ROLE OF MHC CLASS I GENES AND RELATED IMMUNOLOGIC PARAMETERS IN ETIOPATHOLOGY OF SCHIZOPHRENIA

THESIS SUBMITTED TO THE UNIVERSITY OF NORTH BENGAL FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.) IN SCIENCE

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Dedicated

to

My parents

STATUTORY DECLARATIONS

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Degree	:	Doctor of Philosophy (Ph.D.)
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CERTIFICATE

This is to certify that the thesis entitled "**Role of MHC Class I genes and related immunologic parameters in etiopathology of schizophrenia**" is the original investigative study performed by Mr. Bisu Singh, M.Sc. under our guidance and preceptorship and has not been submitted for a degree or diploma of any University. He has carried out the work during the period 2006-2010 and has fulfilled the requirements of the degree of Doctor of Philosophy in Science (Zoology) of the University of North Bengal.

He is conversant with techniques and literature cited in the dissertation and carried out the work thoroughly. In character and disposition Mr. Bisu Singh is fit to submit the thesis for Ph.D. degree.

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STATEMENT BY THE CANDIDATE

I would like to state that the research work embodied in this thesis entitled "Role of MHC Class I genes and related immunologic parameters in etiopathology of schizophrenia" forms my contribution to the research work carried out under the supervision of Dr. T.K.Chaudhuri, Professor, Department of Zoology, University of North Bengal, Dr N.K.Bera, Associate Professor, Department of Psychiatry, North Bengal Medical College and Hospital. This work has not been submitted for any other degree to this or any other University. Whenever references have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.

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ABBREVIATIONS

ARMS	-	Amplification Refractory Mutation System
BMI	-	Body mass index
BPRS	-	Brief Psychiatric Rating Scale
CD	-	Cluster of differentiation
CNS	-	Central nervous system
CRP	-	C- reactive protein
CT	-	Computed tomography
CTL	-	Cytotoxic T lymphocytes
DALY	-	Disability adjusted life-years
DC	-	Dendritic cells
DSM	-	Diagnostic and Statistical Manual of Mental Disorders
DZ	-	Dizygotic
EEG	-	Electroencephalographic
ELISA	-	Enzyme Linked Immuno Sorbent Assay
GM-CSF	-	Granulocyte-Monocyte Colony Stimulating Factor
HLA	-	Human Leukocyte Antigen
ICD	-	Inernational Classification of Diseases
IFN	-	Interferon
Ig	-	Immunoglobulin
IHW	-	International histocompatibility workshop
IL	-	Interleukin
Ir	-	Immune response (genes)
MHC	-	Major Histocompatibility Complex
MRI	-	Magnetic Resonance Imaging
MZ	-	Monozygotic
NMDA	-	N-methyl-D-asparate
OD	-	Optical density
OPD	-	Outpatient department
P1	-	Forward Primer
P2	-	Reverse Primer
PBS	-	Phosphate Buffer Saline
PCR	-	Polymerase Chain Reaction
PET	-	Positron Emission Tomography
PF	-	Phenotype frequency
PS	-	Paranoid Schizophrenia
RA	-	Rheumatoid arthritis
rpm	-	Revolution per minute
RR	-	Relative Risk
SCID	-	Structured Clinical Interview for DSM-IV
SLE	-	Systemic lupus erythematosis
SPET	-	Scanning Positron Emission Tomography
SSP	-	Sequence Specific Primers
TGF	-	Tumor Growth Factor
Th	-	T helper
TNF	-	Tumor Necrosis Factor
UV	-	Ultraviolet
WHO	-	World Health Organization
YLD	-	Years lived with disability
5-HT	-	5-hydroxytryptamine

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Section - 1

Introduction

1. INTRODUCTION

Schizophrenia is a complex psychiatric disorder and is perhaps the most enigmatic, tragic and devastating amongst all the psychiatric disorders. It strikes normally at a young age and is one of the leading causes of disability among young adults. The disease hinders the normal life of the patients such as attending school, working, having children etc. Schizophrenia is ranked as the ninth leading cause of disability among the people of age group between 15-44 years worldwide and fourth in developed countries (Murray and Lopez, 1996). Apart from its effect on individuals and families, schizophrenia creates a huge economic burden for the society. In India, there are an estimated four million people affected with schizophrenia, with different degrees of impact on some 25 million family members. Despite of this, schizophrenia has yet to receive sufficient recognition as a major health concern to initiate the necessary research support to investigate its causes, treatments and prevention. However, some private foundations have been working to meet these needs and for making the public aware regarding schizophrenia (Ho *et al.*, 2003).

The word "schizophrenia" comes from the Greek roots "schizein" meaning "to split" and "phren" meaning "mind", which translates roughly as "splitting of the mind". The term schizophrenia was first coined by Eugen Bleuler, by which he intended to describe the separation of function between personality, thinking, memory, and perception in the patients. Unfortunately, Bleuler's intended meaning is often lost on the lay public, which tends to equate schizophrenia with multiple personality disorder (Calkins and Lacono, 2003). Although defining a complex disorder like schizophrenia is very difficult more comprehensively it is defined as "a psychotic illness, which is manifested in its acute/active phase by delusions, hallucinations, impaired social functioning, loss of drive, neglect of self-care and disturbance of other mental processes". In order to diagnose an individual as a schizophrenic patient, he/she must demonstrate a six-month period of marginal functioning accompanied by a mixture of psychotic symptoms which are classified according to a Negative and Positive Symptom scale. Negative symptoms are characterized by the loss of motivation, impaired concentration and the inability to express emotions, while positive symptoms include delusions, hallucinations, anxiousness and distorted perceptions of

reality (Greer *et al.*, 2005; Schwartz *et al.*, 2004). Hall-mark delusions (false beliefs) involve the conviction that individuals are conspiring to harm the patient and bizarre beliefs such as that one's thoughts are being broadcasted or controlled by an external force. Auditory hallucinations (false sensory experiences) such as hearing voices are typical, but unusual bodily sensations (e.g., the perception that one is being touched) and visions (e.g., the image of an individual) are also possible. No one individual is likely to have all or even most of these symptoms, thus complicating diagnosis and leading to great heterogeneity in the clinical picture. Depression often accompanies schizophrenia, with approximately 10% of patients committing suicide.

Schizophrenia affects 1% of the world population (Sawa and Synder, 2002). Study suggests that when uniform diagnostic criteria are used to identify cases, the prevalence of schizophrenia varies little across cultures as diverse as the United States, Japan and India (Jablensky *et al.*, 1992). However in some cultures the prevalence varies slightly from the averaged estimate. For instance, African Caribbeans in the United Kingdom have higher rates of schizophrenia than other inhabitants of the European country (Calkins and Lacono, 2003). On the other hand the content of delusions may vary across countries, emphasizing regionally popular themes.

Schizophrenia typically begins during adolescence or early adulthood, with females lagging behind the average age of onset in males by about five years. Although the disorder can begin abruptly, most individuals experience an extended period of impairment characterized by mild symptom expression and a decline in social, educational, or occupational functioning. The course is variable, with a minority of individuals recovering whereas most experience recurrent episodes interspersed with periods of partial remission or a chronic course characterized by incoherence, unwavering delusions and recurring hallucinations. Although the prevalence of schizophrenia in men and women is believed to be the same, men appear to experience a more chronic course. The course and outcome of schizophrenia may be more favorable in developing than developed nations perhaps because the cultures of developing countries are characterized by more intact families and community networks, fewer job related demands and greater acceptance of the unconventional beliefs and behavior characteristic of affected individuals (Calkins and Lacono, 2003).

The precise etiology and pathophysiology of schizophrenia remains unknown. Till to date no agreement regarding the pathophysiology of schizophrenia has been reached among experts. However, several etiological theories have been proposed, including developmental (Horning *et al.*, 2002) or neurodegenerative processes (Lieberman *et al.*,1999), neurotransmitter abnormalities (Garbutt *et al.*, 1983; Aghajanian *et al.*, 2000), viral infection and immune dysfunction or autoimmune mechanisms (Noy *et al.*, 1994; Fontana *et al.*,1980). In addition, there is substantial evidence for a genetic predisposition to schizophrenia (Mowry *et al.*, 2001; Mueser *et al.*, 2004). Nevertheless, various designs of genetic studies also indicate the role of environmental factors because the concordance rate for the monozygotic twins who grew up in a very similar surrounding is far less than 100% (Prescott and Gottesman, 1993).

