MOLECULAR AND IMMUNO-GENETIC ANALYSIS OF ALLERGEN-INDUCED TOTAL SERUM IgE LEVELS IN A MURINE MODEL OF ATOPY

BY 63

VENUGOPAL GANGUR

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BY

VENUGOPAL GANGUR

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The present study was undertaken with the hypothesis that non-MHC-linked gene(s) control AL-induced total serum IgE (AL-TSIgE) levels in mouse; and that such non-MHC loci can be mapped to specific chromosomes using the microsatellite based molecular-genetic mapping approach. In order to test these hypotheses, a large MHC-identical mouse colony consisting of F1 hybrids, a backcross-1 (BC1) and F2 intercross progeny was established. The parent strains, A.SW and SJL mice used in the establishment of this colony are identical at the MHC locus (both are H-2^s), and similar in IgG1 Ab responsiveness but differ in IgE isotype-specific responsiveness. The following specific objectives were addressed in this study:

1) determination of the mode of inheritance and the genetic control of AL-TSIgE levels; 2) examination of the role of Tcrvb8, Il4 and Ifg as candidate genes for atopic IgE responsiveness; and 3) molecular-genetic mapping of the loci controlling AL-TSIgE levels to specific chromosomes in these mice.

Inheritance, distribution and segregation analyses of TSIgE phenotype in F1, BC-1 and F2 progenies suggested that AL-induced high TSIgE levels is a Mendelian dominant trait and is under the control of a major non-MHC autosomal locus, with the possibility of existence of additional modifier locus (or loci).

As a second objective, three candidate genes viz., Tcrvb8, Il4 and Ifg, were examined for their role in IgE responsiveness. From a detailed analysis it was concluded that neither the inheritance nor the expression of Tcrvb8 gene was associated with IgE responsiveness in BC1 progeny. Furthermore, microsatellite marker analysis indicated that AL-induced persistent TSIgE levels in BC1 mice are not associated with DNA polymorphisms in the genomic interval containing either Il4 (on chromosome 11) or Ifg (on chromosome 10) genes.

Following exclusion of the three candidate genes a genome sweep was undertaken in an effort to genetically map the loci controlling TSIgE phenotype in these mice. Microsatellite marker-based exclusion mapping in BC1 mice identified three genomic intervals exhibiting significant association with the TSIgE phenotype. A locus on distal chromosome-8 was identified as a major one (tentatively designated as, *Iger (IgE responsiveness)-1*) and two other loci, one each on chromosomes-10 and 11 (designated as, *Iger-2* and *Iger-3*, respectively) were identified as modifiers. Further analysis of these genomic intervals by positional cloning should lead to the identification of the corresponding genes. The knowledge of these genes in mice and of the homologous genes in humans is expected to advance our understanding of the molecular basis of genetic predisposition to atopy.

ABBREVIATIONS

Ab	:	Antibody
Ag	:	Antigen
AHR	:	Airways hyperresponsiveness
AL	:	Allergen
Alum	:	Aluminium hydroxide
APC	:	Antigen presenting cell
BC1	:	Backcross-1
BHR	:	Bronchial heperresponsiveness
bp	:	Base pair
BSA	:	Bovine serum albumin
C	:	Constant
cDNA	:	Complementary DNA
CH	:	Heavy chain constant region
cM	:	centiMorgan
DNP	:	Dinitrophenyl residue
ds	:	Double strand
DZ	:	Dizygotic
ELISA	:	Enzyme-linked immunosorbent assay
FACS	:	Fluorescence activated cell sorter
FcεRI	:	High affinity receptor for IgE
FDC	:	Follicular dendritic cell
GIF	:	Glycosylation inhibitory factor
HDM	:	House dust mite
HLA	:	Human leucocyte antigen

hr	:	Hour
i.p.	:	Intraperitoneal
IFN	:	Interferon
Ig	:	Immunoglobulin
IL	:	Interleukin
Ir	:	Immune response
Kb	:	Kilo base
KBG	:	Kentucky Blue Grass
LTSIgE	:	Log_2(Total Serum IgE)
LSPIgE	:	Log_2(Specific IgE)
LSPIgG1	:	Log_2(Specific IgG1)
Log	:	Log_{10}
MAb	:	Monoclonal antibody
Mb	:	Megabase
mCD23	:	Membrane CD23
MHC	:	Major histocompatibility complex
min	:	Minute
mRNA	:	Messenger RNA
MZ	:	Monozygotic
Nb	:	*Nippostrongylus braziliensis*
OD	:	Optical density
OVA	:	Ovalbumin
PBS	:	Phosphate buffered saline
PCA	:	Passive cutaneous anaphylaxis
PCR	:	Polymerase chain reaction
PNP	:	Para nitro-phenyl Phosphate

sCD23	:	Soluble CD23
SPIgE	:	Specific IgE
SPIgG1	:	Specific IgG1
Taq	:	Thermus aquaticus
TCR	:	T cell receptor
Th	:	Helper T
Ts	:	Suppressor T
TSIgE	:	Total serum IgE
V	:	Variable

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I. GENERAL INTRODUCTION

AND

BACKGROUND

1. ATOPY: ITS SIGNIFICANCE IN TODAY'S WORLD:

The term "Atopy" was introduced in 1923 by Coca and Cooke to define a group of diseases including the common allergic disorders such as allergic rhinitis (hay fever), allergic asthma, conjunctivitis, and atopic dermatitis. Now, atopic disorders also include food allergies and the most dangerous life threatening anaphylactic shock, usually provoked by insects stings or parenteral medication. Although the term "atopy" has no precise definition, it is most often used to refer to a state of genetic predisposition towards development of immediate hypersensitivity reactions against common environmental antigens, characterized by the development of immunoglobulin (Ig) E class of antibodies (Abs) (Woolcock and King 1995).

Atopy is remarkably common in the general population and the prevalence estimated is between 20 and 30% (Wuthrich 1989). The atopic diseases afflict individuals of all ages and without significant gender bias (Barnes 1991, Richard 1993). In fact, the prevalence of young adults with a positive skin-prick test to house dust mite (HDM) or grass pollen has been found to be between 40% and 50% in western populations (Cline and Burrows 1989, Holford-Strevens et al 1984). Asthma in particular is the most serious of the atopic diseases in that

it is disabling and occasionally fatal in children and young adults. It is estimated that 2 to 6 % of the general adult population suffer from asthma-related conditions (Salvaggio 1979). Furthermore, asthma has been reported to complicate about 1% of pregnancies, while allergic diseases in general occur in approximately 20% women of child bearing age (Schatz et al 1993). Thus, atopic diseases are among the most common group of medical conditions that complicate pregnancy. Furthermore, direct and indirect effects of treatment and management of atopic disorders, on economy are considerable.

There is a widespread impression that the atopy-related diseases have become more common during the past 15-20 years. The scientific evidence as it stands at present supports this view that the prevalence of allergic diseases is in fact increasing (Richard 1993). Thus an alarming increase in its prevalence, morbidity and mortality over the past decade has led to its designation as the "number one environmental disease".

2.0 ETIOLOGY OF ATOPY:

Development of a clinically apparent allergic reaction involves exposure to common environmental antigens (Ags), such as those present in pollen, food, HDM, animal danders, fungal spores, insect venoms, etc., among many others, followed by a

variety of host responses such as, the production of IgE Abs, the release of inflammatory mediators following the binding of allergen to cell bound IgE on subsequent exposure, and the relative effects of inflammatory mediators on target organs. Since not every individual exposed to these antigens become allergic, the etiology of atopy includes both the genetic background of an individual and the environmental factors (Marsh and Blumenthal 1990).

The central event in the development of atopic allergy involves synthesis of elevated amounts of specific and non-specific (or of unknown specificity) IgE Abs. Evolution of the ability to synthesize IgE Abs has been a boon to mammals as a defence mechanism against helminth parasites such as schistosomes, that infect about 200 million people in the developing world (Capron and Dessaint 1992). Although every normal individual is genetically programmed to make IgE Abs to parasites, only about 25% of us make IgE Abs to innocuous environmental Ags called "allergens" (ALs). Despite recent advances, the precise reasons for this phenomenon are largely unknown.

2.1. Immunological Basis of Atopy:

Prausnitz and Kustner (1921) first demonstrated the presence of a factor in the blood of allergic subjects which, when

transferred to the skin of non-allergic individuals rendered them sensitive to ALs. This substance which used to be called "reagin" was later identified and characterized in 1966 by Ishizakas as a novel class of Ig, IgE (E, from "erythema" that the ALs produce in sensitized skin) (Ishizaka and Ishizaka 1975).

IgE constitutes a minute fraction of the total Ig in human serum (50-300 ng/ml, as compared with 10 mg/ml of IgG) and in other species as well. Although this molecule has the shortest half-life (~2.3 days) among Igs, its action is amplified by the activities of the receptors to which it binds. The high affinity receptor (FcεRI) on skin mast cells or basophils is responsible for immediate hypersensitivity reactions: thus when a multivalent AL "bridges" the IgE Abs bound to FcεRI, it cross-links the receptor molecules leading to perturbation of the cell membrane. This triggers degranulation of the cell, rapid release of stored mediators, notably histamine, leading to the immediate wheal and flare reaction. The immediate reaction is followed by the synthesis and secretion of cytokines that attract and activate inflammatory cells leading to the late-phase reactions (Ishizaka and Ishizaka 1989, Lichtenstein 1993). Furthermore, in allergic asthma, airways hyper-responsiveness may contribute to these events on exposure to allergens, leading to chronic and occasionally fatal consequences.

2.2. Genetic Basis of Atopy:

Genetic capacity to synthesize high levels of IgE confers an advantage in combatting parasites among populations that have evolved in tropical and subtropical regions. Once these diseases have been eliminated or reduced, the inherited high capacity to synthesize IgE renders a high proportion of the population vulnerable to allergy toward environmental agents (Marsh et al 1981). From the clinical viewpoint, those individuals emigrating from environments with a high host-parasite burden, become "genetically" the most likely to "turn on" with severe allergy-associated disease.

Clinical studies since early in this century have revealed that atopic disorders run in families. The hypothesis that a single major gene might be essential for the expression of atopy can be traced to the earlier part of this century when researchers were trying, without success, to fit allergy to simple Mendelian patterns of inheritance (Marsh and Blumenthal 1990). However, extensive genetic analysis of atopy in families over the past decades has led to the consensus that they are 'complex disorders', which do not exhibit classical Mendelian patterns of inheritance (Marsh and Meyers 1992).

The term 'complex' is used to denote diseases that are multifactorial and/or exhibit genetic heterogeneity (Marsh 1996, Lander and Schork 1994). Complex diseases are described

as multifactorial when they are determined by interactions between multiple major and minor genes, and usually involve important non-genetic factors for their expression. Genetic heterogeneity signifies that different genes or groups of interacting genes, lead to the expression of the clinical phenotype. In allergic diseases, several types of genetic heterogeneity have been postulated (Marsh 1994). First, different groups of genes may be associated with differential expression of IgE and /or inflammatory responsiveness, or with different types of disease manifestations - e.g., asthma, allergic rhinitis and eczema, including different combinations and permutations of these conditions. Furthermore, for the different major genes involved in the expression of allergic disease, it remains probable that a variety of mutations exists which modulate gene function, and therefore disease expression, depending on the environmental context (Marsh 1994, Marsh 1996). Thus, atopic disorders are not due to a single-gene defect, a hypothesis which was favoured by the early researchers on the genetics of allergy. Recently, it has been suggested by Dr. David Marsh, to consider the expression of allergic diseases as "the net result of the effects of overlapping constellations of genes that interact in an infinitely large number of ways, with input from multiple environmental factors" (Marsh 1996).

The genetic determinants of atopy may act at various points

extending from the time of sensitization to allergens, to the complex cellular events involving IgE synthesis, eventually leading to the expression of clinical symptoms. Thus, the genetic factors predisposing to allergies may include those genes that control: i) the IgE production and/or its metabolism; ii) the inflammatory mediator release from basophils and mast cells; and iii) the end organ response such as bronchial hyperresponsiveness (BHR).

2.3. Role of Environmental Factors in Atopy:

Atopy is the end result of a complex series of interactions between the host and a wide variety of environmental factors. Thus, a number of environmental factors have been proposed to influence the expression of atopic diseases in genetically susceptible individuals. Some of them are as follows:

i) increased exposure to inhalant ALs such as HDM, animal danders and protein antigens from pollens (Businco et al 1988, Sporik et al 1990);

2) early antigen exposure due to changes in infant-feeding practices, such as bottle-feeding vs breast-feeding (Host et al 1988);

3) viral infections during childhood may increase the chance of atopic sensitization (Welliver et al 1980);

4) influence of smoking, especially in case of children born to smoking mothers, since there is now a good evidence that

maternal smoking does increase the risk of persistent allergy (Kjelman 1981, Magnusson 1986);

5) increased levels of air pollutants such as SO_2, NO_2 and O_3, which have been demonstrated to increase the permeability of the mucous membrane of the airway tract in animals, appear to favour the development of allergic response (Matsumura 1970).

6) It has been shown in animal models that the diesel exhaust particles have an adjuvant activity in producing IgE Abs *in vivo* (Muranaka 1986);

7) occupational exposure, especially in case of occupational asthma; it is reported that out of 2-6 % of the general adults who are estimated to have asthma, 2-15% of this asthma may be of occupational origin (Bernstein 1989).

Although, a number of factors as listed above have been proposed to influence the expression of clinical atopy, it should be noted that these factors will be important in genetically predisposed individuals.

3.0 INDUCTION OF IgE BIOSYNTHESIS:

IgE has been shown to play a dominant role in allergic inflammation. Therefore, the mechanisms of its synthesis and regulation have become subjects of increasing interest during the last 20 years. Several questions have been addressed regarding the molecular events during T cell and B cell interactions, the molecular basis of the switch recombination

to IgE isotype, and on the role of additional modulatory mechanism involved in the biosynthesis and regulation of IgE Abs (Finkelman 1990, HayGlass 1995).

Our current understanding of the regulation of IgE synthesis has been primarily derived from *in vitro* experiments together with functional studies of recombinant cytokines in humans and *in vitro* and *in vivo* data from rodent studies and specific gene-knock out (-/-) mice studies. These studies have indicated that the induction of IgE biosynthesis requires both the direct T-B cells interaction, including MHC-restricted and non-MHC restricted events, and the generation of cytokines.

3.1 Signals Required for IgE Induction:

a) Two signal model of IgE synthesis:

The induction of IgE synthesis is highly T cell dependent. The process of AL sensitization is initiated by the presentation of ALs to T cells by antigen presenting cells (APCs) including B cells. The TCR binds transiently to its ligand - an AL derived peptide (T-cell epitope) bound tightly in the specialized groove of a MHC molecule on the surface of an APC. This transient binding results in TCR dimerization and consequent signal transduction into the nucleus. In addition to the trimolecular interaction, there is co-stimulator

interaction between T cell, via its CD28 receptor, and its ligand B7 on APCs (Bluestone 1995). Interactions involving both trimolecular ligation and co-stimulation leads to T cell activation and production of cytokines.

Based on current evidence, a two signal model has been proposed for the induction of B cells to synthesize IgE : the first signal critical for switching to IgE production is provided by IL-4 or IL-13, which induces sterile ϵ germ-line transcripts in B cells (Paul and Seder 1994). The second signal is provided by a direct B cell-T cell physical interaction, which induces competence for switching. Although the latter event is essential for switching, it is not Ig class specific. It is now widely accepted that the principal molecules that deliver this signal are CD40 on B cells and its ligand gp39 (CD40L) on activated T cells. This promotes the growth and differentiation of B cells and in concert with the first signal, induces productive ϵ transcripts, thereby initiating IgE synthesis (Rothman et al 1989).

b) Molecular events during isotype switching to IgE:

Switching to different Ig isotypes enables the secretion of Abs with different effector functions but retaining variable-region specificity. IL-4/IL-13 switching to IgE i.e., the generation of VDJ-Cϵ transcripts, could in principle, occur by

one of three possible ways: 1) trans-splicing between VDJ-Cμ and Cε (Durdit et al 1989); 2) splicing of long RNA transcripts to create VDJ-Cε (Mackenzie and Dosch 1989), or 3) deletional switching of DNA resulting in Sμ/Sε recombination, followed by transcription (Shapira et al 1991). However, the bulk of evidence favours deletional switching.

Before the switching event occurs, resting B cell express IgM and IgD on their surface. Immunoglobulin isotype switching to IgG, IgA and IgE is based on a deletion/recombination event. In the case of IgE, rearranged V(D)J genes, encoding the variable Ig portion are positioned downstream from the Cε gene that encodes the heavy chain of IgE. During this process the intervening sequences, which include the CH gene of the previously expressed Ig are deleted. Switch (S) regions flanking each CH gene serve as recognition sequence for joining DNA segments together during the process of switch recombination. Cytokines are thought to control class switching by modulating the accessibility of particular S regions to the recombinase enzyme that recombine the CH regions of the targeted Ig-isotype (Rothman et al 1990).

IL-4 mediated switching is associated with the induction of transcription at a site upstream of the switch region for IgE (Sε), in a region designated as Iε. The immature transcript (Iε/Sε/Cε) is spliced to a mature germline transcript (IεCε).

This transcript is sometimes referred to as a "sterile" transcript since there is no evidence that it encodes a protein product (Shapira 1991). The induction of transcription at Iε may reflect the opening of the chromatin in the Sε region, targeting this region for the S/S recombination events resulting in deletional switching and transcription of VDJ-Cε.

c) Role of surface molecules:

Ligation of a number of surface molecules on T and B cells (e.g., ICAM1-LFA1 and LFA1-ICAM1) are also involved in conjunction with the MHC-II restricted interactions between T and B cell but these interactions are not themselves capable of triggering the B-cell activation signal (Klaus et al 1994, Bonnefoy et al 1993a). Another factor that may play a role in the induction of an IgE response is CD23, the low affinity IgE receptor, which is upregulated on B cells by IL-4 (Conrad 1990). However, there is no evidence that CD23 regulates class switching *per se*.

4. REGULATION OF IgE SYNTHESIS:

4.1. Cytokine Mediated Regulation:

a) Cytokines that promote IgE synthesis:

Three cytokines other than IL-4 viz., IL-2, IL-5 and IL-6, are required for, or contribute to, the induction of an IgE response in some systems. IL-2 acts synergistically with IL-4 to stimulate IgE secretion by human B cells cultured with PMA

(Lundgren et al 1989). IL-5 enhances IgE secretion by mouse B cells cultured with LPS plus IL-4 and by human PBMCs cultured with suboptimal concentration of IL-4 (McHeyzer-Williams 1989). Endogenously produced IL-6 is critical for the generation of the IgE responses made by human PBMC cultured with IL-4 (Vercelli et al 1989) or with IL-4 plus anti-CD40 Ab, since in each case, anti-IL-6 Ab abrogates the response (Jabara et al 1991). All these cytokines have been implicated, however in stimulating the differentiation of B cells into Ab secreting plasma cells, and it is unlikely that they are specifically involved in stimulating B cells to switch to IgE expression. Neutralizing Abs to IL-2, IL-2R, IL-5, IL-6 and IL-6R have failed to interfere with the *in vivo* IgE responses in mice, induced by anti-IgD injection or with *Nippostrongylus braziliensis* (Nb) parasite (Finkelman 1990).

b) Cytokines that inhibit IgE synthesis:

IFN-γ is a potent inhibitor of IgE production by switching to IgG2a. It also inhibits IgE secretion by human PBMCs cultured with IL-4, and human B cells cultured with IL-4 plus EBV (Thyphronitis et al 1989, Thyphronitis et al 1991). IFN-γ however has no effect, on IgE secretion by human B cells cultured with IL-4 plus anti-CD40 Ab (Gascan et al 1991). IFN-α also inhibits IgE secretion by human PBMCs cultured with IL-4 (Gauchat et al 1990, Pene et al 1988). Another cytokine, IL-12, has been reported to suppress IgE secretion by human B

cells stimulated with IL-4 plus cortisol (Kiniwa et al 1992). Prostaglandins (PGs) can also inhibit IgE production in some systems (Pene et al 1988). IFN-γ, IFN-α and PGE2 analog have all been found to selectively inhibit *in vivo* mouse IgE responses to injection of anti-IgD Ab (Finkelman et al 1988, Finkelman et al 1991). TGF-β inhibits IgE production and appears to be involved in switching to IgA (Kimata et al 1992). IL-8 inhibits IgE production in an isotype specific manner (Gauchat et al 1992).

Thus, a number of different cytokines can inhibit IgE production in different systems. However, the inhibitory effects of these molecules vary, depending on the co-stimulus that is employed to induce the IgE response.

c) Th1/Th2 Paradigm:

Studies of T-cell lines and clones indicate that different T cells secrete distinct patterns of lymphokines. At least three patterns have emerged, which are said to be derived from Th0, Th1 and Th2 cells. Th0 cells secrete a wide variety of lymphokines. Th1 cells secrete IL-2 and IFN-γ but not IL-4 or IL5. Th2 clones secrete IL-4 and IL-5 but not IL-2 or IFN-γ (Mossman et al 1986, Mossman and Coffman 1989).

It has been demonstrated that while IL-4 promotes the development of Th2 lymphocytes, IFN-γ promotes that of Th1

cells (Mossman and Coffman 1989). IFN-γ is able to inhibit not only the development of Th2 lymphocytes but also the induction of IgE synthesis. This dichotomy between Th1 and Th2 cells has been well established in the murine system initially and later in humans. In humans such a dichotomy has been observed in cytokine profile analyses of Ag specific long-term cultured T cell clones from atopic patients (Romagnani 1990).

The relationship between the balance of Th1/Th2 cytokine production *in vivo* seems to have a wider relevance with respect to the quality of the immune response generated to a specific antigen, like cell mediated vs humoral immune response (Romagnani 1992, Paul and Seder 1994). A dysregulated Th1/Th2 cytokine production *in vivo*, can potentially inhibit or promote IgE production as has been reported by many groups of workers (Finkelman 1990, Romagnani 1995, Paul and Seder 1994).

However, recently Kelso put forward a hypothesis that cytokine producing T cells cannot be classified into discrete subsets (Kelso 1995). Accordingly, individual T cells in a population display remarkable diversity in their cytokine profiles, collectively forming a continuous spectrum in which Th1 and Th2 cells may be only two possible extreme phenotypes.

d) T-cell independent induction of IgE secretion:

It has been shown that mast cells activated by IgE receptor cross-linking are able to produce a number of cytokines, such as IL-3, GM-CSF, IL-4, IL-5 and IL-6 (Plaut et al 1989, Gordon et al 1990, Brown et al 1987, Wodnar-Filipowcz et al 1989). Thus, activated mast cells are able to generate cytokines acting on B cells, such as those produced by Th2 cells. The interaction of basophils and B cells through CD40-CD40L binding together with basophil derived IL-4 is able to induce human IgE synthesis *in vitro* in the absence of T cells (Gauchat et al 1993). This kind of experiment in the mouse also has given the same results (Plaut et al 1989). These studies raise the strong possibility that besides T cells, basophils and/or mast cells may be involved in the induction of IgE *in vivo* and basophil derived IL-4 might play a role in the differentiation of T cell to the Th2 phenotype. However, physiological relevance of this process in the regulation of IgE synthesis *in vivo* warrants further investigation.

e) IL-4 independent IgE response:

The only *in vivo* system in which an IgE response can be elicited that is not inhibitable by IL-4 antagonists is one in which the response is stimulated in mice by injection of a goat anti-IgE Ab (Katona et al 1991). It is believed that this response is produced by B cells that had switched to expression of mIgE prior to immunization, and that the

resulting lack of a requirement for switching during the course of the response explains the lack of a requirement for IL-4. In man, PBMC from normal non-atopic donors only produce IgE if cultured in the presence of exogenously added IL-4 (Delespesse et al 1989). However, PBMC derived from atopic donors produce IgE spontaneously *in vitro* without the addition of IL-4. The spontaneous IgE synthesis was not affected by the addition of neutralizing anti-IL-4 Ab to the cultures. Whereas the IgE response induced by exogenously added IL-4 was abrogated by this treatment. This suggested that in atopics IgE switched B cells exist that have become IL-4 independent and may persist for longer periods (Delespesse et al 1989).

4.2. IgE-IgE Receptor Network Mediated Regulation of IgE Synthesis:

There is a considerable evidence that implicate the role of high (FcεRI) and the low (CD23) affinity IgE receptors, in the regulation of IgE synthesis by a feed-back network. The complement receptor CR2, by virtue of it being a ligand for CD23, is also implicated in this network (Conrad 1990, Sutton and Gould 1993). Role of these receptors is briefly discussed below:

a) Role of FcεRI:

The potential role of FcεRI in atopy in general and IgE

regulation in particular has gained wider attention recently with the proposed role of the β chain gene of this receptor as a candidate gene for atopy (Shirakawa 1994b). In addition, other studies have implicated FcϵRI in the regulation of IgE synthesis. There is a direct correlation between IgE titers in the serum and the distribution of mast cells and basophils, which bear FcϵRI (Mudde et al 1995). However, since the discovery of FcϵRI on Langerhans cells, as well as on monocytes from atopic individuals and eosinophils in parasitic infections, the dogma that mast cells and basophils are unique in expressing FcϵRI no longer holds. In addition recently, it has been proposed that this receptor may be involved in IgE-mediated Ag/AL presentation, which is believed to amplify the allergic-cascade by focusing AL presentation and IgE upregulation (Mudde et al 1995). Furthermore, FcϵRI may exert a regulatory effect on IgE synthesis through IL-4 production by mast cells (Paul et al 1992). However, it should be noted that the rate of clearance of serum IgE in FcϵRI (α-chain) -/- mice has been shown to be not different from wild type (+/+) mice (Dombrowicz et al 1993). Thus, although this receptor may potentially be involved in regulating the synthesis of IgE, it seems to play no role in its clearance.

b) Role of CD23:

CD23 is the low affinity receptor for IgE, also called as FcϵRII. This receptor is different from all other Fc receptors

because it comprises a single chain, this being a type 2 integral membrane molecule with its C-terminal oriented extracellularly (Conrad 1990). It is a member of the calcium-dependent (C-type) lectin family of proteins. Mouse and human CD23 are structurally related. Both human and mouse membrane-bound CD23 (mCD23) can be cleaved off the cell surface, and form a soluble form of the receptor, sCD23 (Letellier et al. 1989).

In the mouse, CD23 is primarily found on B cells and FDCs and its expression is developmentally regulated (Conrad 1990, Sutton and Gould 1993). In the human, the expression pattern is more diverse. Two forms of CD23 exist which differ in their cytoplasmic amino terminal sequence (Yokota et al 1988). The a form is similar to the mouse CD23 both in structure and expression. The b form represents an inducible form and has been reported on T cells, eosinophils, monocytes, platelets and Langerhans' cells (Delespesse et al 1991). Recently, evidence has been brought forward for the similar existence of two forms of CD23 in the mouse (Richards and Katz 1991) but the issue is still controversial (Conrad et al 1993, Richards, ML and Katz DH 1994).

A clear role for CD23 in IgE regulation was elucidated using CD23-/- mice (Yu et al 1994). The rate of clearance of IgE from serum was identical in -/- and +/+ mice. Immune response

to helminth infection was unaffected. In contrast, immunization with thymus-dependent antigens lead to increased and sustained SPIgE Ab titers and a two-fold higher levels of TSIgE in -/- mice compared to +/+ control mice. This study, suggested that CD23 may act as a negative regulator of serum IgE levels in mice but CD23 is not essential for class switching or for plasma cell differentiation and clearance of serum IgE.

In contrast to the above study, the enhanced IgE responses were not observed in two other CD23-/- mice reports (Stief et al 1994, Fijiwara et al 1994). Steif et al (1994) studied in vitro IgE production and did not find much difference between +/+ and -/- mice. Fujiwara et al (1994) studied T-dependent immune responses, but could not confirm the results of Yu et al (1994) described above. Thus, CD23-/- mice studies have yielded variable results on regulation of IgE levels.

B-cell expressed CD23 has another proposed function, as Ag-IgE-CD23 complexes present Ag very effectively to T cells. Thus, IgE-Ag immune complexes when bind to membrane CD23, the B cells phagocytose the IgE-Ag complex and present Ag to the T cells, a process termed as Ag-focusing or AL-mediated Ag presentation (Mudde et al 1995). This role is supported by the evidence from CD23 knock-out mice. Thus, CD23-/- mice had a defect in IgE mediated Ag presentation (Fujiwara et al 1994).

It has been postulated that this, in turn, could lead to the continuous activation of the immune system by very low concentration of AL, thereby upregulating IgE production.

It has been suggested that binding of IgE-containing immune complexes to CD23 renders the B cell unresponsive to further stimulation or directly suppresses IgE synthesis. The support for this model comes from two studies: 1) anti-CD23 Abs or IgE-containing immune complexes suppress human B cell proliferation and IgE production *in vitro* (Yu et al 1994); and 2) anti-CD23 Ab treatment of rats inhibits specific IgE immune responses by conveying a negative signal to B cells (Flores-Romo et al 1993). This function of CD23 is reminiscent of that of FcγR, which also turns off B cells after binding to specific immune complexes.

c) Role of CR2:

CR2, also known as CD21 is a highly glycosylated membrane protein found on B cells, FDCs and some T cells and basophils. It is a receptor for a diverse array of ligands viz., fragments of complement component C3 (C3dg, iC3b, C3d), the Epstein-Barr virus (EBV) coat protein gp350/220 and IFN-α (Sutton and Gould 1993). The discovery that CR2 is a counter-receptor for CD23 established that CR2 is the ligand for the inferred lectin function of CD23. It appears to mediate two important cytokine activities ascribed to CD23 (Sutton and

Gould 1993. They are, 1) upregulation of IgE synthesis: thus cross-linking of mIgE and CR2 (which forms a signal transduction complex with CD19 on B cells) on the surface of committed B cell by sCD23 results in the upregulation of IgE synthesis; anti-CR2 interacts with B-cell associated CR2 and enhances IgE production. Similarly, sCD23 can trigger CR2 on B cells and enhance IgE synthesis. Furthermore, cross-linking of CD21 by CD23 induces degranulation of basophils; 2) Most recently, the CD23-CR2 interaction has been implicated in the rescue of germinal centre B-cells from apoptosis; it thus participates in the most fundamental event in the immune response, the clonal amplification of B cells selected by Ag (Bonnefoy et al 1993b). Flores-Romo et al (1993) have demonstrated that the interaction between CD23 on T cells and CD21 on B cells may also be important for IgE regulation.

d) Regulation of IgE synthesis: A complex network of IgE, IgE receptors and CR2:

Nature has ascribed to the IgE-IgE receptor system, an important function like the release of inflammatory mediators from mast cells and basophils to protect against parasites. However, in order to prevent the potentially harmful consequences of a dysregulated IgE-IgE-receptor network, homeostasis in the system obviously requires mechanisms that limit IgE production.

Recently Sutton and Gould (1993), proposed a model to explain the regulation of IgE synthesis in humans. The salient features of this model are as follows: IgE itself can exert negative-feedback control as it can bind to CD23 on B cells. Thus, cross-linking of mCD23 by IgE sends a negative signal to B-cells and thus inhibits IgE production. IgE-Ag complexes appear to act by blocking both the release of sCD23 and the binding of soluble CD23 to CR2. In addition, degradation of IgE in the course of Ag presentation mediated by CD23 may also contributes to feed-back control. On the other hand, a positive control also seems to exist: binding of IgE-Ag to FcϵRI on mast cells can induce IL-4 secretion, which can induce CD23 and CD40 expression and class switching to IgE, in Ag activated B cell. Release of sCD23 form the B cell itself or from Langerhans cells and cross-linking of mIgE and CR2 by sCD23 can promote survival of the IgE-committed B cells, leading to upregulation IgE synthesis.

It should be noted that, in view of the variable results from CD23-/- mice, the *in vivo* relevance of this model needs to be confirmed.

4.3. Regulation of IgE synthesis in other models:

In other systems, IgE responses have been shown to be regulated by IgE-binding factors (IgE-BFs) and by T cells bearing γ/δ TCRs or specific Vβ TCR.

a) Role of IgE-binding factors:

This model of IgE regulation, proposed by Ishizaka is based mainly on *in vitro* observations (Ishizaka 1985). In these models, the same IgE-BF performs both as an enhancer and suppressor of *in vitro* IgE synthesis, based on its degree of glycosylation. The IgE-BF peptide is related antigenically to CD23. The factor that inhibits glycosylation of IgE-BF, called glucosylation inhibition factor (GIF), reacts with a MAb specific for lipomodulin, which was also found to recognize T cell suppressor (Ts) factors from two types of hapten specific T suppressor hybridomas. In in *vitro* assays, these Ts factors were found to have IgE-BF inhibitory activity similar to GIF and lipomodulin (Steele, 1989). Both Ag binding and non-Ag binding forms of GIF have been described. The Ag binding factors are produced by hybridomas that express the CD3 α/β TCR complex and the factors themselves were found to be serologically related to this TCR (Iwata 1989).

The weakness of this model is that it is based primarily on *in vitro* observations. To date, there is no convincing evidence in support of these factors in the *in vivo* regulation of IgE responses.

b) Role of T cells bearing specific Vβ or γ/δ TCRs:

A series of reports based on studies in mouse, have implicated a role for TCRVβ8+ and TCRVβ2+ T cells in regulating specific

IgE responses to inhaled ALs like OVA in mouse (Renz and Gelfand 1992, Renz et al, 1992, Renz et al 1993, Renz et al 1994). Thus, it has been reported that while TCRVβ8+ T cells upregulate IgE response to OVA, TCRVβ2+ T cells inhibit OVA specific IgE response. Recently, McMenamin et al (1994) reported the regulation of IgE response to inhaled OVA by antigen specific γ/δ T cells. Thus, adoptive transfer of a small number of γ/δ T cells form OVA-tolerant mice selectively suppressed Th2-dependent IgE Ab production without affecting parallel IgG responses. Thus, these studies implicate T cells bearing specific TCRs in the regulation of SPIgE synthesis in mouse.

5.0 GENETIC DISSECTION OF COMPLEX DISORDERS LIKE ATOPY: THE FOUR-FOLD STRATEGY.

The application of genetic mapping during the 1980s has led to the isolation of genes responsible for simple Mendelian diseases such as cystic fibrosis and myotonic dystrophy (Cooper and Schmitdtke 1992). However, many important medical conditions, including asthma, atopy, heart disease, hypertension, diabetes and schizophrenia, do not follow simple inheritance patterns and hence have been classified as complex disorders (Lander and Schork 1994). The challenge facing medical scientists today is to dissect the multifactorial causes of these diseases.

In principle, genetic mapping of any trait - simple or complex- is dependent upon finding those chromosomal regions that tend to be shared among affected relatives and tend to differ between affected and unaffected individuals. Conceptually, this involves three steps: 1) Scanning the entire genome of a segregating progeny with a dense collection of genetic markers such as microsatellite markers; 2) calculating an appropriate linkage statistic S(X) at each position X along the genome; and 3) identification of the regions in which the statistic S shows a significant deviation from what would be expected under independent assortment (Lander and Shork 1994).

There are four commonly useful approaches to achieve this goal, they are: a) linkage analysis in pedigrees, b) allele-sharing methods, c) association studies (all in humans), and d) genetic analysis in experimental crosses using laboratory animals like mouse or rat.

5.1 Linkage Analyses:

Linkage analysis involves constructing a transmission model based on complex statistical analyses to explain the inheritance of a disease in pedigrees. This model is straight forward for simple Mendelian traits but can become very complicated for complex traits. Linkage analysis has been applied to several simple Mendelian traits, as well as to some

complex diseases like atopy and asthma (Cookson and Hopkins 1989, Marsh et al 1994, Meyers et al 1994), breast cancer (Hall et al 1990) and Psoriasis (Tomfohrde et al 1994).

5.2 Allele-Sharing Methods:

These involve testing whether affected relatives inherit a chromosomal region identical-by descent (IBD) more often than that expected under random Mendelian segregation. Affected sib pair analysis is a special case, in which the presence of a trait-causing gene is revealed by more than the expected 50% IBD allele sharing. The method is more robust for genetic complications than linkage analysis but can be less powerful than a correctly specified linkage model. This method is now popular for genetic analysis of asthma and atopy, eg., for TSIgE levels (Marsh et al 1994, Meyers et al 1994) and BHR (Postma et al 1995). Other examples include type I diabetes (Todd et al 1991), and bone density in postmenopausal women (Amos et al 1986).

5.3 Association Studies:

These test whether a particular allele occurs at higher frequency among affected than unaffected individuals. Association studies thus involve population correlation, rather than co-segregation within a family. Examples include HLA associations with atopy and asthma (Marsh 1989), and in many autoimmune diseases like SLE, rheumatoid arthritis,

apolipoprotein E4 in Alzheimer's and angiotensin converting enzyme (ACE) in heart disease (Lander and Schork 1994).

