chemical method of exponentially increasing the concentration of a specific nucleic acid sequence relative to that of other nucleic acid sequences in the reaction mixture (Bloch, 1991). The principle of the method is shown in Figure 2.4.

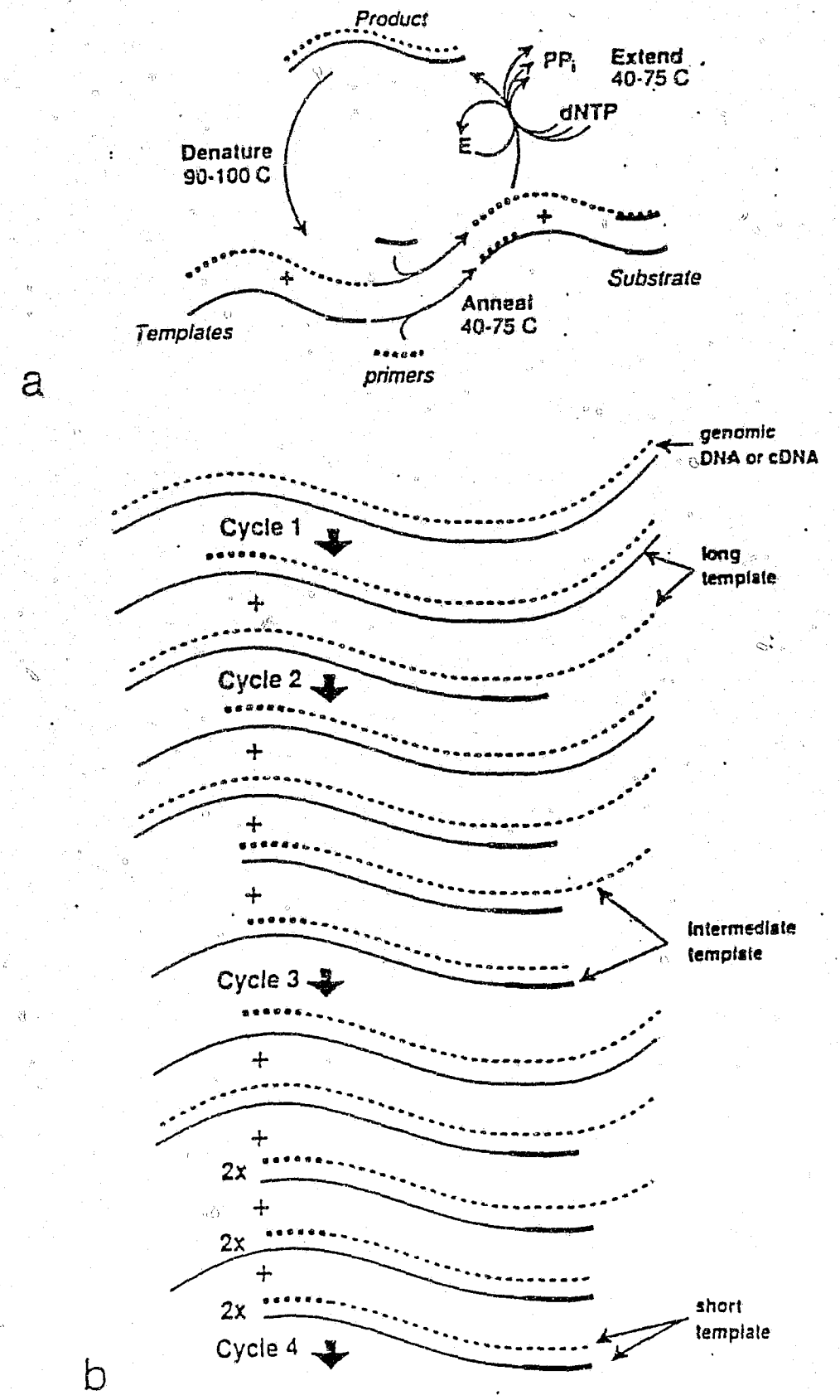


Figure 2.4: Schematic diagram of (a) a single PCR cycle and (b) the generation of the short template over the first few amplification cycles (from Bloch, 1991).

The PCR is an *in vitro* method based on the amplification of specific DNA or RNA fragments of defined length and sequence. Amplification greater than 10^6 fold can be achieved from very small amounts of complex template. This is achieved by means of two flanking oligonucleotide sequences (primers), and repeated cycles of amplification with the enzyme *Taq* DNA polymerase.

The template DNA is added to a reaction mixture containing two synthetic oligonucleotide primers, and is first **denatured;** this is accomplished by heating the double stranded (ds) DNA target to a temperature above its melting point (90-99°C) for up to several minutes. The primers are then **annealed** to complementary strands of the target DNA with their 3' ends pointing towards each other. The temperature is lowered sufficiently (40-75°C) for annealing between the primers and the template, but is left high enough to prevent mismatch hybridization of the primers to similar sequences elsewhere in the genome. Synthesis/**extension** proceeds from the 3' end of each primer as the polymerase adds new residues to the 3' end of the primer, until the reaction is stopped by raising the temperature to above the melting point for a second time. The product of this reaction is known as the long product and is of indefinite length, as only one end of each primer extension is specified by a primer-complementary sequence (Bloch, 1991).

The second cycle is a repetition of the first cycle, but synthesis on the new strand can only proceed as far as the end of the molecule, which corresponds to the 5' end of the opposite primer (Markham, 1993). After the third cycle, synthesis directed by the products of the first 2 cycles will be bounded at both ends by the primer sequences, and

this short template will accumulate exponentially with subsequent cycles, while the long product will accumulate in a linear fashion. The short template rapidly outnumbers the long product, to the extent that it forms the only detectable product molecules when amplification stops (typically after 30 cycles of denaturation, annealing and extension) (Markham, 1993).

After n cycles, the degree of amplification is 2^n, so that after 20 cycles, 10^6-fold amplification results (Markham, 1993). Exponential accumulation of the short template does not continue indefinitely, and usually enters a plateau phase after approximately 20 cycles, when reagent concentrations become limiting, the enzyme becomes exhausted after repeated heating and high concentrations of product favour re-annealing over new primer binding (Saiki *et al.*, 1988). Accumulation stops altogether at a product concentration of about 10^{-7}M (Bloch, 1991; Markham, 1993).

Reaction components

The standard PCR is usually performed in a 50-100µl volume and in addition to sample DNA (typically 10^2 to 10^5 copies of template), contains 50mM KCl, 10mM Tris-HCl (pH 8.4 at room temperature), 1.5mM $MgCl_2$, 100ug/ml gelatin, 0.25µM of each primer, 200µM of each dNTP (dATP, dCTP, dGTP and dTTP), and 2.5 units of Taq DNA polymerase (Saiki, 1989). Gelatin, which is used to protect the polymerase, is preferred over bovine serum albumin as it is less likely to coagulate during denaturation. Some protocols may also include 10% dimetyl sulfoxide (DMSO) to reduce the secondary structure of the DNA, although DMSO can inhibit the

polymerase to a slight degree and reduce the yield of the amplification product (Saiki *et al.*, 1988).

Thermus aquaticus (Taq) DNA polymerase

The earliest PCR experiments utilized the single-subunit Klenow fragment of *Escherichia coli* DNA polymerase I at a temperature of 37°C (Arnheim & Ehrlich, 1992). However, this enzyme was easily denatured by the high temperatures required for melting therefore fresh enzyme had to be added during every cycle. This loaded the reaction with increasing amounts of protein and was extremely tedious (Saiki, 1989). In addition, the Klenow fragment often produced impure target product (Arnheim & Ehrlich, 1992).

The isolation of a heat-resistant DNA polymerase from a thermophilic bacterium inhabiting hot springs, *Thermus aquaticus* strain YT1, changed PCR into a simple, robust reaction that could be automated (Saiki *et al.*, 1988; Saiki, 1989). Taq DNA polymerase allows primer annealing and extensions to be carried out at an elevated temperature, thereby dramatically increasing the stringency of most PCRs by decreasing mismatched annealing to non-target sequences (Saiki, 1989; Arnheim & Ehrlich, 1992). This increase in specificity increases the yield of the target DNA as the higher temperatures reduce the inhibitory secondary structure of DNA that is often experienced (Saiki, 1989). Furthermore, the improved yield is a result of decreased competition by non-target products for enzyme and primers (Saiki, 1989). The plateau phase is reached later in the Taq PCR than in reactions using the Klenow enzyme, due

to the increased specificity of Taq PCRs (Saiki, 1989). The use of Taq DNA polymerase also allows for the amplification of much larger fragments (up to 10-kb) than does the Klenow fragment (<400-bp) (Saiki *et al.*, 1988; Saiki, 1989; Bloch, 1991).

Properties of Taq DNA polymerase

The temperature optimum for DNA synthesis with Taq DNA polymerase is 75-80°C, depending on the template (Tindall & Kinkel, 1988), with a K_{cat} approaching 150 nucleotides/sec/enzyme molecule (Gelfand, 1989). The enzyme has a high rate of processivity under suitable conditions and has been reported to have an extension rate of > 60 nucleotides per second at 70°C (Gelfand, 1989). The extension and processing rates are temperature dependent and are both reduced at lower temperatures (Arnheim & Ehrlich, 1992). The enzyme is relatively stable, and is not denatured irreversibly when exposed to temperatures ≥ 90°C (Gelfand, 1989).

Purified Taq DNA polymerase does not contain measurable 3'→5' exonuclease "proof-reading" activity, but does contain a 5'→3' strand replacement exonuclease activity that is DNA synthesis-dependent (Tindall & Kinkel, 1988; Saiki, 1989). Biochemical studies indicate that the "non-proofreading" Taq DNA polymerase produces single-base substitution errors at a rate of 1 per 9000 nucleotides synthesized and frameshift mutations at a rate of 1/41 000 (Tindall & Kinkel, 1988; Mattila *et al.*, 1991).

Taq DNA polymerase is sensitive to magnesium ion concentrations and to the nature

and concentration of monovalent ions present (Wada *et al.*, 1994). Concentrations of Mg^{++} that are higher than 2.0mM tend to be inhibitory, with 40-50% inhibition at 10mM $MgCl_2$ (Wada *et al.*, 1994). Modest concentrations of KCl (optimum at 50mM), tend to increase the synthesis rate of the enzyme, although higher KCl concentrations are inhibitory (Wada *et al.*, 1994). Low concentrations of urea, DMSO, DMF or formamide do not affect the incorporation activity of Taq DNA polymerase, although the presence of 10% DMSO in a 70°C Taq DNA polymerase activity assay inhibits DNA synthesis by 50% (Gelfand, 1989). DMSO may affect the melting temperature (T_m) of the primers, the thermal activity profile of Taq DNA polymerase and/or the degree of product strand separation achieved at a particular denaturation temperature (Saiki *et al.*, 1988; Gelfand, 1989).

PCR specificity, sensitivity and optimization

Specificity

The specificity of PCR is achieved by 2 factors: firstly, annealing the primers in the correct orientation within a span of less than 10^4 nucleotides (Bloch, 1991). The specificity of the primer-target template versus nonspecific DNA interaction is temperature-and salt concentration-dependent (Arnheim & Ehrlich, 1992). Secondly, specificity is achieved through the use of a thermostable enzyme that allows the annealing and extension temperatures to be raised to the highest value at which the lower melting primer-template hybrid is stable (Bloch, 1991). Specificity can be increased by using "nested priming", that involves 2 different rounds of PCR: after an

initial round of amplification with one set of primers, a small aliquot of the PCR mixture is removed and amplified in a second round with either 2 new primers that are internal to those used in the first round and that define a target that lies within the sequence specified by the original primers (nesting), or with one new internal primer and one of the original primers (hemi-nesting) (Haqqi *et al.*, 1988; Bloch, 1991; Arnheim & Ehrlich, 1992).

Several other factors can potentially affect the specificity of the amplification reaction. The nucleotide concentration must be enough to ensure saturation of the enzyme, as low dNTP concentrations promote misincorporation (Arnheim & Ehrlich, 1992). The primer concentration must be high enough to anneal rapidly to the denatured target DNA and in later stages, the primers must be able to anneal faster than the target-target reassociation (Arnheim & Ehrlich, 1992). Denaturation must be efficient, but not prolonged, as this would lead to instability of the Taq DNA polymerase (Lynch & Brown, 1990).

The reaction can also be affected by the sequence and length of the target. Strong secondary structure in GC-rich areas of denatured strands can prevent the enzyme from reading the template (Saiki, 1989), while the larger the sequence, the less efficient the PCR (Markham, 1993). Robust amplification of target DNA greater than 5-kb has been difficult. However, recent experiments (using a two-polymerase system with 3'→5' exonuclease activity) have shown the successful amplification of targets up to 22-kb (Jeffreys *et al.*, 1990; Barnes, 1994; Cheng *et al.*, 1994). Complementarity between primers can hinder the reaction. When the 3' ends of the primers are complementary,

the formation of primer artifacts (known as "primer dimers") is enhanced (Watson, 1989). In the presence of complementarity, the partially hybridized primers are extended by the enzyme and are amplified, sometimes monopolizing the reaction (Watson, 1989). In the absence of obvious complementarity, it has been proposed that the enzyme may transiently hold two single-stranded DNA molecules such that their 3' ends are adjacent and may be extended. Any extension increase the stability of the complex, resulting in the formation of primer artifacts of dimer size or larger (Watson, 1989).

Sensitivity and contamination

The many cycles needed in PCR for the detection of a unique gene sequence that is present in one or a few cells, increases the opportunities for mispriming and primer artifact formation (Lynch & Brown, 1990). Both mispriming and primer artifacts decrease the sensitivity of PCR because of competition for PCR reagents, especially enzyme (Lynch & Brown, 1990). "Hot start" PCR, which excludes a critical reagent (such as enzyme or magnesium) from the reaction until it has reached denaturation point, is a strategy that minimizes such artifacts (Saiki, 1989).

The immense sensitivity of PCR, which is its greatest asset, can also be a limitation with respect to contamination. Since exponential amplification of target DNA can be achieved from single cells, air-borne cellular debris or contaminated reagents present a serious risk of false results (Saiki *et al.*, 1988). Scrupulous "housekeeping" is therefore p[illegible]t in [illegible]ing contamination. PCR reactions should be prepared and

analysed in separate laboratory areas. Other precautions include the use of dedicated pipettes (i.e. pipettes that are used solely for PCR), aliquotting and autoclaving reagents, and the use of positive displacement pipettes to minimize aerosolization. Enzymatic or photochemical sterilization may also be employed (Kwok, 1989).

Optimization

Primer selection

a) Where possible, select primers with a random base distribution and with an average GC content of 50% - the GC content should be similar to that of the target sequence. Avoid primers with long stretches of polypurines, polypyrimidines or unusual sequences (Saiki *et al.*, 1988; Saiki, 1989).

b) Avoid sequences with substantial secondary structure, especially at the 3' end of the primer (Saiki *et al.*, 1988; Saiki, 1989).

c) Check the primers against each other for complementarity, especially avoiding primers with 3' overlaps, which could form dimers (Watson, 1989). Primers that bind to each other will not amplify the target. Many computer software packages are available that make the task of primer design easy. Most primers should be 20 to 30 bases in length, although longer primers can enhance the specificity of the reaction (Saiki, 1989).

The PCR buffer

The PCR reaction buffer is important in determining the outcome of the amplification. The $MgCl_2$ concentration is crucial and can affect the specificity and yield of the reaction (Markham, 1993). Excess Mg^{++} results in the accumulation of non-specific amplification products, while insufficient magnesium reduces the yield (Saiki *et al.*, 1988). Concentrations of 1.5mM are often optimal. High concentrations of deoxynucleotide triphosphates (dNTPs) promote "thermodynamic infidelity" or misincorporation by the enzyme. Concentrations of 200μM have proved to be sufficient to synthesize 25 μg of DNA. The concentration of dNTPs (which bind Mg^{++} quantitatively) present in the reaction will determine the amount of free magnesium available and as a result, any significant changes in the dNTP concentration should be accompanied by a compensatory change in $MgCl_2$ (Saiki, *et al.*, 1988; Saiki, 1989). Concentrations of Taq DNA polymerase that exceed 4 units per 100μl promote the formation of non-specific PCR products and reduce the yield of target DNA (Saiki *et al.*, 1988).

Cycling parameters

The polymerase chain reaction is performed by incubating the samples at temperatures that correspond to the denaturation, annealing and synthesis/extension steps in an amplification cycle (Williams, 1989). Insufficient denaturation will result in no amplification, while extensive denaturation throughout the reaction may reduce the activity of the enzyme (Saiki, 1989). The annealing temperature is determined by the

length and GC content of the primers, as the T_m of a nucleic acid duplex increases with its length and with increasing (G+C) content. High annealing temperatures (usually > 55°C) increases the specificity of the reaction, although too high annealing temperatures result in too little product, owing to the reduced likelihood of primer annealing (Williams, 1989). The length of the extension is determined by the length of the target sequence - it is useful to begin by allowing 1 minute for each kilobase of sequence (Saiki, 1989). The extension step can be eliminated if the target DNA is ≤ 150-bp, as the polymerase retains sufficient activity at lower temperatures and complete extension will occur during the transition from the annealing step to the denaturation step (Saiki, 1989).