Diverse immune dysfunctions have been reported in schizophrenia for over three decades (Muller *et al.*, 2004). Immune aberrations possibly raised by viral infections during the pre-, peri- or postnatal phase have thus been described in schizophrenia (Munn, 2000). The presence of immunological abnormalities in schizophrenic patients as in proven autoimmune disorders had led many researchers to suspect that schizophrenia may be due to autoimmune processes (Ganguli *et al.*, 1993) triggered by one or more environmental factors in predisposed subjects (Matthysse and Matthysse, 1978). It has been observed that schizophrenia shares similarities to autoimmune disorders in clinical course, epidemiology and inheritance. In addition, many autoimmune disorders, such as systemic lupus erythematosus (SLE) and graves'disease frequently present with psychosis. Such findings have also added to speculation regarding putative autoimmune mechanisms in schizophrenia, such as antibody mediated activation of dopamine receptors (Printz *et al.*, 1999). It was suggested that viral infections and /or autoimmune reactions against central nervous structures may play an important role in the pathogenesis of the disorder (De Lisi, 1986; Kirch, 1993).

With recent advances in technology and an increased understanding of the immune system, autoimmune theories of schizophrenia have once again become a major focus of research. Although direct evidence of autoimmune mechanisms is still lacking, observed alterations in cellular and humoral immunologic elements in patients with

schizophrenia are similar to those documented in known autoimmune disorders (Ganguli et 1993; Kirch 1993). These include increase (Masserini *et al.*, 1990) or decrease (Nyland *et al.*, 1980;Coffey *et al.*, 1983) lymphocyte population, morphological changes in lymphocytes (Fessel *et al.*,1963), altered levels of CD4+, CD45RA+ T cells, CD8+ T cells (Cazzullo *et al.*,1998), CD5+ B cells (Printz *et al.*, 1999), altered levels of IL-2 (Becker *et al.*, 1990; Barak *et al.*, 1995) IL-6 (Ganguli *et al.*, 1994; Akiyama *et al.*, 1999), IFNγ (Rothermundt *et al.*, 2000; Arolt *et al.*, 2000) increased levels of antiviral antibodies (Kaufmann *et al.*,1983) and association of the particular HLA antigens with schizophrenia. Several workers have reported the elevated level of C-reactive protein (Fan *et al.*, 2006; Dickerson *et al.*, 2007; Singh *et al.*, 2008), in schizophrenic patients, thus it has been hypothesized that some kind of inflammatory process may play role in the etiology of schizophrenia.

Since the HLA system governs the immune responses, HLA genes were implicated in the etiology of schizophrenia. Various research groups have observed the association between the class I HLA genes and schizophrenia viz. HLA-A*01 (Lahdelma *et al.*, 1998), HLA- A*02 (Rudduck *et al.*, 1984), HLA-A*9 (Goudemand *et al.*,1991) HLA-A*23 (Ivany *et al.*,1983)HLA-A*24 (Asaka *et al.*,1981) B*17, B*27 and CW*2 (Rudduck *et al.*,1984). Recently HLA-A*03 have been found to be associated with paranoid schizophrenia among the Bengali population of Siliguri (Debnath *et al.*, 2005). HLA class II antigens is also found to be associated with schizophrenia such as DRB1*04 (Wright *et al.*,1996) HLA DR1(Sasaki *et al.*,1999). The results of HLA study have been largely contradictory in studies where schizophrenia has been regarded as a single uniform disorder but more consistent when the patients have been differentiated into specific subtypes of schizophrenia (Goudemand *et al.*, 1981). In such a study heterogeneity between the four clinical subgroups was found for HLA A*03 and BW*35 (Rudduck *et al.*, 1984a). On the other hand statistically significant ethnicity-based variability in the HLA allele frequency and genotype inheritance pattern has been reported, suggesting that polymorphisms within these HLA genes may be responsible for the ethnic-based difference in schizophrenia. Further, the non agreement of results may be due to the fact that schizophrenia is generally considered as a single disease process. Much current research points to the heterogeneity of schizophrenia (Graver *et al.*, 2003). Pulver (2000) has described schizophrenia as a

syndrome with 'genetic heterogeneity' having susceptibility loci at several different chromosomal regions.

Even after the century old research, still a common agreement has not come regarding the etiopathology of schizophrenia. Most of the research in this aspect had been carried out in the Caucasian population and some Mongolian population. In India very few such studies have been reported so far. To our knowledge till to date the investigation has not been carried out to find out the role of immune system in the etiopathology of schizophrenia in Indian schizophrenic subjects.

The present investigation is mainly focused on the circumstantial evidence of autoimmunity and intends to study the role of immune system in the etiopathology of schizophrenia in the schizophrenic patients of Siliguri and edjoing areas. The study also aims to test the validity of the "autoimmune hypothesis" of schizophrenia in the patients. The parameters of immune system such as HLA Class I genes, Th1 and Th2 cytokines IL-2 and IL-6 , CD4+ and CD8+ cells have been considered to shed the light in the autoimmune basis of schizophrenia. Further, C-reactive protein, an inflammatory marker has also been studied to throw light in the role of inflammation in the manifestation of the disorder. Demographic characteristics of the patients were also studied to investigate the role of environmental factors in the etiopathology of the disorder.

Section - 2

Objectives of The Study

2. OBJECTIVES OF THE STUDY

1) To study the incidence of HLA Class I antigens in the patients of the different subtypes of schizophrenia.

2) To analyse the allelic association (positive and / or negative) of class I HLA antigens to different subtypes of schizophrenia.

3) To investigate whether all the subtypes of schizophrenia share the same etiological mechanisms or they have distinct etiological underpinnings.

4) To investigate the serum level of C-reactive protein in schizophrenic patients.

5) To investigate any alteration in the serum level of some cytokines in the patients.

6) To investigate the ratio of CD4+/CD8+ cells in the schizophrenic patients.

Section - 3

Review of The Literature

3. REVIEW OF THE LITERATURE

3.1 HISTORICAL OVERVIEW OF SCHIZOPHRENIA

The symptoms relating to schizophrenia have been noted since the age of antiquity. The history of schizophrenia can be traced back to documents written by the Pharaonic Egyptians as far back as 2000 B.C. In these texts thought disturbances are mentioned that are commonly seen in schizophrenia. At that time it was thought that these mental disturbances were caused by demons and evil spirits and could be cured by exorcising. Many signs and symptoms of schizophrenia have been described in ancient Greek, Roman, and Chinese scripts. Hindu description of schizophrenia dates back to approximately 1400 BC and can be found in the Atharva Veda. It has been posited that health resulted from a balance between 5 elements (Buthas) and 3 humours (Dosas) and that an imbalance between these various elements might result in madness (Kyziridis, 2005).

In the modern times schizophrenia history is better known and recorded since 1700s. It was during this time that more detailed and accurate descriptions of abnormal mental behavior were recorded. These included changes in a person's speech, gestures and emotions. In 1851 Falvet first described a 'Folie Circulaire' or cyclical madness, and some twenty years later Hecker referred to a 'Hebephrenia', or a silly, undisciplined mind after Hebe, goddess of youth and frivolity. In 1874, Kahlbaum referred to both catatonic and paranoid disorders of the mind. The term catatonia is used to describe a movement disorder characterized by a mannequin-like muscle stiffness associated with unusual postures and a pervading fear. Then in 1878 Emil Kraepelin, perhaps auspiciously, combined these various 'disorders' into a single disease entity which he termed dementia praecox, or 'dementia of early onset' reflecting a decline of cognitive processes. This has been divided by him into four subtypes: (1) simple, marked by slow social decline concomitant with apathy and social withdrawal, (2) paranoid, with its attendant fear and 'persecutory' delusions, (3) hebephrenic and (4) catatonic, characterized by a poverty of movement and expression.