5.4 Experimental Animal Crosses:

Experimental crosses of mice or rats, such as Backcross and F2 intercross, offer an ideal setting for genetic dissection of complex disorders. The main advantages of this approaches are: 1) the opportunity to study hundreds of meiosis from a single set of parents selected for disparity in a given trait like TSIgE levels; 2) they can provide a large number of progeny while ensuring genetic homogeneity and hence in such crosses the problem of genetic heterogeneity disappears, which is a major problem for atopy studies in humans and 3) far more complex genetic interactions can be probed than is possible for human families (Lander and Schork 1994). An important drawback on the use of experimental crosses that deserves to be emphasized, is that the genetic mapping results need not be consistent among different crosses. Linkage analysis reveals only those trait-causing genes that differ between the two parental strains used. Thus a locus may thus be detected in an (A X B) cross, but not in an (A X C) cross. Moreover, the effect of a locus allele may change -or even disappear- when bred onto a different genetic background, because of epistatic effects of the other genes (Lander and Schork 1994). Examples where this approach is used include AHR (De Sanctis et al 1995), type I diabetes (Todd et al 1991) and susceptibility to

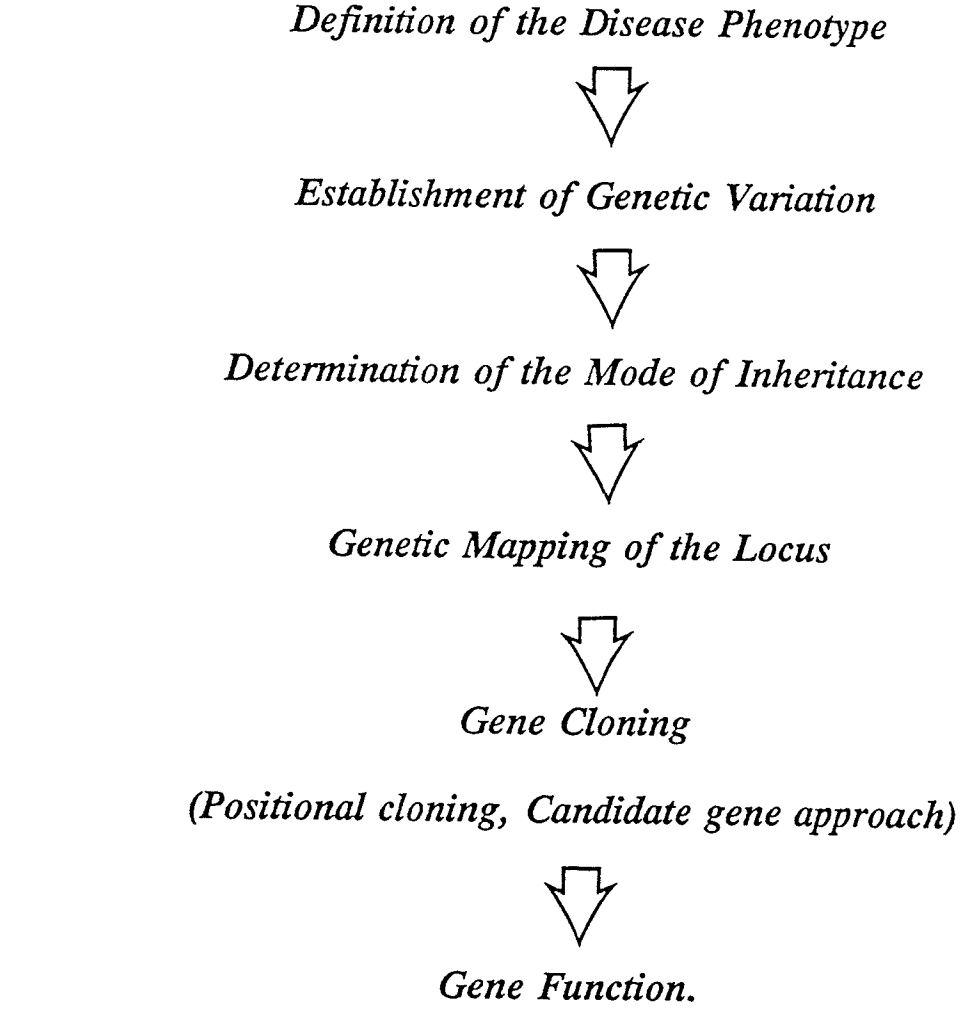
intestinal cancer in mice (Dietrich et al 1993) and hypertension in rats (Jacob et al 1991). This approach was used in the present investigation to dissect the genetic control of TSIgE levels in mouse.

6.0 MAPPING OF DISEASE SUSCEPTIBILITY GENES:

A major breakthrough in Medicine has been the concept of "gene medicine", which is based on the seemingly improbable notion that one can systematically discover the genes causing inherited diseases without any prior biological clue as to how they function or what protein they encode or where they are located in the genome. This has been made possible with the development of a series of molecular genetic procedures collectively referred to as positional cloning (formerly called reverse genetics). The major and the first step in a positional cloning strategy is to "map" the gene of interest, which means to determine which chromosome it is on and where it lies on that chromosome. This strategy is called molecular-genetic mapping, which involves comparison of the inheritance pattern of a trait with the inheritance patterns of polymorphic DNA markers, in segregating populations such as human families or experimental animal crosses (Lander and Schork 1994). The aim is to find two markers on the same chromosome which flank the gene of interest. The various steps involved in molecular-genetic mapping of disease susceptibility loci are schematically shown in Fig. 1.

Fig.1

GENERAL STRATEGY FOR IDENTIFICATION OF DISEASE SUSCEPTIBILITY GENES BY POSITIONAL CLONING APPROACH IN EXPERIMENTAL ANIMAL CROSSES



6.1 Microsatellite Markers: The Key to Success.

In the past genetic mapping has been extensively used to dissect the genetic basis of biological traits in several experimental organisms like frit fly, nematode worm, yeast and maize. However, it was rarely used in mammals due to two reasons: 1) the lack of an abundant supply of genetic markers with which to study inheritance, and 2) the inability to arrange human crosses to suit experimental purposes. The key breakthrough was the recognition that naturally occurring DNA sequence variation can provide a virtually unlimited supply of genetic markers. This idea was first conceived by Botstein and colleagues for yeast crosses (Petes and Botstein 1977) and subsequently used in mammals including humans (Botstein et al 1980). These ideas soon led to an explosion of interest in the genetic mapping of rare human diseases having simple Mendelian inheritance. More than 500 such diseases have been genetically mapped in this manner, and nearly 60 have been positionally cloned (Lander and Schork 1994).

Although a number of strategies have been established over the years to detect polymorphism at the DNA level (Guenet et al 1988), the most interesting are those that take advantage of the polymerase chain reaction (PCR). Because they require very small quantities of template DNA and can be carried out in short time. Two methods have been fervently exploited: (i) Random amplified polymorphic DNA (RAPD) markers, which employ

short random primers to generate anonymous DNA polymorphisms between individuals (William et al 1993, Venugopal et al 1993); (ii) the microsatellite markers also referred to as simple sequence length polymorphisms (SSLPs) which employ two primers to amplify known DNA sequences (Love et al 1990, Weber and May 1989, Cornall et al 1991).

The microsatellite analysis which represent the most popular of these techniques, consist of the amplification of short repeat sequences (<300bp in general) whose polymorphisms are due to variation in length. These sequences are composed of short tandem repeats of 1 to 4 bases in units such as $(T)_n$, $(CA)_n$, $(CT)_n$, $(CAG)_n$ etc., and the number of such repeats vary from individual to individual (Weber 1990, Cornall et al 1991). These represent the most ideal molecular markers for genetic mapping due to number of reasons such as:

1) These markers are abundant in the genomes of many eukaryotes including humans and mice. In mouse and human genomes there are at least $5X10^4$ sequences that contain $(CA)_n$ repeats and thus represent a rich source of genetic variation; 2) they are evenly distributed throughout the genome in humans although not in mouse; 3) generally size difference between alleles is in the order of 4-40 bp so it is easy to resolve the bands on agarose; 4) about 50% of these microsatellites are found to be polymorphic in typical crosses between inbred

laboratory strains and they exhibit high degree of heterozygosity (>70%) in humans; 5) it is relatively easy to process large number of DNA samples and to score polymorphisms on PCR amplification using specific primers designed from the sequences flanking the repeats; 6) the sequences of primers needed for each marker are available in the database and the primers are also commercially available; 7) the advantage of these markers over previous type of markers such as RFLPs is that they can be assayed by the PCR under uniform conditions and are less tedious to perform (Dietrich et al 1994, Gyapay et al 1994).

However, there are some disadvantages for these markers : 1) the rate of polymorphism may not be very high between some laboratory strains; 2) the size variation between alleles can be in some cases very small, which makes scoring of alleles difficult; 3) microsatellite primers occasionally amplify extra PCR products and non-parental bands, which can interfere with genotype scoring; and 4) microsatellites are not evenly distributed throughout the genome in mouse (Dietrich et al 1996).

6.2. Evolution of Genetic Maps:

The discovery of microsatellite markers has revolutionized the construction of genetic maps in humans and mouse (Dietrich et al 1992). The value of genetic map is that an inherited

disease can be located on the map by following the inheritance of a DNA marker present in affected individuals, but absent in unaffected ones, even though the molecular basis of the disease may not yet be understood nor the responsible gene identified. Genetic maps have been used to find the exact chromosomal location of several important disease genes, including cystic fibrosis, sickle cell disease, myotonic dystrophy (Cooper and Schmidtke 1992, Lander and Schork 1994).

A genetic map, or linkage map, is a diagrammatic representation of the linear arrangement of the genes/markers which are located on a given chromosome with an estimation of the distances between them. The establishment of such a map is based on the fact that the genes are positioned along the length of the chromosome and that during meiosis, loci located on different chromosome assort randomly in the gametes while linked ones tend to cosegregate unless a cross-over splits the parental association. In case of linkage, the probability for two genes to be separated by a cross-over event defines the genetic distance between them. This is reflected in the choice of a map unit, the centiMorgan (cM), which is defined as that distance between genes for which one product of meiosis out of 100 is a recombinant, i.e., a recombination frequency of 1% is defined as one cM.

A physical map, on the contrary, is an actual representation

of the linear arrangement of the genes on a chromosome. The gene order is the same as that given by the genetic map but the distance between genes is given in kilo basepair or mega basepairs (kb or Mb) which reflects the close relationship with the primary structure of the DNA. A genetic distance of 1 cM is roughly equal to a physical distance of 1 million bp (1 Mb). It should be borne in mind that there is no strict correlation between the genetic distance (cM) and the physical distance (Mb), as a consequence of the uneven distribution of the cross-over events along the chromatids.

Genetic maps are of particular importance to those who are attempting to isolate genes using positional cloning methods. Using microsatellite markers recently Colette et al (1996), published the last version of Genethon human linkage map. Dietrich et al (1996) reported the final version of mouse genetic map. The final integrated map has 7377 loci, on an average on every 0.2 cM or every 400,000 bps. The microsatellites described here show a polymorphism rate of about 50% among inbred laboratory strains surveyed. The distribution of polymorphism across the genome is not uniform. The average polymorphism rate among *Mus musculus* strains is just under 50% but two chromosome showed substantially lower polymorphism rate: chromosome X at 33% and chromosome 10 at 35% (Dietrich et al 1996).

The availability of dense genetic linkage maps of mouse and humans makes feasible a wide range of studies, including positional cloning of monogenic traits, genetic dissection of complex disorders like asthma and atopy, construction of genome-wide physical maps and evolutionary comparisons (Dietrich et al 1994, Dietrich et al 1996, Colette et al 1996).

7.0 APPROACHES TO STUDY THE GENETIC BASIS OF ATOPY:

In order to study the genetic basis of complex disorders like atopy, different phenotypes have been considered by different researchers. These include: a) intermediate phenotypes such as specific IgE (SPIgE) responses to ALs, total serum IgE (TSIgE) levels, inflammatory mediators release and bronchial hyper responsiveness (BHR); and b) complex phenotypes such as atopy, asthma and allergic asthma.

7.1.0. Intermediate phenotypes:

a) Specific Immune responses to allergens, including SPIgE response:

Role of Major Histocompatibility Complex (MHC):

The pioneering studies by Marsh and associates have demonstrated that specific immune response to ALs, are associated with the HLA system (Marsh et al 1989). These studies involved the use of well characterized, highly purified ALs. Using atopic populations, an association between

the IgE, and to a lesser extent the IgG, responses to *Amb a* V and the HLA DR2 and Dw2 was reported (Marsh and Blumenthal 1990). It has been reported that DR α, β I heterodimers are the principal class II molecules involved in *Amb a* V presentation (Huang et al 1991). Immune responses to other ALs have been associated with DR molecules such as *Amb t* V and *Amb p* V with DR2, *Amb a* VI with DR5 and *Lol p* I, II and III with DR3 (Marsh et al 1992).

Over the past several years, numerous studies have confirmed these findings: they have extended the HLA-D association findings to several ALs, and have demonstrated HLA-D restriction in Ag-specific T cells (Blumenthal et al 1992), including responses to ALs important in asthma such as mite ALs, *Der p* 1 and *Der p* 2 (O'Hehir, 1991).

These studies have demonstrated the role of HLA-D encoded molecules in well defined immune response toward simple AL molecules that appear to possess single major epitopes. HLA associations of IgE responsiveness to multiepitopic ALs have been scarce and debatable. The presence of a particular DR associated class II molecule appears to provide a necessary but not the only condition needed for responsiveness to a particular epitope. It should be noted that the HLA-D association is with the general immune responsiveness of individuals, as assessed by both AL specific IgG (SPIgG) and

IgE Abs, and the HLA-D mediated control is not specific to IgE Ab isotype alone. It has been suggested that HLA associations with atopy in general reflect many aspects of the human immune response, which include not only HLA genes (HLA-A,-B,-C,-DR,-DQ,-DP) but also TNF (α and β), HSP-70 and other 'chaperons', and peptide transporters such as TAP-1 and TAP-2 (Rich 1995).

Role of T cell receptor (TCR) genes:

The role of TCR genes in the regulation of allergic response is not very clear at present. An enormous TCR diversity arises from combinatorial associations involving many variable (V), junctional (J) and diversity (D) gene segments within the TCR loci. However, the use of TCR Vα and Vβ segments by lymphocytes may not be random and may be genetically controlled (Loveridge et al 1991, Moss et al 1993). Furthermore, there is genetic evidence that indicate the existence of allelic polymorphism of human TCRV α and β gene segments (Reyburn et al 1993). Therefore, to find out whether the TCR genes influence susceptibility to particular ALs, Moffatt et al (1994) studied two independent human families for linkage between IgE responses to highly purified major ALs and the TCR-α/δ and TCR β regions on chromosomes 14 and 7 respectively. No linkage of IgE response to TCR β was detected, but significant linkage to TCR α was observed in both families. It is likely that a gene(s) in the TCR α region modifies specific IgE responses. However, more studies are

needed to confirm these results and to arrive at a consensus on the role of TCR α/δ genes as a genetic factor for atopy.

b) Genetic control of TSIgE levels:

Total serum IgE levels in humans have been found to be a good predictor of atopic conditions and atopic individuals have a tendency to exhibit high and persistent TSIgE levels compared to non-atopics. Several studies have shown a strong relationship between asthma and high TSIgE levels (Burrows et al 1989, Freidhoff and Marsh 1993). Bronchial hyper-reactivity in children was also found to be significantly associated with elevated TSIgE levels (Sears et al 1991, Hopp et al 1990).

A number of earlier studies indicated that the TSIgE levels have heritability. Both single, two gene and polygenic inheritance have been suggested (Blumenthal et al 1981, Rao et al 1980, Hasstedt et al 1983, Xu et al 1995) for IgE levels, with a suggestion that IgE expression is associated with dominance (Borecki et al 1985). Twin studies showed that TSIgE level is under genetic control. In one series of 107 twins, the intra-pair correlation coefficient for TSIgE was 82% in monozygotic (MZ) twins compared with 52% in dizygotic (DZ) twins yielding an overall heritability of 61% (Hopp 1984). Thus MZ twins are more concordant than DZ twins for TSIgE levels. Moreover, no significant difference is found between

the TSIgE levels in twins reared together and twins reared apart, indicating that familial or a common breeding environment has very little effect on IgE levels and that there is a significant genetic component regulating IgE levels (Hanson et al 1991). In summary, for TSIgE levels, heritability has been demonstrated in twin, family and population studies.

Recently significant advances have been made in the understanding of genetic control of TSIgE levels in humans: Marsh et al (1994) reported that non-cognate IgE production is linked to a major gene on chromosome 5q31.1 in families of Old order Amish community in Pennsylavania. They found evidence of linkage with TSIgE but not SPIgE, within the 5q31.1 region which contains a number of genes including IL4 that have been shown to have important role in the regulation of IgE biosynthesis and inflammation. They suggested that IL4 or a neighbouring gene(s) in 5q31.1 regulates IgE production in a non-cognate fashion. In a second study, involving Dutch families, Meyers et al (1994) found evidence of linkage of TSIgE levels to 5q32-33, albeit a region near the glucocorticoid receptor (GRL) gene which is different from the above study. However they did not investigate the linkage with IL4 gene. The third study demonstrated that a trait for an elevated level of TSIgE is co-inherited with a trait for BHR and that a gene governing BHR is located near a major locus

that regulates TSIgE levels on chromosome 5q31-33 (Postma et al 1995). This locus was also found to be near GRL gene, away from the IL4 gene.

In contrast to the above studies, other studies did not find evidence of linkage of TSIgE levels with 5q31.1. Cookson et al (1995) investigated chromosome 5 markers for linkage to 5q31.1 in 1000 Australian subjects from 230 families. They did not find evidence for linkage with any marker in 5q31.1, including IL4, and TSIgE levels or with the airways responsiveness to methacholine challenge (Cookson 1995). Similarly, Blumenthal et al (1995) studied five large families and reported that there was no evidence of linkage of TSIgE levels to 5q31.1.

In summary, the genetic control of TSIgE levels in humans, both in terms of the mode of inheritance and the number of genetic factors involved, has been a subject of controversies due to variable, and often contradictory results from various research groups (Table 1). There are a number of potential reasons for this discrepancy, of which, genetic heterogeneity of atopy, is believed to be the major one (Marsh and Meyers 1992, Moffatt et al 1992, Cookson 1995).

c) Release of mediators:

Histamine release from basophils appears to be under genetic control which is independent of the genes that control TSIgE

Table 1:

Chromosomal regions and candidate genes showing evidence of linkage to TSIgE levels in humans

Genomic Interval	*Candidate gene(s)*	*Evidence for*	*Evidence against*
5q31.1	IL3,IL4,IL5,IL13 Csf2	Marsh et al 1994 **(non-cognate IgE)**	Blumenthal et al 1995 Cookson 1995
5q32-33	GRL, ADBR2	Meyers et al 1994 **(TSIgE)** Postma et al 1995 **(TSIgE and BHR)**	
11q13	FCER1B	Cookson et al 1989 **(Total and SPIgE)** Collee et al 1993 Shirakawa et al 1994	Hizawa et al 1992 Lymphany et al 1992 Amelung et al 1992 Rich et al 1992

Gene abbreviations:

IL=interleukin; CSF2, granulocyte-macrophage colony stimulating factor; GRL, glucocorticoid receptor; ADRB2, β-2 adrenergic receptor; FCER1B, high affinity receptor for IgE, β chain.

levels. The MZ twins are more similar than DZ twins in their release of histamine from basophils using standard stimuli including anti-human IgE (Marone et al 1986). Further, a study of 20 nuclear families also showed 55-70% heritability of the releasability of histamine from basophils in response to anti-human IgE (Roitmann-Johnson and Blumenthal 1988).

d) Bronchial hyper-responsiveness (BHR):

The genetic control of BHR was investigated utilizing inhalation challenges with methacholine as a measurable phenotype. A recent study has identified a major locus on chromosome 5q32-33 which is involved in the regulation of BHR (Postma et al 1995). More recently, genes for BHR have been identified in selected inbred mouse strains using genome-wide searches (De Sanctis 1995). However, the location of genes governing BHR in mouse was found to be on chromosomes 2, 15 and 17, which are not syntenic to human chromosome 5q32-33.

7.2 Complex Phenotypes:

The general expression of complex phenotypes such as asthma and atopy have been noted to have heritability. Early investigators emphasized the familial nature of atopic diseases and provided evidence of a genetic predisposition (Marsh and Blumenthal 1990). Because of problems of definition and analysis in these early studies, many investigators directed their efforts at the components of asthma and

allergies, or the intermediate phenotypes as described above. Recently, attempts have been made to study the complex phenotypes for the so called complex diseases such as atopy and asthma. These studies involve the use of linkage and sib pair analysis with different parameters to define the phenotype, and a more sophisticated molecular genetic approach for their analysis.

a) Atopy:

Cookson and Hopkin (1988) defined atopy as at least one positive AL skin test *in vivo* and/or a positive RAST to one or more of a group of common inhalant ALs, and/or a TSIgE level that is more than two standard deviations above the geometric mean for normal subjects. They investigated the genetic basis of this broadly defined phenotype in families. They suggested that atopy as defined by these parameters (at least 2 of the 3), is inherited as an autosomal dominant trait and is linked to a gene on chromosome 11q13 (Cookson et al 1989). They later suggested that the 11q13 linked atopy gene is inherited preferentially from the maternal side, possibly due to either paternal genetic imprinting or maternal modification of the infants's IgE response through the placenta or breast milk (Cookson et al 1992). However, Rich (1992), Hizawa (1992), Lymphany et al 1992, and Amelung (1992) in separate studies could not confirm Cookson's observations although two other small studies provided some support (Collee et al 1993,

Shirakawa et al 1994a).

Sandford et al (1993) reported that the gene of the β chain of (FCER1B) is localized on chromosome 11q13 and it is in close genetic linkage to atopy. Shirakawa et al (1994b) identified a specific abnormality in the trans-membrane portion of the β chain of this receptor and found a strong association between atopy and variants of the β subunit of the FcϵR1 in a 17% of the unrelated nuclear families studied. A common variant of the gene FCER1B was found maternally inherited in each of these families. In a recent study by Marsh et al (1996) in Amish population, found evidence for a gene in 11q13, which is near the region around FGF3 (INT2), rather than FCER1B. However, they did not find evidence for maternal inheritance.

In summary, there are variable results on the presence of a gene on 11q13, linked to atopy in humans.

Holt et al (1992) studied CD+ T cell function in normal children and those at high risk for atopy (defined as a one or more first degree relatives with a positive history of asthma, eczema or allergic rhinitis). They reported that high risk children had markedly reduced immunocompetent T cell precursor frequency and IL-4 production by CD4+ T cell clones was significantly reduced. They suggested that the genetic risk for atopy is associated with delayed postnatal maturation of

T cell competence.

b) Allergic Asthma:

In view of the problems of defining asthma, investigators have looked at those individuals with a specific sensitivity. Caraballo et al (1990, 1991) studied mite sensitive individuals with asthma. Their results point to the existence of an HLA linked recessive gene controlling the IgE immune responsiveness to mite ALs and conferring susceptibility to allergic asthma. The investigations of ragweed allergic rhinitis and asthma with the extended HLA haplotype revealed evidence consistent with a dominant MHC linked gene or a gene on HLA B7, SC31, DR2 that control the IgE immune response to *Amb a* V and predisposition to asthma (Arnaiz-Villena 1993).

c) Asthma:

The complex phenotype of asthma has been investigated in relationship to markers on the chromosome 6. Studies on the relationship between MHC genes, asthma and atopic conditions have yielded conflicting results (Blumenthal et al 1980, Marsh and Blumenthal 1990, Arnon et al 1995). Another candidate locus in asthma is the B2-adrenergic receptor (Ligget 1995). A series of mutations (Thr164-->Ile; Gln27-->Glu; Arg16-->Gly) have been identified and correlations between genotype and asthma phenotype have been explored by Reihsans et al (1993). In particular, the Arg16 mutation appears to play a role in

nocturnal asthma.

There are several reports on the role of variants of the protease inhibitor alpha1-antitrypsin and a partial deficiency of alpha1-antichymotrypsin, as risk factors for asthma (Hoffmann 1981, Lieberman and Colp 1990, Lindmark et al 1990). However, the role of alpha1-antitrypsin remains controversial due to a failure to confirm such associations by other groups (Buist et al 1979, Schwartz et al 1977).

8. BACKGROUND

8.1 Dissection of genetic control of TSIgE levels: Mouse as a model species.

Despite seventy years of search, a molecular understanding of non-MHC genetic factors involved in atopy is lacking. Despite some recent progress in genetic analysis of atopy and TSIgE levels, a majority of these studies has either been subject to controversies or have not been sufficiently replicated. Human studies are hampered by controversies due to a number of inherent confounding problems associated with family studies. Some of these problems are: 1) definition of the phenotype; 2) number of parameters to be studied; 3) influence of polygenic factors and genetic heterogeneity; 4) role of HLA-D genes in specific immune responses; 5) environmental influence on expression of atopy and asthma; 6) study design (size and

number of families) and selection of subjects (sex, age) and 7) statistical analyses (linkage analysis and allele sharing methods) (Moffatt et al 1992, Marsh and Meyers 1992, Cookson 1995).

In contrast to this, the mouse is an excellent animal model for genetic dissection of a number of complex human diseases including atopy and asthma. Humans and mice have more than 80 conserved autosomal segments and many of the complex disorders like type 1 diabetes, have homologous genes in the mouse. Extreme interest in using the mouse for genetic studies is because this species offer a "fast-track to disease gene identification" due to a number of inherent advantages: (i) short generation time, (ii) several embryos per gestation, (iii) high prolificacy, (iv) ease of obtaining recombinant and congenic strains, (v) availability of many mutations and chromosomal rearrangements, (vi) possibility to mutate genes in the germline, (vii) ability to readily produce viable and fertile inter-specific hybrids (Taylor and Reifsnyda 1993, Lander and Schork et al 1994).

Besides this, the mouse is an excellent animal model for genetic studies of various parameters of atopy and asthma in general and TSIgE levels in particular. Although mice do not develop atopy or asthma spontaneously, a number of parameters associated with these disorders can be experimentally induced

in this species. The value of the mouse as a model species for atopy and some aspects of asthma is evidenced by the following arguments :

1) regulation of IgE synthesis is similar in both species as described before; 2) like humans, mouse strains differ in their ability to mount IgE responses when injected with ALs (Levine and vaz 1970) or exposed to ALs intra-nasally (Renz et al 1992); 3) mice develop airways hyper responsiveness (AHR) on inhalation challenge with ALs (Larsen et al 1992), which has similarities to allergic asthma in humans; 4) mice also develop eosinophilia when injected with allergens and IL-5 (Kurup et al 1992); 5) one parameter at a time, like AHR or IgE etc., can be studied using two parent strains of mice that are disparate for a given trait. These similarities provide an animal model of 'some important aspects' of the human disease process.

The most important feature relevant for the present study is the availability of MHC-identical mouse strains, which provided a unique opportunity to dissect the role of non-MHC genes in disease development. Thus, the use of mouse to dissect genetic control of TSIgE levels would be an ideal situation since it overcomes many problems including that of genetic heterogeneity, which is the biggest hurdle in human atopy studies, and which may be responsible for the variable

results reported on the genetic control of TSIgE levels. Furthermore, recent mapping of loci regulating AHR in mouse adds validity to the use of mouse for genetic studies of TSIgE levels (De Sanctis et al 1995).

8.2 Inbred strains of mice differ widely in SPIgE Ab responses:

Levine and Vaz (1970a) systematically studied the strain differences in the ability to produce specific IgE (SPIgE) Abs when injected with antigens. It was found from their studies that persistent and boosterable "reagin" formation in mice could be induced in certain mouse strains (A/He, CBA, C3H, AKR) but not in others (C57BL, C57L, SWR, SJL) by giving suitably spaced repeated minute doses of certain Ags (bovine γ globulin and ovomucoid) with aluminium hydroxide as adjuvant. The ability of mouse strains to produce reagin was found to be only part of a more general ability of immune responsiveness. Thus, these results are consistent with the effects of MHC-linked 'Ir' genes on immune responsiveness, which is well known today.

Later, Watanabe et al (1976). studied SPIgE production to DNP-hapten using mice with different MHC haplotype. Using DNP-KLH and both *Nippostrongylus braziliensis* (Nb) and alum as adjuvants, they found that all strains of mice they used developed persistent and high titers of anti-hapten IgE Ab,

except the SJL strain which had a moderately low titer which was not persistent. They showed that A.SW mice which have the same H-2^s as SJL, mounted a high and persistent DNP-SPIgE response. In contrast mice with H-2^k (AKR, C3H and CBA) produced similar high titers of anti-hapten IgE Abs. Thus, this study provided the first clue to the role of non-MHC genes in SPIgE production in A.SW and SJL strains of mice. However, they did not study the TSIgE levels.

8.3 Genetic control and inheritance of SPIgE responses in mouse:

Studies in murine systems have contributed tremendously to our knowledge of regulation of SPIgE Ab responses. Although the IgE Ab response has been reported to vary with strains of mice (Levine and Vaz 1970a), very little is known on the genetics of IgE immune response in these animals. There are two studies done in 70's in mice which examined the genetic control and inheritance pattern of specific IgE (SPIgE) responses (as measured by PCA titers) to Ags/haptens, as described below:

Levine and Vaz (1970b) studied the genetic control of 'reagin' production in mice and reported that two kinds of genetic control exist in 'reagin' production in mice. Thus in response to immunization with a single large (100 μg) dose of Ag (DNP-BGG in alum), most strains make both SPIgG1 and specific 'reagin'; SJL, AKR and St/b made IgG1 but virtually no reagin.

More detailed studies with RF and SJL mice showed that both produced IgG1, RF but not SJL produced 'reagin' (PCA titer 1:80), and (SJL X RF) F1 produced both IgG1 and reagin like the RF parents. Later they showed that SPIgE responsiveness to DNP hapten in the mouse is under the control of two genes (Levin and Vaz 1971, Levin, 1979). Thus, they reported that (RF X SJL)F1 hybrid mice were all good 'reagin' producers, and the (F1 X SJL) BC1 mice (n=55) segregated out into 50% good serum reagin producers, 25% poor serum reagin producers, and 25% intermediate producers, as measured by PCA titers. Based on these results they suggested that more than one locus or more likely, two loci control reagin production in these mice (Levin and Vaz 1971, Levin, 1979).

In contrast, the second study involved the use of SJL and Balb/c strains, and DNP-KLH (1 ug dose) with both Nb and alum as adjuvants (Watanabe et al 1976). Thus, using (Balb/c X SJL)F1 hybrids and a small number (n=18) of F1 X SJL backcross mice, they reported that the low and transient SPIgE response (as measured by PCA) to 1 μg DNP-KLH, attributable to a suppressor function by T cells of SJL mice, was inherited as a Mendelian recessive trait under the control of a single gene. They further tested the presence of H-2^d Ag on blood cells in BC mice and showed that there was no correlation between this and the IgE Ab production. Based on this result they concluded that the suppressor T cell activity on SPIgE

production was not linked to H-2 complex.

Thus, both one and two locus control of SPIgE response to DNP hapten have been reported in mice. A major caveat in these studies is that both used parent mouse strains which are different in MHC haplotype. There is a wide difference in the dose of antigen used (100 μg in the first study vs 1 μg in the second). It is now well established that specific immune responsiveness to minute doses of protein antigens is governed by Ir genes linked to MHC such as MHC class II molecules (Carbone and Bevan 1989). Therefore, the variable results between above studies could be due to the effect of Ir genes at different doses. Furthermore, use of Nb in the second study as an adjuvant in addition to alum, might have influenced the IgE response non-specifically. It is well known that parasite Ags can induce T-cell independent polyclonal activation of B cells (Lebrun and Spiegelberg 1987), thereby masking the underlying genetic control of IgE responses. In addition, the use of a small number of animals in these studies may also have influenced the conclusion. Furthermore, the use of haptens and parasites may not necessarily be relevant to human atopic IgE responses, which are induced by ALs, mostly in parasite-free regions of the world.

8.4 Characteristics of SJL/J and A.SW/Snj inbred mice:

The inbred strain, SJL has been widely used in experimental

studies on allergies and asthma (Levine and Vaz 1970a, Watanabe et al 1976, Renz et al 1992a,b). Besides this, SJL mice have been used as model animals for studeis on cancer and autoimmune disease, because they develop reticulum cell sarcoma (model for human Hodgkin's disease) and are susceptible to experimental allergic encephalomyelitis (a model for multiple sclerosis). In contrast, A.SW/Snj is less frequently used strain of mouse in experimental studies on atopy. These two strains of mice are identical at the MHC locus (both are of H-2^s haplotype) and have a common Swiss origin (Klein et al 1983).

9.0 SCOPE OF THE PRESENT STUDY:

As reviewed above, there are a limited studies on the genetic control of SPIgE responses in the mouse. However, to our knowledge to date, there have been no studies on the genetic control of AL-induced TSIgE levels in the mouse or any other laboratory models of atopy and asthma. Thus, although the genetic factors controlling TSIgE levels can be investigated with ease in mice, to date they have not been exploited for this purpose.

The present study was therefore undertaken to investigate the genetic control of atopic IgE responsiveness in general and AL-induced TSIgE levels in particular. Furthermore, MHC-identical strains of inbred mice were selected so that the

genetic control by non-MHC genes could be dissected in a 'genetic-environment' where *Ir* genes will have uniform effects. Thus, two strains of inbred mice namely - A.SW/Snj and SJL/J (both are H-2^s haplotype) were selected as parental strains in this study. This combination of strains and this approach, has not been used before for any kind of genetic studies in atopy and asthma.

HYPOTHESIS:

This study was planned based on the following hypotheses:

"Alleles at multiple single-loci are involved in the complex etiologies of atopy and the genetic control of elevated and persistent allergen-induced IgE synthesis constitute a major predisposition factor for atopy"

"The tendency to mount allergen induced persistent and high TSIgE levels is under the control of a gene or genes not linked to the MHC complex in mice"

"Non-MHC locus or loci regulating allergen-induced TSIgE levels could be mapped to specific chromosomes in mouse using microsatellite based mapping techniques"

SPECIFIC OBJECTIVES:

The *specific objectives* of the present study were:

1. Establishment of a large MHC-identical mouse colony consisting of F1, Backcross-1 and F2 intercross progeny, using MHC - identical inbred mouse strains - A.SW/Snj and SJL/J.

2. Elucidation of the genetic control and the inheritance pattern of allergen-induced total serum IgE (TSIgE) levels in F1, Backcross-1 and F2 intercross progeny.

3. Analysis of the role of Tcrvb8, Il4 and Ifg genes as predisposition factors for allergen induced TSIgE levels in Backcross-1 mice.

4. Genetic mapping of non-MHC locus or loci controlling allergen-induced TSIgE levels to specific chromosome(s), in Backcross-1 mice, using microsatellite marker-based mapping approach.

II. MATERIALS AND METHODS

2.1 REAGENTS AND ANIMALS

2.1.1 Chemicals:

All chemicals used in this study were purchased from various suppliers as indicated in the text.

2.1.2 Allergens:

The plant ALs - Kentucky Blue Grass(KBG), short ragweed, white birch were purchased from Hollister Steir (USA). House dust mite allergen extract was purchased from Miles Inc., (Elkhart, IN, USA.) and ovalbumin (OVA) was purchased from Sigma (St.Louis, MO, USA). The AL extracts from KBG, ragweed and birch pollen were prepared as follows: the pollen was suspended in PBS (pH 7.4), vortexed and incubated at 4°C overnight. The suspension was centrifuged at 4°C for 20 min and the supernatant was collected. This was filtered through Whatman filter paper No.1 and the aqueous extract was used for estimation of protein content using Biorad protein assay (Bradford 1976).

2.1.3 Animals:

a) Mice:

Six weeks old SJL/J and A.SW/SnJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in the Central Animal Care services, University of Manitoba. Male A.SW/Snj and female SJL/J, mice were used as founder parents

for establishment of a colony comprising various progenies used in this study.