2.1.6 Aims of this study

The present study was undertaken:

(a) to confirm reported frequencies of α_1-AT variants in asthmatic patients and control individuals in South Africa,

(b) to investigate the frequency of the two haplotypes of the M1 phenotype of α_1-AT, M1(ala^{213}) and M1(val^{213}), in asthmatic and non-asthmatic individuals, and

(c) to find out whether there is any association between the variants other than M1M1 of α_1-AT and the severity of asthmatic patients.

2.2 Materials and Methods

2.2.1 Materials

The following materials were obtained from Promega (Madison, WI): Agarose, acrylamide, dNTPs, Taq DNA polymerase, Bst EII restriction endonuclease and proteinase K; the oligonucleotide primers were synthesized by Genosys Biotechnologies Inc. (Woodlands, TX); the bis-acrylamide, ammonium persulfate and TEMED were from BioRad (Hercules, CA); the Ampholines were supplied by Pharmacia (Uppsala, Sweden); the double-distilled water was from SABAX (Johannesburg); PCR tubes were supplied by Laboratory and Scientific Equipment Co. (Johannesburg); the Coomassie Brilliant Blue and the mineral oil were from Sigma Chemical Co. (St. Louis, MO); the Whatman filter paper was from Whatman International (England); the DNA molecular weight markers were supplied by

BioVentures Inc (Murfreesboro, TN); the 2ml eppendorf tubes were supplied by Treff Laboratories (Switzerland); all other reagents used were of analytical grade (Merck, Darmstadt, Germany).

2.2.2 Subjects

Twenty-nine white asthmatics (10 female) and 30 black asthmatics (12 female) were recruited from the Asthma and Respiratory Clinics of Hillbrow and Johannesburg Hospitals and from Krugersdorp Hospital (Paediatrics). The thirty-six black controls (10 female) and 54 white controls (20 female) were either blood donors who presented themselves at the South African Blood Transfusion Services in Johannesburg or outpatients at the Johannesburg Hospital Paediatric Nephrology Clinic. None of the controls had a history of atopy or any disease process associated with the respiratory system. All individuals who participated in the study were 4-45 years of age, and each subject gave informed consent (or in the case of minors, consent was obtained from parents) to participate in the study. The Committee for Research on Human Subjects of the University of the Witwatersrand approved this protocol.

2.2.3 Asthma and Atopy

Each patient had a history of bronchial asthma as defined by the American Thoracic Society, namely a history of transitory or prolonged episodes of dyspnea, coughing and wheezing, but was otherwise healthy (American Thoracic Society Statement, 1962). The diagnosis was confirmed in each patient by the finding of at least one of the

following: a forced expiratory volume in one second (FEV) < 70% of the predicted value with reversibility of 20% or more following inhalation of a β_2-adrenergic bronchodilator, or a fall in peak expiratory flow rate of at least 20% during a standardised exercise test.

Atopy was defined as a positive skin prick test at least 2mm greater than a negative control and a positive specific IgE titre (>0.35kU/L) or a high concentration of total serum IgE greater than published normal values for children, or greater than 400kU/L in adults. Asthma was classified as mild, moderate or severe using the classification of Scheffer (1991).

2.2.4 DNA extraction

The polymerase chain reaction (PCR) requires a source of nucleic acid (DNA or RNA) for amplification. The technique is highly tolerant of impurities in the DNA sample that is being amplified, and the degree of nucleic acid purification necessary before PCR-amplification depends on the complexity and chemistry of the sample matrix, as well as on the target sequence. It is usually necessary to deproteinase a biological sample to ensure the removal of extraneous proteases, nucleases and phosphatases that might destroy reactants that are crucial for the amplification reaction (Bloch, 1991).

Conventional DNA extraction methods usually employ detergents to solubilize cell components and proteolytic enzymatic degradation of DNA-bound proteins (Maniatis *et al.*, 1982). This step is followed by extraction with organic solvents such as phenol

and precipitation of the nucleic acid with ethanol (Maniatis *et al.*, 1982). Although this standard technique is PCR-compatible, a variety of faster, simpler methods have emerged. The method developed by Higuchi (1989), and which was used in the present study, allows for the efficient release of DNA from nucleated blood cells. It obviates the use of organic extraction and ethanol precipitation, and instead allows for the direct addition of cells or nuclei to a PCR-compatible solution that contains nonionic detergents (which lyse cell membranes by removing integral proteins) and proteinase K (which can be inactivated by heat). This method efficiently releases DNA from $\pm 3x10^5$ nucleated cells, and the DNA can then be amplified by PCR with little or no inhibition (Higuchi, 1989). Porphyrins, which are derived from heme present in blood, may be the most potent cell components inhibitory to PCR and in order to separate porphyrins from nuclear DNA, this extraction method lyses cell membranes and pellets nuclei and cell debris. A number of pelleting and washing steps remove haemoglobin that is released from blood cells, as well as RNA and cytoplasmic DNA. The resulting pellet, containing nuclear DNA, is finally resuspended in proteinase K/detergent solution. The final yield is approximately 20µg of nuclear DNA/1ml of blood (Higuchi, 1989).

Lysis buffer

0.32M sucrose	10.9g
10mM Tris base	0.121g
5mM $MgCl_2$	0.101g
1 % Triton X	1mL

Dissolve the above reagents, except the Triton X, in 90mL double-distilled (dd) H_2O, adjust pH to 7.5 with HCL, add 1mL Triton X and make up to a final volume of 100mL with ddH_2O. Autoclave and store at 4°C (or in aliquots at -20°C for long-term storage).

PCR/Storage buffer

50mM KCl	0.375 g
2.5mM $MgCl_2$	0.050 g
10mM Tris base	0.121 g
0.1mg/ml gelatin	0.010 g
0.45% "Nonidet" P40 (NP40)	45.0 µL
0.45% Tween 20	45.0 µL

Dissolve the above reagents, except the NP40 and the Tween 20, in 90 mL ddH_2O, adjust the pH to 8.3 with HCl, add the NP40 and the Tween 20 and make up to a final volume of 100 mL with ddH_2O. Autoclave and store at 4°C (or in aliquots at -20°C for long-term storage).

Extraction protocol

Ten millilitres of venous blood was collected from all subjects into EDTA-coated tubes and DNA was extracted from the blood using the following modification of the Higuchi (1989) method.

1. Mix 1000 µL EDTA-containing blood with 1000 µL lysis buffer in a 2 mL eppendorf tube.
2. Centrifuge at 6000x*g* for 15 seconds.
3. Carefully pour out the supernatant, add 1 mL lysis buffer and resuspend the pellet by gentle pipetting.
4. Repeat steps 2-3 twice (no more than 4 washes should be used, as more washes result in degradation of the DNA).
5. Centrifuge at 6000x*g* for 15 seconds.
6. Remove supernatant and carefully resuspend pellet in 300 µL of PCR buffer. Add 10 µL proteinase K (10 mg/mL).
7. Incubate at 56°C in a water bath for 1-3 hrs (not longer than 3 hrs).
8. Inactivate the protease by boiling the samples at 95°C for 10 min.

Twenty-five µL of this lysate is equivalent to 1 µg of genomic DNA.

The integrity of the extracted DNA was determined by electrophoresis in 0.7% (w/v) agarose gels in 1xTBE buffer (0.089M Tris-borate, 0.002M EDTA, pH 8.2) at 100V for 20-30 min (Appendix II).

2.2.5 α_1-AT phenotype study

Blood specimens were collected into EDTA-containing tubes. Plasma was separated on the day of collection and stored at -60°C until analysed. α_1-AT phenotypes were identified by isoelectric focussing in polyacrylamide gels. Polyacrylamide gel electrophoresis is one of the most useful methods for the analysis of protein mixtures. Isoelectric focussing (IEF) is used for the fractionation of molecules that only differ in net charge (Andrews, 1987). The technique is performed in non-sieving media (such as polyacrylamide gels) and is capable of very high resolution, especially when shallow, immobilized pH gradients are used (Andrews, 1987). In addition, macromolecules that differ in their isoelectric point by as little as 0.001 pH units can be separated efficiently (Robard *et al.*, 1974).

The technique is based on a moving boundary, and amphoteric substances (e.g. amino acids and peptides) are separated in an electric field which has both pH and voltage gradients (Davis, 1986). The anode region has a lower pH than the cathode area and a suitable pH is maintained between the electrodes - the pH range is chosen such that the molecules being separated have their isoelectric points within that range (Davis, 1986). Substances are positively charged at pH regions below their isoelectric point and they migrate toward the cathode, but as they migrate, the pH increases and the substances become zwitterions with no net charge so that migration stops (Davis, 1986). Similarly, substances which at first occur at pHs above their isoelectric point is reached, at which stage migration ceases (Davis, 1986). As a result, amphoteric substances are focussed into narrow stationary bands (Davis, 1986). IEF is a principal

technique for the analysis and characterization of proteins, peptides, glycoproteins, lipoproteins, phosphoproteins, cell membrane proteins, isoenzyme patterns and studies with nucleic acids and glycosaminoglycans (Andrews, 1987). IEF is thus used in clinical, forensic and genetics laboratories for the separation and identification of serum proteins (Davis, 1986).

Detection of proteins after electrophoresis is most commonly done by staining with the organic dye Coomassie Brilliant Blue, the principle being that electromorphs take up a general protein stain (Constans *et al.*, 1980). This detection method is common due to the fact that it produces little background staining, because the colloidal form of the dye does not enter the gel (Nivinskas & Cole, 1996).

The α_1-AT phenotypes were identified using the following modification of the IEF method by Constans *et al.* (1980). A 0.5mm thick gel was prepared by dissolving 0.97g acrylamide, 0.03g bis-acrylamide and 2.67g sucrose in 19.3 mL of water. One millilitre of Ampholines, pH 4.2-4.9 and 30 µL of TEMED were added and the solution was degassed for 3 min. Thereafter 1 mL of a freshly prepared 10% ammonium persulfate stock solution was added and the gel was poured into a 124x257x0.5mm LKB Multiphor gel mould. The gel was allowed to set for 30 min at room temperature, and thereafter refrigerated (4°C) for 15 min. Electrode strips (17mm Whatman or LKB paper wick 1850-911) were soaked with 0.1 M sodium hydroxide (0.4 g/100 mL distilled water) for the cathode (the negative electrode) and 0.04 M phosphoric acid (0.5 mL/100 mL distilled water) for the anode (the positive electrode). The gel was pre-focussed for 30 min at 800 V, 10 mA, 10 W. The samples

were applied on paper inserts (17 mm Whatman) to the cathode end of the gel and were focussed for 45 min at 500 V, 8 mA, 8 W. The inserts were then removed and the gel focussed for a further 1 hr 45 min at 1200 V, 14 mA, 14 W. Thereafter, the bands were sharpened for 45 min at 2500 V, 14 mA, 8 W. After migration, the gel was stained in a 0.1% solution of Coomassie Brilliant Blue (45% methanol, 10% glacial acetic acid) for 1 hr at 37°C and rinsed in distilled water. Following destaining (45% methanol, 10% glacial acetic acid), the phenotypes were read (Figure 2.5 & 2.6).

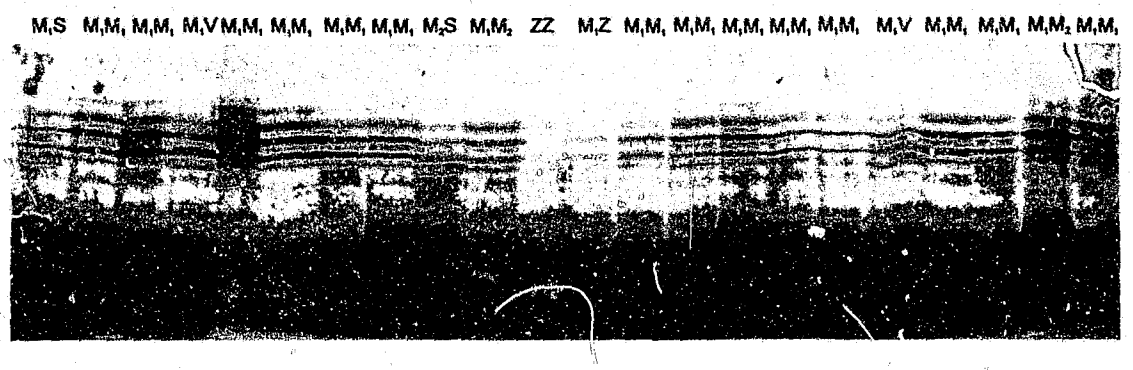


Figure 2.5: Selected α_1-AT variants observed in human sera using isoelectric focussing in ultrathin polyacrylamide gels. The anode is at the top.



Figure 2.6: Selected α_1-AT variants observed in human sera using isoelectric focussing in ultrathin polyacrylamide gels, including the S and V allele. The anode is at the top.

2.2.6 α_1-AT genotype study

Total genomic DNA was extracted from EDTA-containing whole blood according to the Higuchi method (1989), modified as described previously. The polymerase chain reaction (PCR) was used to amplify exon 3 of the α_1-AT gene containing an obligatory and a polymorphic BstE II restriction site, used in order to differentiate the M1 subtypes (Gaillard *et al.*, 1994). The following 30mer primers were used (Schwartz *et al.*, 1989).

153: 5' -CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG- 3'

155: 5' -GGG CCT CAG TCC CAA CAT GGC TAA GAG GTG- 3'

The amplified sequence is 360-bp long. This sequence has an obligatory and a polymorphic BstE II recognition site. The fragment will either be cut into two (310-bp + 50-bp) or three (227-bp + 83-bp + 50-bp) fragments following digestion with the enzyme BstE II (Figure 2.7). Amplification (by PCR) was performed in a reaction volume of 63 µL, containing 1 µg of genomic DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (0.01% gelatin) and 1 µM each appropriate amplification primer (Appendix I).

The reaction mixture containing all the components, except the Taq DNA polymerase, was overlaid with 50 µL mineral oil and heated to 90°C for 10 min in a Hybaid DNA thermal cycler (Hybaid, UK). Following a cooling step, which reduced the sample

temperature to 56°C , 1 unit of Taq DNA polymerase was added. The samples were then processed through 30 cycles of : denaturation at 92°C for 2 min, annealing at 56°C for 2 min and extension at 72°C for 2 min. The final step was one of extension at 72°C for 5 min.

Restriction analysis of the amplified fragments of exon 3 of the α_1-AT gene was performed as follows:

10 µL of the PCR reaction mixture was digested with 2 units of BstE II and incubated at 37°C for 1 hr. Resolution of the restricted products was performed by electrophoresis in 2% agarose gels. Following electrophoresis, gels were stained with ethidium bromide (0.5 µg/mL) in 1xTBE buffer (0.089 M Tris-HCL, 0.002 M EDTA, pH 8.2), viewed under UV illumination (302nm) and photographed with a Polaroid CU-5 Land camera.

Direct sequencing was carried out using the Thermo Sequenase kit and ^{33}P labelled terminators (Amersham Life Science, Cleveland, USA), (Appendix III).

2.2.7 Statistical ana

The comparison of the distribution of the different haplotypes in the various populations and the association of the different variants with severity was done by means of the Fisher's exact test.

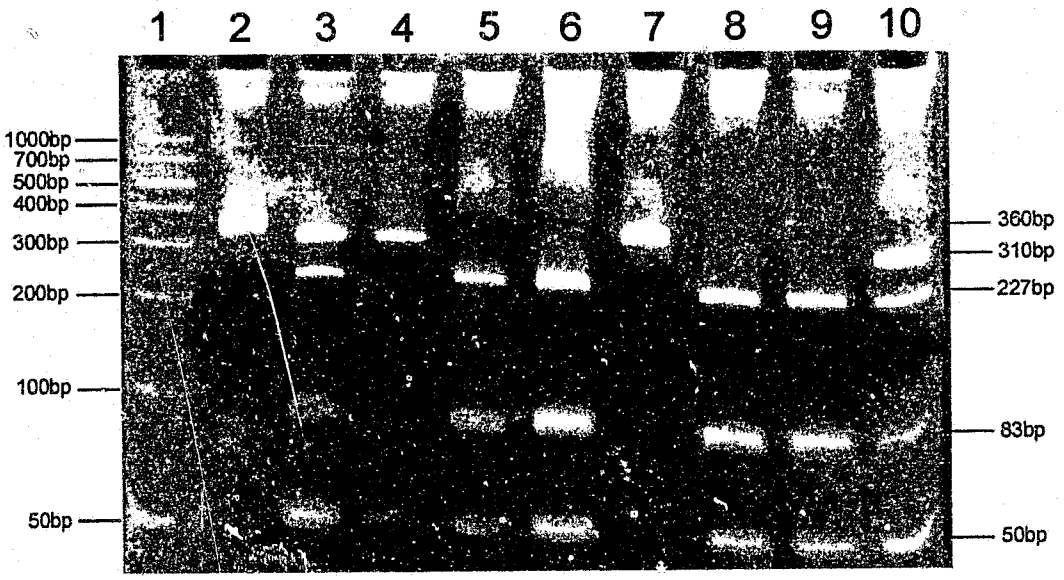


Figure 2.7: Bst EII restriction fragment length polymorphism (RFLP) analysis of exon 3 of the α_1 AT gene, for differentiation of the M1(Ala^{213}) and M1(Val^{213}) subtypes. Lane1, DNA molecular weight markers; lanes 2 & 7, undigested PCR products; lane 4, M1(Ala^{213}) homozygote; lanes 3 & 10, M1(Val^{213})M1(Ala^{213}) heterozygotes; lanes 5, 6, 8 & 9, M1(Val^{213})M1(Val^{213}) homozygotes.