The inevitable inexactitudes of this emerging science continued with the dawn of the 20th Century when in 1908 Eugen Bleuler criticized the use of the term dementia praecox, arguing for an absence of evidence supporting a global dementing process. It was Bleuler who first coined the divisive term 'schizophrenia' in 1911. Bleuler defined schizophrenia with his four "A's", referring to the blunted Affect (diminished emotional response to stimuli), loosening of Associations (by which he meant a disordered pattern of thought, inferring a cognitive deficit), Ambivalence (an apparent inability to make decisions, again suggesting a deficit of the integration and processing of incident and retrieved information) and Autism (a loss of awareness of external events, and a preoccupation with the self and one's own thoughts).

After the publication of his classic textbook "Dementia Praecox, or the Group of Schizophrenias" in 1911, Bleuler's ideas enjoyed increasing acceptance and became an influential description of schizophrenia in most of Europe, England and the United States for decades. Consequently, the influence of Bleuler led to an increasingly broad definition and conceptualization of schizophrenia as psychiatry gathered strength and momentum during the post war years and through the 1950s and 1960s. This phenomenon was particularly apparent in the United States, where concepts such as "latent schizophrenia" and "pseudoneurotic schizophrenia" became popular (Black and Bofelli, 1989). These concepts were reflected in the initial editions of the "Diagnostic and Statistical Manual of Mental Disorders", published by the American Psychiatric Association. The first edition (American Psychiatric Association, 1952) emphasized intrapsychic mechanisms rather than classes of disease, whereas the second edition (American Psychiatric Association, 1968) shifted the emphasis to classification, but without listing specific criteria.

By the late 1960s, a number of factors intervened to introduce a climate of change. Studies of comparative diagnostic practices in the United States, England and other nations alerted American psychiatrists to the fact that their diagnostic habits were out of step. For example, the United States/United Kingdom diagnostic project (Cooper *et al.*, 1972) set out to determine why the prevalence of schizophrenia was greater in New York than London, while the reverse was true for manic depressive illness. The investigators discovered that the same patients received different diagnoses in different countries due to conceptual and theoretical differences between their

respective diagnostic systems. At about the same time, findings were reported from the International Pilot Study of Schizophrenia (WHO, 1975), in which schizophrenia was studied in nine countries. The major finding to emerge was that similar criteria were used in seven of the nine countries, but broader criteria were used in the United States and the Soviet Union.

In the context of these studies, an interest in reliable diagnosis emerged, leading to the development of structured interviews such as the Present State Examination (Wing *et al.*, 1967) and operational diagnostic criteria such as the St. Louis Criteria (Feighner *et al.*, 1972).The Present State Examination helped to introduce the concepts of the German psychiatrist Kurt Schneider (1887-1967) and his emphasis on "first-rank symptoms" to the English-speaking world. These forces helped reshape the concept of schizophrenia into one of a relatively severe psychotic disorder, bringing it closer to the original ideas of Kraepelin, but lacking Kraepelin's emphasis on a longitudinal definition that used course and outcome as a diagnostic guide.

Finally, and perhaps more importantly, the development of effective treatments such as neuroleptics, anti depressants and eventually lithium carbonate made diagnosis an important clinical issue. If a patient with bipolar disorder or major depression were misdiagnosed with schizophrenia because of an excessively broad diagnostic concept, that patient might be deprived of the most appropriate treatment available, potentially condemned to an unnecessarily chronic course of illness and perhaps condemned to needlessly suffer permanent and irreversible medication side effects.

All of these developments led to a reassessment of how schizophrenia and other mental disorders were diagnosed, culminating in the third edition of the "Diagnostic and Statistical Manual of Mental Disorders" (DSM-III)(American Psychiatric Association, 1980), which enumerated specific criteria for all recognized psychiatric disorders. DSM-III and its revision (DSM-III-R) (American Psychiatric Association, 1987) represented a convergence of these various points of view. The criteria included the Kraepelinian emphasis on course through the requirement that the illness be present for at least 6 months, the emphasis on specific delusions and hallucinations thought important by Schneider, and the emphasis on the importance of fundamental

Bleulerian symptoms (thought disorder in the form of associative loosening or incoherence and affective blunting).

The DSM-III and DSM-III-R compromise stirred debate among investigators interested in understanding the pathophysiology and etiology of schizophrenia. New technologies, such as molecular genetics or brain imaging, reemphasized the importance of careful and precise definition of the disease, as has occurred with the introduction of neuroleptics. Geneticists interested in familial patterns of transmission wondered whether the DSM-III and DSM-III-R definitions, with their requirement of florid psychotic symptoms, were too narrow to pick up subclinical cases in family pedigrees. They considered whether the concept of schizophrenia should be expanded to include nonpsychotic forms (e.g., simple and latent schizophrenia and "schizotaxia"), much as Bleuler originally suggested (Tsuang *et al.*, 2000). Studies of the neurobiology of schizophrenia, made possible by brain imaging and postmortem-brain banks, blurred the distinction between schizophrenia and "organic" disorders. Nearly four decades of psychopharmacological treatment of schizophrenia demonstrated that florid psychotic symptoms are probably not the core defining features after all, since crippling negative or deficit symptoms persist and seem fundamental, much as Bleuler observed. These observations have now been given more weight in DSM-IV (American Psychiatric Association, 1994) and DSM-IV-TR (American Psychiatric Association, 2000). Research conducted in the 1980s, as well as field trials conducted specifically for the Task Force on DSM-IV, showed that deficit symptoms can be reliably defined and should be considered as core features of the disorder.

3.2 DIAGNOSTIC CRITERIA FOR SCHIZOPHRENIA

Several sets of operational criteria were developed in the United States during the 1970s to increase the reliability of diagnosis. The St. Louis Criteria developed in 1972 include both longitudinal and cross-sectional criteria designed to identify schizophrenic patients with poor prognosis. The criteria require the exclusion of affective illness, drug abuse, or alcoholism and exclusion of cases of less than 6 months duration. The Research Diagnostic Criteria (RDC)(Spitzer *et al.*, 1978) were introduced later and differ from the St. Louis criteria mainly in emphasis on course of

illness. The St. Louis criteria require only a 2-week history. These two sets of criteria were instrumental in the development of DSM-III in 1980 and DSM-III-R in 1987 and include definitions of schizophrenia requiring both longitudinal and cross-sectional features.

The concept of including both cross-sectional and longitudinal features remains in DSM-IV-TR (Table 1), but more prominence is given to Bleulerian fundamental symptoms, reconceptualized as disorganized or negative symptoms. According to DSM-IV-TR, schizophrenia consists of the presence of characteristic positive or negative symptoms for of at least 1 month (unless successfully treated). The negative symptoms are characterized by the loss of motivation, impaired concentration, and the inability to express emotions, while positive symptoms include delusions, hallucinations, anxiousness and distorted perceptions of reality (Greer *et al.,* 2005; Schwartz *et al.,* 2004).This symptoms are continuous for at least 6 months.

If an illness otherwise meets the criteria but has a duration of at least 1 month but less than 6 months, it is termed a schizophreniform disorder. If it has lasted less than 4 weeks, it may be classified as either a brief psychotic disorder or a psychotic disorder not otherwise specified which is a residual category for psychotic disturbances that cannot be better classified.

Changes made in the diagnostic criteria of schizophrenia from DSM-III to DSM-IV are shown in Table 2. The major changes involve the description of and time requirement for active-phase symptoms, the introduction of the concept of negative symptoms, the deletion of the age at onset criterion, various exclusions, and the expansion of choices for classification of course.

Table 1: DSM-IV-TR diagnostic criteria for schizophrenia.

A.	Characteristic symptoms: Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated):
	(1) delusions
	(2) hallucinations

(3) disorganized speech (e.g., frequent derailment or incoherence)

(4) grossly disorganized or catatonic behaviour

(5) negative symptoms, i.e., affective flattening, alogia or avolition

Note: Only one Criterion A symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behavior or thoughts, or two or more voices conversing with each other.

B. Social/ occupational dysfunction: For a significant portion of the time since the onset of the disturbance, one or more major areas of functioning such as work, interpersonal relations, or self-care are markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, failure to achieve expected level of interpersonal, academic, or occupational achievement).

C. Duration: Continuous signs of the disturbance persist for at least 6 months. This 6- month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences).

D. Schizoaffective and Mood Disorder exclusions: Schizoaffective disorder and mood disorder with psychotic features have been ruled out because either (1) no major depressive, manic, or mixed episodes have occurred concurrently with the active- phase symptoms; or (2) if mood episodes have occurred during active-phase symptoms, their total duration has been brief relative to the duration of the active and residual periods.