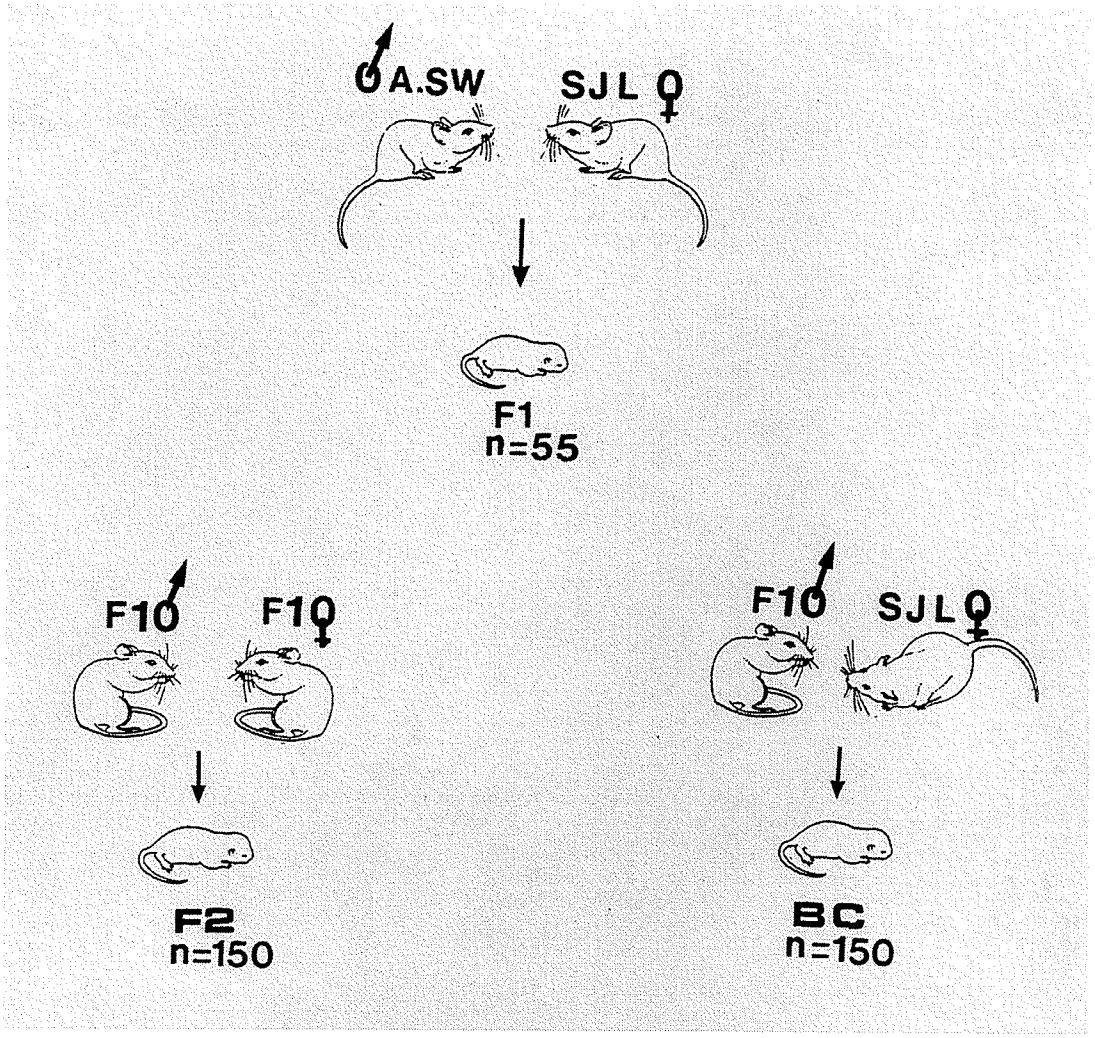
b) Establishment of a mouse colony:

A mouse colony was established using two MHC-matched inbred mouse strains- A.SW and SJL. Male A.SW and female SJL mice were used for breeding. The general breeding protocol was as follows: male mice were kept with females for a week and then the male was removed. The female mice were examined for next few days for signs of pregnancy. Once an animal was found pregnant, it was put in a separate cage and managed until delivery. Once the pups were born, each animal was given an identification number. Toe clipping was done as a method of identification. The pups were kept with the mothers until 3 weeks, at which time each pup was subjected to sex determination and subsequently weaning was done. Male and female mice from each cross were separated and maintained in different cages.

Using this breeding protocol the F1 hybrid progeny was obtained by crossing SJL females with A.SW male. The F1 hybrid animals, were used in the backcross (BC) and F2 intercross breeding. Thus the F1 males were crossed with the SJL females to derive the backcross-1 (BC1) progeny. The F2 intercross progeny was obtained by crossing the F1 male and female animals (Fig. 2). All animals were given identification number

Fig. 2 Establishment of a mouse colony for the study of genetics of atopic IgE responsiveness.

MHC-identical inbred strains of mice - A.SW/Snj and SJL/J ($H\text{-}2^s$) were used as parent founder strains for breeding. Male A.SW mice were crossed with female SJL mice to produce the F1 hybrids; SJL females were crossed with F1 males to produce BC1 progeny; male and female F1 mice were crossed to produce F2 intercross progeny.


♂A.SW
SJL ♀
F1
n=55
F1♂
F1♀
F2
n=150
F1♂
SJL♀
BC
n=150

by toe clipping method. All mice were maintained under identical conditions in a single room at the Central Animal Care and Services, facility of the University of Manitoba. All animals were bled at 4 weeks of age for the purpose of collecting control sera and were used for immunization at the age of 6-10 weeks.

c) Rats:

Six to 8 week old Sprague Dawley (SD) male rats were purchased from Charles River (St. Constant, PQ, Canada) and maintained at the Central Animal Care and Services facility, the University of Manitoba. Rats were used at the age of 10-12 weeks in a passive cutaneous anaphylaxis (PCA) assay.

2.1.4 Primers:

Primers used in this study are listed in the Table 2. The primers for amplification of the Tcrvb8 gene were designed from the published genomic DNA sequence of Tcrvb8 gene (Chou et al 1987). Primers for amplification of Il4 and Ifg gene associated microsatellites, were based on the published sequences (Jacob et al, 1993). However, primers were designed for (CA) and (GT) repeats present in intron-9 of mouse CD23 gene, based on published sequence information of the murine CD23 gene (Richard et al 1991) (Table 2).

All other primers (Mouse Map Pairs) for PCR amplification of

Table 2.

List of Primers used in this Study.

PRIMER		NUCLEOTIDE SEQUENCE
Tcrvb8		forward: 5' TGG CAG TAA CAG GAG GAA
		reverse: 5' GAA GCC AAC TCC AGA ATG
I14 (CA) repeat		forward: 5' GTC TGC TGT GGC ATA TTC TG
		reverse: 5' GGC ATT TCT CAT TCA GAT TC
Ifg (AC)AT(AC) repeat		forward: 5, GGT GAC CTT GTG ACA AGC TC
		reverse: 5' TGC TGT GTG GTC TGT CTG TC
CD23 (CA) repeat	1.	forward: 5'GTA TGT AGT ACG TGT GTG CTC
		reverse: 5'ACA TAC ATA TAC ACC ACA TGC AC
	2.	forward: 5'TCT GAG CTC AGC TCT GGG TCT
		reverse: 5'ACC ACA CAT ATG CAC CCA CAT

various microsatellite markers from mouse chromosomes 1 to 19, were purchased from Research Genetics (Huntsville, AL, USA). The various microsatellite markers used in the molecular-genetic mapping in this study are listed in the Table 19.

2.1.5 Monoclonal Antibodies:

The mouse monoclonal anti-mouse TCR Vβ8 Ab (F23-1, IgG2a) was a gift from Dr.C.S.David (Mayo Clinic, Rochester, USA). Antibodies used in ELISA were as follows: Purified rat anti-mouse IgE MAb, Alkaline phosphatase-rat anti mouse IgE and purified mouse IgE, kappa isotype standard (PharMingen, CA, USA) were used for TSIgE determination. Alkaline phosphatase-Rabbit anti-mouse IgG1 (Zymed, USA) for KBG-SPIgG1 Ab determination.

2.2 IMMUNOLOGICAL METHODS:

2.2.1. Preparation of the allergen-cocktail for immunization.
A cocktail of ALs consisting the extracts of Kentucky Blue Grass(KBG), short ragweed, white birch, house dust mite and OVA, was co-precipitated with alum and used for immunization. Individual animals were injected i.p., with 0.5 ml of inoculum consisting 5 μg each of the ALs plus 5 mg of alum. Animals were boosted after four weeks with the same preparation and bled at days 10, 21 and 31 after the booster.

2.2.2 Enzyme-linked immunosorbent assay (ELISA).

a) Total Serum IgE (TSIgE) measurements:

The TSIgE levels were determined by a modified sandwich ELISA using MAbs against the Ig epsilon heavy chain (Technical Protocols, Pharmingen, San Deigo, CA, USA). Briefly, the ELISA plates (Zymed Laboratories, San Fransisco, CA) were coated with a capture rat anti-mouse IgE MAb overnight at 4°C. The plates were washed three times in phosphate-buffered saline/Tween-20. The plates were then blocked with 3% BSA in PBS for 2 hr at 37°C and washed three times. The serum samples were serially diluted and incubated at 4°C overnight. The plates were washed three times and incubated with 1:2000 dilution of an alkaline-phosphatase conjugated rat anti-mouse IgE MAb for 2 hr at 37°C. The plates were washed five times and developed with p-nitrophenyl phosphate (PNP) (Sigma Chemicals, USA) substrate at 37°C for 1 hr. The optical density (OD) was measured at 405 nm and 690 nm in an automated ELISA reader. A mouse IgE isotype control Ab was serially diluted and used in each plate for constructing standard curves. The TSIgE levels were determined from the titration curves using a curve fitter program. The lower limit for detection was 3.125 ng/ml.

b) KBG-Specific IgG1 Ab measurements:

The KBG-specific IgG1 Ab levels were determined by an indirect-ELISA (Venugopal et al 1995). Briefly, the plates

were coated with 100 ng of KBG extract in coating buffer, overnight at 4°C. After washing, the plates were blocked with 1% BSA, washed and incubated with the serially diluted serum for 2 hr at 37°C. Subsequently, the plates were washed and incubated with 1/2000 dilution of an alkaline-phosphatase conjugated rabbit anti-mouse IgG1 MAb for 1 hr at 37°C. The plates were then washed and developed with PNP substrate at 37°C for 1 hr. The optical density was measured at 405 nm and 690 nm. The KBG-specific IgG1 Ab levels were expressed in terms of OD values obtained using a serum dilution of 1/4000.

2.2.3. PCA assay for Specific IgE Ab measurements:

Anti-KBG, anti-OVA and anti-(OVA + ragweed + white birch) IgE titres was determined by the PCA assay in SD male rats as described (Ovary et al 1975). Volumes of 0.05 ml of serially diluted sera were injected intradermally on the back of anaesthetized rats. Thus, injected animals were rested for 48 hr. Subsequently, the sensitized rats were challenged with the appropriate AL (500 μg) in 1 ml of PBS containing 0.5% Evans Blue, by injecting in to the penile vein. After 20 min, the animals were euthanized and the skin reaction was analyzed. A diameter of $\geq$ 5 mm was considered as a positive skin reaction. The highest dilution of the serum giving a positive skin reaction was regarded as the end point. The reciprocal of this dilution was expressed as PCA titer. The KBG-specific PCA titres were determined for all F1, BC1 and F2 mice at days 10,

21 and 31. A subgroup of BC1 and F2 intercross mice sera (n=59) was assayed for specific IgE against OVA, ragweed and white birch ALs by challenging the sensitized rats with a mixture composed of 500 μg each of these ALs. Another subgroup of BC1 mice sera was assayed for anti-OVA IgE Ab titers, by challenging the sensitized rats with OVA.

2.2.4. Flow cytometry analysis of TCRVβ8 expression.

The TCR Vβ8 protein expression on the peripheral blood mononuclear cells was determined by flow cytometry. Peripheral blood from individual mice was collected by tail incisions into sterile anticoagulant containing tubes. One hundred ul of this blood was pipetted into Falcon 2003 test tube and 10 ul of FITC-conjugated mouse monoclonal anti-mouse TCR Vβ8 Ab was added and incubated for 1 hr on ice. An irrelevant FITC-conjugated mouse IgG2a Ab was used as an isotype control. After the incubation time the samples were placed into the leukocyte preparation system (Coulter Immunoprep Epics), which gently lysed erythrocytes and maintained leukocyte morphology and cell surface markers. Thus prepared samples were analyzed on a flow cytometer. Flow cytometry was performed in the laboratory of Dr. F. Paraskevas, Winnpeg.

2.3. MOLECULAR BIOLOGICAL METHODS.

2.3.1 Preparation of genomic DNA:

Genomic DNA was prepared from mouse tail as described (Venugopal et al 1993). Briefly, about 1 cm of the tail tip was cut into fine pieces in presence of 500 ul TENS buffer (0.1 M NaCl, 10 mM Tris HCl, 1mM EDTA, 1% SDS). The tissue was digested with 50 ul proteinase K (10 mg/ml) overnight at 55°C and treated with 50 ul RNAse A (5 mg/ml) for 2 hr at 37°c. The DNA was extracted twice by phenol-chloroform-isoamyl alcohol (25:24:1), dialysed thrice against TE (10 mM Tris Cl, 1mM EDTA, pH 8.0), and concentrated using N-butanol. The concentration and purity of DNA was determined spectrophotometrically by measurements of optical density at 260 and 280 nm.

2.3.2 Tcrvb8 Gene Analysis:

a) Southern blot hybridization to detect Tcrvb8 gene :

The protocol followed for Southern transfer and hybridization was as per the instructions given in Amersham International P/C, UK, manual (Venugopal et al 1993).

Southern blotting of Eco RI digested genomic DNA:

Ten microgram of genomic DNA was digested with Eco RI restriction enzyme overnight. Enzyme digested DNA was electrophoresed in a 0.8% agarose gel at 22 V overnight, along

with 1 kb DNA ladder as a standard. The gel was stained with ethidium bromide and the gel was examined under UV to verify the enzymatic digestion and to ensure the uniform quantity of DNA in all lanes. A photograph was taken for later comparisons. The gel was then soaked in 0.25 N HCl for 10 minute and rinsed in distilled water. Then Southern transfer was done onto nylon membrane (Hybond, Amersham, Canada) by alkaline transfer method using 0.4 N NaOH for overnight at room temperature.

Preparation of TCRVβ8.1 cDNA probe:

The Tcrvb8.1 probe was prepared from Vβ8 plasmid containing E.Coli clones briefly as follows: bacterial culture was grown in LB medium with appropriate antibiotics overnight and plasmid DNA was prepared using a modified protocol as described (Birnboim and Doly 1979). Briefly, the bacterial cells were lysed in a solution of 1:2:1 ratios of TE (10mM Tris, pH 8.0, 1mM EDTA), lysis buffer (1.25 % SDS, 0.2N NaOH), and 10 M ammonium acetate. The supernatant was precipitated by absolute ethanol. The supernatant was drained and the pellet was dried on "speed vac" and then dissolved in TE buffer. Thus prepared plasmid DNA was subjected to restriction enzyme digestion to excise the Vβ8 insert. Thus, an aliquot of DNA digested with Eco RI and Hind III restriction endonucleases overnight at 37^{o}C. The digested product was analyzed by electrophoresis in a 1% agarose gel. The Vβ8.1 probe was

isolated by cutting the insert DNA containing band and subsequently purified by gene cleaning method (GIBCO BRL). Briefly, the agarose containing DNA was dissolved in sodium iodide at 55°C. Then appropriate volume of glass milk was added to the gel and incubated at room temperature for 5 min. The mixture was then centrifuged and the supernatant was discarded. The pellet was washed (New Wash, BRL) and then dissolved in TE buffer. The purity of the DNA was tested by running the sample on agarose gel.

Thus prepared Vβ8.1 cDNA was used for labelling. Thus, Tcrvb8.1 probe was isotope labelled with [^{32}P]dCTP by the random priming method (GIBCO BRL). Briefly, an aliquot of DNA was boiled in water and cooled, then appropriate amount of random primer mixture was added on ice (Gibco BRL). The isotope and the klenow fragment was added and the labelling was allowed to take place at 37°C for 1 hr. The labelled probe was subsequently cleaned as follows: the cDNA probe was mixed with denatured salmon sperm DNA and an equal volume of 4 M ammonium acetate was added. The DNA was precipitated using absolute alcohol at -20°C. Then it was centrifuged and the supernatant was discarded. The pellet was rinsed in 70% alcohol and dissolved in TE buffer. Thus, cleaned probe was stored at -20°C until used. The specific activity of the labelled probe used in the hybridization was 1.2×10^8 cpm/ug.

DNA hybridization:

The hybridization protocol was briefly as follows: prehybridization was performed for 2-3 hours at 65°C; hybridization was performed at 65°C for 24 hours. Three washings were given using 2X, 1X and 0.1 X SSPE buffer each containing 0.1% SDS. The signal was visualized by autoradiography. The size of the band was deduced by comparing to the 1 kb DNA ladder which was run in parallel.

b) Establishment of a PCR assay for Tcrvb8 gene detection:

To determine the presence of Tcrvb8 genes in individual mouse, the genomic DNA was isolated from tail of each mouse by method described previously. The PCR assay was optimized for annealing temperatures, Mg^{++} ion and DNA concentrations. Thus PCR reactions were performed at different annealing temperature and the optimal annealing temperature of 52°C was determined. Furthermore, titration of Mg^{++} ion concentration was done at three different dilutions and 0.75 mM was found optimal. The genomic DNA was subjected to PCR amplification using Tcrvb8 primers (Table 1) that amplify the second exon of Tcrvb8 genes. For the amplification, an aliquot of 200 ng genomic DNA was used as template in presence of Taq DNA polymerase (Gibco, BRL). Each sample was overlaid with 25 ul of mineral oil and incubated in a DNA thermal cycler (Perkin Elmer Corp.), for a total of 35 cycles. Each cycle consisted of strand denaturation at 90°C for 1 min, primer annealing at

52°C for 2 min and extension at 72°C for 1 min.

The PCR amplified DNA was resolved on a 2% agarose gel prepared in TBE (89 mM Tris, 89 mM Boric Acid, 2 mM EDTA pH 8.0). Electrophoresis was performed at constant voltage. At the end of the run the gel was stained with ethidium bromide and the DNA band was visualized by UV illumination. The size of the amplified band was determined by comparing the size of standard 1 kb DNA ladder which was run in parallel.

2.3.4 Microsatellite Marker Based Molecular-Genetic Exclusion Mapping:

2.3.4.1 Establishment of microsatellite marker polymorphism assay:

The Mouse Map-Pairs purchased from Research Genetics were used to amplify the microsatellite markers from various mouse autosomes 1 to 19 (Table 19). For Il4, Ifg and CD23 gene associated CA repeat amplification primers were either synthesized based on published primer sequences or designed from the published genomic DNA sequence, respectively (Table 2). The tail DNA extracted from A.SW and SJL mice was quantified spectrophotometrically.

Optimization of the PCR conditions for microsatellite marker amplification:

The PCR conditions were optimized with respect to (i) DNA concentration; (ii) the annealing temperature for each primer; and (iii) the Mg^{++} ion concentration. Each of the variables was considered optimal when it amplified a sharp DNA band corresponding to the expected size of the microsatellite marker. Thus, various concentrations of DNA was used in PCR reactions to determine the optimal DNA concentration. Once, optimal DNA concentration was determined, the same concentration was used in all subsequent PCR assays to determine the other variables. Thus different annealing temperatures were tested to arrive at the optimal annealing temperature for a given primer. Subsequently the titration of Mg^{++} ion concentration was performed to determine the optimal one. The annealing temperature and Mg^{++} ion concentration optimal for each primer are listed in the Table 19.

PCR amplification of the microsatellite markers:

Using the optimized PCR conditions, the microsatellite polymorphism assay was conducted. Briefly, each PCR reaction mixture contained 200 ng DNA, 0.2 uM of each primer, 200 uM of each of the four dNTPs, optimal concentration of $MgCl_2$, 0.25 U of Taq polymerase (Promega, Madison, WI, USA) in tris buffer at pH 9.0. The mixture was overlaid with 20 ul of mineral oil. The samples were heated for 3 min at 94°C, and PCRs were

conducted (Hybaid thermocycler, Woodbridge, NJ, USA) with 40 cycles of denaturation (45 s, 94°C), followed by annealing (for 2 min at the optimal temperature for each primer), and extension (45 s, 72°C) and the final extension for 10 min at 72°C.

After amplification of the DNA, an aliquot of the PCR amplified product, mixed with DNA loading buffer, was loaded on to agarose gels. Electrophoresis was performed in Metaphor agarose gels (FMC, Rockland, ME, USA). After electrophoresis, the gel was stained by ethidium bromide and visualized by UV illumination. The gels were photographed, and used in analysis.

2.3.4.2 Molecular genetic mapping:

The molecular-genetic exclusion mapping involved genome-scanning of the BC1 progeny. The genomic DNA from each individual BC1 mice was subjected to microsatellite polymorphism assay as described before. Each individual mouse in the BC1 was scored for genotype at each microsatellite marker locus. Thus, parent strains which are inbred, are homozygous at all loci and hence were designated as either AA for A.SW allele and SS for SJL allele. The F1 hybrids by definition are identical and heterozygous at all loci. Therefore their genotype was designated as AS. Each individual in BC1 progeny were scored as homozygous when it possessed

both alleles from SJL (SS) or heterozygous when it possessed one allele from A.SW and one from SJL (AS). The genotype information was used in the statistical analysis to map the loci linked to the TSIgE phenotype.

2.4 STATISTICAL ANALYSES

The statistical test performed in each set of experiment is indicated in the appropriate result section. The analysis of Ab response between A.SW and SJL strains was compared by Student's t-test. In case of F1, BC-1 and F2 intercross mice, the Ab response at days 10, 21 and 31, after primary immunization were $\log_2$ (L) transformed (for the biological variation and for statistical reasons) and used in the analysis. The distribution of L(TSIgE) levels were used to statistically determine the cut-off values for low and high IgE groups of mice. The difference between gender, and between low and high IgE groups were analyzed by Student's t-test. Correlation between Ab isotypes was determined by Spearman's correlation analyses. The distribution analysis was performed by Komogorov-Smirnov goodness-of-fit test. Association studies were done by Fisher's exact test. The significance level was set at $P<0.05$ in all analyses.

Genotype of low and high TSIgE mice at each of the microsatellite loci were used in statistical analyses. Linkage of the marker loci to TSIgE phenotype was evaluated by Chi-

square (χ^2) tests for goodness-of-fit against an expected 50:50 distribution as if the marker locus was unlinked to TSIgE phenotype (Ghosh et al 1993). The P values of <0.05, and $\chi^2>3.8$, were considered evidence for significant association; and $P<0.001$, and $\chi^2>10.8$ were considered evidence of highly significant association (and evidence of significant linkage, Ghosh et al 1993). No correction for multiple comparisons was applied so that all loci showing significant associations with the TSIgE phenotype would be identified.

III. RESULTS AND DISCUSSION

A. INHERITANCE OF AL-INDUCED PERSISTENT TSIgE LEVELS IN MOUSE: GENETIC CONTROL BY NON-MHC AUTOSOMAL GENES

A.1.0 INTRODUCTION

Immunogenetic analyses of atopy in the past decades have led to the consensus that atopy is a complex genetic disorder, without any consistent mode of inheritance (Marsh 1996). The atopic aetiology is believed to be multifactorial (meaning several genes, along with an array of environmental factors, interact to determine the expression of the disease) and genetically heterogeneous (meaning a number of different genes or combinations of genes determine the same or a very similar clinical phenotype) (Marsh and Meyers 1992, Marsh 1996). Since the inheritance of atopy is multifactorial, it may reasonably be assumed that genetic determinants of serum IgE levels are one relevant variable. Consequently, the genetic analysis of serum IgE levels will be expected to be less complicated than the analysis of allergic disease in general. Therefore, extensive genetic studies have been carried out in the past to elucidate the genetic control of IgE responsiveness in humans as measured by specific IgE (SPIgE) Abs and total serum IgE (TSIgE) levels.

The pioneering studies by Marsh and associates indicated significant associations between the HLA-linked immune response genes and SPIgE Ab responses to low molecular weight ALs (Marsh 1989, Marsh and Freidhoff 1993). Furthermore, it

was also reported that the basal serum IgE level in humans is controlled by a recessively acting autosomal gene, which may modulate the development of SPIgE Ab responses (Marsh et al 1974). Thus, there is a deluge of information on the role of HLA alleles as reviewed in the previous chapter. It should be stressed that HLA alleles are associated with specific immune responsiveness in general and such associations are not restricted to IgE isotype alone.

Studies investigating the genetic control of TSIgE levels are of major importance in understanding basic pathophysiologic mechanism in atopy and asthma because high TSIgE levels correlate with the clinical expression of both allergy and asthma (Burrows et al 1989, Sears et al 1991, Halonen et al 1992, Marsh and Freidhoff 1993). Earlier studies provided evidence for single gene, two gene and polygenic inheritance (Blumenthal et al 1981, Rao et al 1980, Hasstedt et al 1983, Xu et al 1995) for TSIgE levels. Inheritance studies have indicated dominant, co-dominant as well as recessive pattern for TSIgE levels (Marsh and Blumenthal 1990, Morton 1992, Borecki et al 1985, Martinez et al 1994). Twin studies indicated that familial or a common breeding environment has very little effect on IgE levels and that there is a significant genetic component regulating TSIgE levels (Hanson et al 1991).

Taken together the human studies provided evidence that 1) TSIgE levels are under strong genetic control in humans; 2) there is no clear mode of inheritance for TSIgE levels; and 3) there are at least two types of genetic determinants of IgE responsiveness, namely - a) HLA-linked control of specific immune responsiveness which is not isotype specific but includes SPIgE Ab production to common environmental ALs; and b) non-HLA-linked control of the overall production of IgE (of any specificity).

Studies in the murine system, as described in previous chapter, have contributed tremendously to our knowledge of regulation of IgE response. Inbred mouse strains can be classified as low, intermediate and high IgE responders, when injected with ALs (Levine and Vaz 1970a,b). This variability among strains suggest that IgE responsiveness is under genetic control. However, since MHC-linked 'Ir' genes play an important role in immune responsiveness to protein Ags in general (Carbone and Bevan 1989), this can complicate the genetic analysis of IgE responsiveness, by bringing MHC-linked 'Ir'genes as a variable. However, availability of MHC-identical mouse strains provide an unique opportunity to dissect the non-MHC gene(s) mediated control of IgE responsiveness. Furthermore, IgE Ab synthesis in the mouse resembles humans in a number of ways as described before, and therefore, such studies will be relevant to human situation.

Nevertheless, there is little information on the genetic control of IgE isotype responsiveness by non-MHC genes in mice.

In contrast to the plethora of investigations on the genetic control of TSIgE levels in humans, no study as yet has been conducted in any laboratory animal including mice, on the genetic control of AL induced TSIgE levels. Since TSIgE levels correlate well with the clinical expression of allergy, bronchial hyper-responsiveness (BHR) and asthma, it represents a potentially useful quantitative parameter that can be used to map genes for these complex disorders. Therefore, the first objective of this study was to elucidate the inheritance pattern of AL-induced persistent TSIgE levels in mouse. For this purpose, a large mice colony consisting of F1, BC1 and F2 progeny was established using two inbred mice strains, namely A.SW/Snj and SJL/j. The rationale for selecting these strains was that both are MHC-identical ($H\text{-}2^s$ haplotype), similar in IgG1 Ab responsiveness, but differ extensively in IgE isotype response (as measured by AL-SPIgE or TSIgE levels). Since, high levels of TSIgE are not spontaneously detected in these mice, they were immunized with a cocktail of ALs derived from diverse sources like plant, animal and mite, to induce high TSIgE levels. Thus, genetic control of AL-induced persistent TSIgE levels was investigated as described in next section.

A.2 RESULTS

A.2.1 MHC-identical inbred mouse strains - A.SW/Snj and SJL/j, do not differ in IgG1 Ab responsiveness but differ in IgE isotype responsiveness to ALs.

In order to dissect the genetic control of IgE responsiveness by non-MHC genes, two MHC-identical mice strains A.SW/Snj and SJL/j were used in this study. Initially, Ab responsiveness of these mice to ALs was studied. Thus, a group of adult A.SW and SJL mice were sensitized with a cocktail of ALs consisting of KBG, Ragweed, White birch, OVA and HDM extracts in the presence of alum as adjuvant and boosted with the same cocktail after 4 weeks of rest. The day 10 sera was assayed for IgG1 and IgE Ab response to KBG extract by ELISA and PCA, respectively. Whereas both A.SW and SJL mice mounted comparable IgG1 Ab response to KBG ALs, only A.SW mice mounted high KBG-SPIgE Ab response (Table 3). In contrast, most of the SJL mice exhibited no detectable SPIgE Ab as measured by PCA. There was no significant difference in SPIgG1 levels between A.SW and SJL mice ($P<0.62$), while the difference between these strains in SPIgE levels was highly significant ($P<0.00001$). Thus, these results suggest that MHC-identical inbred strains A.SW and SJL do not differ in IgG1 Ab responsiveness but differ in IgE isotype responsiveness.

Table 3:

MHC-identical inbred strains- A.SW/Snj and SJL/J, do not differ in IgG1 responsiveness but differ significantly in IgE isotype responsiveness to ALs.

	KBG-Specific Secondary Ab response (day 10)	
Strain	**Anti-KBG IgG1 level[1] (OD 405-690)**	**Anti-KBG IgE level[2] (PCA titer)**
A.SW/Snj	0.73 ± 0.11	2011 ± 210
	n=14	n=14
SJL/J	0.58 ± 0.27	3.6 ± 3
	n=11	n=11
P value[3]	**0.615**	**0.00001**

1. KBG-SPIgG1 Ab level in serum was measured by ELISA as described in the text; OD (405-690) values are presented as mean ± SE

2. KBG-SPIgE Ab level in serum was measured by PCA assay in Sprague-Dawley male rats as described in the text; reciprocal of serum dilution giving a positive skin reaction is presented as mean ± SE

3. Difference in KBG-SPAb levels between A.SW and SJL mice was analyzed by Student's t test.

A.2.2 A.SW and SJL strains of mice differ significantly in TSIgE levels.

Having established that there is no difference between A.SW and SJL mice in terms of IgG1 Ab response to ALs, the AL induced TSIgE levels were studied in these mice. Thus, groups of adult A.SW and SJL mice were sensitized with the cocktail of ALs, as described before, and the primary response was assessed on days 8, 15 and 41. Low levels of TSIgE were detectable in both A.SW and SJL mice in pre-immune serum and on day 8. In contrast, high levels of TSIgE levels were detectable in the A.SW mice on days 15 and 41, while most of the SJL mice made barely detectable levels of TSIgE (Table 4) The mean TSIgE level in A.SW was significantly different from that of SJL in the pre-immune serum ($P<0.0001$), on days 15 ($p<0.0002$) and 41 ($p<0.00001$), but not on day 8 ($P<0.064$).

After primary immunization, animals were rested for 4 weeks and then boosted with the same cocktail of ALs with alum as adjuvant, and the secondary response was assessed on days 10 and 31 (Table 5). Whereas, extremely high levels of TSIgE Ab levels were detected on both days in A.SW mice, TSIgE level was barely detectable in SJL mice. There was a significant difference in AL-induced TSIgE levels between A.SW and SJL mice on day 10 ($P<0.0002$) and day 31 ($P<0.00001$). Furthermore, high TSIgE levels were detectable in A.SW mice even on day 125 of secondary response (mean for 2 mice examined, 12.9 μg/ml)

Table 4:

TSIgE levels in MHC-identical inbred mice strains, A.SW/Snj and SJL/j (primary response).

	TSIgE level[1] (μg/ml)			
		Primary Response[2]		
Strain	**pre[3]**	**day 8**	**day 15**	**day 41**
A.SW/Snj	0.15 ± 0.02	0.25 ± 0.04	6.78 ± 1.1	8 ± 1.15
	n=14	n=14	n=14	n=14
SJL/J	<0.001	0.13 ± 0.01	0.46 ± 0.19	0.54 ±0.24
	n=11	n=11	n=11	n=11
P value[4]	**0.0001**	**0.064**	**0.0002**	**0.00001**

1. TSIgE level in serum was measured by ELISA as described in text.
2. TSIgE level in μg/ml serum is presented as mean ± SE.
3. TSIgE level in preimmune serum.
4. Difference in TSIgE levels between A.SW and SJL mice was analyzed by student's t-test.

Table 5:

TSIgE levels in MHC-compatible inbred mice strains, A.SW/Snj and SJL/j (Secondary response).

	TSIgE levels[1] (μg/ml)	
	Secondary Response[2]	
Strain	**day 10**	**day 31**
A.SW/Snj	20.6 ± 4.0	23.9 ± 2.0
	n=14	n=14
SJL/J	0.23 ± 0.22	0.38 ± 0.24
	n=11	n=11
P value[3]	**0.0002**	**0.00001**

1. TSIgE level in serum was measured by ELISA as described in text
2. TSIgE level in μg/ml serum is presented as mean ± SE
3. Difference in TSIgE levels between A.SW and SJL mice was analyzed by student's t-test

indicating persistence AL-induced TSIgE levels in this strain.

A.2.3 The AL-induced TSIgE levels in A.SW and SJL mice are not influenced by gender.

In view of the well known influence of gender on TSIgE levels in humans (Marsh et al 1994), it was decided to examine the influence of gender on TSIgE levels in A.SW and SJL mice. A group of male and female mice were immunized as described and the TSIgE levels were assessed on day 15, primary response and on day 31 secondary response (Table 6). As evident from the results, both male and female A.SW mice mounted comparable levels of high TSIgE in both primary and secondary responses. There was no significant difference between gender on day 15, primary response ($P<0.617$), as well as on day 31, secondary response ($P<0.931$) in A.SW mice. Similarly, both male and female SJL mice mounted a comparable and very low levels of TSIgE, primary response on day 15 ($P<0.244$) and secondary response on day 31 ($p<0.271$). It was concluded from these results that AL-induced TSIgE levels in these two mouse strains are not influenced by gender.

The following conclusions were drawn from the results obtained from above described experiments:

1. While A.SW and SJL strains do not differ in their ability to mount an Ab response to ALs with regard to IgG1 isotype, they differ significantly in IgE isotype responsiveness;
2. Whereas, extremely high levels of persistent TSIgE can be

Table 6

TSIgE levels in A.SW/Snj and SJL/j mice are not influenced by gender

	TSIgE level (μg/ml)					
	Primary Response (d15)			Secondary Response[2] (day10)		
Strain	male	female	p[3]	male	female	p[3]
A.SW/Snj	6.8 ± 1.1 n=14	7.5 ± 0.9 n=4	**0.617**	23.92 ± 3.03 n=14	23.50 ± 3.75 n=4	**0.931**
SJL/J	0.18 ± 0.13 n=6	0.46 ± 0.2 n=11	**0.244**	0.96 ± 0.74 n=6	0.01±0.0 n=11	**0.271**

1. TSIgE level in serum was measured by ELISA as described in the text.
2. TSIgE level in μg/ml serum is presented as mean ± SE.
3. Difference in TSIgE levels between A.SW and SJL mice was analyzed by student's t-test.

induced in A.SW mice by injecting a cocktail of ALs, the same treatment generally induced barely detectable levels of TSIgE in the MHC-identical SJL mouse strain; and

3. Gender has no significant influence on AL-induced TSIgE levels in these strains of mice.

Given the complex inheritance of both allergy and asthma in humans, these results suggest that studying a related quantitative measure like TSIgE levels, in these mice, may be a suitable approach to mapping genes for these disorders. Therefore, it was decided to investigate the genetic control of AL-induced TSIgE levels in a F1, BC1 and F2 mice colony, established using these two parent strains, as described in next section.

A.2.4 Establishment of a mice colony consisting of F1, BC1 and F2 progenies, using A.SW and SJL as parents.

In order to study the inheritance pattern of AL induced TSIgE levels, a large MHC-identical mice colony, consisting of F1 hybrids, BC1 and F2 intercross progeny, was established using A.SW and SJL strains as founder parents. Since, gender had no influence on TSIgE levels, and because of poor viability of female A.SW and male SJL mice, A.SW male and SJL female mice were used in breeding. Using the breeding protocol described in previous chapter, various crosses were set up. The mating pairs are pictorially shown in Fig. 2. Thus, 55 (A.SW X SJL)

Table 7:

Summary of the Breeding Data on Mouse Colony Established for the present Study.

Type of cross	Number of Matings	♂	♀	Total	Preweaning mortality
(A.SW X SJL)F1	8	29	26	55	0
Backcross-1[a]	27	95	92	187	18
F2 intercross[b]	23	85	80	165	3

a. Backcross: [(A.SW X SJL)F1 X SJL] progeny

b. F2 intercross: [(A.SW X SJL)F1 X (A.SW X SJL)F1] F2 progeny

F1 mice were produced in 8 matings (n=55), 187 [(A.SW X SJL) F1 X SJL] backcross 1 (BC1) were produced in 27 matings, and 165 [(A.SW X SJL)F1 X (A.SW X SJL)F1] F2 intercross mice were produced in 23 matings (Table 7). The sex ratio in all the three progeny was not significantly different from 1:1 ratio. While there was no pre-weaning mortality in F1 mice, there was 9.6 % mortality in BC1 and 1.8% mortality in F2 progeny. However, this mortality did not affect the sex ratio.

A.2.5 Allergen-induced persistent high TSIgE levels is a Mendelian dominant trait in mice.

To determine the inheritance pattern of AL-induced TSIgE level, (A.SW X SJL) F1 hybrid mice were generated and used in the study. A group of F1 mice (n=29) were control bled at 4 weeks of age and used in the experiments at 6-8 weeks. Four weeks after primary immunization animals were boosted with the AL-cocktail and the secondary response was assayed on days 10, 21 and 31. A low level of TSIgE ranging up to 0.20 μg/ml were detectable in the preimmune serum of 29% of the F1 mice (Table 8). In contrast, all (A.SW X SJL)F1 hybrid mice mounted high levels of TSIgE on days 10, 21, and 31. Distribution analysis of persistent TSIgE levels revealed that distribution pattern of TSIgE levels in F1 hybrids resembled A.SW parent (Fig. 3). There was no difference in TSIgE levels between gender in F1 hybrids (males, 19.92 $\pm$ 2.3; females, 23 $\pm$ 3.9 μg/ml, on day 10, secondary response; Student's t-test, $P<0.5$). Thus, these

Table 8 :

Evidence that AL-induced persistent high TSIgE levels in mouse is a Mendelian Dominant trait.