2.3 Results

Table 2.1: Haplotype frequency of α_1-AT type M1(Ala^{213}) and M1(Val^{213}) among black groups having the M1 allele for α_1-AT.

Asthmatic Patients				
Phenotype	**Number**	**Haplotype**	**Number**	**Haplotype Frequency**
M1(Val^{213})M1(Val^{213})	7	M1(Val^{213})	28	0.46
M1(Val^{213})M1(Ala^{213})	14	M1(Ala^{213})	32	0.53
M1(Ala^{213})M1(Ala^{213})	9			
Total	31		60	
Control Individuals				
Phenotype	**Number**	**Haplotype**	**Number**	**Haplotype Frequency**
M1(Val^{213})M1(Val^{213})	8	M1(Val^{213})	32	0.45
M1(Val^{213})M1(Ala^{213})	16	M1(Ala^{213})	39	0.54
M1(Ala^{213})M1(Ala^{213})	11			
M1(Ala^{213})V	1			
Total	36		71	

Table 2.2: Haplotype frequency of α_1-AT type M1(Ala^{213}) and M1(Val^{213}) among white groups having the M1 allele for α_1-AT.

Asthmatic Patients				
Phenotype	**Number**	**Haplotype**	**Number**	**Haplotype Frequency**
M1(Val^{213})M1(Val^{213})	8	M1(Val^{213})	31	0.69
M1(Val^{213})M1(Ala^{213})	10	M1(Ala^{213})	14	0.31
M1(Ala^{213})M1(Ala^{213})	1			
M1(Val^{213})Z	2			
M1(Val^{213})S	1			
M1(Val^{213})M2	1			
M1(Ala^{213})M2	2			
M1(Val^{213})E	1			
Total	26		45	
Control Individuals				
Phenotype	**Number**	**Haplotype**	**Number**	**Haplotype Frequency**
M1(Val^{213})M1(Val^{213})	26	M1(Val^{213})	77	0.79
M1(Val^{213})M1(Ala^{213})	15	M1(Ala^{213})	20	0.21
M1(Ala^{213})M1(Ala^{213})	2			
M1(Val^{213})S	2			
M1(Val^{213})M2	8			
M1(Ala^{213})M2	1			
Total	54		97	

Three asthmatic patients of the 29 had the following phenotypes containing no M1: 1 M2M2, 1 M2S and 1 SS and were therefore excluded from the table.

Table 2.1 and 2.2 show the haplotype frequencies of α_1-AT in the four different population groups. There was a significant increase in the M1(Ala^{213}) haplotype in the black groups as compared to the white groups (p=0.0029). When comparing the phenotypes of α_1-AT, a significant difference was found between the white asthmatics and the white control group (p=0.0001), whereas no significant difference was found between the corresponding black groups (p=0.2867). A significant difference was found between all white asthmatic patients compared to all black asthmatic patients (p=0.0024).

A significant difference was also found when comparing 16 patients with severe asthma, 7 of whom had the rarer variants of α_1-AT (including 2 M1Z, M1S, M1M2, M2S and SS) as compared with 14 patients with mild or moderate asthma with the M1M1 phenotypes , (p=0.045). A new variant, the $M1E_{(JOHANNESBURG)}$ was also identified (Figure 2.8) in a moderate asthmatic (plasma level 1.51 g/L). Of further interest, his asthmatic mother was also Pi M1E (plasma level 1.60 g/L). The patient with the SS variant (plasma level 0.8 g/L), which is extremely rare, was a severe asthmatic patient. The mutation was confirmed by direct sequencing of exon III of the α_1-AT gene (Figure 2.9). Two patients had the M1Z variant, one of whom was a child with severe asthma and the other was an adult with severe asthma (plasma level 0.5 g/L) on oral corticosteroid medication and who was also suffering with gastroesophageal reflux.

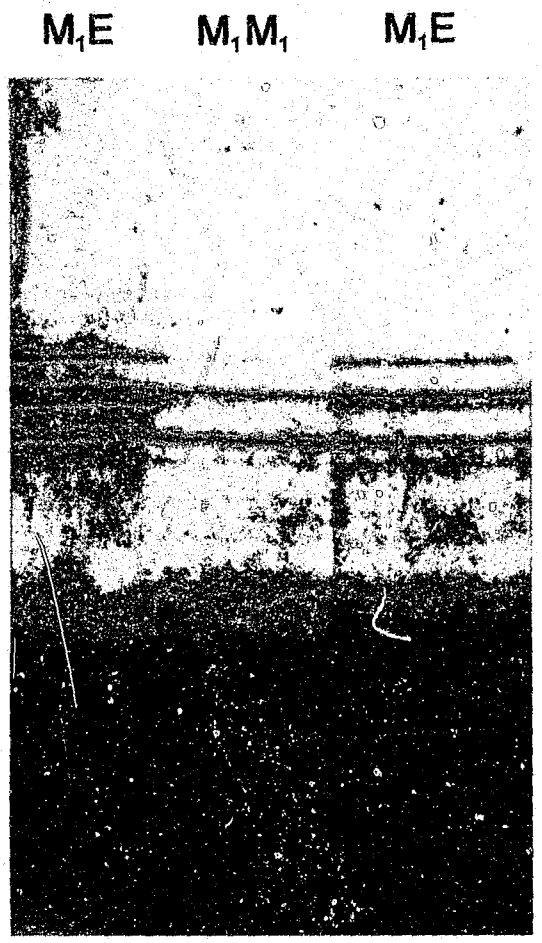


Figure 2.8: Selected α_1-AT variants observed in human sera using isoelectric focussing in ultrathin polyacrylamide gels, including the $M_1E_{(JOHANNESBURG)}$. The anode is at the top.

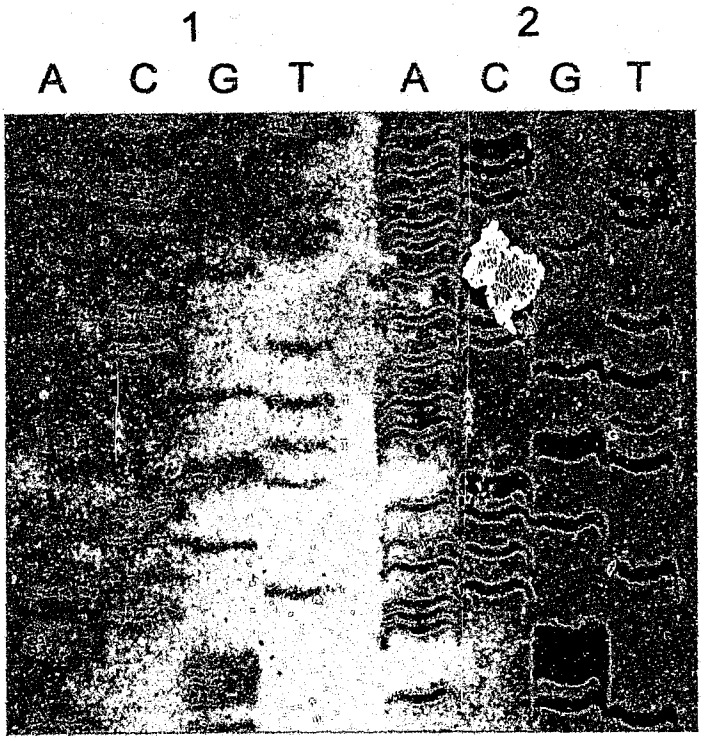


Figure 2.9: Direct sequencing of exon III of the α_1-AT gene indicating a homozygote (1) and a heterozygote (2) individual for the SS mutation indicating the polymorphic site resulting in a Valine ⟶ Glutamate at position 264.

2.4 Discussion

The incidence of the M1(Ala^{213}) haplotype was significantly higher in the black groups than in the white group (p=0.0024). In the black groups there was no difference in the incidence of the M1(Ala^{213}) haplotype between the asthmatic patients and the control individuals. However, in the corresponding white groups, a significant difference was found (p=0.0001). A previous study by Gaillard *et al.*, (1994) showed that the M2 phenotype of α_1-AT in asthmatics was significantly increased and was also associated with significantly lower elastase inhibitory capacity than that of the control group. This population difference confirms the results of Gaillard *et al.*, (1994). This previous study also showed a decrease in elastase inhibitory capacity (EIC) associated with the M1(Ala^{213}) haplotype. The increase in this haplotype in the black population is interesting as asthma is more severe in this group.

The differences between α_1-AT phenotypes involve simple amino acid substitutions. The two most common M1 phenotypes differ by having either valine or alanine at position 213. The normal M2 subtype differs from M1(Val^{213}) by two substitutions: histidine for arginine at the 101 position and aspartic acid for glutamic acid at the 376 position (Crystal *et al.*, 1989). Two important deficiency variants, Z and S, are associated with deficient plasma α_1-AT levels which, in the case of the former, predisposes to emphysema. The Z molecule differs from the M1(Ala^{213}) by the substitution of lysine for glutamic acid at the 342 position, whereas the S molecule differs from the M1(Val^{213}) by the substitution of valine for glutamic acid at the 264 position (Brantly *et al.*, 1988).

The Z allele is associated with markedly reduced plasma levels of α_1-AT, directly as a result of the Glu^{342}→Lys^{342} substitution, which appears to interfere with the salt bridge to Lys^{290} causing a change in the configuration of the molecule (Loebermann *et al.*, 1987). It is also clear that the Z molecule is qualitatively deficient, being unable to maintain a complex with elastase (Ogushi *et al.*, 1987). The precise mechanism of this abnormality is not understood, but it may also relate to the altered three dimensional configuration of the molecule. The S allele is also associated with low plasma α_1-AT levels, apparently due to molecular instability with intracellular destruction (Curiel *et al.*, 1988). It is functionally normal and does not appear to confer a risk of emphysema even in its homozygous (SS) form (Ogushi *et al.*, 1988). The mechanism by which the S mutation results in plasma defiency is unclear and has been attributed to increased clearance, aberrant protein folding and intracellular degradation (Elliott *et al.*, 1997). It has been suggested that the defiency of S antitrypsin results from a slow transition to form dimers and short chain polymers that result in a mild reduction in secretion from the hepatocyte and an increased rate of turnover in the plasma. The crystal structure of S antitrypsin shows that the S mutation breaks the Glu264→Tyr38 salt bridge which would predictably perturb the Phe52→Ser53 region at the commencement of the β helix and hence favour polymerisation as occurs more dramatically with the Siiyama and Mmalton mutants (Elliott *et al.*, 1997). Among populations of asthmatic patients, those heterozygous for the deficient α_1-AT variants may have more severe disease as manifested by worse spirometric results, poor bronchodilator response and steroid dependency (Gaillard *et al*, 1992).

The present study has shown a significant association between asthma severity and those asthmatic patients having a rare variant as compared to the asthmatic patients with the normal M1M1 phenotype. In this study two unusual cases were found. The patient with the SS phenotype is a severe asthmatic with a significantly reduced plasma level and one of the patients with the M1Z was also diagnosed as having gastroesophageal reflux. Gastroesophageal reflux (GER) occurs commonly in asthmatics and in selected patients appears to contribute to the state of bronchospasm. The contribution of GER to the inflammatory state remains to be adequately evaluated. The mechanism by which GER potentiates bronchoconstriction is likely to be multifactorial, with the asthma itself worsening reflux, leading to a vicious cycle (Ducoloné *et al.*, 1987 & Simpson, 1995). This is interesting in the light of the gene for myosin light chain being located on chromosome 14. The identification of a new variant, $E_{(JOHANNESBURG)}$ (Figure 2.8), is a novel finding and strengthens the link between α_1-AT and atopy on chromosome 14. This new variant has not as yet been fully characterised however it is possible that it could play a prominent role in the pathogenesis of disease. It has been reported that a significant proportion of patients with severe α_1-AT deficiency and advanced emphysema show clinical features of asthma, and that asthma appears to be more common in patients with this condition than in those with COPD and a normal Pi phenotype (Eden *et al.*, 1997).

Several deficiency alleles have been reported in which the plasma concentration of α_1-AT is detectable against standards and is generally in the range of about 2 to 15 percent of normal. All have an electrophoretic mobility different from that of the PI Z variant (Cox, 1995), just like the $M1E_{(JOHANNESBURG)}$ variant. PI $Z_{AUGSBURG}$ and PI

$Z_{WREXHAM}$ have the Z mutation as well as another benign amino acid difference. PI M_{MALTON} is associated with PAS-positive hepatocyte inclusions identical with those found in association with PI Z, and, like Z, the protein has a tendency to aggregate (Cox, 1995). PI M_{DUARTE}, first reported in a 48-year-old woman with severe bullous emphysema, migrates in acid starch gel and agarose similarly to PI M, and the isoelectric point is similar to that of M3, as determined by isoelectric focussing. PAS-D globules were present in the liver, indicating that this deficient variant also has, like Z, a defect in secretion from the liver (Coz, 1995). Individuals with rare deficiency alleles have the same risk for obstructive lung diseases as PI ZZ individuals. Furthermore, these PI types will produce α_1-AT inclusions in the liver, particularly in heterozygotes, in the presence of an apparently normal M phenotype. When isoelectric focussing is used, the M_{MALTON} α_1-AT can be identified even in the presence of a normal M allele. M_{DUARTE} can be detected in a heterozygote with an S or Z allele but, depending on the degree of resolution with isoelectric focussing, may not be detectable in the presence of a normal M allele (Cox, 1995). Although the PI*F allele is not associated with a decrease of α_1-AT concentration, there is a suggestion that PI FM heterozygotes may be more susceptible to pulmonary function impairment, particularly when exposed to industrial pollutants. α_1-AT of the F type, which has an extra cysteine residue, appears to have an increased tendency to oxidation, as shown by its altered electrophoretic pattern after aging, and this increased tendency to oxidation may make PI FM individuals susceptible to lung destruction in a polluted environment (Cox, 1995). Further characterisation of the new variant found in this study may produce similar results. This study has shown that α_1-AT plays a role in the pathogenesis of asthma.

CHAPTER 3

THE POSSIBLE ROLE OF CORTICOSTEROID BINDING GLOBULIN IN ASTHMA

3.1 Introduction

Corticosteroid binding globulin (CBG) is the major transport protein for glucocorticoids in the blood of almost all vertebrate species, and > 90% of the cortisol in human plasma is bound by this protein. The remaining fraction is distributed more evenly between albumin and the pool of nonprotein-bound or "free" steroid that is generally assumed to be biologically active (Hammond *et al.*, 1987)

In humans, CBG is an acidic glycoprotein of approximately 58 KDa, comprising five N-linked oligosaccharide chains that collectively represent approximately 23% of the molecule by mass. The binding site for natural glucocorticoids appears to be a hydrophobic pocket containing one of two cysteine residues that have been identified by amino acid composition analyses. Like many other plasma transport proteins, CBG is produced and secreted by hepatocytes, but has also been identified in a number of glucocorticoid responsive cells, and may even interact directly with the plasma membranes of some cells (Hammond *et al.*, 1987).

Several human CBG variants with reduced affinity for cortisol have been examined biochemically. After neuraminidase treatment to remove sialic acid, these variants are

characterized by unique isoelectric points that are indicative of primary structural abnormalities (Robinson *et al.*, 1985; Van Baleen *et al.*, 1982). Furthermore, their transmission within families follows an autosomal pattern of inheritance (Van Baleen *et al.*, 1982) consistent with the presence of a single gene on human chromosome 14 (Underhill & Hammond, 1989; Sealing *et al.*, 1990).

The amino acid sequence of human CBG has been deduced from liver and lung cDNA (Hammond *et al.*, 1987), and confirmed by sequencing human genomic fragments that contain the coding region for CBG. Since tissues used to obtain these DNA sequences were taken from different individuals, and all revealed the same coding sequence for human CBG, they appear to encode the consensus primary structure of the normal protein. Analyses of the genes for CBG variants with abnormal steroid binding affinities may therefore be expected to locate amino acids that contribute to the functional integrity of the steroid binding domain (Smith *et al.*, 1992). In this regard, the physicochemical properties of the CBG steroid binding site have been reviewed with respect to phylogenetic comparisons of its primary structure (Hammond *et al.*, 1991) and the tertiary structure of a related protein, α_1-proteinase inhibitor. This has indicated that cysteine 228 in the human CBG polypeptide is located within a proposed β-barrel that is thought to comprise at least a portion of the steroid binding domain (Smith *et al.*, 1992).

Corticosteroid binding globulin (CBG) is involved in control of inflammation through delivery of cortisol to tissue. Both *et al.* (1994) have shown that the gene for CBG and for alpha-1-protease inhibitor are physically close (approximately 80-kb) on

chromosome 14. These genes, they suggest, lie so close that variants of one could act as a marker for variant forms of the other. Furthermore, Hammond *et al.*, (1987) have demonstrated significant homology between the alpha-1-protease inhibitor gene and the CBG gene and have described potential sites for restriction enzyme analysis for polymorphisms. Alpha-1-antitrypsin and CBG share more than 40% homology of amino acid sequence. The genes for both proteins share 56% homology of coding nucleotide sequence, and the genes have identical organisation and intron-exon junctions (Billingsley *et al.*, 1993). Therefore, disease association for alpha-1-protease inhibitor could be due to genetic variation in CBG that inefficiently controls the inflammatory response.