E. Substance/general medical condition exclusion: The disturbance is not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication) or a general medical condition.

F. Relationship to a Pervasive Developmental Disorder: If there is a history of autistic disorder or another pervasive developmental disorder, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations are also present for at least a month (or less if successfully treated).

Table 1: DSM-IV-TR diagnostic criteria for schizophrenia

Classification of longitudinal course (can be applied only after at least 1year has elapsed since the initial onset of active-phase symptoms):
Episodic With Interepisode Residual Symptoms (episodes are defined by the reemergence of prominent psychotic symptoms); also specify if: **With Prominent Negative Symptoms**
Episodic With No Interepisode Residual Symptoms
Continuous (prominent psychotic symptoms are present throughout the period of observation); also specify if:
With Prominent Negative Symptoms
Single Episode In Partial Remission; also specify if:
With Prominent Negative Symptoms
Single Episode In Full Remission
Other or Unspecified Pattern

Table 2: Differences among DSM-III, DSM-III-R and DSM-IV/DSM-IV-TR criteria for schizophrenia.

DSM-III	DSM-III-R	DSM-IV/DSM-IV-TR
Characteristic active-phase symptoms	Characteristic active-phase symptoms for 1 week or more	Characteristic active-phase symptoms 1month or more (less if treated)
Deterioration in functioning	Impairment in functioning	Social/occupational dysfunction
Duration at least 6 months or more (including active phase)	Duration at least 6 months or more (including active phase)	Duration at least 6 months or more (including active phase)
Depression and mania ruled out	Schizoaffective disorder and psychotic mood disorder ruled out	Schizoaffective disorder and psychotic mood disorder ruled out
Organic mental disorder and mental retardation ruled out	Organic mental disorder ruled out	Effects of substance or general medical condition ruled out

Onset before age 45	Autistic disorder ruled out	If there is a history of pervasive developmental disorder, prominent delusions, hallucinations must also be present for at least 1 month (or less if successfully treated)
Classification of course Subchronic (>6months but<2years) Chronic (>2 years)	Classification of course Subchronic (>6months but<2years Chronic (>2 years)	Classification of course Episodic with interepisode residual symptoms Episodic with no interepisode residual symptoms
Subchronic with acute exacerbation	Subchronic with acute exacerbation	Continuous
Chronic with acute exacerbation	Chronic with acute exacerbation	Single episode in partial remission
In remission	In remission	Single episode in full remission
	Unspecified	Other or unspecified pattern

3.2.1 DIFFERENTIAL DIAGNOSIS

The major task in differential diagnosis involves separating schizophrenia from scizoaffective disorder, mood disorder with psychotic features, delusional disorder, or a personality disorder. To rule out schizoaffective disorder and psychotic mood disorders, major depressive or manic episodes should have been absent during the active phase, or the mood episode should have been brief relative to the total duration of the psychotic episode. Unlike delusional disorder, schizophrenia is characterized by bizarre delusions and hallucinations are common. Patients with personality disorders, particularly those in the "eccentric" cluster (e.g., schizoid, schizotypal, and paranoid personality) may be indifferent to social relationships and display restricted affect. They also have bizarre ideation and odd speech, or may be suspicious and hypervigilant, but they do not have delusions, hallucinations, or grossly disorganized behavior. Furthermore, patients with schizophrenia may develop other symptoms, such as a profound thought disorder, behavioral disturbances and enduring personality

deterioration. These symptoms are uncharacteristic of the mood disorders, delusional disorder, or the personality disorders.

Other psychiatric disorders also must be ruled out, including schizophreniform disorder, brief psychotic disorder, factitious disorder with psychological symptoms, and malingering. If symptoms persist for more than 6 months, schizophreniform disorder can be ruled out. The history of how the illness presents will help to rule out brief psychotic disorder, since schizophrenia generally has an insidious onset and there are usually no precipitating stressors. Factitious disorder may be difficult to delineate from schizophrenia, especially when the patient is knowledgeable about mental illness or is medically trained, but careful observation should enable the clinician to make the distinction between real or feigned psychosis. Likewise, a malingerer could attempt to simulate schizophrenia, but careful observation will help to distinguish the disorders. With the malingerer, there will be evidence of obvious secondary gain, such as avoiding incarceration, and the history may suggest antisocial personality disorder. The differential diagnosis for schizophrenia is summarized in Table 3.

Table 3: Differential diagnosis of schizophrenia.

Psychiatric illness	General medical illness	Drugs of abuse
Psychotic mood disorders	Temporal lobe epilepsy	Stimulants (e.g., amphetamine, cocaine)
Schizoaffective disorder	Tumor, stroke, brain trauma	Hallucinogens (e.g., phencyclidine
Brief reactive psychosis	Endocrine/metabolic disorders (e.g., porphyria)	Anticholinergics (e.g., belladonna alkaloids)
Schizophreniform disorder	Vitamin deficiency (e.g.,B_{12})	Alcohol withdrawal delirium
Delusional disorder	Infectious (e.g., neurosyphilis)	Barbiturate withdrawal delirium
Induced psychotic disorder	Autoimmune (e.g., systemic lupus	
Panic disorder	erythematosus)	
	Toxic (e.g., heavy metal	
Depersonalization disorder	poisoning)	
Obsessive-compulsive disorder		

Personality disorders (e.g., "eccentric" cluster) Factitious disorder with psychological symptoms Malingering		

3.2.2 CLINICAL FINDINGS

Clinical manifestations of schizophrenia and schizophreniform disorders are diverse and can change over time. Because of their variety, it has been said that to know schizophrenia is to know psychiatry. Whereas many symptoms are obvious, such as hallucinations, other symptoms like affective blunting or incongruity are relatively subtle and can be easily missed by a casual observer.

Various methods have been developed to describe and classify the multiplicity of symptoms in schizophrenia. Traditionally, schizophrenia is considered to be a type of "psychosis," yet the definition of psychosis has been elusive. Older definitions stressed the subjective and internal psychological experience and defined psychosis as "impairment in reality testing." More recently, psychosis has been defined objectively and operationally as the occurrence of hallucinations and delusions. Because schizophrenia is characterized by so many different types of symptoms, clinicians and scientist have tried to simplify the description of the clinical presentation by dividing the symptoms into subgroups. The most widely used subdivision classifies the symptoms as positive and negative.

3.2.2.1 Positive and negative symptoms

The concept of positive and negative symptoms was originally formulated by the British neurologist John Hughlings Jakson (1931). Jackson believed that positive symptoms reflected release phenomenon occurring in more phylogenetically advanced brain regions, due to injury to the brain at a more primitive level. Negative symptoms on the other hand, simply represented a "dissolution," or a loss of brain function. Current definitions of positive and negative symptoms are an amplification of these earlier ideas.

The positive symptoms, including hallucinations, delusions, marked positive formal thought disorder (manifested by marked incoherence, derailment, tangentiality, or illogicality) and bizarre or disorganized behavior reflect a distortion or exaggeration of functions that are normally present. For example, hallucinations are a distortion or exaggeration of the function of the perceptual systems of the brain. The person experiences a perception in the absence of an external stimulus.

Negative symptoms reflect a deficiency of a mental function that is normally present. For example, some patients display alogia (i.e., marked poverty of speech, or poverty of content of speech). Others show affective flattening, anhedonia/associality (i.e., inability of experience pleasure, few social contacts), avolition/apathy (i.e., anergia, impersistence at work or school) and attentional impairment. These negative or deficit symptoms not only are difficult to treat and respond poorly to neuroleptics than positive symptoms, but they are also the most destructive because they render the patient inert and unmotivated. The schizophrenic patient with prominent negative symptoms may be able to raise his or her performance under supervision but cannot maintain it when supervision is withdrawn.

Recent research suggests that the positive and negative symptoms reflect dimensions rather than discrete categories of psychopathology and that there are probably three dimensions rather than two (Andreasen *et al.*, 1995; Arndt *et al.*, 1995; Bilder *et al.*, 1985; Liddle *et al.*, 1989). Positive symptoms can be further divided into dimensions of psychoticism (i.e., delusions and hallucinations) and disorganization (i.e., disorganized speech and behavior and inappropriate affect). Negative (or deficit) symptoms represent a third dimension. The relationship between these three dimensions and their underlying pathophysiology continues to be studied and discussed (Andreasen *et al.*, 1995). The positive and negative symptoms of schizophrenia is summarized in Table 4.