		TSIgE levels[1] in (A.SW X SJL) F1 mice			
			Secondary Response		
F1 Mice Number	Sex	Pre[2]	Day 10	Day 21	Day 31
2	male	0	15	11	12
4	male	0.1	14	12	8
8	male	0.1	16	ND	18
10	male	0	36	64	56
22	male	0	ND[3]	ND	10
26	male	0.2	7	8	10
31	male	0	12	16	28
32	male	0	16	16	16
33	male	0.1	28	24	24
35	male	0	25	18	14
36	male	0	14	14	12
39	male	0	28	24	24
41	male	0	20	16	12
45	male	0	28	56	64
1	female	0	32	32	32
5	female	0.2	28	64	56
11	female	0.2	28	24	16
14	female	0	ND	ND	64
16	female	0	4	16	16
17	female	0	40	56	56
18	female	0	ND	56	56
23	female	0	19	20	10
25	female	0.2	23	35	24
28	female	0.1	3.5	14	28
44	female	0	16	21	20
46	female	0	43	36	18
51	female	0	17	70	49

1. TSIgE levels were measured by ELISA as described in the text and values presented are in μg/ml serum.

2. pre, TSIgE levels in preimmune serum.

3. ND, not done.

results demonstrate that all (A.SW X SJL) F1 hybrid mice, irrespective of gender, resembled A.SW parent in their ability to mount high levels of persistent TSIgE. Hence, it was concluded that AL induced persistent TSIgE levels is a Mendelian dominant trait, controlled by autosomal gene(s) in these mice.

A.2.6 Allergen-induced persistent TSIgE levels is controlled by a single major autosomal locus.

In order to precisely determine the genetic control of AL-induced TSIgE levels in A.SW and SJL mice, it was decided to conduct a systematic genetic analysis of this trait in a BC1 and F2 progeny, derived from A.SW and SJL mouse strains. Thus, a BC1 and F2 progeny consisting of ~150 mice in each group, were used in the study. All mice were control bled on 4 weeks age and used for immunization at 6-8 weeks of age. After immunization with the cocktail of ALs as described before, animals were rested for 4 weeks and then boosted with the same cocktail and the secondary response was assayed. TSIgE levels were measured in the pre-immune serum and on days 10, 21 and 31. A low level of TSIgE (ranging up to a maximum of 1.7 μg/ml) was detectable in pre-immune serum of a small proportion (36/150) of BC1 and (42/150) F2 mice. Only about 2% of the mice in BC and F2 progeny showed a transient IgE response, i.e., a considerable TSIgE levels were detectable on day 10 but not on day 31. Therefore, the day 31 values were

regarded as persistent TSIgE level and used in the analysis. All data was $\log_2$ (L) transformed and used in the statistical analysis.

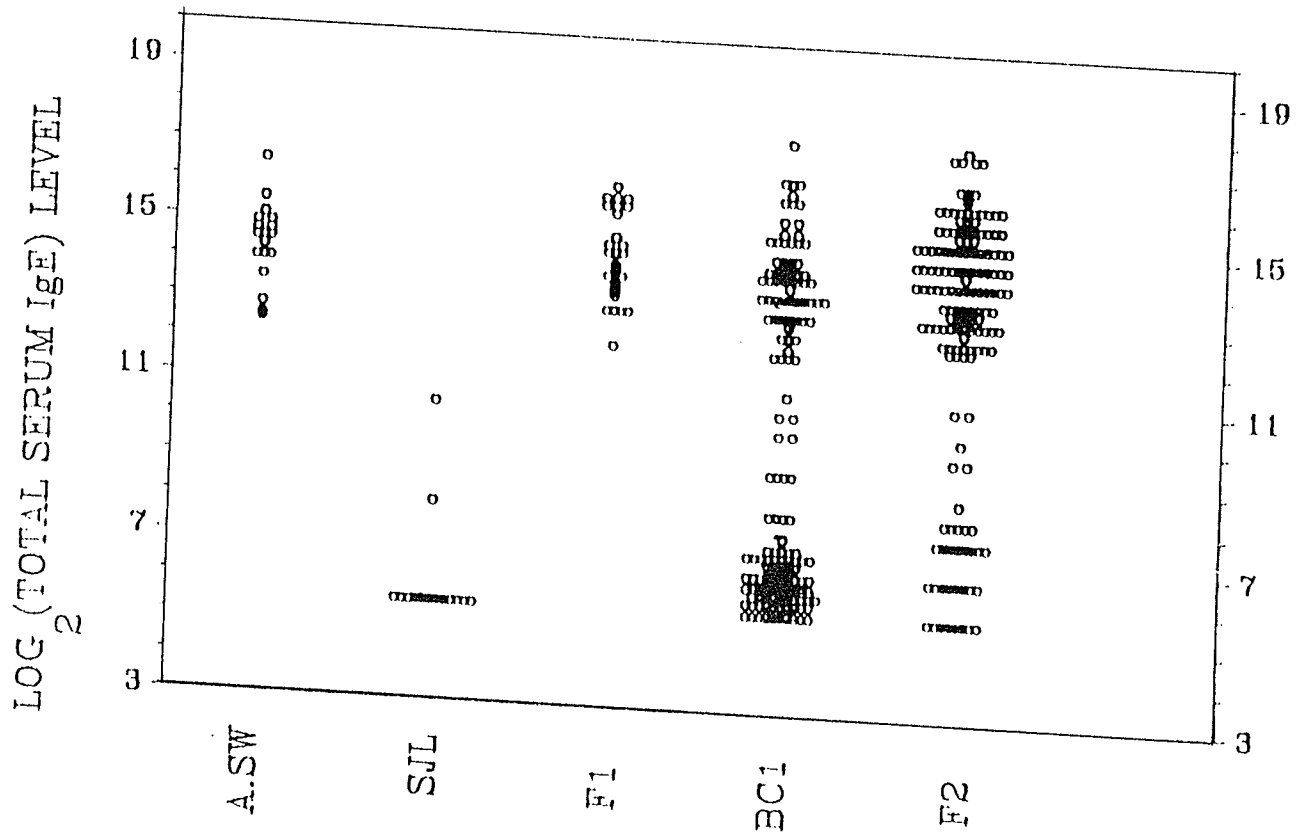
Distribution analysis of allergen induced persistent TSIgE levels in BC1 and F2 intercross mice:

Frequency distribution analysis was performed in both BC1 and F2 mice. The LTSIgE levels of day 31, secondary response were used in these analyses. As evident from the results (Fig 3) distribution of persistent TSIgE levels revealed a clear bi-modal pattern in both BC1 mice and F2 mice. Subsequently, the distribution analyses were performed in a gender-wise fashion. The bi-modal distribution was clearly apparent even in gender-wise distribution analyses of TSIgE levels in both BC1 and F2 progenies. This suggested that the BC1 and F2 progeny each consist of two groups of phenotypically distinguishable individuals with regard to TSIgE levels. Thus one group resembled the SJL parent, whereas the other resembled the A.SW parent (and the F1 hybrid mice) in the distribution of TSIgE levels (Fig. 3). These results therefore, suggested the possibility of genetic control of AL-induced persistent TSIgE levels by a single major autosomal locus.

Segregation analysis of AL induced persistent TSIgE levels in BC1 and F2 mice:

Fig. 3 Distribution of AL-induced TSIgE levels in A.SW, SJL, F1 hybrids, BC1 and F2 intercross progeny.

The TSIgE levels on day 31, secondary response, were measured in ng/ml and the values are shown as Log_2(TSIgE). Data on A.SW (n=18) and SJL (n=14) mice are from two separate sets of experiments. F1=(A.SW X SJL)F1, BC1=(F1 X SJL), F2= (F1 X F1) (number of mice, F1=29, BC1=149, F2=148).


LOG2 (TOTAL SERUM IgE) LEVEL
19
15
11
7
3
A.SW
SJL
F1
BC1
F2

Bi-modal distribution of AL-induced persistent TSIgE levels in both BC1 and F2 progeny suggest the involvement of a single genetic locus. In order to test this hypothesis, segregation analysis was performed in both BC1 and F2 mice. Preliminary analysis of LTSIgE levels from males and females as groups revealed significant difference in both BC1 ($p=0.002$) and F2 ($p=0.0018$) mice. Therefore, segregation analysis was performed separately in a gender-wise fashion. On the basis of distribution of TSIgE levels in the BC1 and F2 progeny, the values above 1 and 5 μg/ml of TSIgE were defined as the high TSIgE responder phenotype for males and females, respectively. Thus, the segregation analysis of BC1 mice revealed that about 46% of males and 49% of females were persistent high TSIgE responders (Table 9). In contrast, segregation analysis of F2 progeny indicated that about 81% of males and 83% of females were persistent high TSIgE responders. The observed frequency of high TSIgE responders was compared to the expected frequency based on the hypothesis that a single major locus controls TSIgE phenotype in these mice by Chi-square analysis. Thus, segregation analysis by χ^2 test provided evidence that the observed frequency of high TSIgE responders was not different from the expected segregation, in both BC1 ($P=0.56$ for males, $P=0.99$ for female mice) and F2 ($P=0.36$ for male, $P=0.16$ for female mice) progenies (Table 9). Therefore, these results suggest that a single major locus controls the AL-induced TSIgE phenotype in these mice.

Table 9:

Segregation Analysis of AL-induced TSIgE levels in BC1 and F2 intercross progeny:

Type of Cross	LTSIgE Levels[1]	Number of mice	Frequency of High TSIgE Responder Mice			
			Observed	Expected	χ^2	P
[(A.SW X SJL) F1 X SJL] BC1						
Male	12.7 ± 1.3	74	34 (46%)	37 (50%)	0.34	**0.56**
Female	14.8 ± 1.1	75	37 (49%)	37.5 (50%)	0.00	**0.99**
(A.SW X SJL) F2 Intercross						
Male	14.0 ± 1.4	73	59 (81%)	54.6 (75%)	1.03	**0.36**
Female	15.0 ± 1.2	75	62 (83%)	56.1 (75%)	1.96	**0.16**

1. Mean Log_2(TSIgE) ± SD, on day 31 after secondary immunization.

2. High TSIgE level was defined using the value above 1000 and 5000 ng/ml as the cut-off value for male and female mice respectively.

3. Expected number, based on the hypothesis that a single major locus controls high TSIgE levels.

Taken together, above results on the inheritance, distribution and segregation analyses suggested that AL induced persistent high TSIgE level is inherited as a simple Mendelian dominant trait and is under the control of a single major non-MHC autosomal locus.

A.2.7 Analysis of variance of TSIgE levels in F1, BC1 and F2 intercross mice.

Although segregation and distribution analysis suggested that the AL-induced TSIgE levels are controlled by a single major locus, there was a wide variation in the relative levels of TSIgE in the IgE responder mice (Fig. 3). Since, genetically identical F1 hybrids also exhibited such variation in the TSIgE levels, the role of environmental influence was assessed. Thus, variance analysis of LTSIgE levels was performed (Table 10). A variance of 0.901 in LTSIgE levels was observed in F1 hybrids, which was attributed to environmental influence (De Sanctis et al 1995, Lander and Botstein 1989). Similarly, variance analysis in BC1 and F2 IgE responder mice indicated a variance of 2.628 and 2.027 in LTSIgE levels, respectively. The fraction of the variance observed in BC1 and F2, that was in excess of the variance observed for F1 hybrid mice, was calculated. This fraction was 65.71% in BC1 and 55.55% in F2 mice, which may be attributed to genetic factors.

Table 10 :

Analysis of variance of AL-induced persistent TSIgE levels in (A.SW X SJL)F1 hybrids and BC1 and F2 intercross high IgE responder mice.

Type of cross	Number of Mice	Log_2(TSIgE)[1] (mean ± SD)	Variance
(A.SW x SJL) F1	27	14.462 ± 0.949	0.901
Backcross-1 mice[2]	71	13.837 ± 1.621	2.628
F2 intercross mice[3]	121	14.558 ± 1.424	2.027

1. TSIgE levels were determined by ELISA as described in text; values in ng/ml were log_2 transformed and used in the analysis.
2. Backcross: [(A.SW X SJL)F1 X SJL] progeny.
3. F2 intercross: [(A.SW X SJL)F1 X (A.SW X SJL)F1] F2 progeny.

A.2.8 Analysis of correlation between TSIgE levels and KBG-SPIgE Ab levels in BC1 and F2 progenies.

In addition to the determination of TSIgE levels, SPIgE Ab levels were also measured by PCA. Initially, day 31 sera from a subset of BC1 and F2 animals (n=59) were assayed for KBG-SPIgE Abs and (OVA + Ragweed + White birch)AL-SPIgE Abs. For this, SD rats sensitized with the sera, were challenged with either KBG extract or the AL mixture. There was a strong correlation between KBG-SPIgE Ab level and the AL mixture-SPIgE Ab levels in both BC1 (r=0.843) and F2 (r=0.818) mice. Therefore, KBG-SPIgE Ab levels were used in all subsequent analysis.

In order to elucidate the relationship between TSIgE and SPIgE Abs in BC1 and F2 progeny, correlation analysis was performed using Spearman test. Correlation between LTSIgE and LSPIgE levels were analyzed at days 10, 21 and 31 after secondary immunization. The correlation between LTSIgE and LSpIgE among IgE responder mice was highly significant at days 10 (r=0.65, p=0.0001), 21 (r=0.88, p=0.0001), and 31 (r=0.72, p=0.0001) after secondary immunization in BC1 progeny. The correlation between LTSIgE and LSPIgE was also found significant in F2 mice on day 10 (r=0.649, p=0.0001), day 21 (r=0.736, p=0.0001) and day 31 (r=0.522, p=0.0001). Subsequently, correlation analyses were performed in a gender-wise manner (Table 11). These analyses also revealed a strong correlation between

Table 11:

Gender-wise Correlation Analysis between TSIgE and SPIgE Abs in BC1 and F2 mice

	Male			Female		
Correlation Analysis[1]	**n[2]**	**r**	**P**	**n**	**r**	**P**
BC1 mice[3]:						
LSPIgE vs LTSIgE (D31)	34	0.806	0.0001	37	0.690	0.0001
F2 mice[4]:						
LSPIgE vs LTSIgE (D31)	58	0.832	0.0001	62	0.722	0.0001

1. Correlation analysis was performed by Spearman's test
2. n, number of mice examined
3. Backcross: [(A.SW X SJL)F1 X SJL] progeny
4. F2 intercross: [(A.SW X SJL)F1 X (A.SW X SJL)F1] F2 progeny
5. Abbreviations: LSPIgE, Log_2(SPIgE Ab); LTSIgE, Log_2(TSIgE) D31, day 31 of secondary response.

LTSIgE and LSPIgE levels in BC1 and F2 mice.

Thus, it was concluded from these results that a strong correlation exists between KBG-SPIgE Ab and TSIgE levels in both BC1 and F2 mice.

A.2.9 Analysis of KBG-SPIgG1 Ab response:

Distribution analysis:

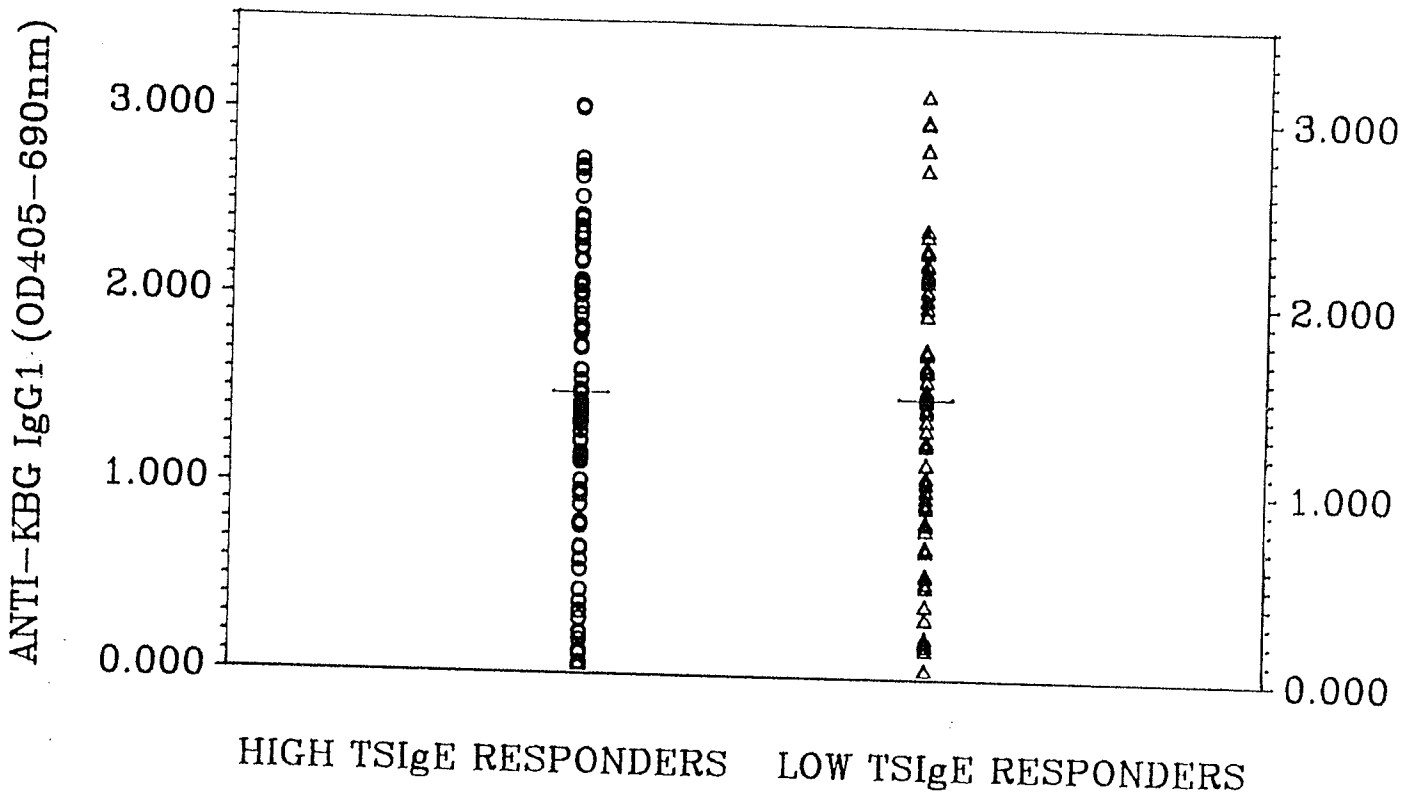
In order to study the IgG1 Ab response in the BC1 and F2 mice, distribution analyses of LSPIgG1 Ab was performed in BC1 and F2 mice. These analysis of LSPIgG1 levels revealed a typical Gaussian distribution in both BC and F2 mice. The unimodal distribution of LSPIgG1 was in contrast to that of LTSIgE levels in both BC1 and F2 mice. Furthermore, the levels of SPIgG1 Abs in the low and high TSIgE mice of BC1 progeny was compared. Interestingly, there was no significant difference in the mean levels of SPIgG1 Abs between high and low TSIgE responder mice (for low IgE group, n=78, mean OD=1.448 ± 0.091, for high IgE group, n=70, mean OD=1.537 ± 0.089; $P<0.5$) (Fig. 4). These results suggest that the low TSIgE response was not due to a lack of Ab response to allergens.

Correlation analysis between SPIgE and SPIgG1 responses:

In order to elucidate the pattern of *in vivo* Ab isotype synthesis and the inter-relationship between IgG1 and IgE

Fig.4 Distribution of KBG-SPIgG1 Ab levels in high and low TSIgE responder BC1 mice.

The high (n=70) and low (n=78) TSIgE responder BC1 mice (defined as described in the text) were examined for KBG-SPIgG1 Ab levels by ELISA (day 10, secondary response). OD values obtained using a constant dilution of serum are presented in the figure. The mean levels are indicated with a horizontal bar.


ANTI-KBG IgG1 (OD405-690nm)
3.000
2.000
1.000
0.000
3.000
2.000
1.000
0.000
HIGH TSIgE RESPONDERS
LOW TSIgE RESPONDERS

isotypes, correlation analysis was performed. For this purpose, gender-wise correlation analyses of LSPIgE and LSPIgG1 was performed by Spearman's test (Table 12). There was no significant correlation between LSPIgE and LSPIgG1 Abs in male (r=0.006, p=0.97) and female (r=0.231, P=0.18) in BC1 mice. Similarly, there was no significant correlation between LSPIgE and LSPIgG1 in male F2 mice (r=0.203, =0.13). However, although the correlation was poor, it exhibited statistical significance in female F2 mice (r=0.254, P=0.04).

In summary, these results together suggested that: i) the distribution of LSPIgG1 was unimodal in both BC1 and F2 mice; ii) the low and high TSIgE, BC1 responders are not significantly different in SPIgG1 Ab responses; and iii) *in vivo* synthesis of IgE Abs was generally not correlated with that of the IgG1 Ab isotype in these mice.

A.2.10 Allergen induced TSIgE levels is long lasting in F1, BC1 and F2 mice.

The duration of persistence of AL-induced TSIgE levels was examined. For this, after secondary immunization with ALs, a subset of F1, and high IgE responder BC1 and F2 mice were bled and TSIgE levels assessed on day 270. As evident, the AL-induced TSIgE levels were long lasting up to 270 days, and the levels were considerably high in some of these mice. (Tables 13, 14 and 15). Thus, 5 out of 8 F1 mice, 8 out of 14 BC1 mice

Table 12 :

Correlation analysis between SPIgE Ab and SPIgG1 Ab synthesis in vivo in BC1 and F2 intercross mice.

Correlation analysis[1]	n[2]	r	P
LSPIgE vs LSPIgG1:			
Backcross-1 mice[3]:			
Male	34	0.006	0.97
Female	36	0.231	0.18
F2 intercross mice[4]			
Male	58	0.203	0.125
Female	62	0.254	0.040

1. Correlation analysis was performed by Spearman's test.
2. n, number of mice examined.
3. Backcross: [(A.SW X SJL)F1 X SJL] progeny.
4. F2 intercross: [(A.SW X SJL)F1 X (A.SW X SJL)F1] F2 progeny.
5. Abbreviations: LSPIgE, Log_2(SPIgE Ab); LSPIgG1, Log_2(SPIgG1).

and 9 out of 16 F2 mice examined, still exhibited very high TSIgE levels. These results suggest that the AL-induced TSIgE levels were long-lasting, up to at least ~9 months, in F1, BC1 and F2 mice.

Table 13:

Persistence of TSIgE levels in (A.SW X SJL) F1 hybrid mice

F1 Mice number	TSIgE level[1]		
		Secondary response	
	pre[2]	day 31	day 270
1	0.0	32.0	16.3
10	0.0	56.0	15.4
11	0.2	16.0	4.6
14	0.0	64.0	27.8
16	0.0	16.0	3.9
17	0.0	56.0	14.2
33	0.1	24.0	15.4
39	0.0	24.0	4.9

1. TSIgE levels were measured by ELISA as described in the text and values presented are in μg/ml serum.

2. TSIgE levels in preimmune serum.

Table 14:

Persistence of TSIgE levels in BC1 high IgE responder mice

BC1 Mice Number	TSIgE level		
		Secondary response	
	Pre[2]	day 31	day 270
134	0.3	64.0	8.9
145	0.4	48.0	10.3
178	1.0	24.0	4.3
186	0.4	96.0	19.5
198	0.2	40.0	27.2
218	0.4	22.0	13.2
220	0.3	14.0	13.9
222	0.0	12.0	4.1
242	0.0	16.0	3.8
243	1.0	36.0	21.2
244	1.2	18.0	8.2
249	0.0	10.0	7.8
255	0.0	24.0	11.2
258	0.0	20.0	15.5

1. TSIgE levels were measured by ELISA as described in the text and values presented are in μg/ml serum.

2. TSIgE levels in preimmune serum.

Table 15 :

Persistence of TSIgE levels in F2 high IgE responder mice

F2 Mice Number	TSIgE level[1] Pre	Secondary response Day 31	Day 270
411	0.1	53.0	12.0
412	0.2	158.0	34.2
426	0.0	34.0	8.8
427	0.2	76.0	14.3
434	1.0	123.0	15.5
490	0.0	41.0	8.8
492	0.0	199.0	11.3
501	0.0	26.0	7.1
503	0.8	115.0	17.9
525	0.0	36.0	7.5
543	0.0	33.0	6.5
546	0.2	46.0	17.6
548	0.4	30.0	8.2
550	0.3	89.0	13.9
554	0.0	72.0	10.9
566	0.0	56.0	7.1

1. TSIgE levels were measured by ELISA as described in the text and values presented are in μg/ml serum.

2. pre, TSIgE levels in preimmune serum.

A.3 DISCUSSION

An important aspect of the experimental design in this study was the elimination of MHC-linked 'Immune response (Ir)' genes as a variable, by using MHC-identical parent strains of inbred mice - A.SW and SJL. Since, 'Ir' genes are crucial in determining immune responsiveness to protein Ags (Carbone and Bevan 1989), these two strains will be expected to be similar in immune response to protein-based ALs as well. This was clearly evident from the results, wherein, A.SW and SJL mice mounted comparable levels of Ab response to KBG ALs as measured by KBG-SPIgG1 levels (Table 3). In contrast, these strains exhibited extreme disparity in terms of IgE responsiveness as assessed by both TSIgE and SPIgE Ab levels during primary and secondary responses (Tables 4,5). These results on specific Ab response are in agreement with a number of previous studies in SJL mice. Thus, it has been reported that SJL mice mounts a good response by making IgG1 Abs to a number of Ags including ovalbumin, but mounts a very poor IgE Ab response to these Ags (Levine and Vaz 1970a,b, Watanabe et al 1976). Furthermore, results on TSIgE levels in SJL mice are in agreement with a recent report by Yoshimoto et al (1995). In this study, using anti-IgD Ab injection they induced substantial amounts of IgG1, and barely detectable TSIgE levels in SJL mice. Therefore, the results from the literature

together with the results from present experiments suggest that the inability of SJL mice to make AL-induced high TSIgE or SPIgE Ab to KBG was not due to a lack of Ab response. Thus, it may be concluded from these data that IgE isotype responsiveness but not IgG1 isotype responsiveness is under the control of non-MHC genes in these strains of mice. Furthermore, lack of a significant influence of gender on IgE responsiveness at the strain level, suggested that the genetic control of this trait is by autosomal gene(s).

As reviewed earlier, there are two previous studies in mouse on the inheritance of DNP-SPIgE Ab response. The first study was done using [(RF X SJL)F1 X SJL] BC1 mice and reported that PCA titers specific to DNP hapten are controlled by two genes in mice (Levine and Vaz 1970b, Levine and Vaz 1971, Levine 1979). In contrast, another study used a small number of (n=18), [(Balb/c X SJL) F1 X SJL] BC1 mice in a model of parasite-induced IgE responsiveness (Watanabe et al 1976). They reported that the low and transient, anti-DNP-SPIgE response observed in SJL mice, attributable to a suppressor T cell function, was inherited by a single Mendelian recessive gene on autosomes (Watanabe et al 1976). The disparity observed between the above two studies may be due to the "Ir" gene effects, (because they did not use MHC-matched strains) which have well known effects on specific Ab responses, or due to the small number of animals used in these studies.

Furthermore, in crosses using different strain combinations, different sets of strain-specific genes will be segregating. Therefore, the use of different strain combinations may also explain the differences between these reports. In contrast to these reports on genetic control of DNP-SPIgE Abs, there is no previous study on the genetic control of AL-induced TSIgE levels in any laboratory species including mouse. It is noteworthy that the mouse colony and the approach used in this study is significantly different from the above studies: 1) a large MHC-identical mouse colony was used here; 2) animals were immunized with a mixture of ALs from diverse sources like plants, mite and animal, which should trigger not only the AL specific responses but also enhance overall genetic propensity to mount high TSIgE levels, as opposed to parasite Ags, which induce a strong T cell-independent and non-specific activation of B cells (Lebrun and Spiegelberg 1987), thereby masking the underlying delicate regulation of IgE isotype responses; 3) mice were immunized with ALs at a low dose via the i.p. route in conjunction with alum as adjuvant, to study atopic IgE response; and 4) most importantly, the genetic control of AL-induced persistent TSIgE levels was investigated. These features of the present model resemble the AL-induced persistent TSIgE synthesis, which is a key feature of atopy in humans.

A major finding of this study is that AL-induced persistent and high TSIgE levels are inherited in these mice as a dominant Mendelian trait. The dominant inheritance of high TSIgE levels in mice is in agreement with a number of previous studies in humans (Marsh and Meyers 1992, Morton 1992, Cookson and Hopkin 1988, Borecki et al 1985). It should be stressed that in humans, there is no clear pattern for inheritance of TSIgE levels, as reviewed before. To our knowledge there is no previous study in the mouse on the inheritance of TSIgE levels. Furthermore, the distribution analysis of TSIgE levels in both BC1 and F2 intercross progeny revealed a clear bimodal pattern (Fig. 3) suggesting the possibility of genetic control of TSIgE levels by a single major locus. This observation was further supported by segregation analysis of LTSIgE levels. However, these analyses cannot exclude the possibility of tandemly linked genes in close proximity to each other or the existence of unlinked modifier gene(s) that may modify the TSIgE levels by acting on the major locus. The latter hypothesis is supported by the observation of extensive variations in TSIgE levels among high IgE responder mice in both BC1 and F2 progeny (Fig. 3). However, variation in the levels of TSIgE in A.SW and F1 mice, which are by definition genetically identical, indicate that there may be some environmental influence. Therefore, this raises the possibility that a fraction of the variation in TSIgE levels in BC1 and F2 progeny may be actually due to the environmental

factor(s).

Using the variance of LTSIgE in F1 hybrids, it was estimated that about 65.71% variance of TSIgE levels in BC and 55.55% variance of TSIgE levels in F2 IgE responder mice was due to the genetic factors (Table 10). These results suggest that genetic factors play a major role in controlling IgE responses, and environmental factors contribute to a lesser extent. The role of environmental factors influencing TSIgE levels is well appreciated in humans (Marsh 1981, Marsh and Blumenthal 1990).

In humans TSIgE levels correlate moderately with SPIgE Ab levels (Marsh et al 1994). In contrast to this, the result from the present study revealed a strong correlation between TSIgE levels and KBG-SPIgE Ab levels (Table 11). This high degree of positive correlation may be due to the reason that these mice do not develop elevated levels of TSIgE spontaneously (Table 4) and that high TSIgE levels can only be induced by AL injection. Therefore it is likely that a significant and major component of TSIgE in BC1 and F2 mice may actually represent AL-induced IgE Abs.

The demonstration that the low and high TSIgE responders are strikingly similar in their ability to mount IgG1 Ab response (Fig. 4), suggest that the low TSIgE levels were not due to a

lack of Ab response *per se* in these animals. Correlation analysis in BC1 mice revealed that the *in vivo* synthesis of SPIgG1 Abs was poorly correlated with that of SPIgE Ab synthesis (r=0.006 to 2.54) (Table 12). Furthermore, unimodal distribution of LSPIgG1 Ab levels in BC1 and F2 mice, and comparable IgG1 Ab levels ($P<0.5$, Fig 4) in both the high and low IgE responders, suggest that these Ab isotypes are differentially regulated at the genetic level in this colony. This observation is consistent with a recent report which provide evidence for independent regulation of DNA recombination and Ig secretion during isotype switching to IgG1 and IgE in mouse (Purkerson and Isakson 1994).

However, observations in the present study of the dissociation between IgG1 and IgE Ab responses, are in contrast to the previous study using (Balb/c X SJL) BC1 mice by Watanabe et al (1976). They reported that 'high' and 'low' DNP-SPIgE responders among BC1 mice were also 'high' and 'low' DNP-SPIgG1 responders respectively. They suggested that the 'high' and 'low' responder characteristic of the BC1 mice for IgE and IgG1 production might be due to suppressor T cell activity or could be due to a second gene governing both IgE and IgG1 Ab production. It should be noted that in contrast to their system, the single major locus governing AL-induced TSIgE levels in this colony is exclusively specific for IgE isotype and it has no effect on IgG1 isotype.

In summary: i) the AL-induced persistent TSIgE levels in these mice is a Mendelian dominant trait under the control of a major non-MHC autosomal locus and possibly by additional modifier loci; ii) TSIgE levels are significantly correlated with the KBG-SPIgE Abs; and iii) IgE and IgG1 Ab isotype synthesis *in vivo* generally exhibited poor correlation, indicating IgE isotype specific genetic control.

B. ANALYSIS OF CANDIDATE GENES FOR ATOPIC IgE RESPONSIVENESS IN MICE

B.I ANALYSIS OF Tcrvb8 GENE AS A PREDISPOSING FACTOR FOR ATOPIC IgE RESPONSIVENESS

B.I.1 INTRODUCTION

Regulation of IgE synthesis is complex, and is under the control of Ag specific and non-Ag specific genetic factors encoded by the MHC and non-MHC genes, respectively (Marsh et al 1994). The AL-specific activation of T cells requires the engagement of the T-cell receptor (TCR) with the AL derived peptide in context of MHC II molecules on APCs (Lichtenstein 1993, Marsh 1989). This Ag-specific, MHC-restricted event induces the effector function of T cell help via the activation of T cells and their expression of cytokines, such as IL-4, IFN-γ etc., which leads to activation and class switching of B cells (Paul and Seder 1994). The class-switched B cells now undergo differentiation and proliferation to become IgE producing plasma cells and memory B cells (Rothman et al 1989). A dysregulated IgE responsiveness to ALs can potentially originate from a defect at any point in this cascade of events. In particular, this may be related to differences in the TCR repertoire or the expression of different MHC II molecules or due to the downstream events occurring after MHC-peptide-TCR trimolecular interaction.

It is well established that AL induced IgE synthesis is

strictly T cell dependent. However, the role of the TCR in the regulation of IgE Ab synthesis is not very clear. Based on the structure of the TCR, T cells have been classified into two subsets. Most of the T cells in periphery of adults express a TCR heterodimer consisting of an α and a β chain, where as about 5-10% of T cells in the peripheral blood and most of the intra-epithelial T cells express TCR made of α and δ chains (Davis and Bjorkamn 1988). An enormous potential for TCR diversity arises from the many variable (V), diversity (D) (in case of β and δ loci) and junctional (J) gene segments within the TCR loci. However, the use of TCR Vα and Vβ elements by lymphocytes may not be random and may be under genetic control (Loveridge et al 1991, Moss et al 1993). The arrangement of the different TCR elements on the α and β chains, which in turn depends on the peripheral T-cell repertoire of an individual, determines which peptide may be recognized by T cells. The Ag specificity of this recognition process is determined, in addition to other TCR elements, by the amino acid sequences of the variable elements of the TCR α and β chains (i.e., TCR Vα and Vβ). The definitive role of certain TCR Vβ elements in determining susceptibility to autoimmune diseases (for eg. TCR Vβ 8.2+ T cells in EAE, a mouse model of multiple sclerosis, Acha-Orbea H et al 1988) further highlights the importance of Ag-TCR interactions in immune responsiveness.

In a series of reports, Renz et al (1992a,b, 1993) have implicated a role for TCRVβ8 elements in IgE Ab regulation in mouse. Thus, using Balb/c mouse system they studied the TCR Vβ repertoire of OVA-responsive T cells. Screening of OVA-responsive T cell hybridomas generated from OVA-sensitized mice, indicated that these T cell hybridomas primarily expressed Vβ8.1 and 8.2 TCRs. Furthermore, sensitization of mice to OVA resulted in an increase in numbers of Vβ8.1/8.2 T cells in the local lymphnodes. Later they reported that *in vitro* production of IgE by primed B cells could be stimulated only in the presence of Vβ8.1/8.2 T cells but not with other Vβ bearing T cells (Renz et al 1992a,b, 1993). Later, they found that SJL/J mice which have genetically deleted Vβ8 T cells from the T cell repertoire (in contrast to Vβ8-expressing Balb/c mice), did not mount an IgE anti-OVA response. In the second *in vivo* system, the sole transfer of TCR Vβ8+ T cells from sensitized Balb/c mice into non-immunized syngeneic recipients stimulated an IgE anti-OVA response. Thus these data demonstrated the functional capacity of Vβ8-expressing T cell population to upregulate IgE production *in vitro* as well as *in vivo*. Thus, T cells bearing Vβ8 TCRs, have been implicated in upregulating Ag specific IgE responses in mouse. In view of these studies and the results of segregation analysis in the present study, which suggested a single gene difference between A.SW and SJL mice; and because the SJL mouse is deficient in Tcrvb8 gene, this gene

has emerged as a major candidate gene controlling differential IgE responsiveness. Hence, a comprehensive investigation on the role of Tcrvb8 gene in IgE responsiveness was undertaken in this mice colony.