3.2 Detection of point mutations

The ability to detect single-base mutations in human DNA is vital in the diagnosis of a number of important genetic diseases. A range of techniques has been developed for analysing and characterizing new genetic mutations. All these techniques rely on the generation by PCR, of large quantities of DNA from normal and mutant alleles (Markham, 1993), as it is much simpler to analyse PCR-amplified fragments than it is to clone each gene fragment (Arnheim & Ehrlich, 1992). The aim of these techniques is to detect as little as a single base change and this is frequently accomplished by creating heteroduplexes between the two alleles and screening for modified properties in: chemical cleavage reactions; ribonuclease digestion of the duplex, in which one strand is RNA; denaturing gradient gel electrophoresis or; single strand conformation polymorphism analysis (Markham, 1993). Once the mutated area of the gene has been

localized, DNA sequencing of both fragments is necessary to identify the exact nature of the mutation.

3.3 Aims of the study

The possible role of the CBG gene in the pathogenesis of asthma prompted the present study in which the second exon of the CBG gene was screened for the Leu→His substitution in black and white asthmatic and control individuals.

3.4 Materials and methods

3.4.1 Materials

The following materials were obtained from Promega (Madison, WI): Agarose, dNTPs, Taq DNA polymerase and proteinase K; the Thermo Sequenase radiolabeled terminator cycle sequencing kit was from Amersham Life Science (Cleveland, Ohio); the oligonucleotide primers that were used were synthesized by Genosys Biotechnologies Inc (Woodlands, TX); the DNA molecular weight markers were supplied by BioVentures Inc (Murfreesboro, TN); the 2 mL eppendorf tubes were supplied by Treff Laboratories (Switzerland); the PCR tubes were from Laboratory and Scientific Equipment Co. (Johannesburg); the double-distilled sterile water was supplied by SABAX (Johannesburg); all other reagents used were of analytical grade (Merck, Darmstadt, Germany).

3.4.2 Subjects

Six white asthmatics (3 female) and 5 black asthmatics (2 female) were recruited from the Asthma and Respiratory Clinics of Hillbrow and Johannesburg Hospitals and from Krugersdorp Hospital (Paediatrics). The 4 black controls (2 female) and 5 white controls (1 female) were either blood donors who presented themselves at the South African Blood Transfusion Services in Johannesburg or outpatients at the Johannesburg Hospital Paediatric Nephrology Clinic. None of the controls had a history of atopy or any disease process associated with the respiratory system.

All individuals who participated in the study were 4-45 years of age, and each subject gave informed consent (in the case of minors, consent was obtained from parents) to participate in the study. The Committee for Research on Human Subjects of the University of the Witwatersrand approved the protocol.

Atopy and asthma were diagnosed as described earlier in this study (Chapter 2).

3.4.3 PCR amplification of the CBG gene

Total genomic DNA was extracted from EDTA-containing whole blood according to the Higuchi method (1989) modified as described previously. The polymerase chain reaction (PCR) was used to amplify exon 2 of the CBG gene.

The following oligonuceotide primers were used:

CBG1: 5'- GGG GTT TGA ATT CCC CAT TGA CTC CAG AGA- 3'

CBG2: 5' -GGG TTG AAT TCG GAT GGG CCT TCA GAT G- 3'

Amplification (by PCR) was performed in a reaction volume of 63 µL, containing 1 µg of genomic DNA, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (0.01% gelatin) and 1 µM each appropriate amplification primer (Appendix I). The reaction mixture containing all the components except the Taq DNA polymerase, was overlaid with 50 µL mineral oil and heated to 90°C for 10 min in a Hybaid DNA thermal cycler (Hybaid, UK). Following a cooling step, which reduced the sample temperature to 52°C , 1 unit of Taq DNA polymerase was added. The samples were then processed through 30 cycles of : denaturation at 92°C for 2 min, annealing at 52°C for 2 min and extension at 72°C for 2 min. The final step was one of extension at 72°C for 5 min.

Following amplification the PCR products were resolved by electrophoresis in 2% agarose gels. Following electrophoresis, gels were stained with ethidium bromide (10 mg/ml) in 1xTBE buffer (0.089 M Tris-HCl, 0.002 M EDTA, pH 8.2), viewed under UV illumination (302nm) and photographed with a Polaroid CU-5 Land camera.

Sequencing was carried out as described in Appendix III.

3.5 Results

Sequencing analysis (Figure 3.1) did not detect any mutation within the amplified exon of the CBG gene in the asthmatic and control subjects that were tested.

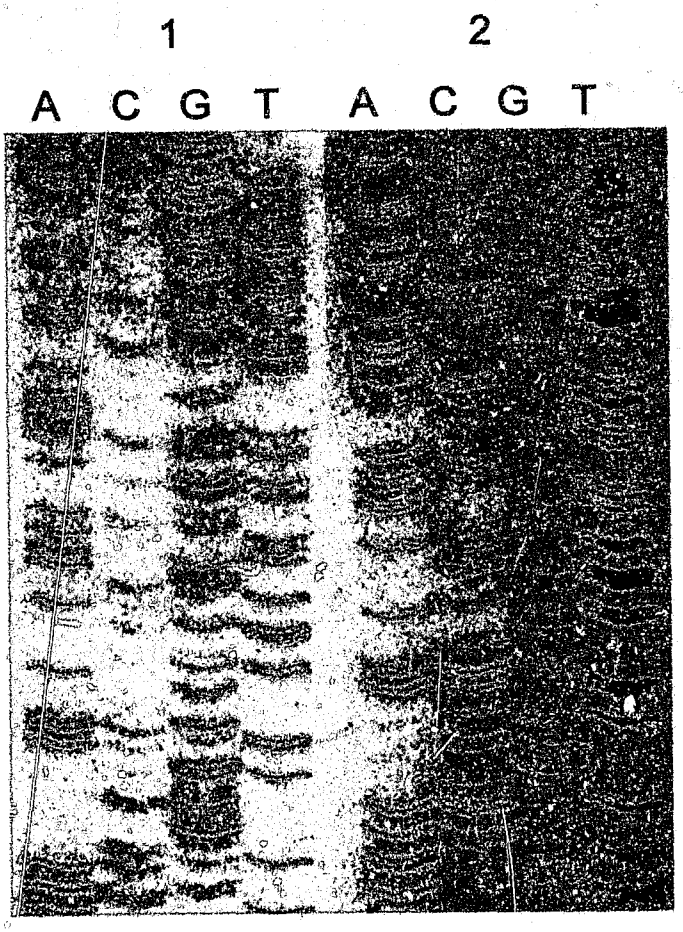


Figure 3.1: Direct sequencing of exon II of the CBG gene, showing no difference between patient (1) and control (2).

3.6 Discussion

CBG and α_1-AT are both members of the serine proteinase inhibitor (serpin) superfamily, and are closely related in terms of primary structure. The primary structures of CBG and α_1-AT are also closely related to thyroxine binding globulin (TBG), and a TBG mutation is located in the same relative position as residue 91 of the CBG molecule (Smith *et al.*, 1992). This mutation (TBG-Asn96) is responsible for a reduction in the thyroxine binding affinity of TBG, and suggests that this region contributes to the ligand binding properties of these serpins, which act as hormone transport proteins (Smith *et al.*, 1992).

The TBG-Asn96 variant is also characterised by reduced stability and this has been associated with the creation of an additional consensus site for N-glycosylation . In a previous study a His93 mutation of the CBG was identified (Smith *et al.*, 1992). This mutation did not appear to influence its stability because the serum CBG concentrations of the carrier increased by approximately 2-fold to within the normal range after recovery from sepsis, in a manner similar to other patients recovering from septic shock. Also, the intracellular processing did not appear to be influenced because the levels of CBG-His93 expression in culture media were similar to those of normal CBG (Smith *et al.*, 1992).

The carrier of this CBG mutation was identified as having a medical history of inflammatory diseases. Therefore, in view of the possible involvement of CBG in the delivery of natural glucocorticoids to sites of inflammation (Hammond *et al.*, 1990),

it is tempting to speculate that these mutations may have in some way contributed to the development of these diseases and the poor control of inflammation resulting from an acute infection in this patient.

It is interesting to note that CBG and α_1-AT, which have 53% amino acid homology (Hammond & Underhill, 1989), are within 50-kb of each other and yet have different functions. α_1-AT is a protease inhibitor that mainly inhibits leukocyte elastase, and CBG transports cortisol, which can be released at site of inflammation, by elastase. There is a precise balance between proteolytic activity and its regulation by inhibitors present at an inflammatory site.

CBG function has apparently evolved as part of this biological complex (Hammond, 1990). The physical proximity of the CBG gene to protease inhibitor suggests that variation in CBG could be implicated in the inflammatory diseases that appear to be associated with α_1-AT. The results in our present study suggest that in our population, mutations of exon 2 of the CBG gene do not appear to play a role in the development and/or severity of asthma. The possibility remains however, that mutations in other areas of the CBG gene exist that might act as markers for mutations in other genes that are closely linked to the CBG gene and which play a role in asthma. Also, the molecular characterization of new CBG variants may further increase our understanding of the structure and function of this protein.

CHAPTER 4

POLYMORPHIC INTERRELATIONSHIPS OF THE INTERLEUKIN-1 GENE CLUSTER AND PLASMA CONCENTRATIONS OF THE INTERLEUKIN-1 RECEPTOR ANTAGONIST IN BLACK AND WHITE SOUTH AFRICAN ASTHMATICS AND CONTROL SUBJECTS

4.1 Introduction

4.1.1 Interleukin-1

IL-1 is a polypeptide with multiple metabolic, endocrine, immunologic, inflammatory, and haematopoietic activities. Originally described in the 1940's as an endogenous pyrogen, it was later described in 1972 as lymphocyte activating factor (LAF), a macrophage product that augmented thymocyte proliferation to mitogens (Gery & Wakeman, 1972). Several substances originally described for their biological activities have been identified as IL-1. These include leukocytic endogenous mediator, mononuclear cell factor, catabolin, oesteoclast activating factor, and haemopoetin-1, among others (Dinarello, 1991).

Although both forms of IL-1 (IL-1α and IL-1β) are products of separate genes on chromosome 2, they recognise the same cell surface receptors and share similar biological activities. There is a dramatic increase in IL-1 production by a variety of cells (macrophages, monocytes, neutrophils, lymphocytes, natural killer cells,

astrocytes, microglia, fibroblasts, vascular endothelial cells, synovial lining cells, dermal dendritic cells and smooth muscle cells) in response to infection, inflammatory agents, microbial toxins, products of activated lymphocytes, thrombin, bile salts, androgen metabolites, and complement components (Dinarello, 1985).

4.1.2 The α and β forms of IL-1

The two forms of IL-1 are synthesised as 31 kDa precursors, and the secreted forms are 17 kDa in size. The genes for the α and β forms show only 26% homology in final amino acid sequences, but they have seven highly conserved exons, and are closely linked on the same chromosome. However, IL-1β represents more than 90% of both the mRNA and the active protein secreted by monocytes in response to stimulation with endotoxin (March *et al.*, 1985). Enzymatic cleavage appears to be a prerequisite for the biological activity of IL-1β, but not of IL-1α. The intracellular high molecular mass IL-1β precursor does not translocate across intact microsomal preparations. However, a specific processing enzyme is found in the cytosol of monocytes/macrophages (Kostura *et al.*, 1989). The kinetics of IL-1 release are unlike those of other secreted proteins: secretion of both forms is delayed after synthesis, allowing pools of protein to accumulate within the cytosol of monocytes. Moreover, the intracellular half-life of the α and β forms varies considerably (Hazuda *et al.*, 1988).

Most IL-1α remains in the cytosol of cells in its precursor form, where it may function as an autocrine messenger. There is also evidence that the precursor is transported to the cell surface and associated with the membrane. This membrane-bound precursor

is biologically active, perhaps serving as a paracrine messenger to adjacent cells. A considerable amount of IL-1β, on the other hand, is released by the cell into the extracellular space and the blood stream. The mechanisms of release include exocytosis from vesicles, active transport by multidrug-resistant proteins, and cell death.

In normal subjects, plasma concentrations of the IL-1β are usually below the limit of detection (40 pg/ml) of the available assays, but are detectable in patients with sepsis, acute organ rejection, or exacerbations of rheumatoid arthritis (Eastgate *et al.*, 1988). Plasma IL-1α is rarely detected in such patients, even though the assays for IL-1α are more sensitive than those for IL-1β. The lack of circulating IL-1α is consistent with the observation that cultured cells do not release the α form under conditions that result in the release of the β form.

4.1.3 Control of transcription and translation

Transcription and translation of IL-1 are distinct and dissociated processes, and regulation of IL-1 gene expression occurs at both levels. Transcription for IL-1 without translation can be initiated in monocytes by adherence to plastic surfaces or exposure to β-glucan polymers (Dinarello, 1991). The half-life of mRNA following these stimuli is unchanged, suggesting that accelerated destruction of mRNA is not the explanation for the failure of translation (Schindler *et al.*, 1990).

Cells containing untranslated mRNA for IL-1 are said to be primed, and small amounts

of other stimuli, such as endotoxin or IL-1 itself, rapidly trigger translation, and usually result in more IL-1 synthesis than non-primed cells. In contrast to cell adhesion, cell activation with lipopolysaccharide (LPS) or through phagocytosis leads to increased transcription as well as translation and posttranslational processing.

Using endotoxin and other microbial products, transcription of IL-1β mRNA is rapid: in macrophages and monocytes or cell lines derived from these cell types, transcription is observed within 15 minutes. Following stimulation with endotoxin, peak accumulation of IL-1β mRNA occurs ~2 to 4 hours, is sustained for 6 to 8 hours, and then decreases rapidly. Using IL-1 as a stimulus of its own gene expression, steady state levels are slower to increase and are sustained for 30 hours (Dinarello, 1991). Under certain conditions, transcription of IL-1 can be enhanced by blocking the synthesis of a suppressor protein. Transcription of IL-1 is retarded by prostaglandin E_2-induced cyclic AMP production, and the protein kinase C inhibitor. In contrast, translation of IL-1 mRNA can be enhanced by calcium ionophores and leukotrienes (Fenton *et al.*, 1987). The regulatory role of prostaglandin E_2 in IL-1 gene expression represents an important autoregulatory control mechanism, since the activation of macrophages triggers IL-1 synthesis and a concomitant increase in endogenous prostaglandin E_2 synthesis (Kunkel *et al.*, 1988). Control of IL-1 transcription and translation is affected by other cytokines. IL-1 is a stimulus of its own gene expression in certain cells, as are TNF, M-CSF and GM-CSF. IL-1 transcription is suppressed by IL-4, IL-8, IL-10 and TGF-β. IL-1- induced IL-1 production is suppressed by IFN-γ at the transcriptional level, as is the IL-1 production by activators of protein kinase C (Dinarello, 1991).

Activators of cytokine production in a variety of cells also increase the synthesis of products derived from arachadonic acid, and some of these products provide a positive signal for IL-1 gene expression. Corticosteroids suppress IL-1 transcription and synthesis when added before initiation of transcription. However, they are less effective when added after transcription. Agents that non-specifically block the lipoxygenase pathway of arachadonate metabolism reduce IL-1 levels (Dinarello, 1985).

The first translational product of IL-1 is the pro-IL-1 31-kDa precursor. It has no clear signal peptide, and hence a considerable amount of the precursor remains cell associated, almost entirely in the cytoplasm (Auron *et al.*, 1987). It is unclear how IL-1 is transported from the cytosol to the extracellular compartment and how it is cleaved to its mature peptides, but secretion of the precursor and processing to its mature peptides appear to be linked events, although it has been suggested that pro-IL-1-β is secreted intact and then later cleaved by various enzymes present in inflammatory tissue (Hazuda *et al.*, 1988).

IL-1 activity has been described after macrophage fixation, and has been ascribed to "membrane-bound" IL-1. "Membrane-bound" IL-1 is active in lymphocytes, macrophages, dendritic cells and fibroblasts, and is almost exclusively IL-1α (Kurt-Jones *et al.*, 1985). It is unclear how "membrane-bound" IL-1 is oriented on the surface of the cell so that its structural components are available for binding to IL-1 receptors.

4.1.4 Receptors for IL-1

Characterisation of the IL-1 receptor has identified two separate moieties (Bromsztyk *et al.*, 1989), which bind both IL-1α and IL-1β, as well as the IL-1 receptor antagonist (IL-1Ra). Molecular cloning first identified an 80-kDa form of the receptor, the type I receptor, which is expressed on T-cells and fibroblasts and displays a single class of high affinity receptors ($K_d = 2x10^{-10}$ M) (Dinarello *et al.*, 1989). However, a very high affinity form has also been reported on murine T-cells and human keratinocytes ($K_d = 5x10^{-12}$ M) (Dower & Sims, 1990). A second smaller receptor, the type II receptor (p60), has been identified on B-cells and macrophages; this receptor appears to display dual affinity binding with high ($K_d = 5x10^{-11}$ M) and low ($K_d = 10^{-9}$ M) affinity. Both types of receptors belong to the Ig superfamily and their binding sites for IL-1 are structurally similar. The type I receptor has a longer cytoplasmic segment and a higher affinity for IL-1α, whereas the type II receptor has a shorter cytoplasmic segment and a higher affinity for IL-1β. The multiple glycosylation sites on the type I receptor may have a role in receptor expression, affinities and specificities. IL-1 bound to the type I receptor is internalised and only a small amount is degraded, whereas that bound to the type II receptor is found in extracellular fluid in a degraded form, thus suggesting different vesicles and acidification mechanisms for the two receptors (Dinarello, 1991).