Table 4: Positive and negative symptoms of schizophrenia.

Positive Symptoms of Schizophrenia
Delusions are firmly held erroneous beliefs due to distortions or exaggerations of reasoning and/or misinterpretations of perceptions or experiences. Delusions of

being followed or watched are common, as are beliefs that comments, radio or TV programs, etc., are directing special messages directly to him/her.

Hallucinations are distortions or exaggerations of perception in any of the senses, although auditory hallucinations (?hearing voices? within, distinct from one?s own thoughts) are the most common, followed by visual hallucinations.

Disorganized speech/thinking, also described as ?thought disorder? or ?loosening of associations,? is a key aspect of schizophrenia. Disorganized thinking is usually assessed primarily based on the person?s speech. Therefore, tangential, loosely associated, or incoherent speech severe enough to substantially impair effective communication is used as an indicator of thought disorder by the DSM-IV.

Grossly disorganized behavior includes difficulty in goal-directed behavior (leading to difficulties in activities in daily living), unpredictable agitation or silliness, social disinhibition, or behaviors that are bizarre to onlookers. Their purposelessness distinguishes them from unusual behavior prompted by delusional beliefs.

Catatonic behaviors are characterized by a marked decrease in reaction to the immediate surrounding environment, sometimes taking the form of motionless and apparent unawareness, rigid or bizarre postures, or aimless excess motor activity.

Other symptoms sometimes present in schizophrenia but not often enough to be definitional alone include affect inappropriate to the situation or stimuli, unusual motor behavior (pacing, rocking), depersonalization, derealization, and somatic preoccupations.

Negative Symptoms of Schizophrenia

Affective flattening is the reduction in the range and intensity of emotional expression, including facial expression, voice tone, eye contact, and body language.

Alogia, or poverty of speech, is the lessening of speech fluency and productivity, thought to reflect slowing or blocked thoughts, and often manifested as short, empty replies to questions.

Avolition is the reduction, difficulty, or inability to initiate and persist in goal-directed behavior; it is often mistaken for apparent disinterest. (examples of avolition include: no longer interested in going out and meeting with friends, no longer interested in activities that the person used to show enthusiasm for, no longer interested in much of anything, sitting in the house for many hours a day doing

nothing.)

Anhedonia is the few recreational interests/activities. Little sexual interest/activity. Impaired intimacy/closeness. Few relationship with friends/peers.

Attention is the social inattentiveness. Inattentiveness during testing.

3.3 SUBTYPES OF SCHIZOPHRENIA

According to the presence of the symptoms (Table 5) DSM-IV-TR recognizes five classic subtypes of schizophrenia i.e., paranoid, disorganized, catatonic, undifferentiated and residual. The main purpose of subtyping schizophrenia are to improve predictive validity, to help the clinician select treatments and predict outcome and to help the researcher delineate homogeneous subtypes. However, these goals remain largely unfilled. The reliability and validity of the classic subtypes are poor. Data from the International Pilot Study of Schizophrenia also failed to substantiate their usefulness (Strauss and Carpenter, 1981). Furthermore, many patients seem to fit several of these subtypes during the course of their illness. Hence, other subtyping strategies have been investigated, including paranoid versus nonparanoid forms, deficit versus nondeficit, Kraepelinian versus non-Kraepelinian, and early versus late onset.

Table 5: DSM-IV-TR subtypes of schizophrenia.

Subtype	Criteria	Associated features
Paranoid	Preoccupation with one or more delusions or frequent auditory hallucinations.	Often associated with unfocused, anger, anxiety, argumentativeness, or violence.
	Relative preservation of cognitive functioning and affect.	Stilted, formal quality or extreme intensity of interpersonal interactions may be seen.
	None of the following is prominent: disorganized speech, disorganized or catatonic behavior, flat or inappropriate affect.	
Disorganised	All of the following are prominent: disorganized speech; disorganized	Silly and childlike behavior is common;

	behavior; flat or inappropriate affect. The criteria are not met for catatonic type.	associated with extreme social impairment, poor premorbid functioning and poor long-term functioning.
Catatonic	The clinical picture is dominated by at least two of the following: Motoric immobility as evidenced by catalepsy (including waxy flexibility)or stupor.	Marked psychomotor disturbance present (stupor or agitation), and unusual motor disturbances may be present.
	Excessive motor activity (that is apparently purposeless and not influenced by extreme stimuli)	May need medical supervision due to malnutrition, exhaustion, hyperpyrexia or self-injury.
	Extreme negativism (an apparently motiveless resistance to all instructions or maintenance of a rigid posture against attempts to be moved) or mutism.	Sodium amobarbital interview may be helpful diagnostically.
	Peculiarities of voluntary movement as evidenced by posturing (voluntary assumption of inappropriate or bizarre postures), stereotyped movements, prominent mannerisms or prominent grimacing Echolalia or prominent grimacing	
Undifferentiated	Symptoms meeting criterion A are present, but criteria are not met for paranoid, disorganized or catatonic types.	Probably the most common presentation in clinical practice.
Residual	Absence of prominent delusions, hallucination, disorganized speech and grossly disorganized or catatonic behavior.	Active-phase symptoms (i.e., psychotic symptoms) are not present, but patient still exhibits emotional blunting, eccentric behavior, illogical thinking and mild loosening of associations.
	Continuing evidence of the disturbance, as indicated by the presence of negative symptoms or two or more symptoms listed in criterion A for schizophrenia, present in an attenuated form (e.g., odd beliefs, unusual perceptual experience).	

In validating the paranoid/nonparanoid subtypes, Kendler *et al.*, (1984) found that across all four diagnostic systems (i.e., DSM-III, RDC, ICD-9 and the Tsuang-Winokur criteria [Tsuang and Winokur, 1974]), patients with the paranoid subtype had better short-and long-term outcomes than nonparanoid patients (hebephrenic or undifferentiated subtypes). There was also moderate stability and reliability of subtype diagnosis at follow-up, with the paranoid subtype being the most stable and reliable (Kendler *et al.*, 1985). However, these subtypes did not appear to "breed true" within families (Kendler *et al.*,1988). Carpenter *et al.*, (1988) proposed dividing schizophrenia into deficit and nondeficit forms based on the presence or absence of "primary enduring negative symptoms." A body of literature has accumulated in support of the reliability and validity of this construct (Roy *et al.*, 2001). The deficit syndrome represents a promising strategy for reducing the heterogeneity of schizophrenia, identifying more homogeneous subgroup(s) and enhancing the power of research design at each level of inquiry (Carpenter *et al.*, 1999).

3.4 EPIDEMIOLOGY OF SCHIZOPHRENIA

Schizophrenia presents a challenge to the epidemiologist due to disagreement about the definition of its core features and the breadth of its spectrum. The development of operational criteria such as those in DSM-IV-TR has provided greater specificity for the diagnosis of schizophrenia and has resulted in a more cautious use of the concept. This has led to a reassessment of earlier epidemiological studies that generated rates based on older conceptualizations of schizophrenia. Despite these advances, case identification remains an ongoing problem among epidemiologists. Efforts to standardize the diagnosis have met with some success, such as with the present state examination used in the international pilot study of schizophrenia and the composite interview diagnostic instrument used in the National Comorbidity Study (Kessler *et al.*,1994).

Schizophrenia occurs equally in male and females but it appears earlier in males. The peak ages of onset are 20-28 years for males and 26-32 years for females (Castle *et al.*, 1991). Negative symptoms tend to predominate in men, whereas depressive episodes, paranoid delusions and hallucinations tend to predominate in women. Onset in childhood is much rarer (Kumra *et al.*, 2001), as is onset in middle or old age

(Hassett *et al.*, 2005). The lifetime prevalence of schizophrenia, the proportion of individuals expected to experience the disease at any time in their lives is commonly given at 1%. However, a systematic review of many studies found a lifetime prevalence of 0.55% (Goldner *et al.*, 2002). Table 6 shows the prevalence of schizophrenia in across the various countries. Despite the received wisdom that schizophrenia occurs at similar rates worldwide, its prevalence varies across the world (Jablensky *et al.*, 1992), within countries (Kirkbride *et al.*, 2006) and at the local and neighborhood level (Kirkbride *et al.*, 2007). One particularly stable and replicable finding has been the association between living in an urban environment and schizophrenia diagnosis, even after factors such as drug use, ethnic group and size of social group have been controlled for (Van Os, 2004).