B.I.2.0 RESULTS:

B.I.2.1. A.SW/Snj but not SJL/J strain of inbred mice possess Tcrvb8 genes in the germ line.

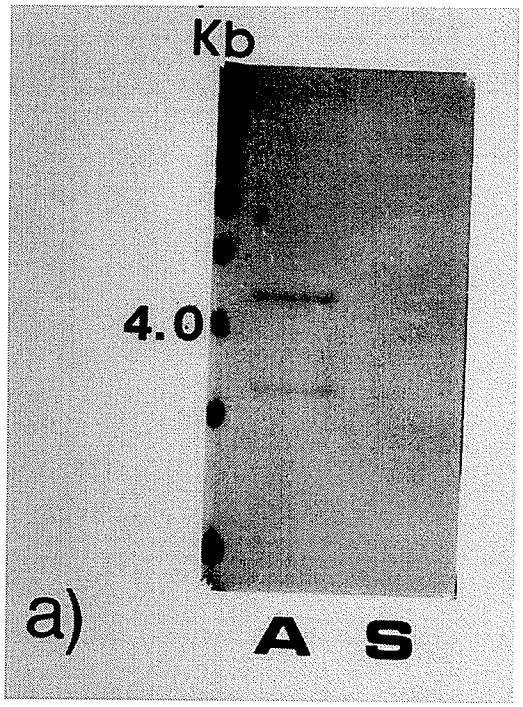
It has been reported that a number of mouse strains including SJL/J are deficient in Tcrvb8 genes, due to a large deletion of the DNA segment on chromosome 6 in the Tcrvb region (Behlke et al 1986, Chou et al 1987). However, no information was available on the status of Tcrvb8 gene in the ASW/Snj mouse strain. Therefore, it was necessary to find out if Tcrvb8 gene was present in the germline of A.SW strain. This objective was accomplished by two approaches as described below:

a) Southern hybridization:

Genomic DNA from A.SW and SJL mice was prepared from tail tissue and used in Southern blot hybridization analysis. As shown in Fig. 5a, Tcrvb8.1 probe detected two bands corresponding to ~4.4 and 3.4 kb in A.SW mouse. The former band was identified as Tcrvb 8.1 and the latter one as Tcrvb 8.2 gene (Chou et al 1987). Furthermore, hybridization of genomic DNA with Tcrvb8 probe did not detect any bands in SJL

Fig.5a Detection of Tcrvb8.1 and 8.2 genes in A.SW but not in SJL mice by Southern blot hybridization analysis.

Eco RI enzyme digested genomic DNA from A.SW and SJL mice were hybridized with isotope labelled TCRVβ8.1 cDNA probe. The probe detected two bands as shown. The upper band (~4.4 Kb) corresponds to Tcrvb8.1 gene and the lower band (~3.4 Kb) corresponds to Tcrvb8.2 gene. The position of 4 kb marker is indicated on the left side. A=A.SW, S=SJL, Kb=1Kb DNA ladder.


Kb
4.0
a)
A S

mice, which was consistent with the previous reports on the absence of Tcrvb8 genes in this strain. Thus, these results demonstrated the presence of Tcrvb8 genes in A.SW strain and confirmed its absence in SJL strain.

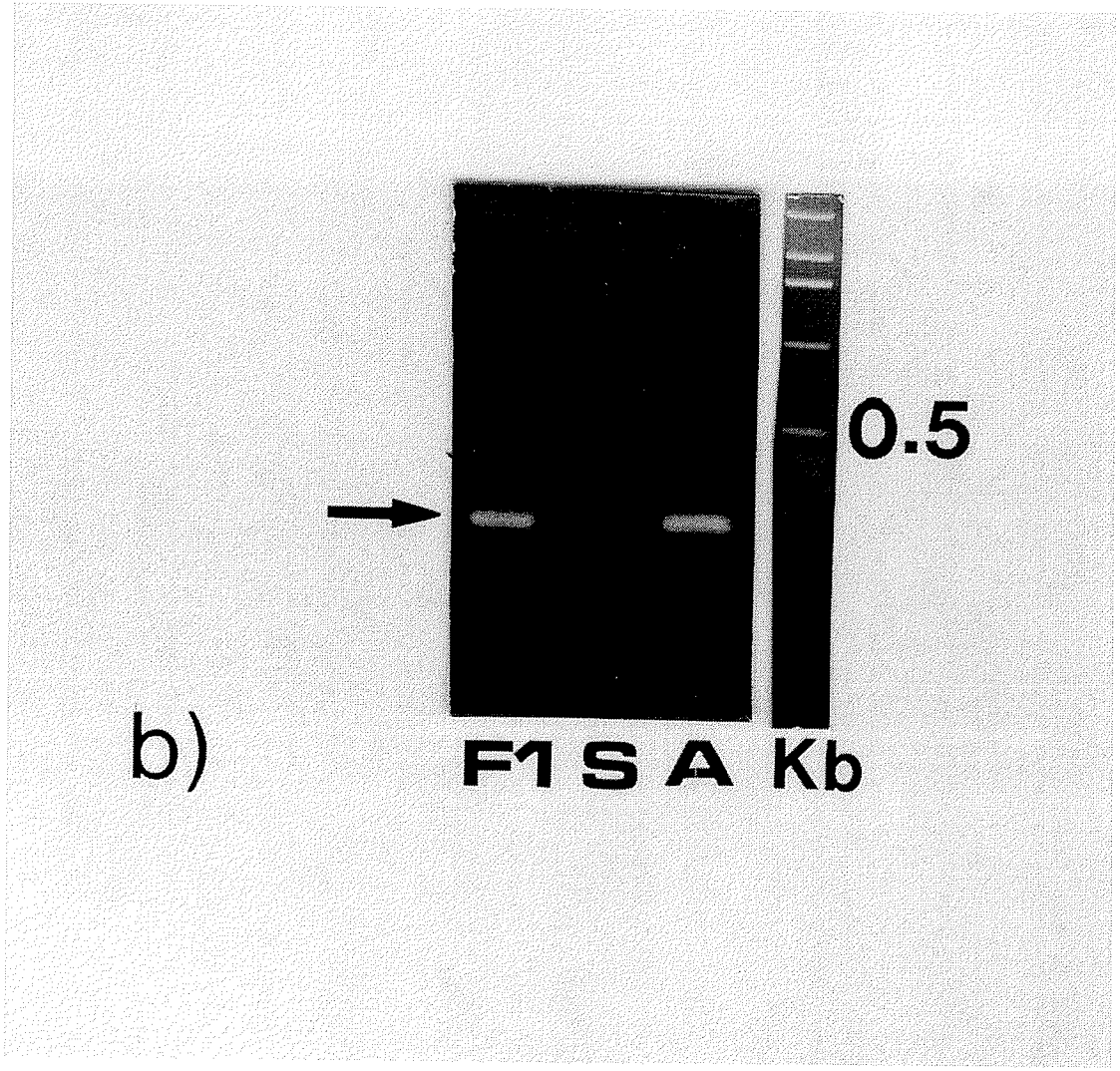
b) PCR analysis of TCR Vb8 gene.

In order to assay the Tcrvb8 gene more easily, a PCR assay was developed for this purpose. Thus primers were designed based on published sequence information, for amplification of the second exon of Tcrvb8 gene. Genomic DNA PCR assay was optimized with respect to annealing temperature and the Mg^{++} ion concentration. Thus, an annealing temperature of 52^{o} C and Mg^{++} concentration of 0.75 mM were found optimal. Using the optimized assay, genomic DNA from A.SW and SJL mice were amplified with the vb8 primers. Analysis of PCR product identified a single, amplified DNA band of ~150 bp in A.SW strain (Fig. 5b). This band was sequenced and confirmed as Tcrvb8 gene by comparing to the published sequence (data not shown). In contrast, genomic DNA-PCR analysis of SJL mice failed to amplify the Tcrvb8 gene, which was consistent with the results from Southern hybridization analysis. Furthermore, examination of DNA from (A.SW x SJL) F1 hybrid mice, revealed the presence of Tcrvb8 gene, indicating its inheritance from A.SW parent (Fig. 5b).

The results of the above experiments demonstrated i) the

Fig.5b Detection of Tcrvb8 gene in A.SW and F1 mice but not in SJL mice by PCR analysis.

The genomic DNA from individual A.SW, SJL and F1 hybrid mice was subjected to PCR amplification using Tcrvb8 primers. The position of the expected band is indicated with the arrow. The position of 0.5 Kb is shown on the right margin. A=A.SW, S=SJL, F1=(A.SW X SJL)hybrid.


b)
F1 S A Kb
0.5

presence of Tcrvb8 gene in A.SW/Snj mouse; ii) the inheritance of Tcrvb8 gene by the (A.SW x SJL)F1 hybrid mice; and ii) confirmed absence of Tcrvb8 gene in SJL/J mouse.

B.I.2.2 A.SW/Snj but not SJL/j mice possess T cells in the periphery expressing TCR Vβ8 proteins.

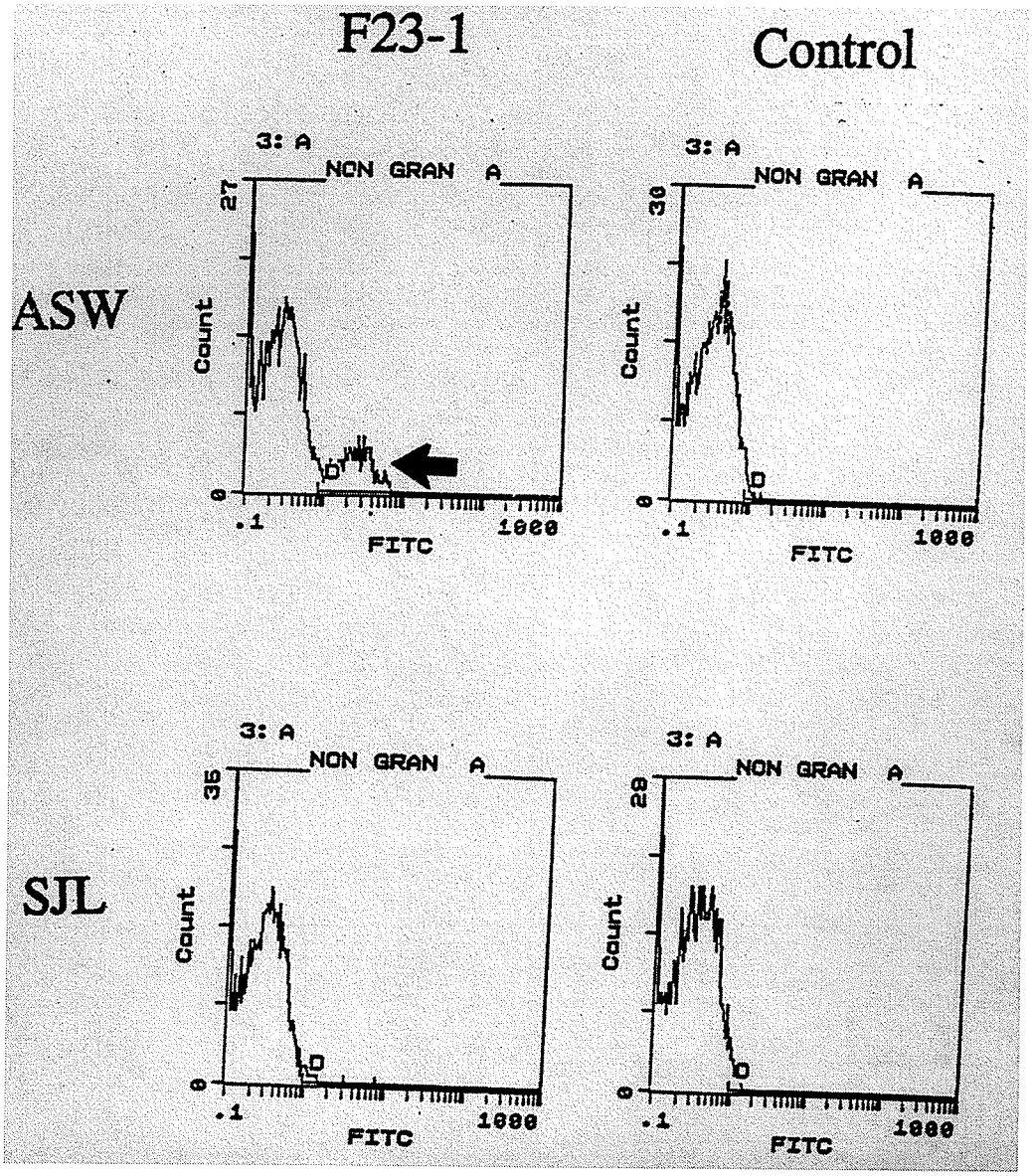
Having established at the genetic level the presence of Tcrvb8 gene in A.SW mice, its expression as protein on peripheral blood T cells was studied. A group of adult A.SW (n=5) and SJL/J (n=5) mice were examined for the presence of T cells bearing TCR Vβ8 proteins by flow cytometry analysis. Thus, the peripheral blood from A.SW and SJL mice was stained with FITC-conjugated anti-TCRVβ8 Mabs (F23-1) as described. All analysis was done against an irrelevant FITC-conjugated mouse IgG Ab as an isotype control. Flow cytometry analysis using this MAb identified T cells ranging from 3.2% to 10.2 % in the PBMCs of A.SW mice. These results indicated the presence of an average ~8.1% TCRVβ8+ T cells in the periphery of A.SW mice. In contrast, the per cent of T cells expressing TCRVβ8 proteins was negligible (ranged from 0-0.5%) in SJL mice. This suggested the absence of TCRVβ8+ T cells in the peripheral T cell repertoire of SJL mice (Fig. 6).

B.I.2.3 IgE Responsiveness does not co-segregate with Tcrvb8 genes in BC1 mice.

Having established the presence of Tcrvb8 gene in A.SW strain,

Fig.6 A.SW but not SJL strain possess T cells expressing TCRVβ8 proteins in the periphery.

The presence of TCRV$\beta 8^+$ T cells in the peripheral blood of A.SW and SJL mice (n=5 each) was examined by flow cytometry analysis. Fig. shows a representative analysis. Upper panel shows the presence of T cells expressing TCRVβ8 in A.SW mice (indicated with the arrow) detected using FITC conjugated F23-1 MAb by flow cytometry analysis; the lower panel shows its absence in SJL mouse. The flow cytometry analysis with the control Ab is shown on the right side.


F23-1
Control
ASW
SJL
3: A
NON GRAN A
Count
FITC
.1
1000
0
27
30
35
28

and its inheritance by (A.SW x SJL)F1 hybrids, its role in IgE responsiveness was investigated in BC1 mice. A subgroup of BC1 mice (n=24) was examined. Thus genomic DNA from individual mice was assayed for the presence of Tcrvb8 gene in the germline, using a previously established PCR assay. Thus amplification and detection of the ~150 bp band on the agarose gel, indicated the presence of Tcrvb8 gene. While the Tcrvb8 gene was detectable in ~ 50% of the mice examined, it was absent in the rest of BC1 mice, which was consistent with the expected random 1:1 segregation in a BC1 progeny (Table 16). Association between IgE responder phenotype (both TSIgE and KBG-SPIgE) and inheritance of Tcrvb8 gene was analyzed by Fishers exact test. There was no significant association between either TSIgE or SPIgE phenotype and the presence of Tcrvb8 gene (Tables 16 & 17). These results therefore revealed that neither SPIgE nor TSIgE phenotype co-segregated with the inheritance of Tcrvb8 gene, in the BC1 progeny.

B.I.2.4 Neither SPIgE nor AL-induced TSIgE levels are associated with the presence of TCR Vβ8⁺T cells in the periphery of BC1 mice.

In order to examine the relationship between IgE responsiveness and the presence of TCRVβ8⁺ T cells in peripheral blood lymphocytes, flow cytometry was performed as described. Peripheral blood from a subgroup of BC1 mice (n=24) was examined. PBMCs from each BC1 mouse were stained with

Table 16:

Inheritance or expression of Tcrvb8 gene is not associated with AL-induced persistent TSIgE levels in BC1 mice.

BC1 Mice Number	Tcrvb8 gene[1]	Vβ8$^+$ T cells[2]	TSIgE level[3] (μg/ml)
BC1 mice having Tcrvb8 gene:			
101	+	7.4	0
104	+	3.4	0
108	+	6.7	0
109	+	5.6	32
113	+	2.5	44
118	+	4.7	0.16
134	+	0	64
139	+	0	0.17
143	+	7.2	1.5
146	+	2.1	12
148	+	4.3	2.0
150	+	3.4	96
154	+	7.6	24
157	+	5.5	40
BC1 mice lacking Tcrv8 gene:			
103	-	0.5	0
110	-	0.3	0
111	-	0	0.17
115	-	0	88
125	-	0	0
132	-	0.7	20
136	-	0	1.5
137	-	0.1	6
145	-	0	48
147	-	0	48

1. The presence of Tcrvb8 was assayed by DNA PCR using Vb8 primers.

2. The number of Vβ8$^+$ T cells was assayed by flow cytometry analysis using F23-1 MAb-FITC conjugate;

3. The TSIgE levels measured on day 31, secondary response is presented; it was measured by ELISA.

Table 17:

Inheritance or expression of Tcrvb8 gene is not associated with AL-SPIgE response in BC1 mice.

BC1 Mice Number	Tcrvb8 gene[1]	Vβ8⁺ T cells[2]	SPIgE (PCA titer)[3]	
			Anti-OVA	Anti-KBG
BC1 mice having Tcrvb8 gene:				
101	+	7.4	<40	<40
104	+	3.4	40	<40
108	+	6.7	<40	<40
109	+	5.6	<40	2560
113	+	2.5	<40	2560
118	+	4.7	<40	<40
134	+	0	320	2560
139	+	0	<40	<40
143	+	7.2	<40	<40
146	+	2.1	640	1280
148	+	4.3	<40	160
150	+	3.4	640	2560
154	+	7.6	640	1280
157	+	5.5	320	640
BC1 mice lacking Tcrv8 gene:				
103	-	0.5	<40	<40
110	-	0.3	<40	<40
111	-	0	<40	<40
115	-	0	<40	2560
125	-	0	<40	<40
132	-	0.7	<40	640
136	-	0	<40	<40
137	-	0.1	640	160
145	-	0	1280	2560
147	-	0	640	2560

1. The presence of Tcrvb8 was assayed by DNA PCR using Vb8 primers.
2. The number of Vβ8⁺ T cells was assayed by FACS analysis using F23-1 MAb-FITC conjugate;
3. Specific IgE titers were measured on day 31, secondary response by PCA using KBG extract or OVA as challenge Ag.

anti-Vβ8 Mab (F23-1) and the fraction of T cells expressing TCRVβ8 protein was determined by flow cytometry. Based on the results from SJL/J mouse, a cut-off of 1% was set-up to indicate the presence of TCRVβ8$^+$ T cells. As is evident from the results, ~50% of BC1 mice expressed TCRVβ8 protein (Table 16). Thus expression of this protein in BC1 segregated into 1:1 ratio. The per cent of T cells possessing TCRVβ8 proteins ranged from 2.1% to 7.6% (Table 16). There was a good concordance between inheritance of the Tcrvb8 gene and its expression. Thus TCRVβ8 protein expression was seen only in those mice that inherited the gene, with the exception of mice #134 and #139, which although inherited, did not express the gene. The association between TCRVβ8$^+$ T cells and IgE responder phenotype (both TSIgE and KBG-SPIgE) was examined by Fishers exact test (Table 17). There was no significant association between either TSIgE or SPIgE levels with the presence of TCRVβ8$^+$ T cells ($p=0.66$). Thus, these results were consistent with the co-segregation study of Tcrvb8 gene as described before.

From these results it was concluded that neither the inheritance nor the expression of Tcrvb8 gene was associated with IgE responsiveness in BC1 progeny. This paved the way for examination of other candidate genes as described below.

B.II ANALYSIS OF Il4 AND Ifg GENES AS PREDISPOSING FACTORS FOR ATOPIC IgE RESPONSIVENESS.

B.II.1 INTRODUCTION

The events downstream of the MHC-peptide-TCR trimolecular interaction, that are critical for IgE regulation include the cytokine IL-4/IL-13 mediated class switching of B cells to IgE secreting plasma cells (Paul and Seder 1994, Finkelman et al 1990). Of particular interest for the regulation of IgE production is the reciprocal functional activities of IL-4 and IFN-γ in mouse. IL-4 has two major effects on activated B cells: it stimulates B-cell proliferation (Howard et al 1982), and it acts as a switch factor for IgE and IgG1 Ab synthesis. In contrast, IFN-γ inhibits IL-4-induced IgE production (Coffman and Carty 1986; Snapper and Paul 1987). Direct evidence of an *in vivo* role for IL-4 is provided by the findings that IgE production is virtually abolished by treatment with anti-IL-4 MAb or with MAb to anti-IL-4 receptor to naive mice, before challenge with the parasite *Nippostrongylus brasiliensis* (Nb), which usually induces massive IgE production (Finkelman et al 1990). Salvelkoul et al (1991) examined the SJA mice which have the Balb/c IgH locus on an SJL background. Unlike SJL mice the SJA do not produce IgE even to parasites and this defect can be corrected with injections of IL-4. Furthermore, injection of high doses of recombinant IFN-γ leads to suppression of polyclonal IgE

responses (Finkelman et al 1988) and *in vivo* induction of IFN-γ synthesis (via immunization with *Brucella abortus*) has been shown to reduce substantially IgE levels *in vivo* (Finkelman et al 1988). Thus, the cytokines IFN-γ and IL-4 reciprocally regulate IgE synthesis in mouse.

A significant evidence, as outlined below has implicated IL-4 and IFN-γ, as potential genetic factors for atopic disorders in general and for dysregulated IgE responsiveness in particular.

1) Central role for IL-4 in the Th2 the cytokine response and in particular IgE synthesis, was confirmed in Il4 gene knockout (Il4-/-) mouse studies (Kopf et al 1993). The salient findings from this study were: i) naive Il4-/- mice showed no detectable IgE (<0.015 ug/ml) and 20-fold decreased IgG1 levels in the serum compared to Il4+/+ controls, while the serum levels of other isotypes were not significantly altered; ii) anti-IgD treatment or infection with Nb, both of which are potent inducers of IgG1 and IgE response, failed to induce IgE levels and reduced IgG1 levels by 12-fold in Il4-/- mice compared with +/+ mice.

2) Recently Marsh et al (1994), reported that a gene on chromosome 5q31.1, is linked to the overall IgE production in non-atopic individuals. The likelihood of a direct role for

Il4 as a genetic predisposition factor for atopy attained high significance by the demonstration of linkage between markers surrounding this gene but not for three markers lying just outside this region.

3) There have been several reports in adults and in children with atopic diseases that correlate the expression of IL-4 positively and IFN-γ negatively with the disease (Romagnani 1994, HayGlass 1995). These studies implicate IL-4 and IFN-γ in the process of inappropriate IgE synthesis.

4) Patients with hyper-IgE syndrome have been shown to have a defect in *vitro* IFN-γ production, which has been attributed as a cause of abnormally high serum IgE levels (Del prete et al 1989). This hypothesis is supported by the evidence that treatment of patients with hyper-IgE syndrome with IFN-γ, reduced the serum IgE level by ~50% (Finkelman et al 1990).

Thus, either alterations of molecular mechanisms directly involved in the regulation of Il4 gene expression, or a deficient regulatory activity of IFN-γ or both may potentially account for the dysregulated IgE responsiveness in atopic patients. Therefore, it was of obvious interest to investigate the role of these two genes as predisposing genetic factors for AL-induced IgE responsiveness in this study.

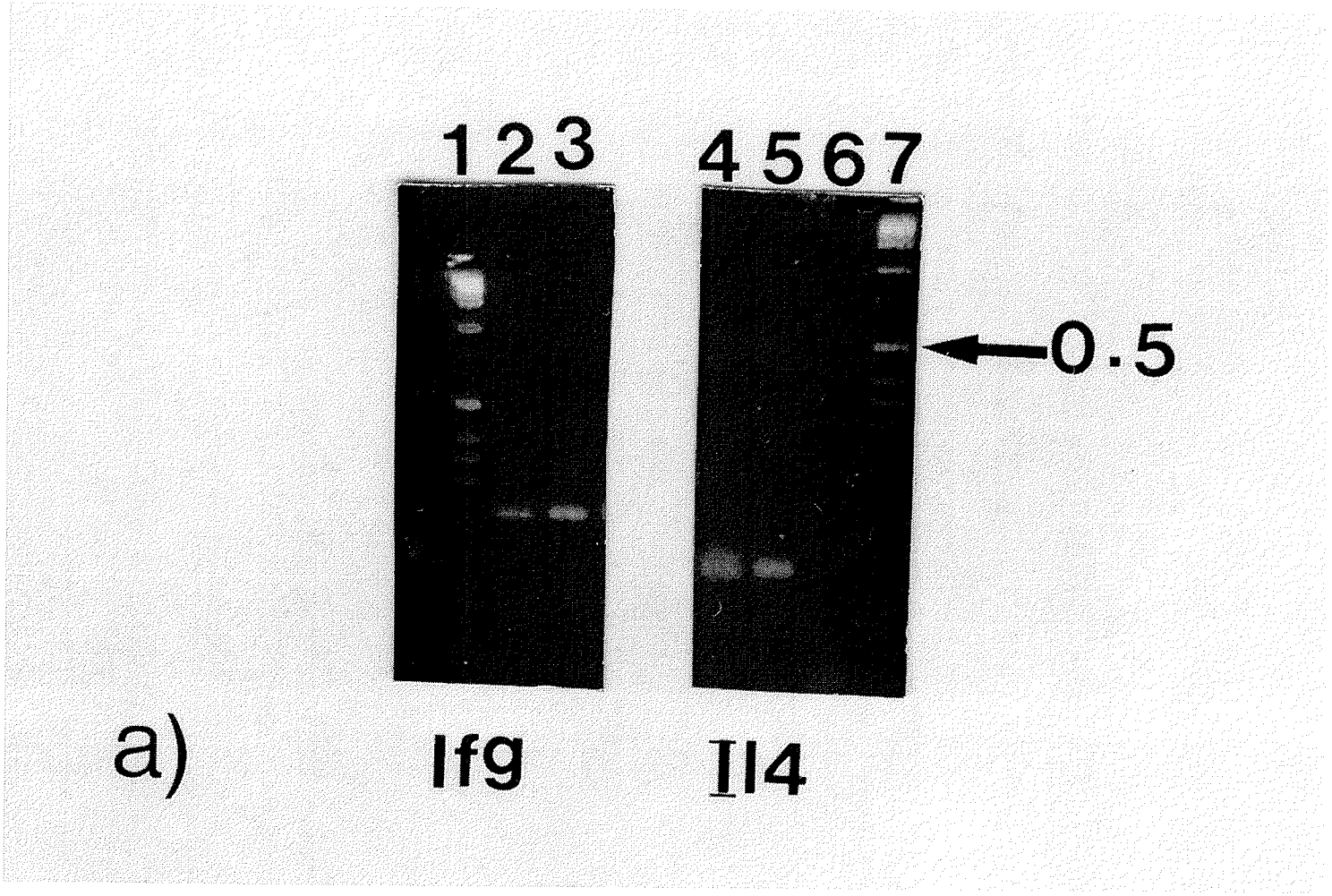
B.II.2.0 RESULTS

B.II.2.1 Identification of microsatellite markers linked to Il4 and Ifg genes that are polymorphic between A.SW and SJL strains of mice.

In order to examine the role of Il4 and Ifg genes as genetic predisposition factors for IgE responsiveness, the approach of examining the DNA polymorphism at the microsatellite repeats, was used. Initially, primers were designed for microsatellite repeats from Il4 and Ifg genes themselves, which have been reported to be polymorphic between a number of mouse strains (Jacob et al 1993). These primers were designed to amplify: 1) a 'perfect-dinucleotide CA repeat' from the Intron-2 of Il4 gene, which is known to harbour a mast-cell specific enhancer; and 2) an 'imperfect-dinucleotide repeat from Intron-3 of Ifg gene. The mice strains A.SW and SJL were examined for polymorphism at both of these loci. Thus, a PCR assay was developed after optimizing for annealing temperature and Mg^{++} concentration. The genomic DNA from both strains was PCR amplified using the above primers for Il4 and Ifg genes. The analysis of the PCR product revealed a DNA band of size ~110 bp for IL4 and ~230 bp for Ifg gene (Fig. 7a). These product sizes were consistent with the published reports in SJL mice strain (Jacob et al 1993). However, there was no apparent polymorphism in the CA repeat between A.SW and SJL mice at both Il4 and Ifg loci. Thus microsatellite markers located within the Il4 and Ifg genes were found not to be polymorphic

Fig.7a Microsatellite markers in the intron of Il4 and Ifg genes are not polymorphic.

The genomic DNA from A.SW and SJL mice was PCR amplified using primers designed for microsatellites associated with Ifg and Il4 genes. The position of 0.5 Kb marker is shown on the right side. 1=1Kb ladder; 2,4=A.SW; 3,5=SJL; 6=PCR control.


1 2 3
4 5 6 7
0.5
a)
Ifg
II4

between A.SW and SJL mouse strains.

Subsequently, another set of microsatellite markers (Res. Genetics) which are known to be linked to Il4 and Ifg genes were examined for polymorphism between A.SW and SJL mice. This analysis identified four microsatellites which exhibited polymorphism between A.SW and SJL strains. They are :D11Nds9 and D11Mit24 linked to Il4 gene; and D10Mit14 and D10Mit74 linked to Ifg. The polymorphic microsatellites, D10Mit74 and D11Nds9 are shown in the Fig. 7b. These microsatellites were used in subsequent assays.

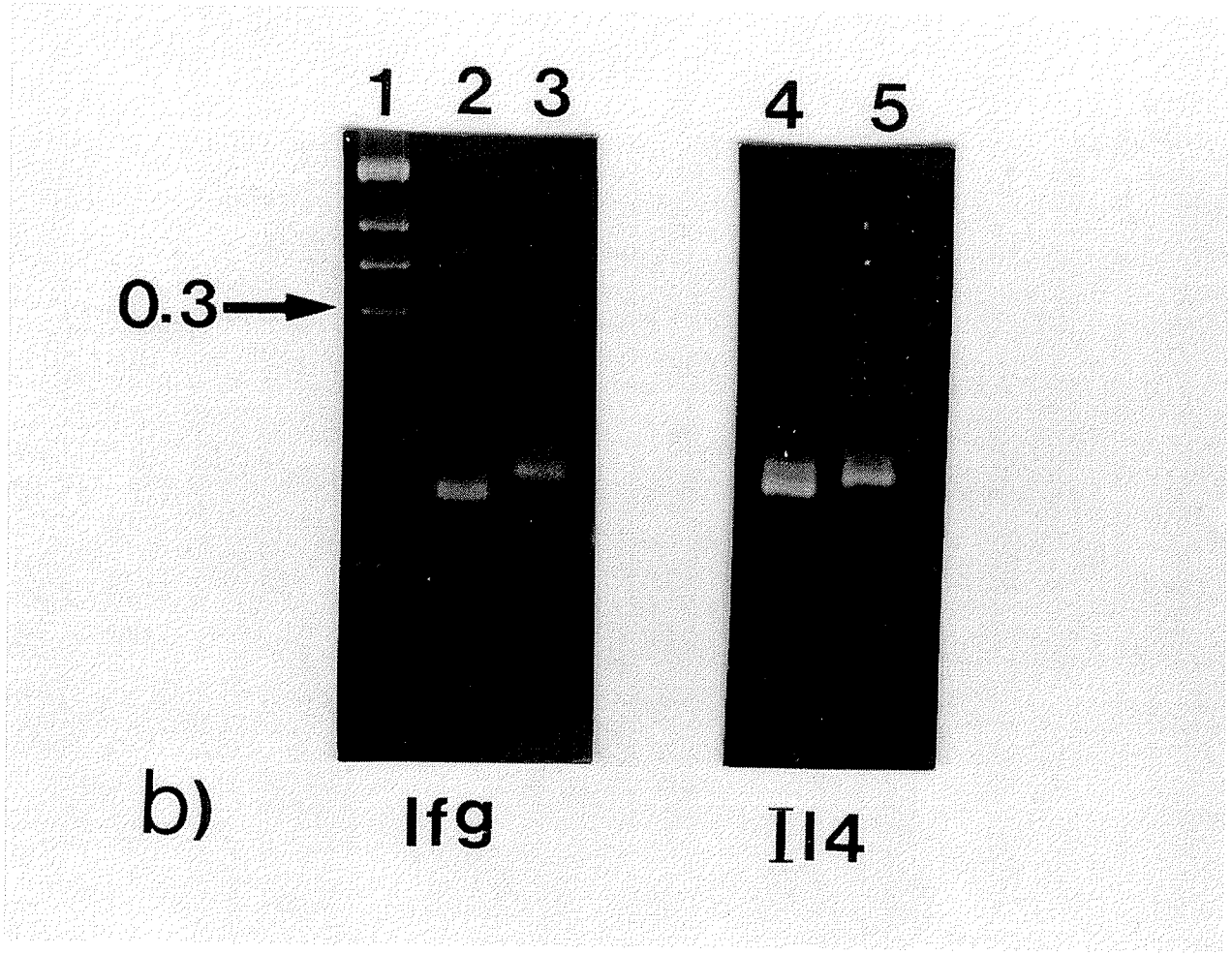
B.II.2.2 Al-induced TSIgE levels are not associated with microsatellite markers linked to Il4 and Ifg genes in BC1 mice.

a) Analysis of a randomly chosen BC1 progeny:

Having identified the polymorphic microsatellite markers linked to Il4 and Ifg genes, genetic analysis of examining the segregation of these markers was performed initially in a subgroup of randomly chosen BC1 mice (n=30), consisting of both high and low IgE responders (Fig. 8). Genomic DNA from each individual mouse was amplified using two pairs of primers each linked to Il4 (D11Mit24 and D11Nds9) and Ifg (D10Mit14 and D10Mit74) genes, one at a time. The PCR amplified product was analyzed for the presence of alleles at these loci. Thus, individual BC1 mice were genotyped at each of these loci for

Fig.7b Microsatellite markers linked to Il4 and Ifg genes are polymorphic between A.SW and SJL mice.

The genomic DNA from A.SW and SJL mice was PCR amplified using Mouse MapPairs for D10Mit74 and D11Nds9. The position of 0.3 Kb marker is shown on the left side. 1=1Kb ladder; 2,4=A.SW; 3,5=SJL.


1 2 3
4 5
0.3
b)
Ifg
I14

the presence of either only SJL allele (and therefore, homozygous at the locus, SS) or for both A.SW and SJL alleles (and therefore, heterozygous at the locus, AS). The segregation of alleles in BC1 mice corresponding to D11Mit24 and D11Nds9 was identical to each other and so were the alleles at D10Mit14 and D10Mit74. This indicated that the former two markers are tightly linked with each other on chromosomes 11 and the latter two markers on chromosome 10. There was no significant association between marker alleles at these loci and the TSIgE levels in these mice (These experiments were performed in the laboratory of Dr. David Marsh, Johns Hopkins University, MD, USA).

b) Analysis of extreme phenotype BC1 progeny:

Subsequently, a larger group of BC1 mice (n=57) selected for exhibiting extreme phenotypes,i.e, either having no detectable TSIgE or very high levels of TSIgE (>18,000 ng/ml) was examined in the present laboratory, to verify the lack of association between TSIgE and Il4 as well as TSIgE and Ifg genes. Thus, individual BC1 mice in this group were genotyped at D11Nds9 and D10Mit74 loci, as described above. Each mouse was identified as homozygous (SS) or heterozygous (AS) at the marker alleles. The association between marker loci and the TSIgE phenotype was examined by Chi-square analysis (Table 18). The results indicated that there was no significant association between either Il4 linked D11Nds9 marker (χ^2=1.02,

Mouse #	Ifg D10Mit74	Ifg D10Mit14	Il4 D11Mit24	Il4 D11Nds9	TSIgE (10^3ng/ml)	SpIgE (PCA titer)
HER:Female						
105	□	□	□	□	120	2560
113	□	□	□	□	72	2560
115	□	□	■	■	68	2560
120	■	■	□	□	96	2560
127	■	■	■	■	22	80
133	□	□	□	□	40	2560
134	■	■	□	□	52	2560
HER: Male						
109	□	□	□	□	36	2560
114	■	■	■	■	14	320
132	□	□	□	□	20	640
135	□	□	■	■	20	640
LER:Female						
107	■	■	□	□	0	0
111	■	■	■	■	0	0
112	□	□	■	■	0	0
116	■	■	■	■	0	0
118	■	■	□	□	0	0
126	□	□	■	■	0	0
129	■	■	□	□	0	0
130	□	□	□	□	0	0
131	■	■	□	□	0	0
LER:Male						
101	□	□	■	■	0	0
103	■	■	□	□	0	0
104	□	□	□	□	0	0
106	□	□	■	■	1	0
108	■	■	□	□	0	0
110	■	■	□	□	0	0
119	□	□	□	□	0	0
121	■	■	■	■	0	0
122	□	□	■	■	0	0
125	■	■	■	■	0	0

FIGURE 8 Evidence for lack of association of *Il4* and *Ifg* gene polymorphisms with IgE response. Polymorphisms were assessed by microsatellite marker analysis as shown. The heterozygote and homozygote mice are shown respectively by filled and empty squares. HER, High IgE responder; LER, Low IgE responder.