4.1.5 Biological effects of IL-1

The pyrogenic effect of IL-1 is now well known, as it is produced in the course of acute or chronic inflammatory reactions, and mediates its pyrogenic effect in the

hypothalamic thermoregulatory centre, principally by release of prostaglandin E_2 (Dinarello *et al.*, 1986). Another key effect of IL-1 in the central nervous system is the release of corticotropin-releasing factor and adrenocorticotropin, which leads to steroid production by the adrenal glands (Besedorsky *et al.*, 1986).

Corticosteroids down-regulate IL-1 production at the level of mRNA transcription. In small doses, IL-1 stimulates insulin production, but at high concentrations it is cytotoxic to the β cells of the pancreas. Other endocrine effects of IL-1 include suppression of progesterone secretion from granulosa cells after induction with follicle stimulating hormone (Gottschall *et al.*, 1987), a cytotoxic effect to thyroid cells (Dinarello, 1988a), and a natriuretic effect (C[illegible]erzasio *et al.*, 1987). The variety of these effects of IL-1 serves to support a link between the endocrine and the immune systems.

IL-1 has numerous other metabolic effects. It is known to induce production of prostaglandins E_2 and I_2 in endothelial and smooth muscle cells. These substances are potent vasodilators causing hypotension (Dinarello, 1988b). In the liver, IL-1 induces increased synthesis of acute phase proteins (Perlmutter *et al.*, 1986), decreased albumin production, and decreased activity of cytochrome P-450 (Dinarello, 1988b). IL-1 also has catabolic effects that include induction of collagenase production in synovial cells and cartilage, and in the bones, where it also affects calcium resorption. IL-1 has also been detected in synovial fluid, and has been implicated with a possible role in the pathogenesis of rheumatoid arthritis (Eastgate *et al.*, 1988). Gulik *et al.* (1989) showed that IL-1 and TNF inhibit β-adrenergic agonist-mediated myocyte contractility

and intracellular accumulation of cyclic AMP, and suggested a mechanism for congestive heart failure accompanying inflammatory myocarditis and allograft rejections. This study is the first in which a cytokine is implicated in the functional regulation of cardiac muscle cells.

IL-1 has a vital role to play in the activation of the immune system. It acts on macrophages and monocytes, inducing its own production and the production of other cytokines such as TNF and IL-6. It also induces the cytotoxic activity of these cells against tumour cell lines. It acts on T-lymphocytes, stimulating them to express interleukin-2 receptors (IL-2Re) and to produce IL-2 (Herrman *et al.*, 1988). It induces the production of growth factors such as GM-CSF and IL-4 from activated T-cells, and IL-6 from thymocytes. IL-1 also has a direct effect on B-lymphocytes, inducing their activation, growth, differentiation, and Ig synthesis. During the activation of normal human B-lymphocytes, it is interesting to note that they express IL-1α on their membrane, and secrete IL-1β, thus suggesting an important role of IL-1 in the antigen-presenting capacity of B-lymphocytes to T-cells (Bonnefoy *et al.*, 1989).

IL-1 exhibits anti-tumour effects probably via three different mechanisms. The first mechanism is a direct cytolytic effect of IL-1 on malignant cells, observed *in vitro* by using tumour cell lines as targets (Onozaki *et al.*, 1985). The second mechanism involves activation of cells of the immune system, such as natural killer cells, that exhibit direct anti-tumour properties when activated (Herberman, 1981). Lastly, IL-1 has indirect anti-tumour effects by either inducing the production of other tumouricidal lymphokines or by facilitating and enhancing the effect of these lymphokines on

malignant cells, i.e. synergism with other mediators.

4.1.6 IL-1 receptor antagonism

Given the widespread presence of IL-1 in different organs, its production by a variety of cells, and its putative pro-inflammatory role in human disease, many investigators had long sought the existence of naturally occurring inhibitors of IL-1. This endeavour culminated in the description of a specific receptor antagonist of IL-1, the IL-1 receptor antagonist (IL-1Ra). Lipoproteins, lipids and α_2-M are examples of naturally occurring substances that inhibit IL-1 activity, but these molecules also inhibit other cytokines, such as IL-2 and IL-6 (Dinarello, 1991).

The genes for the IL-1Ra has been mapped to chromosome 2. Most interestingly, the same region of the human chromosome 2 contains the genes for IL-1α, IL-1β and IL-1 receptors type I and II (Arend, 1993). The IL-1Ra was originally named the "IL-1 inhibitor". This region is also known as the IL-1 gene cluster and was identified as a 25-kDa protein which was purified from the urine of patients with monocytic leukemia (Seckinger *et al.*, 1987). The cDNA sequence codes for a polypeptide of approximately 17-kDa, whereas the 25-kDa molecular mass is due to glycosylation. The amino acid sequence deduced from the cDNA showed a 26% homology with IL-1β and a 19% homology to IL-1α (Dinarello, 1991). Two structural variants of the IL-1Ra exist: sIL-1Ra, a secretory molecule produced by monocytes, macrophages, neutrophils and other cells; and icIL-1Ra, an intracellular molecule produced by kerat[illegible]ther epithelial cells, macrophages and fibroblasts. It has been

suggested that icIL-1Ra may play additional roles inside cells, such as in the regulation of IL-1Re expression or of IL-1-induced gene transcription (Arend, 1993).

The IL-1Ra blocks IL-1 activity *in vitro* and *in vivo*. *In vitro*, it appears to occupy the type I IL-1 Re on T-cells and fibroblasts with almost the same affinity as IL-1, but without demonstrable agonist activity i.e. without inducing any discernible intracellular responses. The IL-1Ra was initially reported not to recognise type II IL-1Re's, but recent studies have shown that it can recognise these receptors on a number of cells (Dinarello & Thompson, 1991).

To date, there are no examples where IL-1Ra has failed to block the anticipated biological response to IL-1 added to cultured cells. Recombinant IL-1Ra blocks IL-1 augmentation of thymocyte proliferation; the ability of IL-1 to activate protein kinase in fibroblasts; and the production of IL-1-induced IL-1, TNF and IL-6 in human monocytes (Dinarello & Thompsom, 1991).

IL-1Ra is synthesised in septic animals and humans with a variety of infectious or inflammatory diseases. The balance between the amount and secretion of IL-1 and its receptor antagonist may be critical in some diseases. The production of IL-1 and IL-1Ra is differentially regulated, even in the same cell. In cells stimulated with endotoxin, the production of IL-1β precedes that of IL-1Ra (Arend *et al.*, 1991). Endogenously produced IL-1Ra is likely to contribute to limiting the severity of the disease, but may be inadequate in overwhelming infection or acute inflammation. This is largely due to the fact that in most cells with IL-1Re's there is partial occupancy of

the receptors by IL-1, and partial occupancy by the IL-1Ra, thus allowing IL-1 to still trigger a response.

The interleukin-1 receptor antagonist (IL-1 Ra) has been shown to be a powerful anti-inflammatory agent as it inhibits the activities of IL-1α and IL-1β (Dinarello & Thompson, 1991; McIntyre *et al.*, 1991). The balance of cytokine production, receptor expression and inhibitor levels seems to be a major factor in determining the outcome of the inflammatory response. IL-1 Ra does not trigger signal transduction, but acts as a competitive inhibitor. It may, therefore, play a crucial role in many IL-1 mediated diseases, acting as an important endogenous regulator of inflammation (Tarlow *et al.*, 1993).

There is a variable number tandem repeat (VNTR) of an 86 base pair (bp) sequence in intron 2 of the IL-1 Ra gene. Five alleles of this system have been found representing 240, 325, 410, 500 and 595-bp copies of the repeat sequence. This population study also showed an increased frequency of the 410 bp allele (73%) and a lower frequency of the 240 bp allele (21%) in a Caucasian population (Tarlow *et al.*, 1993). Recently associations of the 240-bp allele of the IL-1 Ra with several inflammatory diseases have been reported (Clay *et al.*, 1994; Blakemore *et al.*, 1994; Mansfield *et al.*, 1994).

Blakemore *et al.* (1994) have reported an increase in both frequency and carriage rates of the 240-bp allele in white patients with systemic lupus erythematosus (SLE). They have also found associations of this allele with severity of the disease. A Pst I

polymorphism in the promoter region of the IL-1 Re gene has been associated with insulin dependent diabetes mellitus (IDDM) (Bergholdt *et al.*, 1995), and a Taq I polymorphism in exon V of the IL-1β has been shown to correlate with increased secretory capacity and has also been associated with IDDM (Pociot *et al.*, 1992).

4.2 Aims of this study

The present study was undertaken in order to determine the different frequencies of the VNTR polymorphism of the IL-1 Ra gene in black and white South African patients with asthma and control individuals and to correlate these with the Pst I (-) allele of the IL-1 Re and the Taq I (-) allele of the IL-1β genes. Furthermore, we investigated whether a particular allele of the IL-1 Ra influenced its circulating levels and the severity of the disease in these patients.

4.3 Materials and methods

4.3.1 Subjects

Thirty-six white asthmatics (11 women) and 39 black asthmatics (26 women), 61 White controls (23 women) and 46 black controls (18 women) were screened for the VNTR's in intron 2 of the IL-1 Ra gene. Twenty-one white individuals (5 women), of these 14 were homozygous for the 410 bp allele, 5 were heterozygous for the 410/240 bp alleles and 2 were homozygous for the 240 bp allele, and 15 black individuals (4 women), of these 11 were homozygous for the 410-bp allele, 4 were

heterozygous for the 410/240 bp alleles, were screened for the Pst I polymorphism of the IL-1 Re. Fourteen white individuals (2 women), of which 9 were homozygous for the 410 bp allele and 5 were heterozygous for the 410/240 bp alleles, and 13 black individuals (3 women), of which 10 were homozygous for the 410-bp allele and 3 were heterozygous for the 410/240 bp allele, were screened for a Taq I polymorphism of the IL-1 β gene. The black and white asthmatic individuals were recruited from the Asthma and Respiratory Clinics of Hillbrow and Johannesburg Hospitals and from Krugersdorp Hospital (Paediatrics). The controls were either asymptomatic individuals who presented themselves as blood donors at the South African Blood Transfusion Services in Johannesburg, or outpatients from the Krugersdorp Hospital Paediatric clinics. None of the controls had a history of atopy or any disease process associated with the respiratory system. All individuals who participated in the study were 4-45 years of age, of these the following were <16 years: 18 (6 girls) white asthmatics, 10 (5 girls) black asthmatics, 10 white controls (4 girls) and 10 black controls (3 girls). Each subject gave informed consent (where children were involved, their parents gave informed consent) to participate in the study, which was passed by the Committee for Research on Human Subjects of the University of the Witwatersrand. Atopy and asthma were diagnosed as described earlier in this study (Chapter 2).

4.3.2 Genotype studies

VNTR's of the IL-1 Ra

Total genomic DNA was extracted from EDTA-containing whole blood according to the Higuchi method (1989). All PCR amplifications were performed in a Hybaid Omnigene™ thermal cycler. The polymorphic region of the IL-1 Ra intron 2 was amplified using oligonucleotide primers described by Tarlow *et al.* (1993). Genomic DNA samples (1 μg) were amplified in a total volume of 57 μL, containing 0.2 mM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (0.01% gelatine) and 1 μM each appropriate amplification primer, overlaid with mineral oil. The reaction mixture containing all the components except the Taq DNA polymerase was heated to 96°C for 10 min. Following a cooling step which reduced the sample to 55°C, 1 U Taq DNA polymerase was added to each sample. The samples were then processed through 30 cycles of: denaturation at 94°C for 1 min., annealing at 55°C for 1 min. and extension at 72°C for 1 min. The final step was one of extension at 72 °C for 1 min. Following amplification, the PCR products were resolved by electrophoresis in 4% (w/v) agarose gels, stained with ethidium bromide and viewed under UV illumination (302nm) (Figure 4.1).

Pst I polymorphism of the IL-1 Re promoter

The prevalence of the Pst I polymorphic site of the promoter of the IL-1 Re was determined according to Bergholdt *et al.* (1995). Genomic DNA samples (1 µg) were amplified in a total volume of 57 µL, containing 0.2 mM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (0.01% gelatine) and 1 µM each appropriate amplification primer, overlaid with mineral oil. The reaction mixture containing all the components except the Taq DNA polymerase was heated to 96°C for 10 min. Following a cooling step which reduced the sample temperature to 58°C , 1 U Taq DNA polymerase was added. The samples were then processed through 30 cycles of: denaturation at 94°C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. The final step was one of extension at 72 °C for 1 min. After confirmation of successful PCR amplification, 5 µL PCR product was digested in a final volume of 10 µL with 1 U Pst I enzyme, with the recommended buffer, for 1 hr at 37°C. Digested products were then separated on 4% (w/v) agarose gels, stained with ethidium bromide and viewed under UV illumination (302nm). Three bands potentially resulted from the Pst I digestion of the PCR products : a 362, a 256 and a 108-bp band (Figure 4.2).

Taq I polymorphism in exon 5 of the IL-1 β

The prevalence of a Taq I polymorphism in exon 5 of the IL-1 β gene was determined using the following oligonucleotide primers according to the sequence published by Bensi *et al.* (1987).

(a) 5'-GTC AGG TCC ATG TTC TTA GCC AC- 3'

(b) 5'-GCA AGC TGC CAG GAG GCC AGG CA- 3'

Genomic DNA samples (1 µg) were amplified in a total volume of 57 µL, containing 0.2 mM each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (0.01% gelatine) and 1 µM each appropriate amplification primer, overlaid with mineral oil. The reaction mixture containing all the components except the Taq DNA polymerase was heated to 96°C for 10 min. Following a cooling step which reduced the sample to 65°C, 1 U Taq DNA polymerase was added. The samples were then processed through 30 cycles of: denaturation at 94°C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min. The final step was one of extension at 72 °C for 1 min. After confirmation of successful PCR amplification, 5 µL PCR product was digested in a final volume of 10 µL with 1 U Taq I enzyme, with the recommended buffer, for 1 hr at 65°C. Digested products were then separated on 4% (w/v) agarose gels, stained with ethidium bromide and viewed under UV illumination (302nm). Three bands potentially resulted from the digestion of PCR products : a 380, a 196 and a 184-bp band (Figure 4.3).

4.3.3 IL-1 Ra plasma assay

Blood specimens were collected into EDTA containing tubes. Plasma was separated on the day of collection and stored at -60°C until analysed. Plasma IL-1 Ra concentrations were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The groups studied consisted of ten black adult asthmatics (3 women), 11 white adult asthmatics (4 women), 10 black adult controls (2 women) and 10 white adult controls (2 women). The adult patients were being treated with a variety of conventional anti-asthmatic agents, consisting of: an inhaled β_2-adrenergic agonist (95.4% of the patients); inhaled beclomethasone dipropionate (72.7%); or a theophylline derivative (29.5%). A subset of children with asthma were also studied prior to therapeutic intervention. These consisted of 10 black asthmatic children (5 girls), 18 white asthmatic children (6 girls), as well as 10 black control children (3 girls) and 10 white control children (4 girls). All controls were matched for age.

4.3.4 Statistical analysis

The comparisons of the allelic frequencies of the IL-1 Ra, IL-1 Re and IL-1 β between patients and controls were obtained by means of the 2-tailed Chi-Square test. The comparison of the plasma concentrations of the IL-1 Ra in each group was done by means of the Mann-Whitney non-parametric test. A comparison of the IL-1 Ra plasma concentrations with severity was done by means of the Fischer's exact test.

4.4 Results

4.4.1 Genotype studies.

We studied the different distributions of the various VNTR's of the Il-1 Ra. The frequency of the 5 different 86-bp tandem repeats in intron 2 of the IL-1 Ra gene in the black and white asthmatic groups and the black and white control groups is given in Table 4.1. There was a significant difference in the frequency of the 240-bp allele between the white asthmatics (28%) and black asthmatics (8%) ($p=0.001$), and a significant difference was also observed in the frequency of the 240-bp allele between the white controls (25%) and the black controls (12%) ($p=0.02$) A significant difference was also observed in the frequency of the 410-bp allele between the white controls (71%) and the black controls (85%) ($p=0.02$).

There was no significant difference between the white asthmatics and white controls for either allele and between black asthmatics and black controls. There was a significant difference in the frequency of the Pst I (-) allele of the IL-1 Re between all white individuals (47%) as compared to the all black individuals (70%) ($p<0.00001$), (Table 3.2). The association between the 410-bp allele of the IL-1 Ra gene and the Pst I (-) allele of the IL-1 Re gene in black and white individuals is shown in Table 4.2. The 410-bp allele was significantly associated with the Pst I (-) allele in black individuals (100%) as compared to white individuals (64%) ($p=0.002$). Table 4.3 shows the allelic frequencies of the Taq I (-) allele in exon 5 of the IL-1 β gene in the different groups. There was no allelic association between the IL-1 Ra polymorphisms

and the Taq I (-) allele of the IL-1 β gene nor were there population differences in the frequency of this allele.