Among the epidemiological studies on psychoses conducted in India, one of the largest has been the longitudinal Study of Functional Psychoses in an Urban Community (SOFPUC) in Chennai carried out by SCARF and the Department of Psychiatry, Madras Medical College. A population of over 100,000 was screened. The age corrected prevalence rate of schizophrenia was 3.87/1000. Other studies in India have reported prevalence of 0.7/1000 to 14.2/1000. However comparability among studies has been limited by variations in population size, geographical area and diagnostic criteria. The SOFPUC study also reported a higher prevalence of the illness in urban slums, in those living alone, with no schooling and unemployed (Rajkumar *et al.*, 1993). This study did not report any difference in male-female incident rates in contrast to other studies, which had reported a higher preponderance among males. The paucity of incidence studies in India could be due to the absence of demarcated catchment areas for health service delivery and lack of case registers and costs involved in conducting community surveys.

Table 6: Prevalence of schizophrenia across the continents and countries (Extrapolated Statistics).

Schizophrenia in America	Extrapolated Prevalence	Population Estimated Used
USA	2,375,154	293,655,405[2]
Canada	262,931	32,507,874[2]
Belize	2,207	272,945[2]

Brazil	1,489,053	184,101,109[2]
Chile	127,987	15,823,957[2]
Colombia	342,219	42,310,775[2]
Guatemala	115,504	14,280,596[2]
Mexico	848,937	104,959,594[2]
Nicaragua	43,350	5,359,759[2]
Paraguay	50,077	6,191,368[2]
Peru	222,784	27,544,305[2]
Puerto Rico	31,527	3,897,960[2]
Venezuela	202,346	25,017,387[2]
Schizophrenia in Africa	**Extrapolated Prevalence**	**Population Estimated Used**
Angola	88,797	10,978,552[2]
Botswana	13,258	1,639,231[2]
Central African Republic	30,270	3,742,482[2]
Chad	77,149	9,538,544[2]
Congo Brazzaville	24,248	2,998,040[2]
Congo kinshasa	471,681	58,317,030[2]
Ethiopia	576,986	71,336,571[2]
Ghana	167,887	20,757,032[2]
Kenya	266,767	32,982,109[2]
Liberia	27,424	3,390,635[2]
Niger	91,886	11,360,538[2]
Nigeria	143,569	12,5750,356[2]
Rwanda	66,636	8,238,673[2]
Senegal	87,774	10,852,147[2]
Sierra leone	47,590	5,883,889[2]
Somalia	67,169	8,304,601[2]
Sudan	316,639	39,148,162[2]
South Africa	359,509	44,448,470[2]
Swaziland	9,457	1,169,241[2]
Tanzania	291,749	36,070,799[2]
Uganda	213,450	26,390,258[2]
Zambia	89,178	11,025,690[2]
Zimbabwe	29,698	1,2671,860[2]

Schizophrenia in Australasia and Southern Pacific	Extrapolated Prevalence	Population Estimated Used
Australia	161,062	19,913,144[2]
New Zealand	32,302	3,993,817[2]
Schizophrenia in Europe	**Extrapolated Prevalence**	**Population Estimated Used**
Austria	66,119	8,174,762[2]
Belgium	83,699	10,348,276[2]
Britain (United Kingdom)	487,483	60,270,708[2]
Czech Republic	10,079	1,0246,178[2]
Denmark	43,784	5,413,392[2]
Finland	42,176	5,214,512[2]
France	488,725	60,424,213[2]
Greece	86,119	10,647,529[2]
Germany	666,669	82,424,609[2]
Iceland	2,377	293,966[2]
Hungary	81,144	10,032,375[2]
Liechtenstein	270	33,436[2]
Ireland	32,106	3,969,558[2]
Italy	469,582	58,057,477[2]
Luxembourg	3,742	462,690[2]
Monaco	261	32,270[2]
Netherlands (Holland)	131,985	16,318,199[2]
Poland	312,418	38,626,349[2]
Portugal	85,121	10,524,145[2]
Spain	325,800	40,280,780[2]
Sweden	72,684	8,986,400[2]
Switzerland	60,264	7,450,867[2]
United Kingdom	487,483	60,270,708[2]
Wales	23,601	2,918,000[2]
Azerbaijan	63,641	7,868,385[2]
Belarus	83,393	10,310,520[2]
Bulgaria	60,807	7,517,973[2]
Estonia	10,851	1,341,664[2]

Georgia	37,965	4,693,892[2]
Kazakhstan	122,485	15,143,704[2]
Latvia	18,653	2,306,306[2]
Lithuania	29,181	3,607,899[2]
Romania	180,816	22,355,551[2]
Russia	1,164,496	143,974,059[2]
Slovakia	43,867	5,423,567[2]
Slovenia	16,269	2,011,473[2]
Tajikistan	56,711	7,011,556[2]
Ukraine	386,068	47,732,079[2]
Uzbekistan	213,613	26,410,416[2]
Schizophrenia in Asia	**Extrapolated Prevalence**	**Population Estimated Used**
Bangladesh	1,143,195	141,340,476[2]
Bhutan	17,677	2,185,569[2]
China	10,505,385	1,298,847,624[2]
East Timor	8,243	1,019,252[2]
Hong Kong	55,445	6,855,125[2]
India	8,614,541	1,065,070,607[2]
Indonesia	1,928,663	238,452,952[2]
Japan	1,029,899	127,333,002[2]
Laos	49,080	6,068,117[2]
Macau	3,601	445,286[2]
Malaysia	190,255	23,522,482[2]
Mongolia	22,253	2,751,314[2]
Philippines	697,543	86,241,697[2]
Papua New Guinea	43,840	5,420,280[2]
Vietnam	668,596	82,662,800[2]
Singapore	35,215	4,353,893[2]
Pakistan	1,287,617	159,196,336[2]
North Korea	183,583	22,697,553[2]
South Korea	390,125	48,233,760[2]
Sri Lanka	160,997	19,905,165[2]
Taiwan	184,006	22,749,838[2]
Thailand	524,647	64,865,523[2]

Afghanistan	230,625	28,513,677[2]
Egypt	615,655	76,117,421[2]
Gaza strip	10,716	1,324,991[2]
Iran	545,981	67,503,205[2]
Iraq	205,236	25,374,691[2]
Israel	50,139	6,199,008[2]
Jordan	45,384	5,611,202[2]
Kuwait	18,259	2,257,549[2]
Lebanon	30,551	3,777,218[2]
Libya	45,549	5,631,585[2]
Saudi Arabia	208,643	25,795,938[2]
Syria	145,724	18,016,874[2]
Turkey	557,230	68,893,918[2]
United Arab Emirates	20,414	2,523,915[2]
West Bank	18,693	2,311,204[2]
Yemen	161,965	20,024,867[2]

3.5 ETIOLOGY OF SCHIZOPHRENIA

In his classic monograph on schizophrenia published early in the 1900s, Eugen Bleuler said, "We do not know what the schizophrenic process actually is." Today a century later, Bleuler's comment is equally true. The problem of etiology in the schizophrenia is complicated by the fact that this group of disorders includes a variety of conditions. It is unlikely that there is a single cause common to all the schizophrenias. One would hardly expect, on the basis of the symptom picture, to find the same causative factors responsible for such widely divergent clinical types of schizophrenia.

While the reliability of the diagnosis introduces difficulties in measuring the relative effect of genes and environment, evidence suggests that genetic and environmental factors can act in combination for the causation of schizophrenia (Harrison and Owen, 2003). Evidence suggests schizophrenia has a significant heritable component but that onset is significantly influenced by environmental factors or stressors (Day *et al.*, 1987). The various theories of causations of schizophrenia are mentioned below.