Table 18 :

Lack of association of Il4 and Ifg gene linked polymorphisms with TSIgE level in extreme phenotype BC1 mice

Marker	Linked gene	Locus	Backcross mice with Low TSIGE SS	Low TSIGE AS	High TSIGE SS	High TSIGE AS	χ^2	P
			n	n	n	n		
D10Mit74	**Ifg**	10(67)	13	17	17	10	**2.2**	**0.19**
D11Nds9	**Il4**	11(26)	15	15	14	13	**1.02**	**1.0**

1. TSIgE level was measured by ELISA on day 31, secondary response. A group of BC1 mice (n=30) having no detectable TSIgE levels were included in the low group and a group of BC1 mice (n=27) having >18,000 ng/ml TSIgE were included in the high group.

2. Positions of the markers are designated by chromosome and centiMorgans (cM) distal to the centromere. The location of the marker loci were based on the Encyclopedia of the Mouse Genome, genetic map v3.0a.

3. Known genes linked to a marker are indicated with the gene symbol for mouse; this information is from MIT mouse genome data base, 1995 directory.

4. The difference in the frequency of SS and AS alleles compared to random expectations in low TSIgE and high TSIgE groups was estimated by χ^2 analysis by 2 X 2 contingency tables.

5. Abbreviations: SS, homozygous; AS, heterozygous.

P<1.00) or the Ifg linked D10Mit74 marker (χ^2=2.2, P<0.19).

These results together indicate that AL-induced persistent TSIgE levels in BC1 mice are not associated with DNA polymorphisms in the genomic interval containing either Il4 (on chromosome 11) or Ifg (on chromosome 10) genes.

B.III ANALYSIS OF CD23 GENE.

B.III.1 INTRODUCTION

It has been reported that CD23 exerts a negative feedback control on TSIgE levels induced by T-dependent antigens but has no significant effect on TSIgE levels induced poly clonally by parasite infections in mice (Yu et al 1994, Gould and Sutton 1993). Therefore, the role of CD23 in regulating AL-induced TSIgE levels was examined here.

B.III.2.0 RESULTS

B.III.2.1 Microsatellite markers from CD23 gene are not polymorphic between A.SW and SJL mice:

Analysis of a large number of microsatellite markers (~20) from chromosome-8 in the vicinity of CD23, failed to detect polymorphism between A.SW and SJL strains. Therefore, two pairs of primers were designed for two microsatellites located on intron-9 of the mouse CD23 gene. These two microsatellites were examined for polymorphism between A.SW and SJL mouse

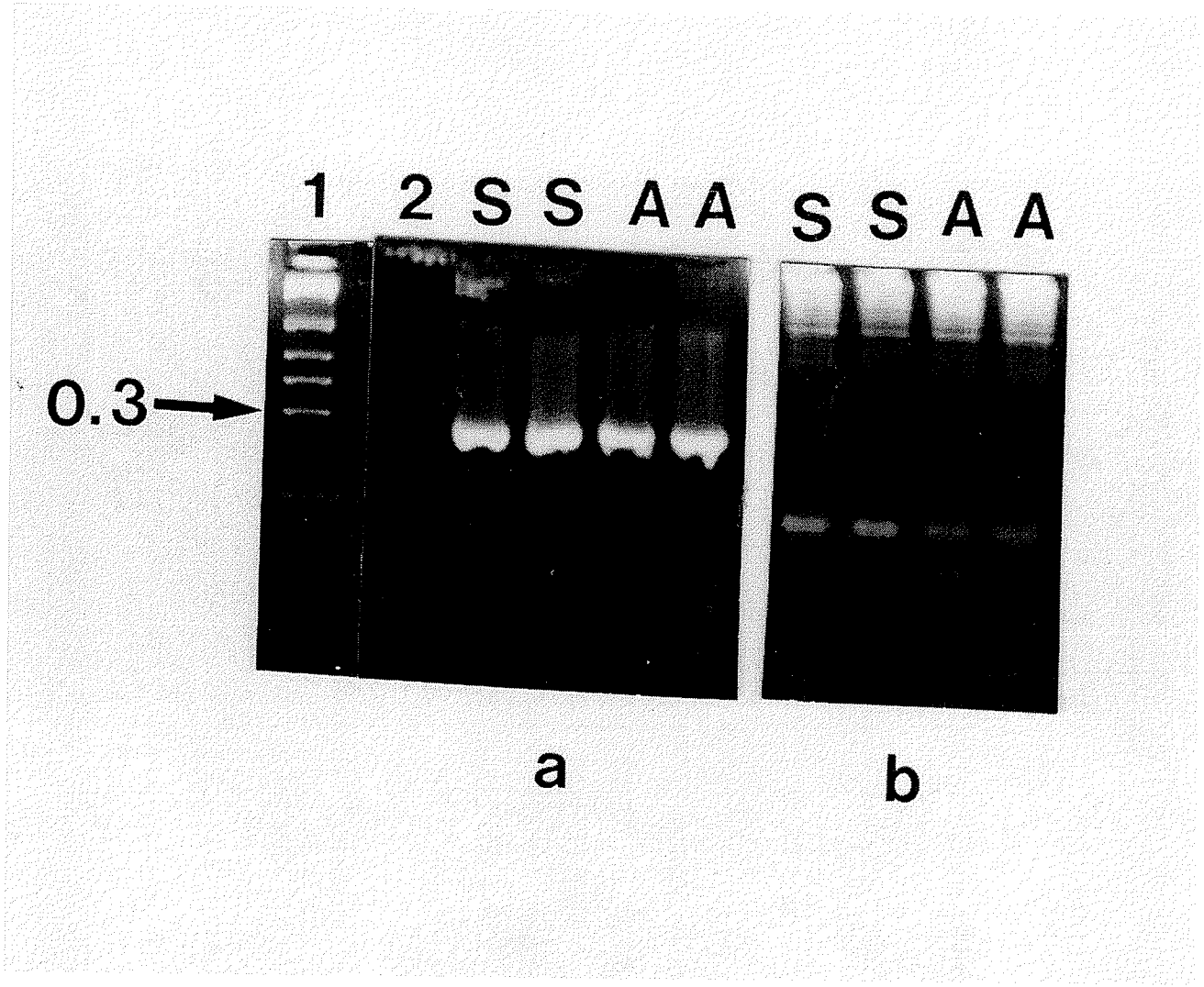
strains. After optimization of PCR conditions, the microsatellite marker assay was performed using the genomic DNA from A.SW and SJL mice. No polymorphism was apparent in either of the Intron-9, associated microsatellites on CD23 gene, between A.SW and SJL mice (Fig. 9).

B.IV DISCUSSION

In view of the proposed role of Tcrvb8 genes in upregulation of IgE responses in mouse (Renz et al 1992a,b, 1993), and because SJL mice is genetically deficient in Tcrvb8 genes (Behlke et al 1986), it was important to examine the contribution of Tcrvb8 gene towards IgE responsiveness in these mice. The results of the present study clearly demonstrated that A.SW strain of mouse which exhibits high IgE responses, was found to possess Tcrvb 8.1/8.2 genes in the germ line and also express the TCRVβ8 protein on T cells in the periphery. The lack of Vb8 genes in the germline and TCR Vβ8+ T cells in the periphery in SJL mice was also confirmed. Although the role of Tcrvb8 gene has been suggested in specific IgE responses to ALs, association with both SPIgE and TSIgE levels was examined in this study. Analysis of BC1 mice revealed that there was clearly no significant association of IgE responder phenotype with either the inheritance or the expression of Tcrvb8 gene. These results are in sharp contrast to the reports by Renz et al (1992a,b, 1993) implicating TCRVβ8+ T cells in the upregulation of IgE responses to OVA.

Fig. 9 Microsatellite markers from CD23 gene are not polymorphic between A.SW and SJL mice.

The (CA) and (GT) repeats from intron 9 of CD23 gene was amplified by PCR assay using appropriate primers. The amplified samples are loaded in duplicates. The position of 0.3 Kb is shown on the left side. The (CA) repeat is shown in the left panel (a); the (GT) repeat is shown in the right panel (b). A=A.SW; S=SJL; 1=1Kb ladder.


1
2
S
S
A
A
S
S
A
A
0.3
a
b

Although the reasons for this disparity are not clear at this time, there are a number of differences between two studies. Thus, Renz et al (1992a,b, 1993). studied the role of TCRVβ8+ T cells in Balb/c strain of mice, which were immunized with OVA by aerosol route. Therefore, the immunization protocol, the mouse strain used and the use of a single antigen in those studies are remarkably different from the present study.

However, the discordance between these two studies was not due to the antigen used, because there was no association between OVA-SPIgE and TCRVβ8 in this study (Table 17). Furthermore, the lack of association was not due to a bias in the selection of a subset of BC1 mice, because segregation of Tcrvb8 gene (and protein) in this BC1 group was consistent with the expected random 1:1 ratio.

A major difference between two studies is that we used A.SW strain of mice while Renz et al (1992a,b, 1993) used Balb/c mouse strain. It should be noted that Vβ8 T cells in A.SW encounter OVA peptides in context of H-2^s MHC class II molecules in contrast to Vβ8 T cells of Balb/c which interact with OVA peptides in association with H-2^d MHC class II molecules. Therefore, it is possible that the OVA-specific epitopes presented with H-2^s may be different from those of H-2^d class II molecules and consequently Balb/c and A.SW Vβ8T cells might recognize different epitopes of OVA. One

immunogenic epitope for Balb/c Vb8 T cells is the OVA peptide 323-339 (Buss et al 1987). However, the immunogenic epitopes for A.SW Vβ8 T cells on OVA are unknown. Furthermore, since the route of immunization is different between two studies, OVA-presenting APCs in the peritoneal lymphnodes in A.SW may be quite different from those of Balb/c APCs in the air-ways, and hence might initiate different kinds of immune responses in these strains. It is not known whether Vb8+ T cells differ functionally between inbred strains of mice, and there is no evidence that Vβ8+ T cells always stimulate specifically IgE production. Based on the current understanding of TCR-biology, it appears unlikely that the expression of the Vβ element goes beyond the antigen recognition unit and determines the functional capacity of T cells to modulate Ig isotype production.

In addition, although SJL mice lack Tcrvb8 gene, it can mount an IgE response, albeit a low and transient IgE response, when infected with Nb or on irradiation (Watanabe et al 1976, Watanabe and Ovary 1983). Thus, the presence of Tcrvb8 gene in A.SW and it's absence in SJL mice cannot explain by itself, the genetic basis of disparity in IgE responsiveness between these two strains of mice. In essence, these results prompted the examination of other two candidate genes, Il4 and Ifg, which play pivotal roles in IgE class switching and synthesis.

IL-4 and IFN-γ are known to reciprocally regulate IgE Ab synthesis in mice and humans (Finkelman et al 1990) and several pieces of evidence, as described before, implicate these two genes in the etiology of atopic diseases in general and dysregulated IgE synthesis in particular. Therefore, the role of Il4 and Ifg genes as potential genetic factors for IgE responsiveness was examined in this mouse colony. Results from microsatellite marker analysis in two independent sets of experiments, demonstrate that the genomic interval containing Il4 on chromosome 11 and Ifg on chromosome 10, is not linked to TSIgE levels in these mice. It should be stressed that these results, do not question the well established role of these two cytokines in IgE regulation. However, they do suggest that the low and high responder IgE mice in the BC1 progeny do not differ in this region of the genome and hence these genes do not constitute a predisposition factor for AL-induced persistence of high IgE levels in the serum.

It is to be stressed that whereas the role of IL-4 cytokine in IgE regulation is incontrovertible, the evidence in support of its role as a genetic predisposition factor for atopy is not so clear. Studies by Marsh et al. of Amish families using sib-pair analysis suggested linkage of the 5q31.1 markers, including IL4 with log(TSIgE) but not with log(IgE Ab) to common aero-ALs, suggesting genetic control of overall IgE production in a non-cognate fashion (Marsh et al 1994). Also

among atopics i.e., those who were IgE Ab-positive to common aero-ALs, no evidence of linkage of any of 5q31.1 markers with either log(TSIgE) or log(multiallergen IgE Ab) was found in this study. The findings in this murine model, whereby elevated IgE response can only be induced after immunization with ALs, are also not linked to the Il4 gene. Thus, the AL-induced, i.e., cognate IgE response, measured as TSIgE or SPIgE is not associated with the Il4 gene. Furthermore, the AL-SPIgE response in this model was highly correlated with the TSIgE levels ($r=0.72$, $p=0.0001$; and see Table 11), as opposed to a moderate correlation ($r=0.45$) seen in humans (Marsh et al 1994).

The relationship of IL-4 and IFN-γ may be different in terms of TSIgE levels in humans compared to the murine model. This may depend on a number of factors including, but not limited to, the choice of AL, the route of sensitization with the AL, and the use of adjuvant in the animal model compared to the human scenario where AL (at least that associated with asthma) is usually delivered by inhalation.

Results of the present study are in agreement with two other studies in human families. Thus, Blumenthal et al (1995) reported that there was no evidence of linkage of TSIgE levels with IL4 gene in families. Similarly, Cookson (1995) did not find evidence of linkage of either TSIgE or SPIgE to IL4 gene

in 230 Australian families.

To our knowledge there are no genetic studies undertaken in mice or other laboratory models to examine the role of IL4 and Ifg genes as genetic predisposing factors for IgE responsiveness. However, there are two reports investigating the role of Il4 gene-containing region of chromosome 11, in BHR in mouse. This issue is important in view of the recent findings that a gene on human 5q31-33 regulates both BHR and TSIgE levels (Postma et al 1995). Genetic mapping studies to identify the genes determining atracurium-induced BHR in recombinant inbred strains derived from C57BL6/J and DBA/2J, did not find evidence of linkage on chromosome 11 near the Il4 gene cluster with BHR (Levitt et al 1995). A second study on the genetic control of methacholine-induced BHR in mouse also did not find evidence of linkage with the chromosome 11 or chromosome 10 in a different combination of inbred mice strains (De Sanctis et al 1995). Thus results from the present study on IgE responsiveness and results from the above two studies on BHR together suggest that Il4 gene does not represent a genetic-predisposition factor for either IgE responsiveness or BHR in mouse.

Polymorphism analysis using a large number of microsatellite markers in the vicinity of CD23 gene on chromosome 8 did not reveal any polymorphisms between A.SW and SJL mice (Table 19).

Furthermore, microsatellite repeats found within the intron-9 of mouse CD23 gene revealed no apparent polymorphism on PCR analysis between A.SW and SJL strains (Fig. 9). These results together suggested that this genomic-interval on chromosome-8 including CD23, does not account for the differences in AL-induced TSIgE levels between A.SW and SJL mice. It should be noted that CD23 gene knock-out mice studies have yielded variable results on the genetic control of TSIgE levels by CD23 (Yu et al 1994, Steif et al 1994, Fujiwara et al 1994) .

C. MOLECULAR-GENETIC MAPPING OF LOCI CONTROLLING ALLERGEN-INDUCED TOTAL SERUM IgE LEVELS IN MICE

C.1 INTRODUCTION

As alluded to earlier, recently significant progress has been made in the analysis of genetic factors involved in controlling IgE responsiveness, using the candidate gene/positional candidate analysis. However, the results to date are not definitive and/or often controversial. It has been suggested that the discrepancies in human studies could be due to a number of confounding factors which have been discussed in previous chapters. One of the major problems is the influence of HLA encoded Ir gene products (class II molecules), which contribute significantly to TSIgE levels in humans (Marsh and Meyers 1992, Cookson 1995). Thus, despite some recent progress, a systematic genetic analysis, for example a genome-wide search for genes predisposing to allergies and asthma in general, and genes controlling TSIgE levels in particular has not been reported so far in humans.

In contrast to human studies, the mouse model provides an excellent opportunity to identify the genetic factors controlling AL-induced TSIgE levels. Furthermore, identification of non-MHC gene(s) controlling persistence of TSIgE levels, may be best demonstrated in combinations of selected crosses, excluding MHC as a variable. Therefore, as described in previous chapters, a large mouse colony consisting of F1, BC1 and F2 progeny, using two MHC-identical strains of mice- A.SW/Snj and SJL/J, was established for this

purpose. It was deduced from the segregation and distribution studies that elevated and persistent AL-induced TSIgE level in this colony is under the control of a major non-MHC autosomal locus, with the possibility of existence of an additional modifier locus or loci. Subsequently, using a number of approaches, the role of Tcrvb8, Il4 and Ifg genes were excluded as candidate genes regulating TSIgE levels in this colony. The results from these studies paved the way for molecular-genetic mapping of the loci controlling AL-induced TSIgE levels in mouse.

A genome sweep of A.SW and SJL mice was performed using state-of-the-art mapping technology based on microsatellite markers also called simple sequence length polymorphisms (SSLP) (Love et al 1990, Weber and May 1989, Cornall et al 1991). The large number of microsatellite markers spaced throughout the genome and the relatively high rate of polymorphism (averaged at 50%) between different laboratory strains make such a mapping approach feasible (Dietrich et al 1994, Lander and Schork 1994, Dietrich et al 1996). A similar approach has been successful in mapping a number of disease susceptibility loci in mouse models of human diseases (Todd et al 1991, De Sanctis 1995). The strategy for molecular-genetic mapping of the loci regulating AL-induced TSIgE levels employed in this study is schematically shown in Fig. 10. The details on the genetic mapping are described in next section.

Fig. 10

STRATEGY FOR MOLECULAR-GENETIC MAPPING OF THE LOCI CONTROLLING AL-INDUCED TSIgE LEVELS IN MOUSE

Step 1:

Genome screening to identify Polymorphic Microsatellite markers between A.SW and SJL mouse strains

Step 2:

Selection of Polymorphic Markers from Autosomes

Step 3:

Genetic Analysis of BC1 mice with Extreme TSIgE Phenotype

Association of genomic intervals

Step 4:

Fine-Mapping of the genomic intervals

Step 5:

Confirmation of Association in the Entire BC1 Progeny

C.2.0 RESULTS

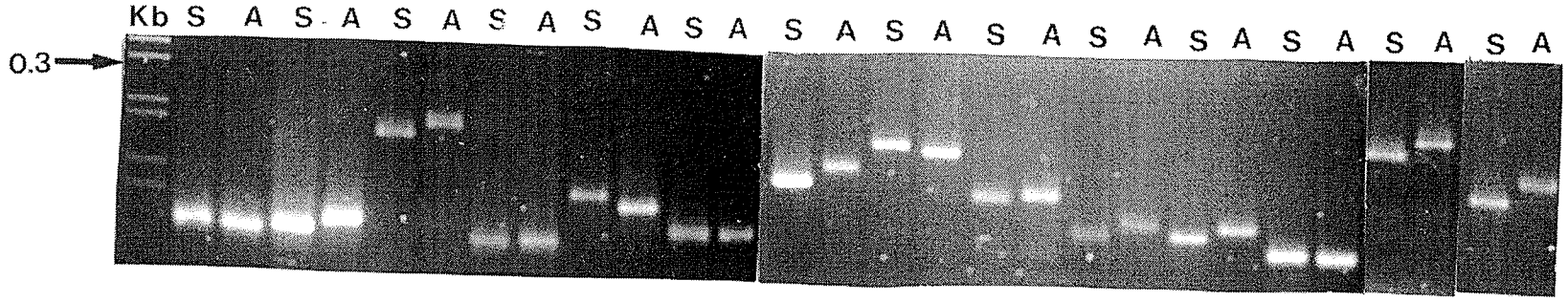
C.2.1 Genome screening to identify the microsatellite markers that are polymorphic between A.SW/Snj and SJL/J inbred strains of mice.

The first step in the molecular-genetic mapping is the identification of polymorphic DNA markers that differ between the inbred parent strains used as founders for establishment of the mouse colony. Identification of such markers is essential, because they serve as informative 'sign post's for construction of molecular-genetic linkage map. The efficiency of such a map increases with the availability of markers from across the mouse genome. Therefore, to obtain a large number of informative markers, the genome of A.SW and SJL mice (excluding sex chromosomes) was screened using a large number of microsatllite markers (a total of 416) originating from chromosome 1 to 19. Representative examples of marker polymorphism analysis are shown in Fig. 11. Microsatellite markers for a given chromosome were selected such that they cover the entire chromosome uniformly. On an average ~22 markers per chromosome were screened in this study. These markers are spaced, evenly on an average at every 3.6 cM in the genome.

The screening of genomic DNA from A.SW and SJL/J mice was performed using the microsatellite marker assay, which was

Fig. 11 Representative microsatellite markers from the autosomes of A.SW and SJL mice.

The various microsatellite DNA markers were PCR amplified using the Mouse MapPairs. The figure shows polymorphic, as well as, non-polymorphic markers between A.SW (A) and SJL (S) strains. The position of 0.3 Kb marker is indicated on the left side.


Kb S A S A S A S A S A S A S A S A S A S A S A S A S A S A S A S A
0.3

established during this study. The PCR conditions were optimized and the results of the optimal Mg^{++} and annealing

temperature for each of the primers used is listed in the table 19.

Thus, a total of 416 microsatellite markers derived from autosomes 1 to 19, were screened for polymorphisms between the A.SW and SJL strains. The genome screening led to the identification of 86 polymorphic microsatellite markers. These polymorphisms were represented in all autosomes. However, the rate of polymorphism was not uniform across the genome (Table 20). Thus the rate of polymorphism varied from 7.7% (chromosome 6) to 57.1% (chromosome 12). The genome-wide rate of microsatellite DNA polymorphism between A.SW and SJL mouse strains was 20.8%. The chromosomes 2, 7, 11, 12, 13 and 14 represented a rate of polymorphisms of more than 30%. In contrast, the rate of polymorphism in rest of the genome varied from 7.7% to 27.3%. The chromosome-wise distribution of the rate of microsatellite polymorphism is listed in the table 20.

Table 19:

Results of Genome Screening for Microsatellite Polymorphisms between A.SW/Snj and SJL/J Strains of Inbred Mice.

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} conc (mM)
Chromosome 1:					
D1Mit59		7	NO	55	1.5
D1Mit120		12	NO	55	1.5
D1Mit70		16	NO	55	1.5
D1Mt71	Il1r1	18	NO	55	1.5
D1Mit228		22	NO	55	1.5
D1Mit213		26	NO	55	1.5
D1Mit74	Cd28,Ctla4	31	NO	55	1.5
D1Mit282	Il8rb	38	YES	54	1.5
D1Mit77		38	NO	55	1.5
D1Mit44	Bcl2	51	YES	55	1.5
D1Mit218	Il10	72	YES	55	1.5
D1Mit34	Fas1,Cd3z	82	NO	55	1.5
D1Mit35		86	NO	55	1.5
D1Mit36		90	NO	55	1.5
D1Mit207	Fcer1a,g	98	NO	55	1.5
D1Mit362	Cr2	110	NO	55	1.5
Chromosome 2:					
D2Mit60	Il2r a	6	NO	55	1.5
D2Mit80		12	YES	53	1.5
D2Mit120		23	NO	55	1.5
D2Mit238		29	YES	54	1.5
D2Mit37		47	YES	55	1.5
D2Mit158		47	NO	55	1.5
D2Mit17	Rag1,2	59	YES	55	1.5
D2Nds3		63	YES	55	1.5
D2Mit135		63	NO	55	1.5
D2Mit402		68	NO	55	1.5
D2Mit194		69	NO	55	1.5
D2Mit47	Adra2b	71	NO	55	1.5
D2Mit59	Il1a,b	74	NO	55	1.5
D2Mit195		74	NO	55	1.5
D2Mit409		77	NO	51	1.5
D2Mit225		77	NO	55	1.5
D2Mit143		79	NO	55	1.5

Table 19:(continued)

Marker[1]	Linked gene[2]	Locus[3]	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D2Mit196		79	NO	55	1.5
D2Mit226		81	YES	55	1.5
D2Mit227		83	NO	55	1.5
D2Mit52	Cd40	86	YES	55	1.5
Chromosome 3:					
D3Mit1	Il7	10	NO	55	1.5
D3Mit46		12	YES	53	1.5
D3Mit21		15	NO	55	1.5
D3Mit6	Il2	18	NO	55	1.5
D3Mit51		27	NO	51	1.5
D3Mit9		29	NO	51	1.5
D3Mit40		30	NO	51	1.5
D3Mit29		35	NO	55	1.5
D3Mit28		35	NO	55	1.5
D3Mit10		38	YES	51	1.5
D3Mit142		39	NO	51	1.5
D3Mit103		39	NO	51	1.5
D3Mit42		39	NO	55	1.5
D3Mit104		40	NO	55	1.5
D3Mit251		43	NO	55	1.5
D3Mit288		43	NO	55	1.5
D3Mit16	Il6r	47	NO	55	1.5
D3Mit146		49	NO	51	1.5
D3Mit196		49	NO	51	1.5
D3Mit38		50	YES	51	1.5
D3Mit18	Vcam1	56	NO	55	1.5
D3Mit86		56	NO	55	1.5
D3Mit45		59	NO	55	1.5
D3Mit44		59	NO	55	1.5
D3Mit87		65	NO	51	1.5
D3Mit58		66	NO	58	1.5
Chromosome 4:					
D4Nds3		NA	NO	55	1.5
D4Mit1		7	NO	55	1.5
D4Mit196		15	YES	53	1.5
D4Mit112		20	NO	55	1.5
D4Mit271		20	NO	55	1.5
D4Mit17		26	NO	55	1.5
D4Mit9		34	YES	53	1.5
D4Mit205		35	NO	55	1.5
D4Mit58	Ifa,Ifb	41	NO	55	1.5

Table 19:(continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D4Mit175		42	NO	55	1.5
D4Nds2		50	NO	55	1.5
D4Mit12		50	NO	55	1.5
D4Mit157	Csf,g	57	NO	55	1.5
D4Mit48		64	YES	53	1.5
D4Mit33		68	YES	55	1.5
D4Mit14		68	NO	55	1.5
D4Mit190	Tnfr2	69	NO	55	1.5
D4Mit62		69	NO	55	1.5
Chromosome 5:					
D5Mit248		0	NO	55	1.5
D5Mit180		6	NO	55	1.5
D5Mit294		6	NO	55	1.5
D5Mit44		13	NO	55	1.5
D5Mit148		15	NO	55	1.5
D5Mit31	Il6	16	YES	55	1.5
D5Mit55		19	NO	55	1.5
D5Mit256		27	NO	55	1.5
D5Nds2		28	YES	53	1.5
D5Mit7		34	NO	51	1.5
D5Mit91		37	NO	55	1.5
D5Mit10		38	NO	55	1.5
D5Mit24		47	NO	55	1.5
D5Mit158		50	NO	55	1.5
D5Mit65		60	NO	55	1.5
D5Mit99		75	NO	51	1.5
D5Mit51		77	NO	51	1.5
D5Mit62		78	NO	51	1.5
D5Mit50		79	NO	55	1.5
D5Mit329		79	NO	55	1.5
D5Mit43		80	NO	55	1.5
D5Mit284			NO	51	1.5
Chromosome 6:					
D6Mit1		3	NO	55	1.5
D6Mit50		3	NO	55	1.5
D6Mit116		6	NO	51	1.5
D6Mit46		7	NO	51	1.5
D6Mit77		8	NO	55	1.5
D6Mit117		9	NO	51	1.5
D6Mit76		10	NO	51	1.5
D6Mit33		12	NO	51	1.5
D6Mit241		17	NO	55	1.5

Table 19:(continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D6Mit73		17	NO	55	1.5
D6Mit95	Tcrb	19	NO	51	1.5
D6Mit16		21	NO	55	1.5
D6Mit126		22	NO	51	1.5
D6Mit3		23	NO	55	1.5
D6Mit6		26	NO	55	1.5
D6Mit8		26	NO	51	1.5
D6Mit9		28	NO	51	1.5
6Mit213		30	NO	55	1.5
D6Mit284	Igk,v,c	31	YES	51	1.5
D6Mit31		32	NO	49	1.5
D6Nds5		40	NO	55	1.5
D6Mit10		41	NO	55	1.5
D6Mit24	Il15ra	46	NO	55	1.5
D6Mit109		47	NO	55	1.5
D6Mit294	Tnfr1	61	YES	55	1.5
D6Mit14	Cd4	61	NO	55	1.5
Chromosome 7:					
D7Mit76	Pkcg	6	YES	49	1.5
D7Mit57		7	NO	55	1.5
D7Mit294		8	NO	55	1.5
D7Mit81		20	NO	55	1.5
D7Mit90		29	NO	55	1.5
D7Mit40		44	YES	55	1.5
D7Mit132		44	NO	55	1.5
D7Mit291	Il4r	64	YES	55	1.5
Chromosome 8:					
D8Mit58		0	NO	55	0.75
D8Mit155		1	NO	55	1.5
D8Mit59		2	NO	55	1.5
D8Mit217		5	NO	55	0.75
D8Mit218		5	NO	55	1.5
D8Mit254		5	NO	55	0.75
D8Mit141		5	NO	55	1.5
D8Mit253		5	NO	51	1.5
D8Mit286		5	NO	55	1.5
D8Mit95		6	NO	55	0.75
D8Mit61		6	NO	55	0.75
D8Mit3		7	NO	55	1.5
D8Mit190		7	NO	55	1.5
D8Mit20		8	NO	49	1.5
D8Mit289	Fcer2a	9	NO	55	1.5

Table 19: (continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D8Mit94		11	NO	55	1.5
D8Mit4		13	NO	55	1.5
D8Mit63		14	NO	55	0.75
D8Mit291		15	NO	51	1.5
D8Mit64		16	NO	55	0.75
D8Mit224		17	NO	55	1.5
D8Mit24		19	NO	55	1.5
D8Mit292		20	YES	55	1.5
D8Mit293		23	NO	55	1.5
D8Mit178	Il15	36	YES	53	1.5
D8Mit179		36	YES	53	1.5
D8Mit194		36	NO	55	1.5
D8Mit81		44	NO	55	1.5
D8Mit15		45	NO	55	1.5
D8Mit182		46	NO	55	1.5
D8Mit33		46	NO	55	1.5
D8Mit84		46	NO	55	1.5
D8Mit11		46	NO	55	1.5
D8Mit242		48	NO	55	1.5
D8Mit85		48	YES	55	0.75
D8Mit110		53	NO	55	1.5
D8Mit198		54	NO	55	1.5
D8Mit12		55	NO	55	1.5
D8Mit12		55	NO	55	1.5
D8Mit213		56	NO	55	1.5
D8Mit87		57	NO	55	1.5
D8Mit271		58	YES	55	1.5
D8Mit116		60	NO	55	1.5
D8Mit272		61	NO	55	1.5
D8Mit35		61	NO	55	1.5
D8Mit119		62	NO	55	1.5
D8Mit120		63	NO	55	1.5
D8Mit168		63	NO	55	1.5
D8Mit188		63	NO	55	1.5
D8Mit274		63	NO	55	1.5
D8Mit49		63	YES	53	1.5
D8Mit168		63	NO	55	1.5
D8Mit55		64	NO	55	1.5
D8Mit55		64	NO	55	1.5
D8MiT201		66	NO	55	1.5
D8Mit36		69	YES	53	1.5
D8Mit140		71	NO	55	1.5
D8Mit42		73	YES	55	1.5

Table 19: (continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D8Mit92		73	NO	55	1.5
D8Mit280		74	NO	55	1.5
D8Mit93		75	NO	55	1.5
Chromosome 9:					
D9Mit59	Icam1	0	YES	49	1.5
D9Mit286		18	NO	55	1.5
D9Mit193	Il10r	25	NO	55	1.5
D9Mit162	Cd3d,e,g	26	NO	55	1.5
D9Mit27	Thy1	25	NO	55	1.5
D9Mit8		42	NO	55	1.5
D9Mit156		42	NO	55	1.5
D9Mit12		52	YES	51	1.5
D9Mit182		53	NO	55	1.5
D9Mit279		62	NO	55	1.5
D9Mit99			NO	50	1.5
Chromosome 10:					
D10Mit49		0	NO	55	1.5
D10Mit75		0	NO	55	1.5
D10Mit28		2	NO	55	0.75
D10Mit246		3	NO	55	0.75
D10Mit83		4	NO	55	1.5
D10Mit169		7	NO	49	1.5
D10Mit16		10	NO	51	1.5
D10Mit86	Ifgr1	12	NO	51	1.5
D10Mit3		14	NO	55	1.5
D10Mit55		17	NO	55	1.5
D10Mit109		18	NO	51	1.5
D10Mit40		19	NO	51	1.5
D10Mit15		23	NO	55	1.5
D10Mit20		23	NO	55	1.5
D10Mit264		47	NO	55	1.5
D10Mit265		49	NO	55	1.5
D10Mit43		51	NO	55	1.5
D10Mit96		52	NO	55	1.5
D10Mit144		53	NO	55	1.5
D10Mit98		54	NO	55	1.5
D10Mit99		57	NO	55	1.5
D10Mit178		54	NO	55	1.5
D10Mit162		55	YES	51	1.5
D10Mit70		55	NO	55	1.5
D10Mit150		56	YES	49	1.5
D10Mit122		58	NO	55	1.5
D10Mit266		60	YES	55	1.5
D10Mit71		60	NO	49	1.5

Table 19: (continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D10Mit135		60	NO	55	1.5
D10Mit71		60	NO	55	1.5
D10Mit33		61	NO	55	1.5
D10Mit136		61	NO	55	1.5
D10Mit100		62	NO	55	1.5
D10Mit101		63	NO	55	1.5
D10Mit74	Ifg	67	YES	55	1.5
D10Mit14		67	YES	55	1.5
Chromosome 11:					
D11Mit1		2	NO	55	1.5
D11Mit79		8	NO	55	1.5
D11Mit173		14	NO	55	1.5
D11Mit53		13	NO	55	1.5
D11Mit83		13	NO	55	1.5
D11Mit270		20	NO	55	1.5
D11Nds9	Il4	26	YES	54	1.5
D11Mit24		26	YES	55	1.5
D11Mit64		26	NO	55	1.5
D11Mit315	Il3,Csf,gm	28	NO	55	1.5
D11Mit164	Il5,Irf1	28	NO	55	1.5
D11Mit26		32	YES	55	1.5
D11Mit90	Asg,r1	39	NO	55	1.5
D11Mit220		45	NO	49	1.5
D11Mit195		45	NO	49	1.5
D11Mit35		45	YES	55	1.5
D11Mit38		46	NO	49	1.5
D11Mit39		46	NO	49	1.5
D11Mit41		49	YES	49	1.5
D11Mit212		49	NO	55	1.5
D11Mit284		50	NO	49	1.5
D11Mit179		50	NO	49	1.5
D11Mit285		50	NO	49	1.5
D11Mit122		51	YES	55	1.5
D11Mit70		54	YES	53	1.5
D11Mit288		55	YES	55	1.5
D11Mit213		55	NO	55	1.5
D11Mit289		58	YES	55	1.5
D11Mit263		59	YES	55	1.5
D11Mit54		60	NO	49	1.5
D11Mit67	Csf,g	61	YES	49	1.5
D11Mit328		61	YES	53	1.5
D11Mit98		61	YES	55	1.5
D11Mit132	Icam2	64	YES	55	1.5
D11Mit258	Pkca	67	NO	55	1.5

Table 19 : (continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D11Mit224		72	YES	55	1.5
D11Mit100		73	NO	55	1.5
D11Mit167		77	YES	55	1.5
D11Mit203		81	NO	55	1.5
Chromosome 12:					
D12Mit168		4	YES	53	1.5
D12Mit124		12	YES	53	1.5
D12Mit54		20	YES	55	1.5
D12Mit52		29	NO	55	1.5
D12Mit203	Hsp,a1b	34	NO	55	1.5
D12Mit78		44	YES	53	1.5
D12Mit8	IgH,C,e	60	NO	55	1.5
Chromosome 13:					
D13Mit1	Tcrg	7	NO	53	1.5
D13Mit59		13	YES	55	1.5
D13Mit91		22	NO	55	1.5
D13Mit156		25	NO	55	1.5
D13Mit222		26	NO	55	1.5
D13Mit20		28	NO	55	1.5
D13Mit21		28	NO	55	1.5
D13Mit23		28	YES	55	1.5
D13Mit54		28	YES	55	1.5
D13Mit96		28	NO	55	1.5
D13Mit113		28	YES	55	1.5
D13Mit184		28	YES	55	1.5
D13Mit185		28	NO	55	1.5
D13Mit13	Il9	28	YES	55	1.5
D13Mit65		29	YES	55	1.5
D13Mit39		30	YES	53	1.5
D13Mit280		30	NO	55	1.5
D13Mit159		39	YES	53	1.5
D13Mit149		46	NO	55	1.5
D13Mit170		46	NO	55	1.5
D13Mit45		46	NO	55	1.5
D13Mit76		48	NO	55	1.5
D13Mit292		49	NO	55	1.5
D13Mit32		56	NO	55	1.5
D13Mit122			NO	55	1.5

Table 19: (continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
Chromosome 14:					
D14Mit206	Il3ra	3	YES	53	1.5
D14Mit44	Pkc,d	11	NO	55	1.5
D14Mit61		21	YES	53	1.5
D14Mit21		24	NO	55	1.5
D14Mit183		24	NO	55	1.5
D14Mit28		33	NO	55	1.5
D14Mit34		43	YES	55	1.5
D14Mit42		59	NO	55	1.5
Chromosome 15:					
D15Mit13		0	NO	55	1.5
D15Mit179	Il7r,Pger2	8	NO	55	1.5
D15Mit6		17	NO	55	1.5
D15Mit26		22	NO	55	1.5
D15Mit209		27	NO	55	1.5
D15Mit71		27	NO	55	1.5
D15Mit122		29	YES	55	1.5
D15Mit29		34	NO	55	1.5
D15Mit1	Il2rb	36	NO	55	1.5
D15Mit33		39	YES	53	1.5
D15Mit171		50	NO	55	1.5
D15Mit242		51	NO	55	1.5
D15Mit41		55	NO	55	1.5
D15Mit14		59	NO	55	1.5
D15Mit16		61	YES	55	1.5
Chromosome 16:					
D16Mit9	Ifgr2	4	YES	53	1.5
D16Mit145	Ig lamba	16	NO	55	1.5
D16Mit2		19	NO	55	1.5
D16Mit156		22	NO	55	1.5
D16Mit103		24	NO	55	1.5
D16Mit36		24	NO	55	1.5
D16Mit3		25	NO	55	1.5
D16Mit58		26	NO	55	1.5
D16Mit4		27	NO	55	1.5
D16Mit5		35	YES	55	1.5
D16Mit19		42	NO	55	1.5
D16Mit158		42	NO	55	1.5
D16Mit7		48	NO	55	1.5
D16Mit6		48	NO	55	1.5

Table 19: (continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
D16Mit94		48	NO	55	1.5
D16Mit106	Ifabr	51	NO	55	1.5
Chromosome 17:					
D17Mit19		0	NO	55	1.5
D17Mit18		1	NO	55	1.5
D17Mit113		2	NO	55	1.5
D17Mit46		3	NO	55	1.5
D17Mit80		4	NO	55	1.5
D17Mit44		5	NO	55	1.5
D17Nds3		10	NO	55	1.5
D17Mit34		10	NO	55	1.5
D17Mit66		20	YES	55	1.5
D17Mit20		31	NO	55	1.5
D17Mit39		42	YES	55	1.5
D17Mit130		51	NO	53	1.5
Chromosome 18:					
D18Mit19		1	NO	55	1.5
D18Mit92		4	NO	55	1.5
D18Mit20		4	NO	55	1.5
D18Mit17		15	YES	55	1.5
D18Mit37		15	YES	54	1.5
D18Mit24		17	NO	55	1.5
D18Mit122		21	YES	53	1.5
D18Mit124		22	YES	55	1.5
D18Mit205		22	YES	53	1.5
D18Mit152		26	NO	55	1.5
D18Mit50		27	NO	55	1.5
D18Mit81		27	YES	53	1.5
D18Mit103		29	NO	55	1.5
D18Mit142		32	NO	55	1.5
D18Mit8	Adrb2	32	NO	55	1.5
D18Mit79		32	NO	55	1.5
D18Mit153		32	NO	55	1.5
D18Mit162		36	NO	55	1.5
D18Mit3		38	NO	55	1.5
D18Mit44	InV	38	NO	55	1.5
D18Mit4		38	NO	55	1.5
D18Mit16		40	NO	55	1.5

Table 19: (continued)

Marker[1]	Linked gene[2]	Locus[3] (cM)	Polymorphism[4]	AT[5] (°C)	Mg^{2+} (mM)
Chromosome 19					
D19Mit77		4	NO	55	1.5
D19Mit56		5	NO	55	1.5
D19Mit44		6	NO	55	1.5
D19Mit68	Cd20, Adrbk1	7	YES	53	1.5
D19Mit16		17	NO	55	1.5
D19Mit73	Fas ag	22	NO	55	1.5
D19Mit97		22	NO	55	1.5
D19Mit106		22	NO	55	1.5
D19Mit13		26	NO	55	1.5
D19Mit17	TdT	36	NO	55	1.5
D19Mit4		37	NO	55	1.5
D19Mit3		40	NO	55	1.5
D19Mit54		42	NO	55	1.5
D19Mit1		46	NO	55	1.5
D19Mit34		48	YES	53	1.5
D19Mit104		48	YES	55	1.5
D19Mit33		49	YES	53	1.5
D19Mit105		50	NO	55	1.5
D19Mit92		60	NO	55	1.5
D19Mit76		60	NO	55	1.5
D19Mit194		NA	NO	55	1.5
D19Mit291		NA	NO	55	1.5
D19Mit260		NA	NO	55	1.5

Legend to Table 19:

1. Microsatellite marker is designated by the chromosome number followed by the marker number on that chromosome

2. Known gene of importance in immunobiology, in linkage with the marker is indicated by the gene symbol for mouse

3. The distance of a marker from the centromere is indicated in centiMorgans (cM); this information is based on mouse genome database, MIT directory, 1995.