4.4.2 Plasma IL-1 Ra concentrations in different groups of asthmatic patients and controls.

Table 4.4 shows the comparison of the mean plasma concentrations of the IL-1 Ra between black and white adult asthmatic patients and black and white adult controls. Table 4.5 shows the comparison of the mean plasma concentrations of the IL-1 Ra between black and white patients younger than 16 years of age as compared to black and white controls younger than 16 years of age. The plasma IL-1 Ra concentrations were determined in 21 adult asthmatic patients (10 blacks) and 20 adult control individuals (10 blacks).

There was a significant difference in mean plasma IL-1 Ra concentrations between the white adult asthmatic patients (5 women), (mean ± SD 1390.98 ± 1113.89 pg/mL) versus the white adult controls (4 women), (486.12 ± 341.33 pg/mL) (p=0.03). There was no difference in the mean plasma IL-1 Ra concentration between the black adult asthmatic patients (4 women), (1473.53 ± 1443.30 pg/mL) as compared to the black adult controls (3 women), (723.59 ± 485.40 pg/mL) (p=0.15).

The plasma IL-1 Ra concentrations were also determined in 28 asthmatic children (10

blacks) and 20 control children (10 blacks). There was a significant difference in mean plasma IL-1 Ra concentrations between the white asthmatic children (6 girls), (mean ± SD 3236.12 ± 1093.33 pg/mL) compared to the white control children (4 girls), (876.06 ± 547.23 pg/mL) ($p<0.00001$). There was also a significant difference in the mean plasma IL-1 Ra concentration between the black asthmatic children (5 girls), (2649.98 ± 1804.68 pg/mL) compared to the black control children (3 girls), (755.62 ± 458.69 pg/mL) ($p<0.003$). Due to these differences between the respective clinical and control groups, we compared the plasma IL-1 Ra levels with severity of asthma in these children as shown in Table 4.6.

Significant differences were observed when we compared the levels of IL-1 Ra in 5 severe patients (3590.48 ± 1547.66 pg/mL) with 9 patients classified as having mild asthma (1673.18 ± 1127.83 pg/mL) ($p=0.04$). Significant differences were also observed when comparing 14 moderate asthmatic children (3174.54 ± 1468.23 pg/mL) with the mild asthmatic children (1673.18 ± 1127.83 pg/mL) ($p=0.013$).

Comparisons were also made in 14 asthmatic children with the 240-bp allele (2 were homozygous for this allele, while 12 were 410/240 bp heterozygous) and 10 patients homozygous for the 410 bp allele. There was no significant difference in mean plasma IL-1 Ra concentrations between the 2 groups (2573.32 ± 1600.39 pg/mL in patients with at least 1 copy of the 240 bp allele versus 2596.99 ± 1918.29 pg/mL in patients homozygous for the 410 bp allele, ($p=0.97$).

Table 4.1 : Frequency of the VNTR of the IL-1 Ra in black and white asthmatics and in black and white controls.

Alleles	Black Asthmatics		White Asthmatics		Black Controls		White Controls	
	No.	Frequency	No.	Frequency	No.	Frequency	No.	Frequency
240-bp	6	0.08*	20	0.28*	11	0.12•	30	0.25•
325-bp	4	0.05	0	0.00	2	0.02	1	0.03
410-bp	67	0.86	52	0.72	78	0.85♦	87	0.71♦
500-bp	1	0.01	0	0.00	1	0.01	4	0.01
525-bp	0	0.00	0	0.00	0	0.00	0	0.00

*Comparison of the 240-bp allele between white asthmatics and black asthmatics by the 2-tailed Chi-square test showed a significant difference (p=0.001).

Comparison of the ♦410 and •240-bp alleles between the white and black controls by the 2-tailed Chi-square test showed a significant difference (p=0.02 and p=0.02 respectively).

Table 4.2: Association of the 410-bp allele of the IL-1 Ra gene in black and white homozygous control adult individuals with the Pst I (-) allele of the IL-1 Re gene.

	No. of individuals	Pst I (-) allele		Pst I (+) allele	
		No.	Frequency	No.	Frequency
Black Individuals	11	22	1.00	0	0.00
White Individuals	14	18	0.64	10	0.36

Comparison of white individuals and black individuals by the 2-tailed Chi-square test showed a significant difference (p=0.002).

Table 4.3: Association of the 410-bp allele of the IL-1 Ra gene in black and white homozygous control adult individuals with the Taq I (-) allele in exon 5 of the IL-1 β gene.

	No. of individuals	Taq I (-) allele		Taq I (+) allele	
		No.	Frequency	No.	Frequency
Black Individuals	10	6	0.30	14	0.70
White Individuals	9	7	0.39	11	0.61

No significant differences were observed in the two groups.

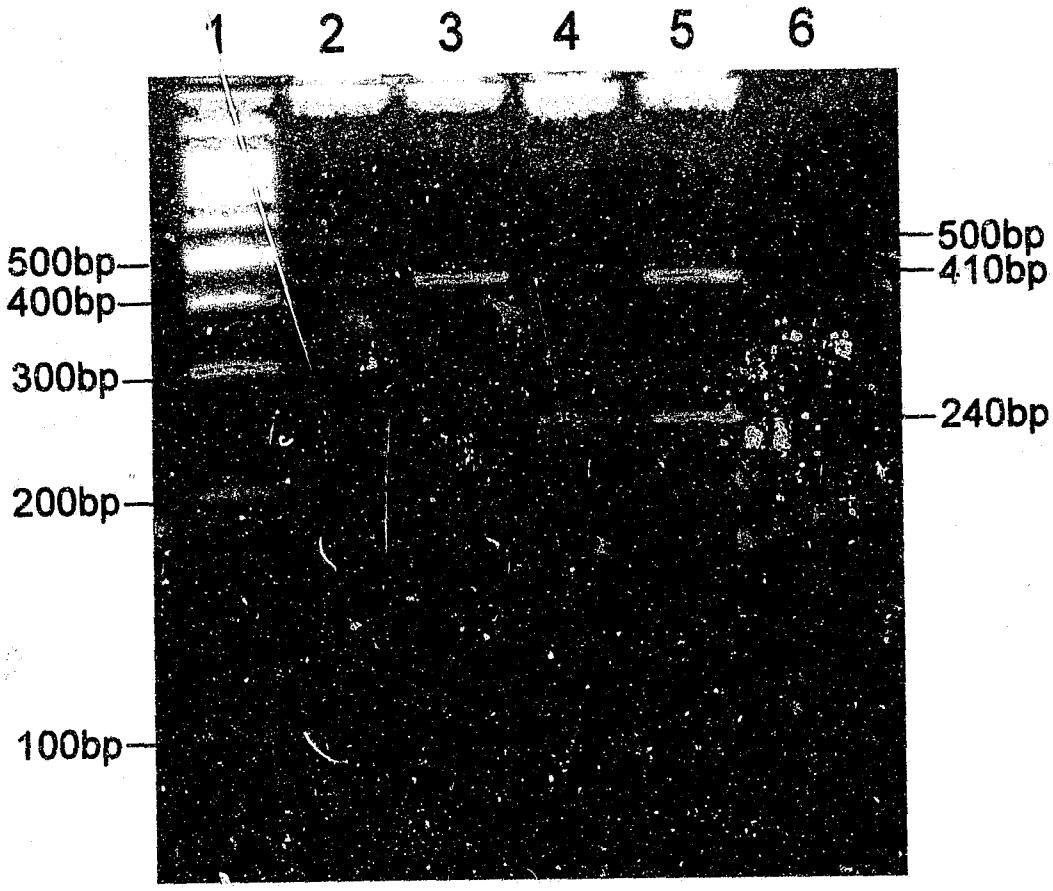


Figure 4.1: VNTRs of intron 2 of the IL-1 Ra gene showing individuals who are homozygous for the 240 bp allele (lane 4), and the 410 bp allele (lane 3), heterozygous for the 410 and 240 bp alleles (lane 5) and the heterozygous for the 410 and 500 bp alleles (lane 2). Lane1, 100 bp molecular weight marker. Lane 6 is a control with no DNA added in the PCR reaction.

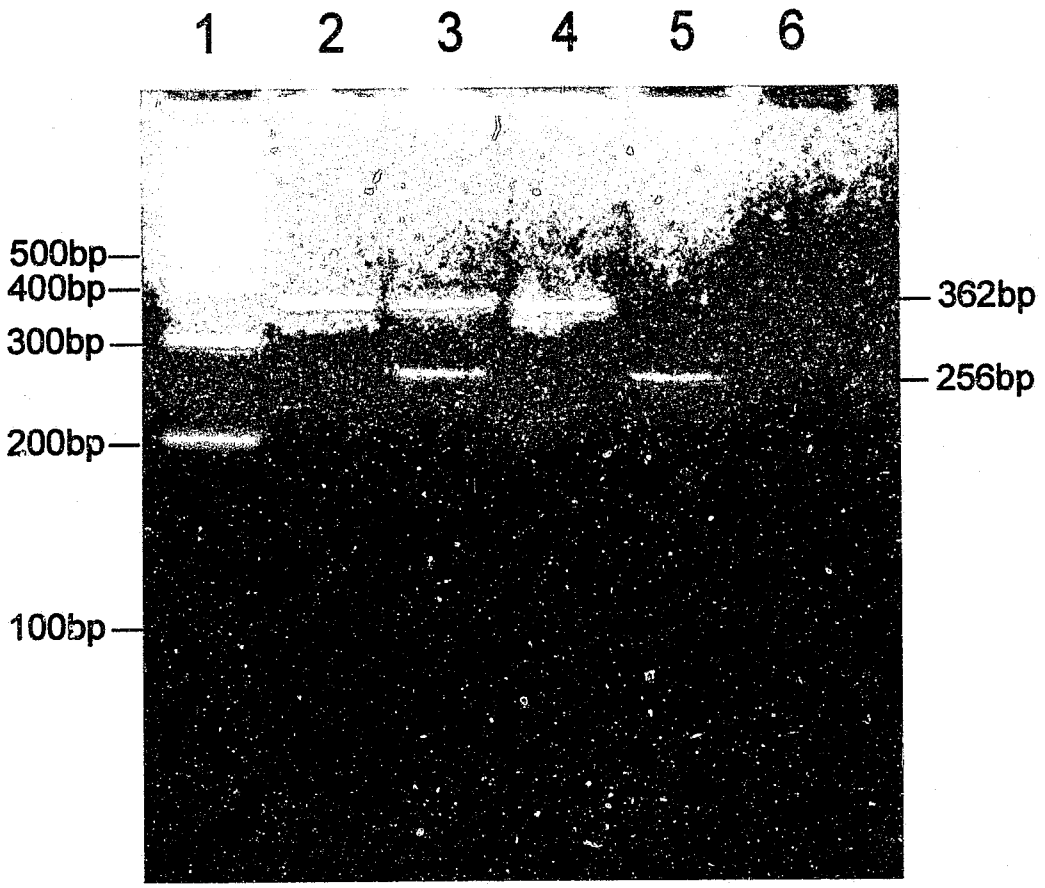


Figure 4.2: *Pst* I restriction of the promoter of the IL-1 Re gene showing individuals who are homozygous for the *Pst* I (-) allele (lane 5), homozygous for the *Pst* I (+) allele (lane 4) and heterozygous (lane 3). Lane 2 is an unrestricted PCR product. Lane 1, 100 bp molecular weight marker. Lane 6 is a control with no DNA added in the PCR reaction.

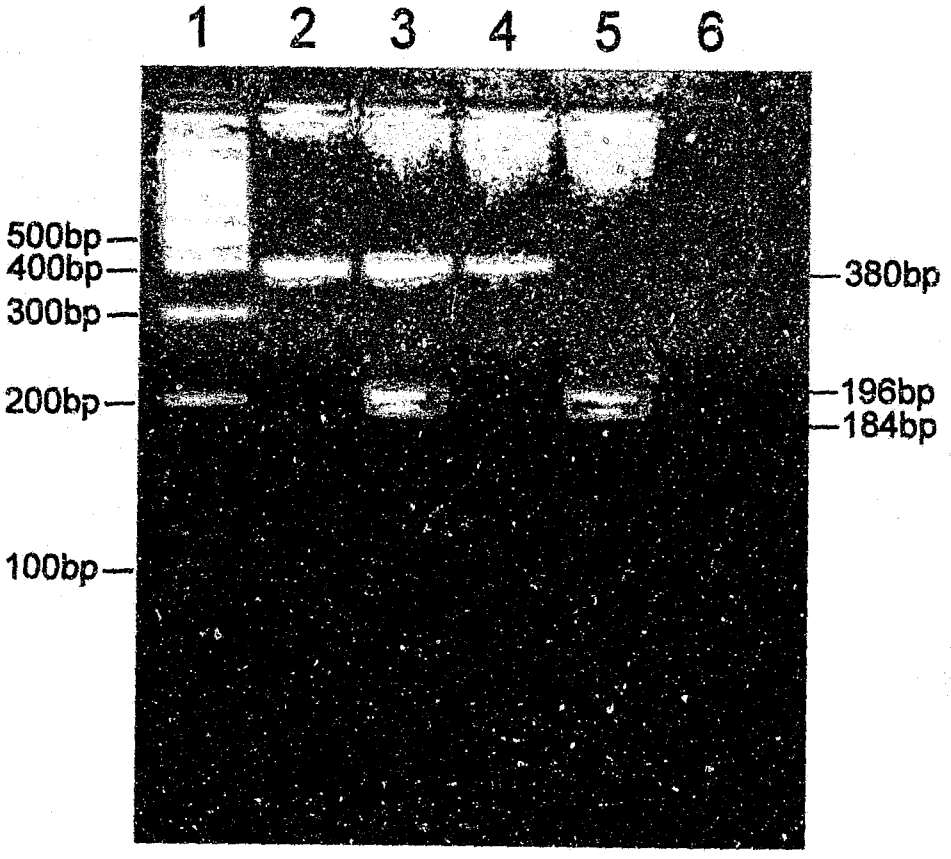


Figure 4.3: *Taq I* restriction of exon V of the IL-1β gene showing individuals who are homozygous for the *Taq I* (+) allele *Taq I* (-) allele, (lane 4 and 5) and heterozygous (lane 3). Lane 2 shows an unrestricted PCR product. Lane 1, 100bp molecular weight marker. Lane 6 is a control with no DNA added in the PCR reaction.

Table 4.4: Mean plasma concentrations of the IL-1 Ra in black and white adult asthmatics and black and white adult control individuals.

	No. Of Individuals	Mean Plasma Concentration pg/mL
Black Adult Asthmatics	10	1473.53 ± 1443.30
White Adult Asthmatics	11*	1390.98 ± 1113.89
Black Adult Controls	10	723.59 ± 485.40
White Adult Controls	10*	486.12 ± 341.33

*Comparison of the white adult asthmatics and white adult controls by the 2-tailed Chi-square test showed a significant difference (p=0.03). No significant differences were observed between the black adult asthmatics and the black adult controls.

Table 4.5: Mean plasma concentrations of the IL-1 Ra in black and white asthmatic children and black and white control children.

	No. Of Individuals	Mean Plasma Concentration pg/mL
Black Asthmatic Children	10*	2649.98 ± 1804.68
White Asthmatic Children	18•	3236.12 ± 1093.33
Black Control Children	10*	755.62 ± 458.69
White Control Children	10•	876.06 ± 547.23

*Comparison of the black asthmatic children and the black control children by the 2-tailed Chi-square test showed a significant difference, (p=0.003). •There was also a conclusive difference between the white asthmatic children and the white control children ($p<0.00001$).

Table 4.6 : IL-1 Ra plasma concentrations related to severe, moderate or mild asthma in black and white asthmatic children.

	No. of individuals	Mean plasma concentrations pg/mL
Severe	5	3590.48 ± 1547.66
Moderate	14	3174.54 ± 1468.23
Mild	9	1673.18 ± 1127.83

Comparison of severe asthmatic children and mild asthmatic children by the 2-tailed Chi-square test showed a significant difference ($p=0.04$). There was a significant difference between the moderate asthmatic children and the mild asthmatic children ($p=0.013$).

4.5 Discussion

Recent studies have linked the 240-bp allele of IL-1 Ra with various inflammatory diseases including SLE (Blakemore *et al.*, 1994; Suzuki *et al.*, 1997), lichen sclerosis (Clay *et al.*, 1994) and ulcerative colitis (Mansfield *et al.*, 1994). The frequency of the different VNTR's in the white control population in this study is similar to that reported by Tarlow *et al.* (1993) however we show a significant decrease of the 240-bp allele in black control individuals as compared to white control individuals and a corresponding increase in the 410-bp allele in the black population. Similarly the 240-bp allele was also significantly less frequent in black asthmatics compared to white asthmatics. The allelic frequencies for our black population are similar to those of Suzuki *et al.* (1997) who found a lower incidence of the 240-bp allele in Japanese patients with SLE (9.7%) as well as healthy Japanese controls (4.1%).

Our results suggests that the VNTR's in intron 2 of the IL-1 Ra gene do not influence the expression of IL-1 Ra since there was no correlation between the genotypes and plasma concentrations in black and white children with asthma prior to therapeutic intervention. However, the IL-1 Ra plasma concentrations were significantly correlated with the severity of the disease in these patients, thus the IL-1 Ra plasma concentration could be a marker for disease severity. It is interesting to note that there was no difference in the IL-1 Ra plasma concentration levels between black adult asthmatic patients and black adult controls. The mean values for the black adult control individuals was higher than for the white adult controls.