3.5.1 GENETIC

Twin, family and adoption studies have suggested that genetics play a major role in the transmission of schizophrenia. Irwing Gottesman compiled over 40 studies in order to work out the risks of developing schizophrenia among the people with different familial relationships to the schizophrenic person. Two classes of relatives have especially high risks of developing schizophrenia. These are the offspring of two schizophrenic parents and a monozygotic (MZ) co-twin (Fig.1). Apparently, people who share the greatest number of genes with the people who have schizophrenia, have an increased risk of developing schizophrenia themselves.

Gottesman and Shields reviewed the results of 5 twin studies looking for concordance rates for schizophrenia (Gottesman and Shields, 1976). It was found that in MZ twins there was a concordance rate of 35-58% compared with dizygotic (DZ) twin rates that ranged from 9-26%. They also found a concordance rate in MZ twins of 75-91% when the sample was restricted to the most severe form of schizophrenia (Gottesman and Shields, 1982). The milder forms of schizophrenia had concordance rates of 17-33% suggesting that there may be greater genetic loading with severe forms of schizophrenia. The twin studies have all assumed that the shared environmental effects for MZ and DZ twins are equal which may be incorrect.

Environmental factors is thought to influence the development of schizophrenia. On the other hand adoption studies support the genetic theory of transmission. In 1994, a study looked at schizophrenia in the biological and adoptive relatives of schizophrenic adoptees and compared this to a demographically matched group of control adoptees (Kety *et al.*, 1994). In the sample of adoptees with chronic schizophrenia, the disorder was found exclusively in their biological relatives and not their adoptive relatives. The prevalence of the disorder was 10 times higher in the biological relatives of the schizophrenic adoptees than in the biological relatives of the control group. These studies make a clear case for the involvement of genetics in schizophrenia.

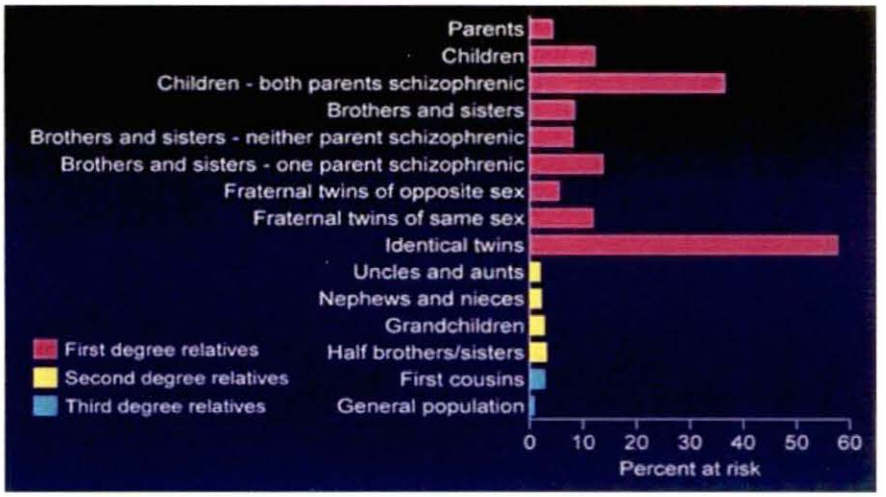


Figure 1: Risk of developing schizophrenia in percentage among relatives of schizophrenic patients.

3.5.1.1 Modes of Transmission

Since the concordance rate among MZ twins is not 100% and people can apparently carry the genotype for schizophrenia without ever developing the disease, there probably is not a single dominant gene for schizophrenia.

One model of transmission that has been suggested is the single gene model with incomplete penetrance (O'Rourke *et al.*,1982). Another model of transmission is the polygenic model which suggests that the 'liability to develop the disorder' is continuously distributed within the population (Bebbington and McGuffin, 1988). Only those individuals whose liability exceeds a certain threshold show symptoms of the disorder. This model is appealing because it could explain why concordance in twins increases with severity of illness and why the risk of schizophrenia increases with the number of relatives affected. Resolving the mode of inheritance of schizophrenia is complicated. Studies using genetic models have not been able to exclude either the single gene or polygenic models. This is most probably due to the problems in defining the schizophrenic phenotype.

3.5.1.2 Schizophrenia Loci

Genetic marker studies can greatly improve the prospect of detecting a major gene effect. A study published in 1998, conducted a genome wide search for evidence of

loci linked to schizophrenia (Shaw *et al.*, 1998). Genetic maps were constructed for each chromosome using genotype data. The genome wide search did not find evidence for a major genetic loci for schizophrenia but, it did find 12 chromosomes that had one statistically significant region at a 5% level (Shaw *et al.*, 1998; Nolen-Hoeksema *et al.*, 1998; Gottesman and Shields, 1976; Gottesman and Shields, 1982; Bebbington and McGuffin, 1988; Bogerts, 1993; Bogerts *et al.*,1985; Harrison, 1999; Crow, 1997; Powchik, 1998; Verdoux, 1997) and 2 of these chromosomes had statistical significance to a 1% level (Harrison, 1999; Powchik, 1998).

From these results, it seems unlikely that one major locus exists for schizophrenia. It could be that schizophrenia belongs to a class of complex disorders that have a genetic predisposition. This could be due to more than one gene that could produce illnesses independently in different families or, genes that act together to cause illnesses in susceptible people.

3.5.2 NEUROPATHOLOGY

A number of brain-imaging and post-mortem studies have shown that abnormal brain morphology and physiology appear to be involved in the development of schizophrenia. Figure 2 shows the specific areas of the brain involved with schizophrenia.

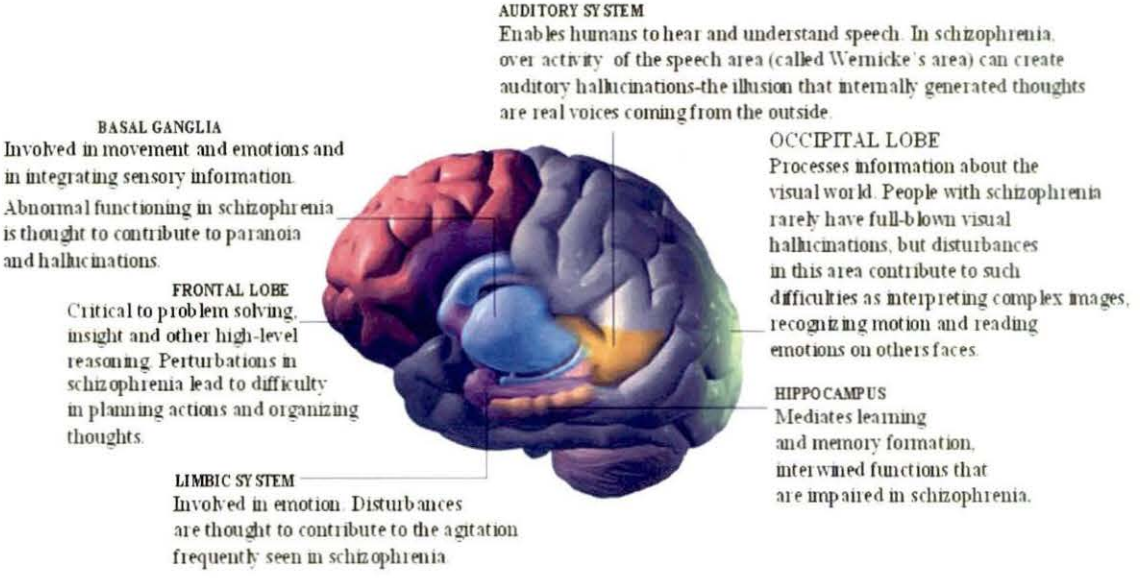


Figure 2: Brain structures involved in schizophrenia.

3.5.2.1 Limbic system

Limbic and paralimbic regions are highly organised regions of the brain and are involved in association and integration functions. If there is a structural and functional deficit in this region it will cause associative and integrative problem, as are experienced by schizophrenic patients. These problem leads to distorted interpretations of reality. The hippocampus and amygdala are the key regions in sensory interpretation, processing and comparing past and present experiences. They also control the basic drives and emotions that are generated in the neuronal networks of the septum hypothalamus complex (Harrison,1999). In addition to disturbed sensory information processing, limbic pathology could explain the dyscontrol syndrome of basic drives and emotions that are seen in schizophrenic patients. There is a particularly strong association between the left temporolimbic pathology and the positive symptoms of schizophrenia (Crow, 1997).Therefore it appears that the limbic system pathology is involved in the pathology of schizophrenia.