4. Marker that is polymorphic between A.SW and SJL mouse strains.

5. AT, annealing temperature of the primer used in PCR.

Table 20:

Rate of DNA polymorphism for microsatellite markers across the genome between A.SW/Snj and SJL/J inbred mice strains.

Chromosome	No. of markers screened	No. of polymorphic markers	Rate of polymorphism (%)
1	16	3	18.7
2	21	7	33.3
3	27	3	11.1
4	18	4	22.2
5	22	2	9.1
6	26	2	7.71
7	8	3	7.5
8	63	6	9.5
9	11	2	8.2
10	36	5	3.9
11	39	16	40.0
12	7	4	57.1
13	25	9	36.0
14	8	3	37.5
15	15	3	20.0
16	16	2	12.5
17	12	2	16.7
18	22	6	27.3
19	24	4	16.7
Total:	**416**	**86**	**20.7%**

C.2.2. Microsatellite marker-based exclusion mapping of the loci controlling AL-induced persistent TSIgE levels.

The set of 69 polymorphic microsatellite markers were selected from autosomes 1 to 19, and used for molecular-genetic mapping. The genomic DNA from BC1 mice was used in these analysis, because in BC about 50% of the progeny resemble each parents thereby providing an uniform number of individuals for genetic analysis. A two-stage strategy was used for mapping the loci controlling TSIgE levels (Fig. 10). This consisted of: i) an initial genotyping of a subset of BC1 mice (n=57) exhibiting extreme phenotypes to establish a putative association of chromosomal regions with TSIgE levels; and ii) subsequent confirmation of association in the entire BC1 progeny (n=136).

C.2.2.1 Genetic analysis of BC1 mice with extreme phenotypes:

In order to quickly establish association with the markers, initial genotyping was done in extreme phenotype individuals. A subgroup of 57 BC1 mice exhibiting extreme TSIgE phenotype were selected for genetic analysis. This group of BC1 mice consisted of thirty animals in the low TSIgE group, which had no detectable TSIgE levels and 27 animals in the high TSIgE group, that had extremely high TSIgE levels (≥18,000 ng/ml,on

day 31, secondary response). Each individual BC1 mouse was genotyped using the previously established microsatellite assay.

The parental A.SW and SJL were laboratory inbred, and hence are homozygous at every locus. These genotypes were designated as AA and SS, respectively. The F1 animals are heterozygous (AS), and the BC1 mice are either homozygous (SS) or heterozygous (AS) at any given genetic locus. The observed frequency of alleles in the low and high TSIgE groups was compared to the random expectations, by Chi-square analysis. A $\chi 2$ value of >3.8 (with P value of <0.05) is normally considered as evidence for significant association. A $\chi 2$ value of >10.8 (with P value of <0.001) is considered as evidence for significant linkage (Ghosh et al 1993). A significant association between the genotype at a microsatellite marker locus and TSIgE phenotype indicates that the microsatellite marker is linked to a locus involved in controlling the TSIgE phenotype.

Table 21, shows the summary of the genotyping results obtained with 55 markers that span the entire mouse genome. Only those markers that exhibited evidence of significant association are indicated with the χ^2 and P values. In comparison with an expected random distribution of A.SW and SJL alleles, three genomic intervals one each on chromosomes 8, 10 and 11

Table 21 .

A linkage map of the mouse genome and association of markers with TSIgE levels in extreme phenotype BC1 mice.

Marker	Chromosome Locus[2] (cM)	Gene[3]	TSIgE level[1] Low SS : AS	High SS : AS	$(\chi^2)^4$	P
			n n	n n		
D1Mit282	1(38)	Il8r	18 : 12	12 : 15		
D1Mit218	1(72)	Il10	13 : 17	15 : 12		
D1Mit207	1(98)	Fcer1a,g	13 : 17	17 : 10		
D2Mit80	2(12)		15 : 15	14 : 13		
D2Mit238	2(29)		14 : 15	12 : 15		
D2Mit17	2(59)	Rag1,2	13 : 16	11 : 16		
D2Mit135	2(63)		13 : 14	10 : 17		
D2Mit226	2(81)		12 : 17	8 : 19		
D2Mit52	2(86)	CD40	16 : 14	10 : 17		
D3Mit46	3(12)		15 : 15	16 : 11		
D3Mit10	3(38)		9 : 18	13 : 14		
D3Mit38	3(50)		12 : 18	15 : 12		
D4Mit196	4(15)		12 : 10	12 : 13		
D4Mit9	4(34)		13 : 16	12 : 15		
D4Mit48	4(64)		12 : 18	16 : 11		

Table 21:(continued)

Marker	Chromosome Locus[2] (cM)	Gene[3]	TSIgE level[1] Low SS : AS	High SS : AS	$(\chi^2)^4$	P
			n n	n n		
D5Mit31	5(16)	Il6	16 : 14	14 : 13		
D5Mit2	5(28)		13 : 13	13 : 14		
D6Mit284	6(31)	Igk	15 : 15	11 : 15		
D6Mit294	6(61)	Tnfr1	14 : 16	12 : 15		
D7Mit76	7(6)	Pkc,g	10 : 20	12 : 15		
D7Mit40	7(44)		10 : 20	8 : 19		
D7Mit291	7(64)		9 : 20	12 : 15		
D8Mit292	8(20)		16 : 14	16 : 11		
D8Mit179	8(36)	Il15	17 : 12	12 : 15		
D8Mit49	8(63)		20 : 9	11 : 16	**4.51**	**0.034**
D9Mit59	9(0)	Icam1	14 : 16	11 : 15		
D9Mit12	9(52)		16 : 13	14 : 13		
D10Mit162	10(55)		12 : 18	13 : 14		
D10Mit266	10(60)		13 : 17	19 : 8	**4.22**	**0.040**
D10Mit74	10(67)	Ifg	13 : 17	17 : 10		

Table 21: (continued)

Marker	Chromosome Locus[2] (cM)	Gene[3]	TSIgE level[1] Low SS : AS	High SS : AS	$(\chi^2)^4$	p
			n n	n n		
D11Nds9	11(26)	Il4	15 : 15	14 : 13		
D11Mit288	11(55)		18 : 12	6 : 21	**8.32**	**0.0068**
D11Mit289	11(58)		18 : 12	6 : 21	**8.32**	**0.0068**
D11Mit167	11(77)		18 : 12	13 : 14		
D12Mit124	12(12)		12 : 17	12 : 15		
D12Mit52	12(29)		11 : 19	13 : 14		
D12Mit78	12(44)		14 : 15	15 : 12		
D13Mit59	13(13)		14 : 15	14 : 13		
D13Mit13	13(28)	Il9	15 : 15	12 : 15		
D13Mit159	13(39)		13 : 16	10 : 18		
D14Mit206	14(3)	Il3ra	18 : 12	15 : 12		
D14Mit61	14(21)	Tcra,d	20 : 9	15 : 12		
D14Mit34	14(43)		19 : 10	14 : 13		
D15Mit122	15(29)		13 : 16	13 : 14		
D15Mit33	15(39)		16 : 13	13 : 14		
D15Mit16	15(61)		12 : 17	13 : 14		

Table 21: (continued)

Marker	Chromosome Locus[2] (cM)	Gene[3]	TSIgE level[1] Low SS : AS	High SS : AS	(χ^2)[4]	p
			n n	n n		
D16Mit9	16(4)	Ifgr2	17 : 12	14 : 13		
D16Mit5	16(35)		18 : 12	12 : 15		
D17Mit66	17(20)		14 : 16	8 : 19		
D17Mit39	17(42)		14 : 15	8 : 19		
D18Mit37	18(15)		15 : 15	14 : 13		
D18Mit122	18(21)		14 : 15	16 : 11		
D18Mit81	18(27)		17 : 12	17 : 10		
D19Mit68	19(7)	CD20	16 : 14	11 : 16		
D19Mit33	19(49)		15 : 15	10 : 17		

Legend to Table 21:

1. TSIgE level was measured by ELISA on day 31, secondary response. BC1 mice exhibiting no detectable TSIgE levels were included in the low group (n=30), and BC1 mice having ≥ 18,000 ng/ml TSIgE were included in the high group (n=27). For occasional markers, certain individual genotypes were not discernible. For such markers less than the total group are presented.

2. Positions of the markers are designated by chromosome and centimorgans (cM) distal to the centromere. The location of the marker loci were based on the Encyclopedia of the Mouse Genome, genetic map v3.0a.

3. Known genes linked to a marker are indicated with the gene symbol for mouse; this information is from MIT mouse genome data base, 1995 directory.

4. The difference in the frequency of SS and AS alleles compared to random expectations in low TSIgE and high TSIgE groups was estimated by χ^2 analysis using 2 X 2 contingency tables. The markers showing significant associations are only indicated with the χ^2 and P values.

5. Abbreviations: SS, homozygous; AS, heterozygous.

exhibited evidence of significant association. One marker each from chromosome-8 (D8Mit49) and chromosome-10 (D10Mit266), exhibited evidence of association (χ^2=4.51, P<0.034, χ^2=4.22, P<0.040). The chromosome 11 markers that exhibited significant association were, D11Mit288 and D11Mit289 (X^2=8.32, P<0.0068 at both loci). All other markers examined did not indicate evidence of association.

C.2.2.2 Fine-mapping of the putative loci on chromosomes 8, 10 and 11.

For the purpose of 'fine-mapping' the loci on the chromosomes-8, 10 and 11, the BC1 mice were genotyped with additional microsatellite markers from the putative linkage regions. This consisted of five markers from chromosome-8, one marker from chromosome-10 and seven markers from chromosome-11. The results from the fine-mapping are shown in Table 22. The chromosome-8 marker D8Mit36, in addition to D8Mit49, exhibited evidence of association in the extreme phenotype mice (χ^2=4.51, P< 0.034). The markers, D8Mit271 and D8Mit42 approached the values of significant association (χ^2=2.91, P<0.088, at both loci). The chromosome-10 marker D10Mit150, exhibited association in the extreme phenotypes (χ^2=8.01, P<0.0046). The chromosome 11 marker D11Mit41 exhibited evidence of association (χ^2=7.01, P<0.014). The markers D11Mit122 and D11Mit70 exhibited significance levels equal to

Table 22:

Fine-mapping of the putative loci controlling TSIgE levels on chromosomes-8, 11 and 10.

Marker	Chromosome Locus[2] (cM)	Gene[3]	TSIgE level[1] Low SS : AS	High SS : AS	$(\chi^2)^4$	P
D8Mit178	8(36)		17 : 11	13 : 14		
D8Mit85	8(48)		16 : 14	10 : 16		
D8Mit271	8(58)		19 : 11	11 : 16	2.91	0.088
D8Mit49	8(63)		20 : 9	11 : 16	4.51	0.034
D8Mit36	8(69)		20 : 9	11 : 16	4.51	0.034
D8Mit42	8(73)		19 : 11	11 : 16	2.91	0.088
D10Mit150	10(56)		11 : 19	20 : 7	8.01	0.0046
D10Mit266	10(60)		13 : 17	19 : 8	4.22	0.0400
D11Mit35	11(45)		16 : 14	7 : 20	4.44	0.0580
D11Mit41	11(49)		17 : 13	6 : 21	7.01	0.0140
D11Mit122	11(51)		18 : 12	6 : 21	8.32	0.0068
D11Mit70	11(54)		18 : 12	6 : 21	8.32	0.0068
D11Mit288	11(55)		18 : 12	6 : 21	8.32	0.0068
D11Mit289	11(58)		18 : 12	6 : 21	8.32	0.0068
D11Mit67	11(61)	Csf,g	16 : 14	8 : 19		
D11Mit132	11(64)	Icam2	17 : 13	8 : 18	3.78	0.052
D11Mit224	11(72)		18 : 12	11 : 16		

Legend to Table 22 :

1. TSIgE level was measured by ELISA on day 31, secondary response. BC1 mice exhibiting no detectable TSIgE levels were included in the low group (n=30), and BC1 mice having ≥ 18,000 ng/ml TSIgE were included in the high group (n=27). For occasional markers, certain individual genotypes were not discernible. For such markers less than the total group are presented.

2. Positions of the markers are designated by chromosome and centimorgans (cM) distal to the centromere. The location of the marker loci were based on the Encyclopedia of the Mouse Genome, genetic map v3.0a.

3. Known genes linked to a marker are indicated with the gene symbol for mouse; this information is from MIT mouse genome data base, 1995 directory.

4. The difference in the frequency of SS and AS alleles compared to random expectations in low TSIgE and high TSIgE groups was estimated by χ^2 analysis using 2 X 2 contingency tables. The markers showing significant associations are only indicated with the χ^2 and P values.

5. Abbreviations: SS, homozygous; AS, heterozygous.

that of D11Mit288 and D11Mit289 (χ^2=8.32, P<0.0068 at all the four loci). In addition the chromosome-11 markers, D11Mit35 and D11Mit132 approached the level of significance (χ^2=4.44, P<0.058 and χ^2=3.78, P<0.052 respectively).

From these analyses it was concluded that three genomic intervals on chromosomes-8, 10 and 11, exhibited evidence of association with the TSIgE levels in the extreme phenotype BC1 mice. Furthermore, fine-mapping of the putative chromosomal intervals revealed that the regions on chromosomes-8, 10 and 11 spanned a genomic interval of ~ 6, 4 and 9 cM, respectively.

C.2.2.3 Genetic analysis of the entire BC1 progeny for confirmation of association to chromosomes-8, 10 and 11.

Once initial evidence for association was obtained in the extreme phenotype BC1 mice, these genomic intervals were examined in the entire BC1 progeny for confirmation. The BC1 progeny genotyped consisted of 72 mice with a low TSIgE levels and 64 mice with high TSIgE levels (definition of 'low' and 'high' TSIgE levels were as described in the chapter-A). A total of 12 microsatellite markers from the putative linkage regions were examined. Thus, three markers from chromosome-8, six markers from chromosome-11 and two markers from chromosome-10 were examined (Table 23). The number of markers

Table 23:

Confirmation of association of the markers on chromosomes-8, 11 and 10, with the TSIgE phenotype in the entire BC1 progeny (n=136)

Marker	Chromosome Locus[2] (cM)	TSIgE level[1] Low SS : AS	High SS : AS	$(\chi^2)^3$	P
		n	n n	n	
D8Mit271	8(58)	45 : 26	30 : 34	3.71	0.054
D8Mit49	8(63)	47 : 24	24 : 40	11.12	0.0009
D8Mit36	8(69)	48 : 23	27 : 37	8.81	0.0030
D8Mit42	8(73)	45 : 27	24 : 40	8.47	0.0036
D11Mit35	11(45)	39 : 33	24 : 40	3.79	0.06
D11Mit41	11(49)	39 : 33	21 : 43	6.27	0.016
D11Mit70	11(54)	40 : 32	22 : 42	6.13	0.016
D11Mit288	11(55)	40 : 32	22 : 42	6.13	0.016
D11Mit289	11(58)	39 : 33	22 : 42	5.37	0.025
D11Mit67	11(61)	38 : 34	24 : 40	3.19	0.086
D10Mit150	10(56)	29 : 43	39 : 25	5.78	0.016
D10Mit266	10(60)	32 : 40	38 : 26	3.02	0.082

Legend to Table 23:

1. TSIgE level was measured by ELISA on day 31, secondary response. Based on the distribution analysis of TSIgE levels in BC1 mice a cut-off of >1000 and >5000 ng/ml was used to define the high TSIgE phenotype for male and females respectively, as detailed in the text.

2. Positions of the markers are designated by chromosome and centimorgans (cM) distal to the centromere. The location of the marker loci were based on the Encyclopedia of the Mouse Genome, genetic map v3.0a.

3. The difference in the frequency of SS and AS alleles compared to random expectations in low TSIgE and high TSIgE groups was estimated by χ^2 analysis using 2 X 2 contingency tables. The markers showing significant associations are only indicated with the χ^2 and P values.

5. Abbreviations: SS, homozygous; AS, heterozygous.

examined from each genomic interval was based on the availability of polymorphic microsatellite markers in that region.

Each BC1 mice was genotyped at all the 12 marker loci. Evidence of significant association was observed for markers D11Mit41 (χ^2=6.27, P<0.016), D11Mit70, D11Mit288 (χ^2=6.13, P<0.016 at both loci) and D11Mit289 (χ^2=5.37, P<0.025). This region spanned 9 cM and located 49 cM from the centromere. All BC1 mice were genotyped using four markers from the putative region of chromosome 8. Interestingly, the marker D8Mit49 exhibited evidence of significant linkage in the entire BC1 progeny (χ^2=11.12, P<0.0009). Whereas, the markers, D8Mit36 exhibited evidence of significant association (χ^2=8.81, P<0.0030), the marker D8Mit271 approached the level of significance (χ^2=3.71, P<0.06) in the entire BC1 progeny. This region on chromosome 8, spanned an interval of ~10 cM, and was located 63 cM from the centromere. Similarly two markers from the chromosome 10 region were examined in the entire BC1 mice. As evident from results, marker D10Mit150 exhibited evidence of significant association (χ^2=5.78, P<0.016), while D10Mit266 did not (χ^2=3.02, P<0.08).

Thus, the genetic analysis in the entire BC1 progeny confirmed association of AL-induced persistent TSIgE levels to markers on chromosomes-8, 10 and 11.

Fig. 12 Association of microsatellite marker - D8Mit49, loci with TSIgE levels in extreme phenotype BC1 mice .
(A representative marker showing significant association with TSIgE phenotype).

Microstellite marker from individual BC1 mice was amplified using the D8Mit49 primer pairs. Figure shows the homozygous or heterozygous nature of the marker locus in each of the BC1 mice. The upper panel shows the BC1 individuals having low TSIgE levels; the lower panel shows the BC1 mice having high TSIgE levels. The position of 0.2 Kb is indicated with the arrow mark. Kb=1Kb ladder; C=PCR control.

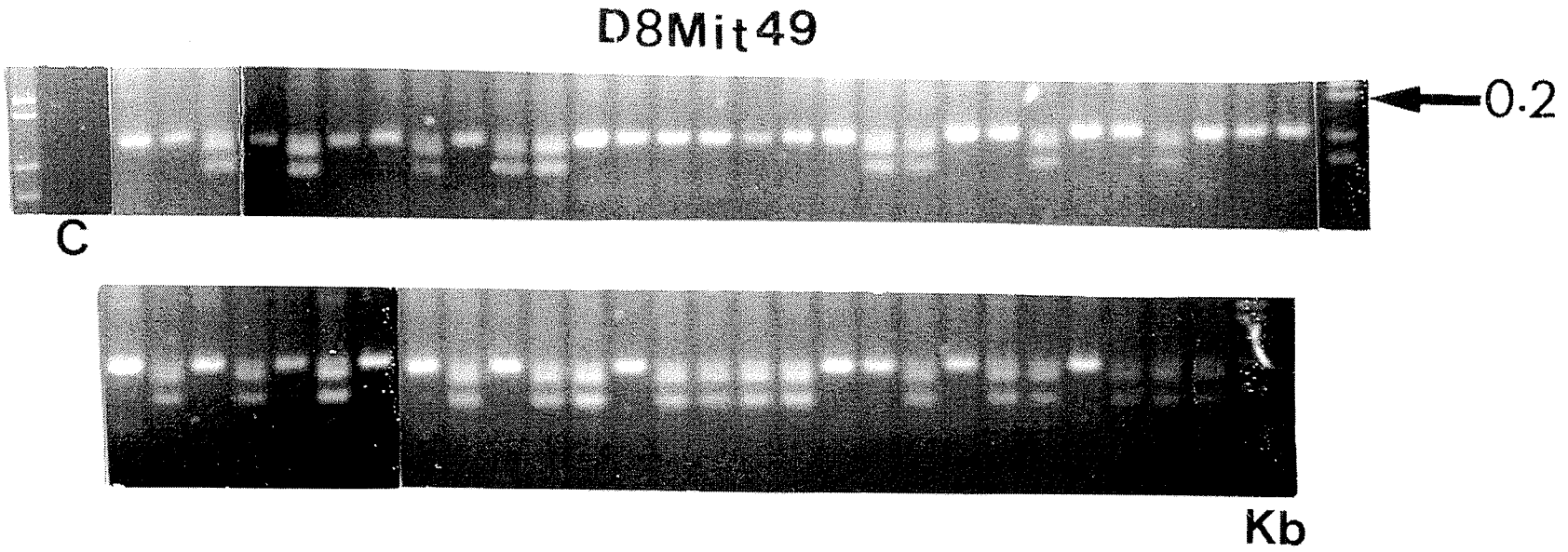

D8Mit49
0.2
C
Kb

Fig. 13 Lack of association of D13Mit13 (I19) marker with TSIgE phenotype in the extreme phenotype BC1 mice.
(A representative marker showing lack of association with TSIgE phenotype).

Microstellite marker from individual BC1 mice was amplified using D13Mit13 primer pairs. Figure shows the homozygous or heterozygous nature of the marker locus in each of the BC1 mice. The upper panel shows the BC1 individuals having low TSIgE levels; the lower panel shows the BC1 mice having high TSIgE levels. The position of 0.2 Kb is indicated with the arrow mark. Kb=1Kb ladder; C=PCR control.

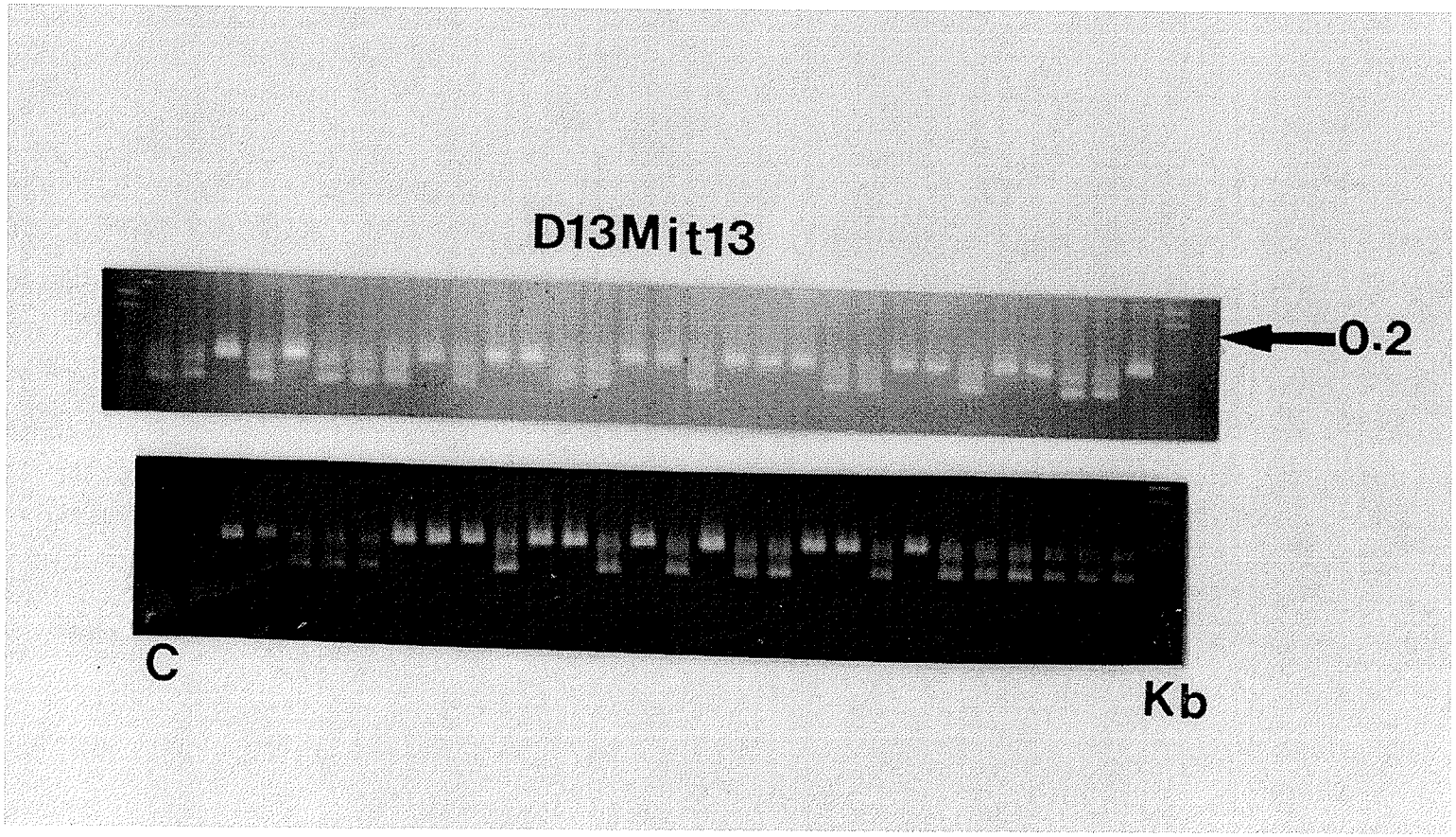
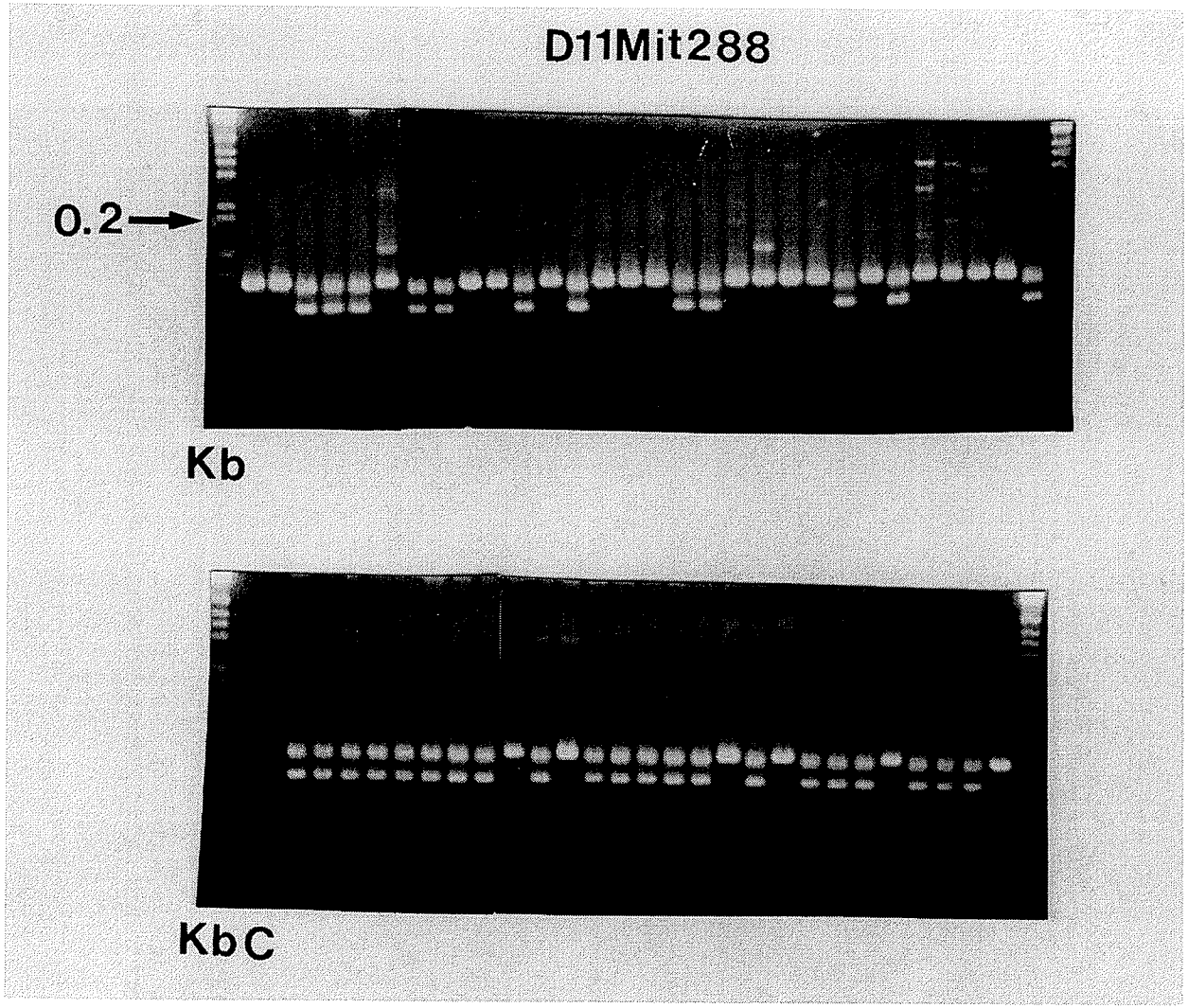

D13Mit13
0.2
C
Kb

Fig. 14 Association of D11Mit288 marker with TSIgE levels in the extreme phenotype BC1 mice. (A representative marker showing significant association with TSIgE phenotype).

Microstellite marker from individual BC1 mice was amplified using D11Mit288 primer pairs. Figure shows the homozygous or heterozygous nature of the marker locus in each of the BC1 mice. The upper panel shows the BC1 individuals having low TSIgE levels; the lower panel shows the BC1 mice having high TSIgE levels. The position of 0.2 Kb is indicated with the arrow mark. Kb=1Kb ladder; C=PCR control.


D11Mit288
0.2
Kb
KbC

C.3 DISCUSSION

The existence of inbred mouse strains differing in their ability to mount IgE, but not IgG1 Ab responses, itself is an indicator of a strong genetic component that regulates IgE isotype responses (Levine and Vaz 1970a,b, Watanabe et al 1976). Furthermore, the demonstration in this study, that the MHC-identical mouse strains, A.SW and SJL, differ widely in the AL-induced persistent TSIgE levels, provides strong evidence that non-MHC gene(s) play an important role in regulating TSIgE levels. Examination of candidate non-MHC genes such as Tcrvb8, Il4 and Ifg clearly indicated that they do not represent the genetic factors regulating TSIgE levels in this mouse colony. This prompted for a genome sweep using microsatellite markers to map the locus or loci that control the AL-induced TSIgE levels in mouse.

The primary requirement for molecular-genetic mapping of the loci controlling a given trait is to establish a linkage map covering most of the genome of the relevant species (Lander and Schork 1994). Therefore, a large number of microsatellite markers (a total of 416 markers) originating from various autosomes were screened in order to identify the polymorphic markers useful for mapping the loci controlling AL-induced TSIgE levels in mouse. This elaborate genome screening

procedure for DNA polymorphisms consisted on an average of one marker at every 3.4 cM length, across the genome.

Microsatellites have been shown previously to exhibit high degree of polymorphism (35-56%) in several combinations of inbred mouse strains (Love et al 1990, Cornall et al 1991, Dietrich et al 1996). However, these studies did not use the combination of A.SW and SJL mouse strains. The results from genome-wide screening in the present study failed to detect high degree of polymorphism of microsatellites between these two strains. Thus, only about 21% of the microsatellite markers screened were found to be polymorphic between A.SW and SJL mice. This suggested that these two strains may have common evolutionary origin and have conserved much of the genomic-intervals between them. The low frequency of polymorphism, however had an impact on the screening efforts, which had to become much more extensive in order to obtain the informative set of markers used for constructing the linkage map in this study.

One problem associated with the microsatellite mapping is the doubt that the distribution of polymorphic markers may not be uniform across the genome, especially in murine species (Dietrich 1996). The data from the present study supports this view. Thus the chromosome-wide rate of polymorphisms reported in mouse is on an average ~50% for all autosomes except

chromosome 10. In the present study, the rate of polymorphism in chromosome 10 was low at 14% (Table 20). It should be noted that in addition to chromosome-10, the chromosomes-3,5,6,8 and 16 also exhibited a significantly low rate of polymorphism at <15%. However, two chromosomes exhibited expected high rate of polymorphism viz 57% (chromosome 12) and 40% (chromosome 11). The rate of polymorphism in rest of the genome was moderate (18-37%). Thus, the genome-wide low rate of DNA polymorphism between A.SW and SJL mouse strains was a major drawback in using this combination of strains for molecular-genetic mapping of the loci controlling TSIgE levels in this study. It was estimated that ~90% of the genome was scanned in this study.