A recent study has shown that the South African black population has an increased frequency of the E237G mutation in the beta chain of the high affinity IgE receptor ($Fc_{\epsilon}RI$-β) (Green *et al.*, 1998). This population also has an intrinsically high level of the IgE antibody. Consequently, this population might be in a low grade state of inflammation, as reflected by higher levels of IL-1 Ra compared to the white adult controls. Although this study did not show an association of a specific VNTR allele of the IL-1 Ra and asthma in the two population groups, there was a significant association between the 410-bp allele and the Pst I (-) allele of the IL-1 Re in black individuals. The significance of this association is not known, but the possibility exists that the Pst I (-) allele of the IL-1 Re could reflect changes in the expression of the receptor since the polymorphic site might influence the binding of the transcription factors to their relevant sequence motifs. However, this association was not found in white individuals.

No association was found between the VNTR's of the IL-1 Ra and the Taq I (-) allele of the IL-1 β in the two population groups. Previous studies have determined that less than 5% of IL-1 receptors are needed to be occupied by IL-1 in order to induce biological responses. However, 10 to 500-fold (0.002-0.1 IL-1β/IL-1 Ra ratio) excess amounts of IL-1 Ra over IL-1β are required to decrease IL-1β stimulation of target cells by 50% (Arend *et al.*, 1990). Taking all these factors into account, our results suggest that the IL-1 Ra is an anti-inflammatory molecule and the polymorphic association between the antagonist and the IL-1 Re might indicate that this gene cluster is important in the pathogenesis of asthma, particularly in black asthmatics who tend to be more severely affected than white asthmatics.

CHAPTER 5

THE GLY 16 POLYMORPHISM OF THE B_2 ADRENERGIC RECEPTOR IN SOUTH AFRICAN ASTHMATIC CHILDREN.

5.1 Introduction

In 1968, Szentivanyi proposed the "β adrenergic theory" of asthma - that is, that asthma results from reduced function of the β adrenergic system. Systemic responses to catecholamines, such as a rise in blood sugar level, free fatty acid, lactate, pyruvate and plasma cyclic AMP (cAMP), have been shown to be reduced in asthmatic patients (Szentivanyi *et al.*, 1985). Reduced cAMP responses to lymphocytes from asthmatic patients have also been reported. In addition, two studies have shown that the relaxant potencies of β stimulants were significantly attenuated in bronchial preparations from asthmatic patients. These studies support the "β adrenergic theory." However, responses to β stimulants have also been shown to be affected significantly by systemic or local treatment with β agonists and by viral respiratory tract infections (Cerrina *et al.*, 1986; Goldie *et al.*, 1986).

The presence of these factors, which modify β adrenergic functions, has made it difficult to confirm a clear relation between abnormal β adrenergic function and asthma. The gene encoding the human β adrenergic receptor has recently been cloned and sequenced. It is an intronless gene that has been localised to q31q32 on chromosome 5. The structure of the β adrenergic receptor is typical of other G

protein coupled receptors, with its amino terminus localised extracellularly, seven transmembrane spanning domains and an intracellular carboxyl terminus (Figure 5.1). Understanding the structure indicated that small changes in the amino-acid sequence (including single amino-acid substitutions) could result in significant alterations in receptor function. Naturally occurring mutations in the population, if they were functionally different from wild-type, might play a role in asthma or its response to therapy. The Gly 16 polymorphism of the β adrenergic receptor appears to impart enhanced down-regulation of receptor number and has been found to have a higher prevalence in nocturnal asthmatic patients (Ohe *et al.*, 1995).

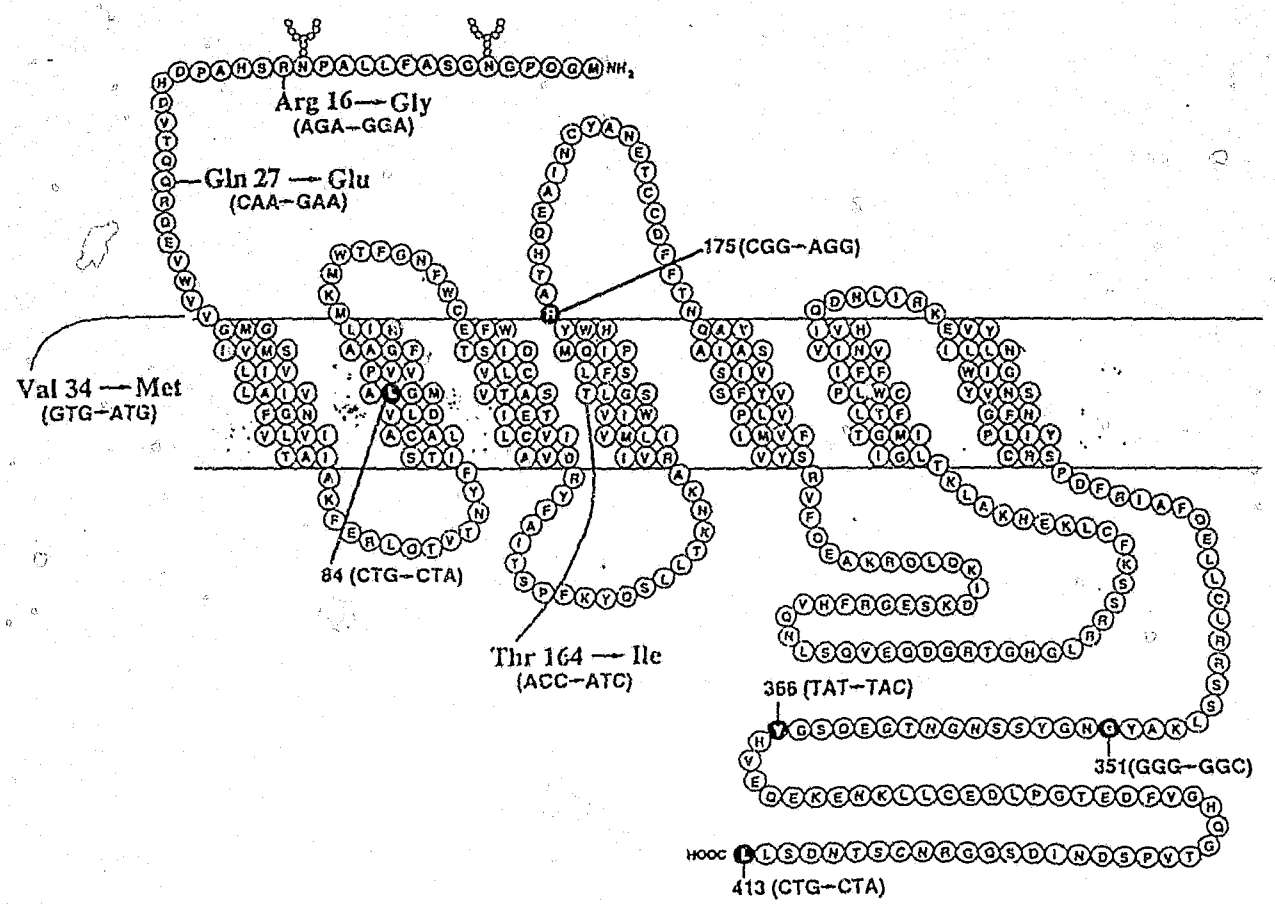


Figure 5.1 Structure of β_2 adrenergic receptor

5.2 Aims of this study

The prevalence of the Gly 16 polymorphism was investigated in nocturnal vs non-nocturnal and in severe vs moderate South African asthmatic children.

5.3 Materials and methods

5.3.1 Materials

Genomic DNA was extracted from 1 ml venous blood using the Higuchi method (1989). An allele specific PCR based approach was used. Allele specific PCR is based on the premise that under the appropriate conditions, a match between template and primer at the most 3' nucleotide is necessary for the generation to a PCR product and that mismatches result in no product. The allele specific PCR was performed to assess the polymorphism at nucleic acid 46 which results in a change in the encoded amino acid at position 16.

The following PCR primers were used:

5' -CTT CTT GCT GGC ACC CAA TA- 3' (sense)

and 5' -CCA ATT TAG GAG GAT GTA AAC TTC- 3' (antisense)

or the same antisense primer and

5' -CTT CTT GCT GGC ACC CAA TG- 3' (sense)

PCR using these primers generated an amplicon of 913 bp. Temperature cycling was 94^{0}C for 1 min, 60^{0}C for 2 min and 72^{0}C for 2 min for 30 cycles. 10 µL of the PCR reactions were then electrophoresed on 4% agarose gels and visualised with ethidium bromide staining and ultraviolet illumination (Figure 5.2). These results were confirmed by DNA sequence analysis (Figure 5.3).

5.3.2 Subjects

Allele frequencies were determined in 30 black and white (10 black) asthmatic children (12 girls) attending the Krugersdorp hospital as well as 54 black and white (20 black) control individuals (10 women). Informed consent was obtained from all subjects (in the case of minors, informed consent was obtained from their parents). All control individuals were obtained from the South African Blood Transfusion Services.

5.3.3 Statistical analysis

Statistical analysis was performed using the Chi-square test. A probability value of 0.05 was considered to be threshold for statistical significance.

5.4 Results

There was no significant difference between the healthy individuals and the asthmatic group. There was also no significant difference between the white control group and the black control group, thus we combined the black and white subjects. No significant difference was observed in the frequency of the Gly 16 polymorphism and the wild type Arg 16 in the control group with a probability of 0.558 obtained (Table 5.1).

From Table 5.2 it can be seen that the Gly 16 polymorphism is over-represented in the severe asthmatic patients. Of the 14 severe asthmatic patients (10) 71% of them had the Gly 16 polymorphism compared to only (4) 40% having this polymorphism in the 10 moderate asthmatic patients. When comparing the nocturnal asthmatic patients to the non-nocturnal patients, we find an almost equal distribution between the two groups of the Gly 16 polymorphism with 65% in the nocturnal group compared to 61% in the non-nocturnal group (Table 5.3).

We also found a significant increase in the prevalence of the Gly 16 polymorphism in those patients who required a long acting β stimulant to gain symptomatic control with a probability of 0.01.

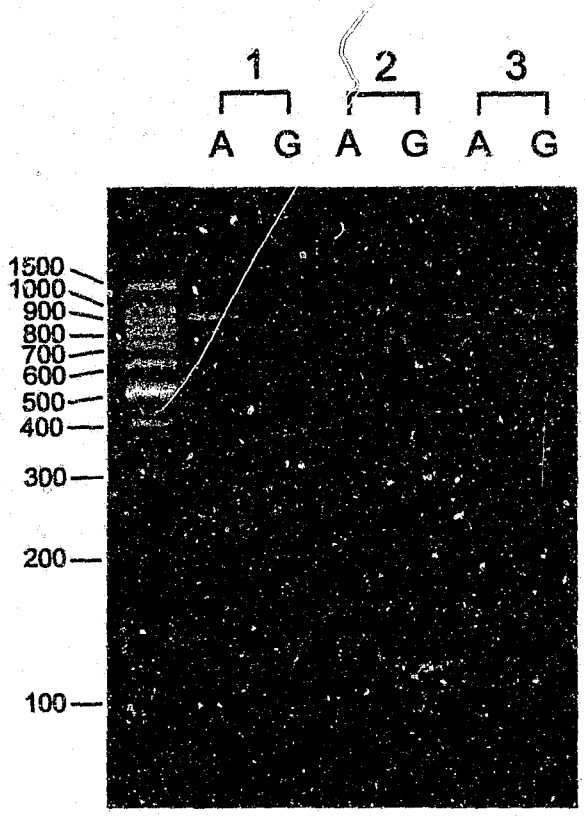


Figure 5.2: Shows the results of allele specific PCR from three patients who were either homozygous for Arg 16, homozygous for Gly 16 or heterozygous at this locus. Primer pairs (labelled as A or G) were designed to provide PCR products when adenosine or guanine, respectively, are at nucleic acid position 46 of the antisense strand.

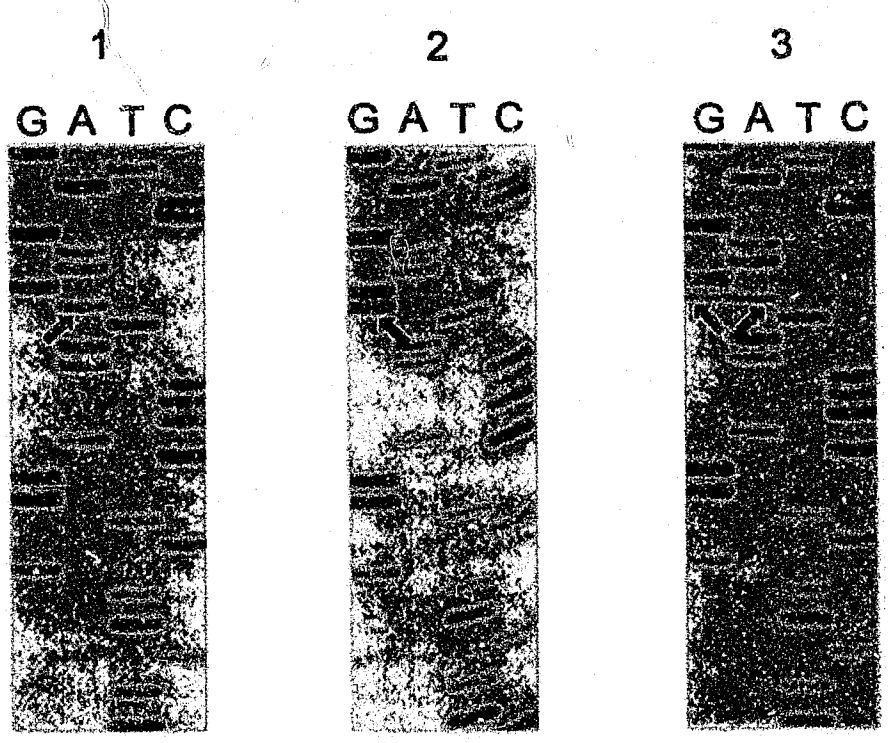


Figure 5.3: Indicates the polymorphisms as depicted by dideoxy sequencing (from Turki *et al.*, 1995)

Table 5.1: Allele frequencies of β_2AR polymorphism at amino acid position 16 in South African control individuals

Genotype	Number of individuals	Control Individuals	
		No.	Frequency
Gly 16	54	52	0.48
Arg 16	54	56	0.52

Table 5.2: Allele frequencies of β_2AR polymorphism at amino acid position 16 in severe and moderate South African asthmatic patients

Genotype	Number of individuals	Severe asthmatics		Moderate asthmatics	
		No.	Frequency	No.	Frequency
Gly 16	24	20	0.71	8	0.40
Arg 16	24	8	0.29	12	0.60

Table 5.3: Allele frequencies of β_2AR polymorphism at amino acid position 16 in nocturnal and non-nocturnal South African asthmatic patients

Genotype	Number of individuals	Nocturnal		Non-nocturnal	
		No.	Frequency	No.	Frequency
Gly 16	22	17	0.65	7	0.61
Arg 16	22	9	0.35	11	0.39

5.5 Discussion

β_2 adrenergic receptors are localised in a number of regions of the lung and thus may be involved in the pathophysiology of asthma. Clearly, the better characterised of these is bronchial smooth muscle, where activation of β_2 adrenergic receptors results in relaxation and increased airway diameter. While such bronchodilation is the primary therapeutic goal for administering β-agonists, the role of the β_2 adrenergic receptor in the absence of exogenous agonist in modulating airway tone is not clear. Relevant to asthma, β_2 adrenergic receptors are also present on epithelial cells, submucosal glands and vascular smooth muscle. In addition, these receptors are expressed on a number of immune cells such as lymphocytes, neutrophils and macrophages (Turki *et al.*, 1995). From many studies, it is clear that β_2 adrenergic receptor expression and function is dynamically regulated by a number of factors.

Mutations of the β_2 adrenergic receptor may not be a primary cause of asthma, however we do note that the severe asthmatic patients and those requiring a long acting β stimulant had a higher frequency of the Gly 16 polymorphism. It is important to note that the Gly 16 polymorphism of the β_2 adrenergic receptor appears from our study to be associated with one particular phenotype of asthma.

The factors that contribute to fatal or near fatal asthma include the severity of airway inflammation and structural changes, the degree of exposure to allergen, the appropriateness of therapy, the compliance of the patient, access to the health care system, and socioeconomic status. These factors or other as yet unidentified genetic

and environmental modifiers of severity could play a role in overshadowing the effects of the β_2 adrenergic receptor genotype. It has been previously reported distinct differences in the allelic frequencies of the β_2-AR polymorphisms between different ethnic groups (Weir *et al.*, 1998). These differences are significant for several reasons; (a) they underscore the necessity of accounting for ethnic background in clinical studies; (b) one might hypothesize that these differences may contribute to differences in asthma severity or the response to β_2-agonists. For example the Gln27 allele which is associated with greater bronchial hyper-responsiveness, higher IgE levels, and more severe asthma (Weir *et al.*, 1998), is more common in blacks than in caucasians, with the former group representing a portion of the U.S. population that is at greatest risk for asthma morbidity (Weir *et al.*, 1998).

It is clear from several studies that asthma and/or atopy may be the result of multiple gene defects. Recently several reports have indicated linkages with markers on chromosome 5q31 and allergy, IgE levels, bronchial hyper-responsiveness and asthma. Interestingly, the β_2 adrenergic receptor is localised to this region on chromosome 5, as are several cytokines [IL-3, IL-4, IL-5], (Liggett, 1997). Taking all these factors into account, the involvement of the β_2 adrenergic receptor polymorphism in a multi gene defect scenario for asthma or certain asthmatic phenotypes appears reasonable.

CHAPTER 6

MAJOR FINDINGS AND CONCLUSIONS

6.1 The Role of Alpha-1-Proteinase Inhibitor in Asthma

In this study no increase was found in the prevalence of the Z and S alleles of α_1-AT (the alleles commonly associated with the disease state) in the asthmatic population as compared to the control group. A novel finding was the identification of a new variant, the $M1E_{(JOHANNESBURG)}$. Although this new variant was not fully characterised (R. Mahadeva, personal communication), the finding of functionally abnormal α_1-AT in asthmatic patients may be of considerable importance in light of the fact that asthma has been recognised as an inflammatory disorder. Further evidence for the importance of characterising these new variants comes from the finding in this study that, a significant increase in severity was noted in those asthmatic patients who had rare variants as compared to those who had the normal M1M1 variants.

6.2 Polymorphic Interrelationships of the Interleukin-1 Gene Cluster and Plasma Concentrations of the Interleukin-1 Receptor Antagonist in Black and White South African Asthmatics and Control Subjects

Our results suggest that the VNTR's in intron 2 of the IL-1Ra gene do not influence the expression of IL-1Ra since there was no correlation between the

genotypes and plasma concentrations in black and white children with asthma prior to therapeutic intervention. However, the IL-1Ra plasma concentrations were significantly correlated with severity of the disease in these patients, thus the IL-1Ra plasma concentration could act as a marker for disease severity. Although this study did not show an association of a specific VNTR allele of the IL-1Ra and asthma in the two population groups there was a significant association between the 410-bp allele and the Pst I (-) allele of the IL-1Re in black individuals. The significance of this association is not known, but the possibility exists that the Pst I (-) allele of the IL-1Re could reflect changes in the expression of the receptor since the polymorphic site might influence the binding of the transcription factors to their relevant sequence motifs. Taking all these factors into account, our results suggest that the IL-1Ra is an anti-inflammatory molecule and the polymorphic association between the antagonist and the IL-1Re might indicate that this gene cluster is important in the inflammatory response which characterizes asthma, particularly in black asthmatics who tend to be more severely affected than the white asthmatics.

6.3 The Gly 16 Polymorphism of the β_2 Adrenergic Receptor in South African Asthmatic Children.

Our current study indicates that the severe asthmatic patients and those requiring a long acting β stimulant had a higher frequency of the Gly 16 polymorphism. It is important to note that the Gly 16 polymorphism of the β_2 adrenergic receptor appears from our study to be associated with one particular phenotype of asthma and the involvement of the β_2 adrenergic receptor polymorphism in a multi gene

defect scenario for asthma or certain asthmatic phenotypes appears reasonable.

6.4 The Possible Role of Corticosteroid Binding Globulin in Asthma

Sequencing analysis of exon 2 of the CBG gene did not detect any mutations in the atopic asthmatics and control subjects (this is most likely due to the small sample size). These findings would suggest that in our population, mutations within exon 2 of the CBG may not be involved in the pathogenesis of asthma. However, these findings do not exclude the possibility that other exons of this gene might carry mutations which could play a role in the development of asthma. Nor do the findings of the present investigation rule out the alternative possibility that the CBG gene could act as a marker for other mutations in other candidate genes that lie in close proximity to the CBG gene.

The present investigation by no means presumes to be exhaustive and other regions of the genes studied in this investigation remain to be searched for mutations that might be associated with atopic disorders and/or asthma severity. In addition, several other plausible candidates for atopic asthma have been proposed and there are complete genome searches being conducted by many investigators that are systematic attempts to identify and characterise all the genes that are involved in susceptibility to atopy and asthma. Such searches will not only identify additional candidate genes that play a role in atopic asthma, but are likely to also identify previously unknown genes that could predispose to asthma, or that could influence the severity of the disease. Such studies should also considerably advance existing

knowledge of the mechanisms that lead to asthma and could have implications for the development of effective therapies for this disorder.

In conclusion, the present investigation demonstrates the extent and complexity of genetic susceptibility to atopic asthma. In this study, serpins, in particular alpha-1-antitrypsin variants, polymorphisms of cytokines (IL-1 gene cluster) and β_2-adrenergic receptor have shown a definite association with asthma. This study further demonstrates the polygenic nature of asthma.

Appendix I

PCR Protocol

Materials:

The Taq DNA polymerase and the dNTPs were from Promega (Madison, WI); the primers used were synthesised by Genosys Biotechnologies Inc (Woodlands, TX); the sterile mineral oil was supplied by Sigma Chemical Co (St louis, MO); the sterile double-distilled water was supplied by SABAX (Johannesburg); the filtered pipette tips and the 0.5 mL PCR tubes were from Laboratory and Scientific Equipment Co (Johannesburg); all other reagents were of analytical grade (Merck, Darmstadt, Germany).

Reagent buffer

1.5 mM $MgCl_2$	0.031 g
10 mM Tris base	0.121 g
50 mM KCl	0.355 g

Dissolve in 95 mL of double-distilled water (ddH_2O), adjust the pH to 8.3 with HCl and adjust the volume to 100 mL with ddH_2O. Autoclave and store at 4°C, or aliquot and store at -20 °C (long-term storage).

Standard PCR protocol

The following was the standard PCR protocol used in this study, with modifications as described in the relevant chapters.

To a 0.5 mL PCR tube, the following reagents were added:

25 µL reagent buffer

10 µL dNTP mix

1 µL (typically 100 pmol) each appropriate primer

20-25 µL (~1 µg) DNA

Each sample was overlaid with 50 µL of lightweight mineral oil and the tubes were placed in a Hybaid DNA thermal cycler. Following the "hot start" and the subsequent reduction of the sample temperature to the appropriate annealing temperature, 2 units of Taq DNA polymerase were added to each sample. The samples were then processed as described in the relevant chapters.

APPENDIX II

Agarose gel electrophoresis

Electrophoresis through agarose gels is the standard method used to separate, identify and purify DNA fragments (Maniatis *et al.*, 1985). The technique is rapid, simple and resolves mixtures of DNA fragments that cannot be separated efficiently by other sizing procedures. The technique is based on the principle that negatively charged DNA fragments migrate toward a positive pole at different rates, depending on the following parameters:

(a) the molecular size of the DNA-linear duplex DNA molecules migrate in an end-on position through the gel matrix at rates that are inversely proportional to the $\log_{10}$ of their molecular weights (Maniatis *et al.*, 1982; Stine, 1989).

(b) the agarose concentration - a DNA fragment of a particular size migrates at different rates through gels containing different agarose concentrations. The relationship between the logarithm of the electrophoretic mobility of DNA (μg) and the gel concentration (τ) is linear, and is described by the following equation:

$$\log_{\mu} = \log_{\mu 0} - K_r \tau$$

where μ_0 is the free electrophoretic mobility and Kr is the retardation co-efficient, a constant that is related to the gel properties and the size and conformation of the migrating DNA molecules (Maniatis *et al.*, 1982).

(c) the applied current - at low voltages, linear DNA molecules migrate at a rate that is proportional to the applied voltage. As the strength of the electric field increases, the mobility of the high molecular weight DNA fragments increases differentially. Consequently, the effective separation range of agarose gels decreases as the voltage is increased-maximum resolution of DNA is obtained by running gels at no more than 5 V/cm (Maniatis *et al.*, 1982; Brown, 1989).

(d) base composition and temperature - the base composition of the DNA and the gel temperature during electrophoresis do not affect DNA migration through the gel significantly, which means that the relative electrophoretic mobilities of DNA molecules of different sizes do not change between 4°C and 30°C (Maniatis *et al.*, 1982).

The location of DNA within the gel can be determined directly by staining the DNA in the gel with a flourescent, intercalating dye such as ethidium bromide (EtBr); as little as 1 ng of DNA can be detected by direct examination of the gel upon ultraviolet (UV) illumination (Maniatis *et al.*, 1982), typically at a wavelength of 302nm. Ethidium bromide is a phenanthridine derivative and contains a planar group that intercalates between the stacked bases of DNA (Williams, 1989). The fixed position of the planar group and its proximity to the bases causes dye that is bound to DNA to flouresce at a greater intensity than dye that is in free solution (Williams, 1989). UV irradiation absorbed by the DNA at 260nm and transmitted to the dye, or irradiation that is absorbed at 300nm and 360nm by the bound dye, is emitted at 590nm in the red-orange region of the visible spectrum (Maniatis *et al.*, 1982; Stine, 1989).

Although EtBr is used in the detection of single-stranded (ss) and double-stranded (ds) DNA and RNA, the dye has a relatively lower affinity for ss nucleic acid and the fluorescence is poor (Williams, 1989). Furthermore, the presence of the dye (0.5 µg/mL) in the gel and in the running buffer tends to reduce the mobility of linear duplex DNA by approximately 15% (Maniatis *et al.*, 1982).

The ionic strength of the buffer used during electrophoresis affects the mobility of the DNA molecules. The more concentrated the buffer, the less current is carried by the actual DNA fragments and as a result, the slower the migration of these fragments (Davis, 1986). Tris-acetate is the most commonly used buffer, especially when the DNA fragments are to be excised from the gel for further manipulation, but its low buffering capacity and the fact that it becomes exhausted during extended runs (the anode becomes alkaline and the cathode becomes acidic), (Maniatis *et al.*, 1982), means that it is often not the best buffer to use. Tris-borate gives good resolution of DNA and has a significantly higher buffering capacity than tris-acetate (Maniatis *et al.*, 1982).

A permanent record of the agarose gel is kept by photographing the gel, using a transmitted or incident UV light (Maniatis *et al.*, 1982). The most sensitive film is high speed Polaroid Type 57 or 67 (ASA 3 000) and with the use of an efficient UV source (>2 500 µW/cm^2) and a good lens, an exposure of a few seconds is sufficient to obtain images of bands containing 10 ng of DNA (Maniatis *et al.*, 1982). Longer exposure times and a stronger UV source, enable the visualization of as little as 1 ng of DNA (Maniatis *et al.*, 1982).

Materials:

The agarose and low melting temperature agarose were supplied by Promega (Madison, WI); the Ficoll (Type 400) was from Sigma Chemical Co. (St Louis, MO); the sterile distilled water was from SABAX (Johannesburg); all other reagents were of analytical grade (Merck, Darmstadt, Germany).

Gel concentration (%)	Agarose (g)	5xTBE, pH8.2 (mL)	dH_2O (mL)
0.7	0.20	6	24
1	0.33	6	24
2	0.67	10	24
4	3:1*	10	90

*3 g agarose:1 g low melting temperature agarose

1. Add buffer at room temperature to a beaker that is 3-4 times the volume of the final solution.
2. Sprinkle agarose powder into the solution while it is being stirred in order to prevent the formation of lumps.
3. Boil the slurry in a microwave oven (100% power, 2-4 min), stirring at intervals, to liquefy the agarose.
4. After agarose is dissolved, cool to 50°C (higher for the 4% gels) add 1.5 µL/30 mL or 5-10 µL/100 mL EtBr (from a 10 mg/mL stock solution) to a final concentration of 0.5 µg/mL and pour the solution into a clean, sealed gel cassette with the comb in place (there should be a space of 0.5-1.0 mm between the bottom of the teeth and the base of the cassette, so that the sample wells are sealed).

5. After the gel sets at room temperature for 30 min, over with a 2-3 mm layer of running buffer and refrigerate for another 30 min.
6. Mix 10 µL of each PCR reaction with 8 µL of a 6x loading dye and load 15 µL of this mixture per well.
7. Run at 4.5 V/cm (for a gel of 12.5x20 cm) or 8.8 V/cm (for a gel of 5.5x9 cm); for the purposes of the present study, all agarose gels were run at 80 V in 0.5XTBE buffer for 45 min, (1% and 2% gels) and 1½ hour (3% and 4% gels).

Electrophoresis buffers

Buffer	Working solution (1x)	Concentrated stock (per litre) 50x
Tris-acetate EDTA (TAE)	0.04 M Tris-acetate	242 g Tris base
		57.1 mL glacial acetic acid
	0.002 M EDTA	100 mL 0.5 M EDTA, pH 8.0
		10x
Tris-borate EDTA (TBE)	0.089 M Tris-borate	108 g Tris base
		55 g boric acid
	0.002 M EDTA	40 mL 0.5 M EDTA, pH 8.0

6x Gel loading dye

bromophenol blue	0.25 g
xylene cyanol FF	0.25 g
Ficoll Type 400	15.00 g

Dissolve in 100mL sterile water, aliquot and store at 4°C.

APPENDIX III

Direct sequencing of PCR products

The most commonly use method for DNA sequencing is the chain termination method developed by Sanger *et al.* (1977). This method involves the *in vitro* synthesis of a DNA strand by a polymerase, using a single-stranded DNA template (Sanger *et al.*, 1977), with modern methods, double-stranded PCR products can be sequenced directly. Synthesis is only initiated at the site at which a primer binds to the template and the synthesis is terminated upon the incorporation of a nucleotide analogue that does not allow continued DNA strand elongation (Sanger *et al.*, 1977; Sanger *et al.*, 1982; Brown, 1989). These nucleotide analogues are 2',3'-dideoxynucleoside 5'-triphospates (ddNTPs) which lack the 3'-OH (hydroxyl) group that is essential for polymerase recognition and strand elongation, so that termination occurs specifically at places where the deoxynucleotide should be incorporated (Sanger *et al.*, 1977; Sanger *et al.*, 1982). Therefore, if a primer is incubated with a template with a DNA polymerase in the presence of a mixture of a ddNTP and the four dNTPs (one of which is radioactively labelled), a mixture of fragments all having the same 5' end and ddNTP residues at their 3' ends is obtained (Sanger *et al.*, 1977; Sanger *et al.*, 1982).

When using direct sequencing, most of the dNTPs and primers used during the PCR remain intact and will interfere with normal sequencing methods which also utilise primers and nucleotides. Two hydrolytic enzymes, shrimp alkaline phosphatase (SAP) and exonuclease I (exo I) can be used to remove the unwanted molecules. The exo I removes residual single-stranded (ss) primers and any ssDNA produced by the PCR,

while the SAP removes remaining dNTPs from the PCR, which would affect the labelling step of the sequencing reaction. It is essential that high quality and good yield PCR is performed in order to obtain high quality sequence information.

Materials:

The Thermo Sequenase radiolabelled terminator cycle sequencing kit, the shrimp alkaline phosphatase , the exonuclease and the 20x glycerol tolerant buffer (GTB) were obtained from Amersham Life Science Inc. (Cleveland, Ohio); the acrylamide and the urea were from Promega (Madison, WI); the ammonium persulfate, bis-acrylamide and TEMED were supplied by BioRad (Hercules, CA); the Sigmacote was from Sigma Chemical Co. (St. Louis, MO); the 0.5 mL microcentrifuge tubes were supplied by Laboratory and Scientific Equipment Co. (Johannesburg); the sterile double-distilled water was from SABAX (Johannesburg); the 3 MM Whatman filter paper was from Whatman International (England).

Sequencing protocol

1. Template preparation

To a 0.5 mL microcentrifuge tube add:

5 µL (0.5 pmol) PCR amplification mixture

1 µL (1.0 U/µL) Exonuclease I (exo I)

1 µL (2.0 U/µL) Shrimp alkaline phosphatase (SAP)

Spin the samples briefly to collect all the reagents at the bottom of the vial and incubate at 37°C for 15 min and inactivate the exo I and SAP by heating to 80°C for 15 min (performed in a Hybaid DNA thermal cycler).

2. Termination mixes

Prepare the termination mixes on ice. Mix 2 µL of nucleotide master mix (dGTP) and 0.5 µL of [α-^{33}P] ddNTP (G, A, T, or C - one of each per sequence) to produce a termination mix for each ddNTP. Label, fill and cap four tubes ('G', 'A', 'T', 'C') with 2.5 µL of each termination mix. To prepare termination mixes for (n) reactions, mix:

	G	A	T	C
Nucleotide master mix (µL)	(2 x n)	(2 x n)	(2 x n)	(2 x n)
[α-^{33}P] ddNTP (µL)	(0.5 x n)	(0.5 x n)	(0.5 x n)	(0.5x n)
Total(µL)	**(2.5 x n)**	**(2.5 x n)**	**(2.5 x n)**	**(2.5xn)**

3. Reaction mixture

For multiple (n) reactions with different primers and/or templates, prepare a n+1 batch of reaction buffer, water, polymerase and aliquot; then add the unique primer and/or template in the appropriate concentration and volume to the aliquots.

Reaction buffer	2 µL
DNA	_ µL (50-500 ng)
Primer	_ µL (0.5-2.5 pmol)
H_2O	_ µL (to adjust total volume to 20 µL)
Thermo Sequenase polymerase (4U/µl)	2 µL (8 units polymerase-add last)
Total	**20 µL**

4. Cycling termination reactions

Transfer 4.5 µL of reaction mixture (prepared in step 3) to each termination tube ('G', 'A', 'C', 'T') from step 2. Mix well and overlay with 10-20 µL of mineral oil (if needed). Cap and place the tube in the thermal cycler (Hybaid DNA thermal cycler). The following cycling parameters were used: 95°C for 1 min, 55°C for 1 min and 72°C for 2 min for a total of 30 cycles.

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