Using a set of 69 polymorphic markers, molecular-genetic exclusion mapping of the loci controlling AL-induced TSIgE levels was performed. The use of extreme phenotypes for initial genetic analysis was based on the following rationale: i) extreme phenotypes may represent groups of individuals with uniform genotypes, viz., individuals within a group are likely to be homogenous than between groups, at the genetic locus regulating the TSIgE phenotype; ii) since, occasionally some SJL/J mice do develop a TSIgE levels of up to 4-5 μg/ml, some BC1 animals with intermediate levels of TSIgE levels may actually carry the SJL alleles at both loci; and iii) genotyping a subset of animals would be advantageous with

respect to the economy of time and efforts, which otherwise would be required to genotype all BC1 mice with the complete set of markers. Furthermore, a number of previous studies (Vitaterna et al 1994, Asada et al 1994, De Sanctis et al 1995, Morel et al 1994), have used such an approach successfully to quickly map the loci associated with a phenotype.

The major finding from genome-scanning of the BC1 mice was the mapping of three non-MHC loci associated with AL-induced TSIgE levels to chromosomes 8, 10 and 11 in mouse. Thus, for three markers on the distal chromosome-8 highly significant associations were found between the genotype and the phenotype in the entire BC1 mice (n=136). Furthermore, evidence of significant linkage was obtained for the marker D8Mit49 (χ^2=11.2, P<0.0009) (Table 23). This suggests that a locus or loci controlling TSIgE levels is/are in linkage with the microsatellite markers in this region. For markers, D8Mit49, and D8Mit36, starting from 63 cM distal to the centromere, spanning about 6 cM, there was a strong association with the TSIgE phenotype, in the entire BC1 progeny. The loci on chromosome 11 which showed significant association in the entire BC1 progeny, also included multiple markers, spanning a genetic length of 9 cM, starting from 49 cM distal to the centromere. In contrast, the loci on chromosome-10 exhibited, association over a very short distance of 4 cM in the extreme

phenotypes. Whereas, the marker D10Mit150 exhibited evidence of association in the entire BC1 progeny, the marker D10Mit266 did not exhibit significance in the entire BC1 progeny.

Both loci on chromosomes 10 and 11 showed evidence of association with a greater significance in the extreme phenotypes ($P< 0.0046$ and $P<0.0068$ respectively) than the significance found in the entire BC1 progeny ($P<0.016$, for both loci). This suggested the possibility that these two loci may be modifiers of TSIgE level in the extreme phenotype mice. In order to test this hypothesis, analysis was performed in the BC1 mice excluding the extreme phenotype mice. Chi-square analysis of association between genotypes and the TSIgE phenotype exhibited evidence of significant association with the chromosome 8 locus ($\chi^2=6.69$, $P<0.0097$) (Table 24). In marked contrast, neither of the chromosomes 10 and 11 loci exhibited significant association ($\chi^2=0.57$, $P<0.45$ for D10Mit150; $\chi^2=0.66$, $P<0.42$ for D11Mit288). Thus, the results from this analysis argue that the chromosome 8 locus may be a major one and the other loci on chromosomes 10 and 11 may be modifiers (Table 25).

Taken alone, the observed frequency and distribution of TSIgE levels in the BC1 progeny, although suggestive, is insufficient evidence to deduce the involvement of single locus in controlling differential TSIgE levels. This is

Table 24:

Genotype Analysis of BC1 mice excluding the extreme TSIgE phenotype mice.

Marker	Chromosome Locus[2] (cM)	TSIgE level[1] Low SS : AS	High SS : AS	χ^2	P
		n	n	n	n		
D8Mit271	8(58)	26 :	15	19 :	18	1.16	0.280
D8Mit49	8(63)	27 :	15	13 :	24	6.69	0.0097
D8Mit36	8(69)	28 :	14	16 :	21	4.37	0.037
D10Mit150	10(56)	18 :	24	19 :	18	0.57	0.45
D10Mit266	10(60)	19 :	23	19 :	18	0.29	0.59
D11Mit35	11(45)	23 :	19	17 :	20	0.61	0.43
D11Mit41	11(49)	22 :	20	15 :	22	1.11	0.29
D11Mit70	11(54)	22 :	20	16 :	21	0.66	0.42
D11Mit288	11(55)	22 :	20	16 :	21	0.66	0.42
D11Mit289	11(58)	21 :	21	16 :	21	0.36	0.55
D11Mit67	11(61)	22 :	20	16 :	21	0.66	0.42

Legend to Table 24:

1. TSIgE level was measured by ELISA on day 31, secondary response. Based on the distribution analysis of TSIgE levels in BC1 mice a cut-off of >1000 and >5000 ng/ml was used to define the high TSIgE phenotype for male and females respectively, as detailed in the text. The mice with extreme TSIgE phenotype, as defined for the analysis presented in table 21, were excluded in this analysis to test the hypothesis of major and modifier loci.

2. Positions of the markers are designated by chromosome and centimorgans (cM) distal to the centromere. The location of the marker loci were based on the Encyclopedia of the Mouse Genome, genetic map v3.0a.

3. The difference in the frequency of SS and AS alleles compared to random expectations in low TSIgE and high TSIgE groups was estimated by χ^2 analysis using 2 X 2 contingency tables.

Table 25:

Evidence that chromosome-8 locus is the major locus and loci on chromosomes-10 and 11 are modifiers of TSIgE levels.

Locus	Effects on TSIgE levels in						Inferred Action of the Locus
	Extreme Phenotype Mice[1]		Total minus extreme Phenotype mice[2]		Entire BC1 progeny[3]		
	$(\chi^2)^4$	P	$(\chi^2)^4$	P	$(\chi^2)^4$	P	
D8Mit49	4.51	0.034	6.69	0.0097	11.12	0.0009	**Major effect**
D10Mit150	8.01	0.0046	0.57	0.4500	5.78	0.016	**Modifier effect**
D11Mit288	8.32	0.0068	0.66	0.4200	6.13	0.016	**Modifier effect**

1. Extreme phenotype mice consisted of 30 low IgE responders and 27 high IgE responders; low group mice had no detectable TSIgE levels while high group mice had TSIgE level ranging from 18000 to 145000 ng/ml.
2. Total minus extreme phenotype mice included all BC1 mice except the extreme phenotype individuals.
3. TSIgE level was measured by ELISA on day 31, secondary response. Based on the distribution analysis of TSIgE levels in BC1 mice a cut-off of >1000 and >5000 ng/ml was used to define the high TSIgE phenotype for male and females respectively, as detailed in the text. The entire BC progeny included 136 mice.
4. The difference in the frequency of SS and AS alleles compared to random expectations in low TSIgE and high TSIgE groups was estimated by χ^2 analysis by 2 X 2 tables.

because, there is a wide variation in the levels of TSIgE in the high IgE responder animals, despite the evidence of bimodal distribution (Fig.3). Furthermore, analysis of variance of TSIgE levels in BC1 mice suggested that the variation in TSIgE levels was not entirely due to environmental factors (Table 10). Therefore, this suggested the possibility of existence of modifier gene(s). However, when an extensive genetic analysis, evaluating 69 microsatellite markers from all over the genome, was performed, the results strongly supported the concept of a major locus on chromosome-8, involved in the control of AL-induced TSIgE levels. Furthermore, results from genetic analysis of BC1 mice excluding the extreme phenotype, argued that the loci on chromosomes-10 and 11 are modifiers. Thus, the results from genome scanning are consistent with the segregation and distribution analysis described in the previous chapter.

Studies to date in humans have suggested the existence of TSIgE regulating locus on human chromosome 5 near IL4 (Marsh et al 1994), and near the GRL gene (Meyers et al 1994) and the existence of a locus regulating both TSIgE and BHR near the GRL gene (Postma et al 1995) and the existence of a 'atopic IgE responsiveness' gene on chromosome 11q (Cookson and Hopkin 1989). However, these results are considered controversial because two other reports found no evidence for a locus linked

with TSIgE levels on human 5q (Cookson 1995, Blumenthal et al 1995). A number of studies have reported that there is no evidence for a locus on human 11q linked to atopic IgE responsiveness (Rich et al 1992, Lympany et al 1992, Amelung et al 1992, Hizawa 1992).

Results from the present study involving genome-scanning in the mouse do not provide any evidence for a locus regulating TSIgE levels in the mouse syntenic regions, on chromosomes 11, 13, 18 (syntenic to human 5q31-33) and 19 (syntenic to human 11q) (Table 26). It should be noted that two previous reports clearly indicated that these syntenic regions in mouse also do not contain genes regulating BHR (De Sanctis et al 1995, Levitt et al 1995). Thus linkage studies in mouse appears to have found loci involved in controlling IgE and BHR which differ from those found for humans so far. Whether these new loci represent additional loci involved in human atopy and asthma remains to be elucidated.

A major limitation of the molecular-genetic linkage mapping approach is that it does not have sufficient resolution to allow precise identification of the genes themselves (Lander and Schork 1994). Rather, it indicates the genomic-intervals containing the putative genes of interest. A number of known genes reside in the vicinity of markers associated with TSIgE phenotype, as per the gene information available at present

(Table 26). However, none of these genes have been shown to be associated with the regulation of IgE response or TSIgE levels in mouse or humans. Therefore, the precise identity of the genes mapped in this study will be revealed after their characterization by positional cloning approach.

Table 26:

List of known genes and human synteny at the putative Iger loci:

Marker	Locus[1]	Known genes[2]	Human synteny
Iger1:			
D8it49	8(63)	Cox4	16q24.1
	8(66.5)	Hfh8	UN[3]
	8(67)	Acta1	1q42.1-q42.3
		Aprt	16q24.2
		Cdh3	16q22.1
	8(68)	Agt	1q42-q43
		Mc1r	16q24.3
D8Mit36	8(69)	UN	16q
Iger2:			
D10Mit150	10(56)	Don	12q21.3-q23
Iger3:			
D11Mit70	11(54)	UN	17q
D11Mit288	11(55)	UN	17q

1. Locus in indicated as chromosome number followed by the location in cM from the centromere. Locus and gene information is from the MIT mouse genome database, 1995 Jan. directory.

2. Gene abbreviations: Cox4, cytochrome C oxidase, subunit IV; Hfh8, HNF-3/forkhead homolog 8; Acta1, actin,alpha1, skeletal muscle; Aprt, adenine phophoribisyl transferase; Cdh3, cadherin 3; Agt, angiotensinogen; Mc1r, melanocortin 1 receptor; Don,decorin.

3. UN, unknown

D. GENERAL DISCUSSION

Clinical manifestation of common atopic disorders involves multiple gene-environmental interactions and hence, these disorders have been designated as 'complex' genetic diseases (Marsh 1994, Lander and Schork 1994, Marsh 1996). Presence of an elevated and persistent TSIgE level is not only a hallmark of atopy but also is an important predictor of atopic predisposition (Halonen et al 1992, Magnusson 1988). Several studies involving children have suggested that TSIgE levels correlate very well with allergy, BHR and asthma (Burrows et al 1989, Sears et al 1991, Freidhoff and Marsh 1993). Hence, genetic determinants of TSIgE levels may constitute one of the important genetic factors predisposing to atopy.

The dissection of the genetic basis of differential TSIgE levels in humans is complicated by a number of confounding factors as described in previous chapters. The variable and often contradictory results from human studies on the genetic control of TSIgE levels has been attributed predominantly due to the genetic heterogeneity (Table 1). The present investigation was undertaken to elucidate the genetic determinants of AL-induced TSIgE levels in a mouse model, which can be performed under controlled environmental conditions and uniform genetic background.

It is known for several decades that inbred strains of mice

differ widely in their ability to mount SPIgE Ab response when injected with Ags (Levine and Vaz 1970a,b). However, earlier studies in mouse on the genetic control of SPIgE Ab response have yielded variable results implicating both one or two gene control of SPIgE responses (Watanabe et al 1976, Levine and Vaz 1979). It is difficult to make definitive conclusions from these studies on the genetic control of IgE isotype *per se*, because of the influence of MHC-linked immune response (Ir) genes, which control the general immune responsiveness of different mouse strains to minute doses of purified Ags (Carbone and Bevan 1989). Moreover, inheritance of TSIgE levels and the role of non-MHC genes as genetic factors regulating AL-induced TSIgE levels in mouse has not been examined before.

On the basis of this background information, the present study was designed to test the hypotheses that non-MHC linked gene(s) may control AL-induced TSIgE levels in mouse; and that such non-MHC loci can be mapped to specific chromosomes using the microsatellite-based molecular genetic mapping approach. In order to examine these hypotheses a large MHC-identical mouse colony consisting of F1 hybrids, a BC1 and F2 intercross progeny was established. The parent strains, A.SW and SJL mice used in the establishment of this colony are identical at the MHC locus (both are $H\text{-}2^{s}$), and mount a similar IgG1 Ab response but differ in IgE isotype response. As described

before, these two strains were also found to be disparate in the TSIgE levels, both in the pre-immune serum and on immunization with ALs. However, since the levels of TSIgE in the pre-immune sera were very low, and because TSIgE levels in atopics are induced by the exposure to ALs, it was decided to study the genetic control of AL-induced TSIgE levels. The following specific objectives were addressed in this study: (i) determination of the mode of inheritance and the genetic control of AL-induced persistent TSIgE levels; (ii) examination of the role of Tcrvb8, Il4 and Ifg as candidate genes regulating AL-induced TSIgE levels; and (iii) molecular-genetic mapping of the loci controlling the AL-induced persistent TSIgE levels to specific chromosomes, in these mice.

There are three major findings from this study:
i) the AL-induced persistent TSIgE levels in mouse is under the control of a major, dominant non-MHC autosomal locus with the possibility of existence of additional modifier gene(s);

ii) Tcrvb8, Il4 and Ifg genes *per se* do not represent genetic factors responsible for the observed TSIgE phenotype in A.SW/Snj and SJL/J mouse strains; and

iii) molecular-genetic mapping of three loci- consisting of a major one on chromosome 8 and two modifier loci on chromosomes

10 and 11, that are associated with AL-induced persistent TSIgE levels in these mice.

1. Inheritance of AL-induced persistent TSIgE levels and genetic control by non-MHC genes.

It is well known that non-atopic individuals make a low and transient IgE responses, in contrast to atopics, where the persistence of high IgE levels is frequently observed. Many decades ago Sherman et al (1940) determined reaginic Abs in the sera of hay fever patients by Prausnitz-Kustner reactions, and observed that the Ab titers in the sera of ragweed-sensitive patients were persistent. This observation was later confirmed by the radioimmunoassay. Quantitative measurements of serum IgE in ragweed-sensitive patients who had not received immunotherapy showed that the Ab level persists throughout the year and that most patients showed secondary IgE Ab response after the ragweed season (Ishizaka and Ishizaka 1975). The exposure to ALs during pollen season induces AL-specific IgE formation, which contributes to post-season peak in serum IgE levels. Although, the serum IgE levels may decrease subsequently, still the levels of TSIgE remain significantly high through out the year in atopics. Because the average half-life of IgE in the serum is 2.3 days in humans (Ishizaka and Ishizaka 1975), persistent and elevated IgE in the serum of atopic individuals would suggest

that IgE Abs are being formed continuously in these subjects. (Marsh et al 1981, Ishizaka and Ishizaka 1989). Thus, genetic control of persistently high TSIgE levels, which is one of the important factor that differentiates atopics from the normal individuals, was investigated as a phenotype of atopy, in this study.

Evidence has accumulated over the past decades in support of the concept that the TSIgE levels are under strong genetic control in humans. However, there has been no consensus on the pattern of inheritance of TSIgE levels in humans. Both dominant, recessive, codominant and polygenic models have been suggested (Marsh 1981, Marsh and Blumenthal 1990, Marsh 1994). One of the many factors that complicates genetic analysis in humans is the considerable influence of HLA-linked *Ir* genes on the general immune response of individuals to protein antigens (Marsh and Freidhoff 1993, Marsh and Meyers 1992, Moffatt et al 1992). In addition to gender, environmental factors such as exposure to ALs, air pollution and smoking also influence the TSIgE levels in a given individual (Marsh et al 1981, Richard 1993, Jarvis et al 1995). Therefore, the best alternative to study the inheritance pattern of TSIgE levels would be to use experimental animals, where many of these variables can be controlled.

A unique feature of the experimental design in the present

study was the use of MHC-matched strains of mice that differ in IgE but not IgG1 responses. This idea clearly simplified the problems associated with the genetic study of IgE responses in mouse. Thus, in the present approach not only the genetic control by MHC genes was kept constant but also, the genetic control of IgG1 and IgE isotype responses were dissociated. This strategy facilitated experimental demonstration of IgE isotype specific control by non-MHC gene(s).

Using two inbred mouse strains (A.SW and SJL), which are MHC-identical (both H-2^s), but differ extensively in the AL induced TSIgE levels, not only the mode of inheritance of TSIgE levels, but also the genetic control by non-MHC genes was demonstrated. Thus, AL-induced persistent and high TSIgE levels was found be a dominant Mendelian trait, under the control of a major autosomal locus, with the possibility of existence of additional modifier locus (or loci). This is the first demonstration of the inheritance pattern and the genetic control of TSIgE levels in any experimental animal model of atopy.

These results on the inheritance of TSIgE levels, are in agreement with a number of reports in human studies suggesting the dominance of high TSIgE levels over that of low TSIgE levels (Borecki et al 1985, Cookson and Hopkin 1989, Morton

1992, Marsh and Blumenthal 1990). Given the complexity of genetic factors in humans, it is likely that more than a single major gene is involved in precipitation of atopic conditions in humans. Despite these complications, however, recent studies suggest that TSIgE levels may be controlled by a few major/modifier gene(s). For instance, Xu et al (1995) reported that a two-gene model best described the segregation of TSIgE phenotype in some Dutch families.

2. Role of Tcrvb8, Il4, Ifg, CD23 and Il9, as genetic predisposition factors for atopic IgE responsiveness.

Dysregulated IgE biosynthesis leading to elevated levels of TSIgE in the serum can potentially originate from a defect at the time when an immune response is initiated. In particular, this may be related to differences in the TCR repertoire or the expression of different MHC II molecules. Since MHC matched founder strains of mice were selected for this study, the role of TCR as a non-MHC gene influencing IgE responses was considered. The Tcrvb8 gene was investigated as a candidate gene due to a number of reasons as described before.

Analysis of the role of the Tcrvb8 gene and its product (as Vβ8+ T cells), clearly excluded this gene as a candidate for the differential TSIgE and SPIgE Ab levels between A.SW and SJL mice strains. This result is in marked contrast to the

finding of TCRVβ8$^+$ T cells in upregulating IgE responses, in Balb/c mice. However, their studeis did not provide evidence that TCRVβ8$^+$ T cells always exert an IgE isotype specific action.

In humans, one study using MZ twins with or without asthma suggested association of TCRVβ8$^+$ T cells with asthma (Davey et al 1994). However, the expansion of TCRVβ8$^+$ T cells in the periphery of one of the MZ twins who had asthma, suggested the role of environmental factors (like superantigens/viral infections) and not the genetic factors. The results from the present study, which clearly demonstrate lack of co-segregation of Tcrvb8 and TSIgE/SPIgE phenotype, are in agreement with another human study using nuclear families. Thus, Moffatt, et al (1994), reported that there is no evidence of linkage of SPIgE or TSIgE levels to TCRVB gene complex in two independent sets of families (Moffatt MF et al 1994). Thus, there is no substantial genetic evidence to implicate TCRVB genes in humans or the Tcrvb8 gene in mice, as genetic factors for IgE responsiveness.

Having excluded the Tcrvb8 gene, the role of Il4 and Ifg as candidate genes for the regulation of AL-induced TSIgE levels was investigated. These two cytokines respectively, represent the prototypic products of Th1 and Th2 subsets of T cells, which have been shown conclusively to regulate the IgE Ab

responses in mouse (Mossman and Coffman 1989, Finkelman 1990) and in humans (Romagnani 1994). IL-4 acts as a switch factor for class switching of activated B cells to IgG1 and IgE isotypes. IFN-γ inhibits the IL-4 dependent class switching to IgG1 and IgE isotypes (Snapper and Paul 1987, Finkelman 1990). The class switched B cells undergo differentiation to Ab secreting plasma cells and memory B cells (Paul and Seder 1994).

The role of IL-4, as a regulator of non-cognate IgE production has been recently proposed, by Marsh et al (1994). As reviewed before, they reported that IL4 or a nearby gene on chromosome 5q31.1, is responsible for the regulation of non-cognate IgE production as measured by TSIgE levels. This result is in not in agreement with two other studies: i) Blumenthal et al (1995) studied large human families and reported that the TSIgE levels are not linked to the IL4 gene on chromosome 5q; and ii) Cookson et al (1995) reported a lack of linkage of TSIgE and SPIgE levels to the IL4 gene in 1000 subjects from 230 Australian families.

Furthermore, other studies on the IL4 promoter region polymorphism in humans have suggested the potential role of IL4 as a genetic factor for atopy (Rosenwasser et al 1995, Song et al 1996). However, the former study involved a small number of subjects and it remains to be verified in a larger

population (Rosenwasser et al 1995). The latter study was done on T cell lines and hence it remains to be seen whether similar findings apply to a normal human cells and, if so, whether such variants are associated with elevated IgE levels. Besides this, studies in hyper-IgE syndrome patients revealed a deficiency of IFNγ production, which is associated with elevated TSIgE levels in these patients (Del Prete et al 1989, Finkelman et al 1990). A recent human study suggested that *in vitro* TSIgE levels are not significantly correlated with the in vitro IL-4 production in atopics (r= -0.04) (Van der Pouw Kraan et al 1995). Another study reported that TSIgE levels in children are not correlated with *in vitro* IFN-g production (Tang et al 1993). Therefore, the role of Il4 and Ifg genes as genetic factors for AL-induced TSIgE levels was examined in this study. At the time this study was undertaken, the Il4 and Ifg genes were known to be polymorphic (albeit, in the non-coding regions) in different strains of mice (Jacob et al 1993), however, the association, if any, between these genes and IgE responsiveness had not been reported.

Results of the present study involving the microsatellite marker polymorphism analysis in BC1 progeny clearly revealed that the differential TSIgE levels (low vs high) are not associated with the polymorphisms linked to either Il4 or Ifg genes in these strains. Furthermore, other studies in this laboratory involving the Il4 gene promoter sequence analysis

between A.SW and SJL mice, although revealed several polymorphisms, these polymorphisms were not associated with IgE responsiveness in BC1 and F2 mice (Venugopal et al 1995). Moreover, a subgroup of BC1 mice of the present study were also examined for IL-4 and IFN-γ production. Cytokine production by the spleenocytes of BC1 mice, indicated that IL-4 and IFN-g secretion by these mice were moderately and poorly correlated with TSIgE levels respectively (r=0.319 for IL-4 and r=0.185 for IFN-g) (Venugopal G et al 1995). It is to be pointed out that a recent study (Yoshimoto et al 1995) involving a cellular approach suggested that defective IgE production in SJL mouse is linked to the absence of $NK1.1^+$, $CD4^+$ T cells, that produce IL-4. This study however, did not investigate the association between Il4 gene polymorphism and IgE phenotype. It should be noted that in this study regulation of IgE response induced by anti-IgD Ab injection was examined, which does not resemble AL-induced IgE responses, a key feature of human atopy.

In summary, whereas the reciprocal role of IL-4 and IFN-γ cytokines in the class-switching of B cells to IgE is unequivocal, the results from the present study together with other human studies question their role as genetic predisposition factors for atopy. Although promoter polymorphisms have been found in humans, the implication of such polymorphism in AL-induced elevated TSIgE levels needs

further investigation.

In adddition to Il4 and Ifg, two other candidate genes, CD23 and Il9 were investigated for their polymorphism and association with TSIgE levels respectively. The CD23 gene associated microsatellites were not polymorphic between A.SW and SJL mice. The cytokine IL-9 has mast cell proliferation activity and hence, has been implicated in allergic disorders (Rosenwasser et al 1995). Rosenwasser et al studied and identified one polymorphism (A->C) in the IL9 5' region. However, this polymorphism was relatively infrequent, present in only 12/72 subjects they studied. The results from the present study, using a marker from the mouse Il9 gene (D13Mit13) did not provide evidence of significant association with the TSIgE phenotype (χ^2=0.18, P<0.79) (Table 21, Fig. 13).

Taken together, the candidate gene analysis revealed that none of the genes examined here, constitute a genetic predisposition factor for AL-induced differential IgE response in these strains of mice.

3. Three non-MHC loci; a major locus on chromosomes 8, and two modifier loci on chromosomes 10 and 11, control AL-induced differential TSIgE levels in A.SW and SJL mice.

A systematic exclusion of the three important candidate genes

viz., Tcrvb8, Il4 and Ifg, paved the way for molecular-genetic mapping of the loci controlling AL-induced TSIgE levels in mouse. Microsatellite marker-based genetic mapping of disease susceptibility loci has been an asset in a number of diseases, including type 1 diabetes, epilepsy, hypertension, SLE and BHR in experimental animals (Todd et al 1991, De Sanctis et al 1995, Lander and Schork 1994). A similar strategy was employed in this study to map the loci regulating AL-induced TSIgE levels to specific chromosomes. Molecular-genetic exclusion mapping of the BC1 progeny was performed using a set of 69 polymorphic microsatellite markers spaced throughout the genome (excluding sex chromosomes). Thus, a detailed and systematic genetic analysis led to the identification of three genomic intervals exhibiting evidence of significant association with the TSIgE phenotype in these mice. It should be pointed out, however, that the markers used in the genome sweep in this study encompassed about 90% of the genome. Therefore, it remains a possibility that other loci may be found when markers covering the remainder 10% of the genome are examined.

A locus on distal chromosome-8 was identified as a major locus (tentatively designated as *Iger (IgE responsiveness)-1*), because it exhibited evidence of significant linkage (χ^2=11.12, P=0.0009) in the entire BC1 progeny. This locus spans a distance of ~6 cM (Fig. 15). Two other loci one each

on chromosomes-10 and 11 (tentatively designated as *Iger-2* and *Iger-3* respectively) were identified as modifier loci, because: i) they exhibited much stronger evidence of association in the extreme phenotype mice (χ^2=8.01, P=0.0046 and χ^2=8.32, P=0.0068, respectively) than in the entire BC1 progeny (χ^2=5.78, P=0.016 and χ^2=6.13, P=0.016); and ii) they did not exhibit association in the BC1 analysis, excluding the extreme TSIgE phenotype mice (χ^2=0.55, P=0.57 and $\chi^{2=}$0.66, P=0.42, respectively) (Table 24). The *Iger-2* locus spans a distance of ~1 cM (Fig.16). Thus, results from molecular-genetic exclusion mapping are consistent with the segregation analysis results, which indicated the existence of a major locus, with the possibility of additional modifier loci, as described in previous chapters.

As pointed out earlier, several human studies involving the candidate gene approach have reported variable and controversial results on the existence of loci on human chromosomes 5q31.1, and 11q13 which have been proposed to regulate IgE levels (Table 1). It should be noted however, that the proposed existence of loci on human chromosomes 11q13 and 5q31.1 regulating IgE levels is a subject of extensive debate and controversy, because of failures to replicate these findings by independent groups. Moreover, a systematic genome-scanning approach to map the TSIgE regulating loci has not been reported so far in humans.

Fig. 15

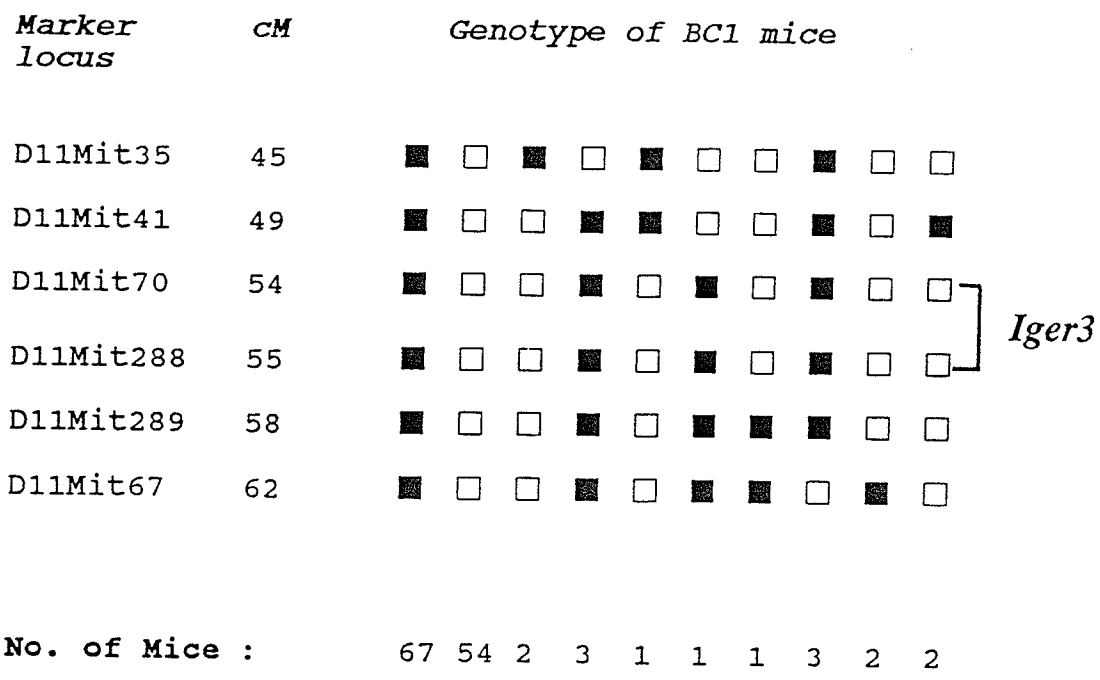
Genotype of [(A.SW x SJL)F1 x SJL]BC1 mice in the vicinity of the putative Iger1 locus on chromosome 8.

Marker Locus	*cM*	*Genotype of BC1 mice*							
D8Mit271	58	■	□	□	■	□	■	□	
D8Mit49	63	■	□	■	□	□	■	■	*Iger1*
D8Mit36	69	■	□	■	□	□	■	□	
D8Mit42	73	■	□	■	□	■	□	■	
No. of Mice :		50	59	9	8	3	1	4	

Symbols: open square, homozygous; solid square, heterozygous.

Fig. 16

Genotype of [(A.SW x SJL)F1 x SJLJBC1 mice in the vicinity of the putative Iger3 locus on chromosome 11



Symbols: open square, homozygous; solid square, heterozygous.

The syntenic region of human 5q31-33 corresponds to portions of mouse chromosomes 11, 13 and 18. However, there was no evidence of association of AL-induced TSIgE levels with these regions in the present study (Table 21). The *Iger-3* locus on mouse chromosome 11 does not correspond to human 5q31-33. The syntenic region of human 11q13 is on mouse chromosome 19, which again did not show association with TSIgE levels (Table 21).

A number of known genes exists in the vicinity of the *Iger* loci mapped in this study (Table 26). However, there are apparently no known genes involved specifically, in IgE regulation in these genomic intervals. Therefore, the identity of the three loci controlling AL-induced TSIgE levels, discovered in this study will be revealed after positional cloning and characterization.

There are a number of limitations of the present study: (1) the exclusion mapping approach used in this study identifies the genetic difference between the two inbred mouse strains used as parents; therefore the loci controlling AL-TSIgE levels mapped in this study need to be extended to other strain combinations; (2) the inbred strain SJL/J was selected as a parent, because it exhibits a very low AL-TSIgE levels, which is phenotypically similar to non-atopic humans. However,

it should be noted that SJL mice, in contrast to non-atopic humans, exhibit a number of characteristics like - high incidence (~91%) of reticulum cell sarcoma, short life span (~1.1 year), NK cell defect and susceptibility to autoimmune disease (Klein et al 1983); since there was no other suitable mouse strain available at that time, SJL mice were used as parents; (3) the AL-TSIgE phenotype was studied by i.p. injection of ALs with adjuvant, which does not resemble human atopy, where individuals are exposed to ALs by nasal route. However, the intranasal exposure of mice to ALs does not result in dramatic elevations of TSIgE levels, compared to i.p., injections (Renz et al 1992a,b) and therefore i.p., route was used in this study. One should keep these limitations in mind before extrapolating the findings of this study to human situation.

The syntenic regions corresponding to *Iger-1*, *-2* and *-3* in humans is located on chromosomes 16q/1q, 12q, and 17q respectively (Table 26). It is to be noted that in a recent preliminary report, a genomic interval on chromosome 12q in humans was found linked to TSIgE phenotype (K. Barnes, presentation in AAAA&I Conference,1996), which is consistent with the findings in these mouse strains. However, the precise identification of the gene(s) needs further investigation. However, to date the other two genomic loci, have not been reported in humans to be associated or linked with TSIgE

levels or atopy. Therefore, a rationalized search can be undertaken now to see the relevance of murine *Iger*-1, -2 and -3 loci to human TSIgE regulation.

V. ORIGINAL CONTRIBUTIONS TO KNOWLEDGE:

The specific objectives of the present investigation were to determine the inheritance pattern and the genetic control by non-MHC genes of AL induced TSIgE levels in mouse. This objective has been successfully achieved as outlined below:

1. The inheritance of high TSIgE levels in the F1 hybrids obtained using low and high TSIgE parent mice indicated that the high TSIgE phenotype is a dominant trait over that of low TSIgE levels.

2. The segregation and the distribution analysis of AL-induced persistent TSIgE levels in both the BC1 and F2 intercross mice suggested the genetic control by a major autosomal locus with the possibility of existence of modifier locus or loci. Deducing the genetic control of AL-induced TSIgE levels in this study is the first of its kind in any experimental animal model of atopy.

3. The exclusion of Tcrvb8, Il4 and Ifg as candidate genetic factors for persistent AL-induced TSIgE levels is a unique finding by a genetic approach which has not been undertaken so far in experimental models of atopy.

4. The molecular-genetic exclusion mapping of the loci

controlling AL-induced persistent TSIgE levels led to the identification of a major and two modifier loci, which have been named here as *Iger-1, 2* and *3*. However the genomic intervals on chromosomes-8, 10 and 11, do not contain any of the known genes involved in IgE regulation (according to the gene information available at present). Therefore, it is possible that these loci may represent new genes controlling specifically persistent TSIgE levels, in mouse.

5. Since fine-mapping of the *Iger* loci has been successfully achieved in this study, it should pave the way for positional cloning and characterization of these genes controlling TSIgE levels.

6. In view of the extensive synteny across the genome between mouse and humans, the results from the present study should provide a rational approach to investigate the relevance of homologous genomic intervals in the TSIgE regulation in humans.

VI. PUBLICATIONS RELATED TO THE PRESENT STUDY

A. PAPERS:

1. **G. Venugopal** and S.S. Mohapatra. Genetic control of allergen induced total serum IgE (TSIgE) levels in mice: Mapping of three loci associated with TSIgE levels to chromosomes 8, 10 and 11 (Manuscript in preparation).

2. **G. Venugopal,** Yang M, Luo Z, Salo D, Cheang M and S.S. Mohapatra. Analysis of Tcrvb8, Il4 and Ifg as genetic predisposition factors for atopic IgE response in a murine model. **J. Immunol. 155, 5463-70 (1995).**

3. **G. Venugopal**, Trivedi HN and S.S. Mohapatra. Arbitrary single short primers identify polymorphic DNA markers that distinguish inbred strains of mice. **Biochem. Biophys. Res. Commns. 203, 1: 659-65 (1994).**

4. **G. Venugopal**, Mohapatra S, Salo D and S.S. Mohapatra. Multiple mismatch annealing:Basis for Random Amplified Polymorphic DNA fingerprinting. **Biochem. Biophys. Res. Commns. 197, 3: 1382-87 (1993).**

B. WORKSHOP PRESENTATIONS:

1. **G. Venugopal,** Yang M, Luo Z, Salo D, Cheang M and S.S. Mohapatra. Genetics of IgE response in a murine model: Analysis of Tcrvb8, Il4 and Ifg genes. 9th International Congress of Immunology, San Francisco, **1995.**

2. **G. Venugopal,** Z.Luo and S.S.Mohapatra. Inheritance of IgE immune responsiveness in mouse. AAAA&I conference, New York **(1994).**

C. ABSTRACTS:

1. G. Venugopal and S.S. Mohapatra. Molecular-Genetic analysis of allergen induced total serum IgE levels in a murine model of atopy. Abst.#47, Student's Research Day, Faculty of Medicine, University of Manotoba (1996).

2. **G. Venugopal,** Yang M, Luo Z, Salo D, Cheang M and S.S. Mohapatra. Genetics of IgE response in a murine model: Analysis of Tcrvb8, Il4 and Ifg genes. ABS #1183, 9th International Congress of Immunology, San Francisco, U.S.A. **(1995)**

3. **G. Venugopal,** Z.Luo and S.S.Mohapatra. Inheritance of IgE

immune responsiveness in mouse. **J. Allergy and Clin. Immunol. 178, 151 (1995).**

4. **G. Venugopal** and S.S.Mohapatra. Identification of genetic markers for IgE responsiveness in murine system. **J. Allergy and Clin. Immunol. 93: 252 (1994).**

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