3.5.2.2 Neurodevelopment

The neurodevelopmental hypothesis states that the origins of schizophrenia are in abnormal brain development and that the pathology originates in the middle stage of intrauterine life (Murray, 1987). An earlier timing for the pathology of schizophrenia can be ruled out since abnormalities in the structure of the cerebral cortex would be expected if neurogenesis were affected. The absence of gliosis can be taken to mean that the changes must have occurred prior to the third trimester (Harrison, 1999). However, this hypothesis is weak because (a) gliosis is difficult to recognise and (b) the cytoarchitectural changes that have been found have not been proven to be a feature of schizophrenia (Arnold, 1996). Overall however, the cytoachitectural abnormalities and the lack of gliosis appear to be indicative of neurodevelopmental problems rather than a neurodegenerative process.

The neurodevelopmental hypothesis can only account for a minority of cases of schizophrenia. Most of the evidence in support of this theory is circumstantial, but the neuropathological findings in the brains of schizophrenics fit in well with this theory.

Another way of studying the etiology of schizophrenia is to use neuropathology to investigate cortical architecture. Abnormal architectural arrangements of single nerve cells, cell clusters or cortical layers are strong indicators of disturbed early brain development. Abnormal cell clusters have been found, more frequently in the left hemisphere of schizophrenic patients, but these abnormalities are not as extensive as in other disorders of cortical development like developmental dyslexia (Bogerts, 1993).

3.5.3 BRAIN ASYMMETRY

Studies of cerebral asymmetry are also used to study the etiology of schizophrenia. In the normal brain, there is a structural asymmetry which includes larger right frontal and temporal lobes. Several CT and MRI studies suggest that in schizophrenia, the normal structural asymmetry is absent (Fig.3), and left temporal horn and left ventricular enlargement have been reported (Fig.4 and 5) (Bogerts,1993; Crow, 1989).

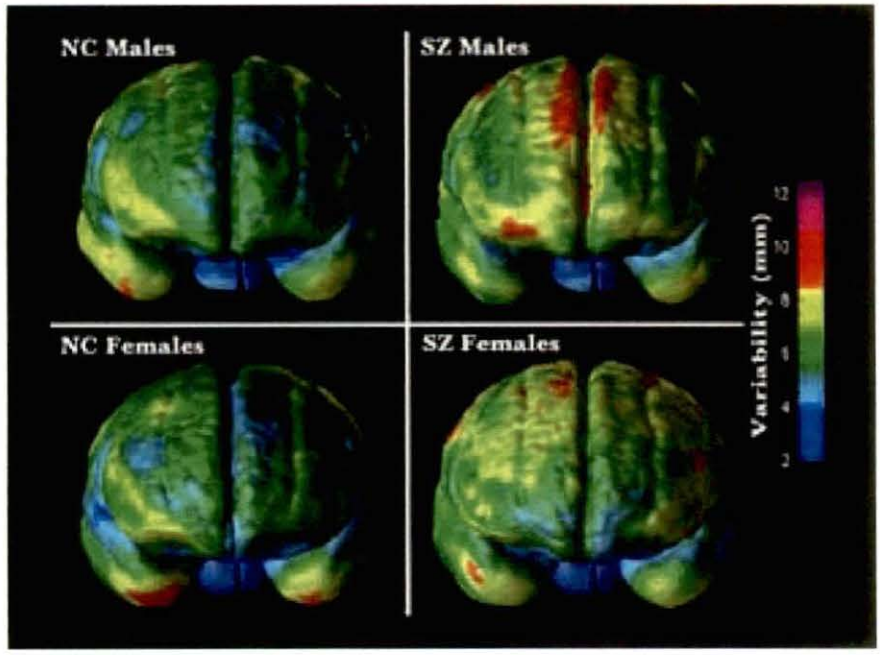


Figure 3: Frontal composite variability and cortical surface variability maps of normal and schizophrenia brains by gender.

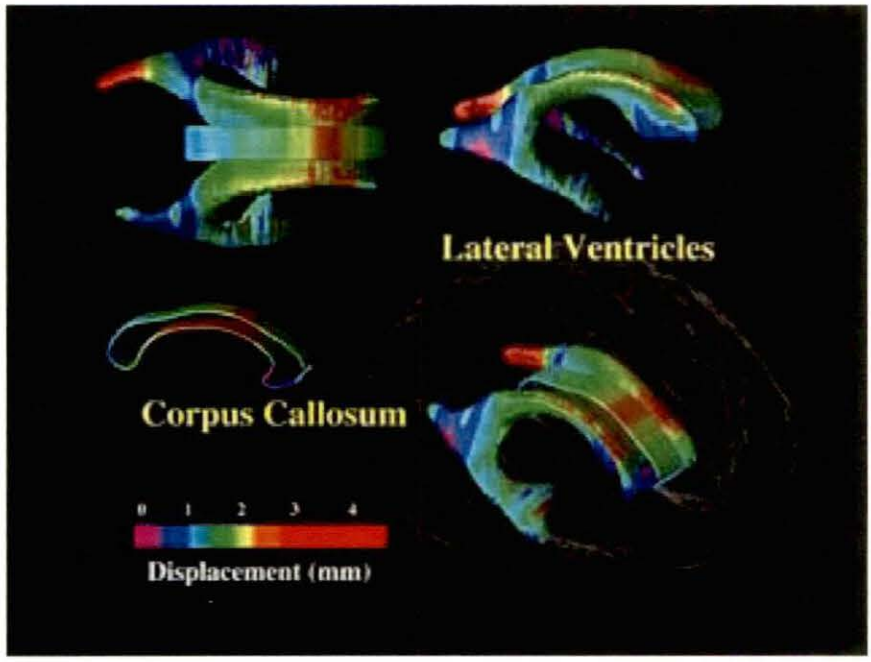


Figure 4: 3D average surface representation and variability maps of the lateral ventricles.

Variability maps are similar in both groups with highest variability in the posterior horns (NC = normal controls, SZ = schizophrenic patients). Increases in LH ventricle length and volume were determined. The color bar encodes the root mean square magnitude of variability in millimeters.

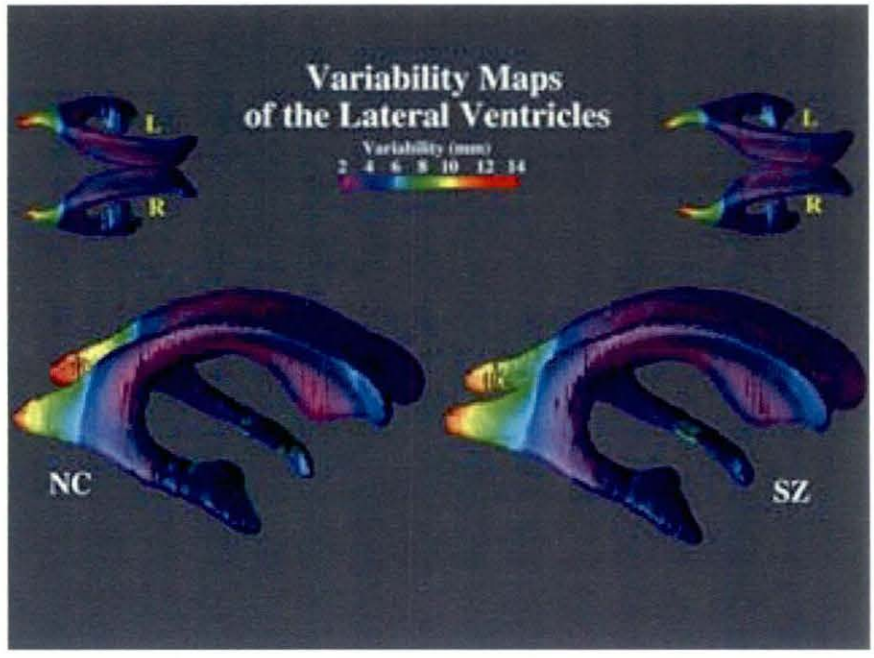


Figure 5: Displacements of the lateral ventricles and corpus callosum.

Displacement maps show the magnitude of displacement (mm) between schizophrenic patients and normal controls as represented by the color bar for the lateral ventricles and corpus callosum. A significant vertical displacement of the lateral ventricles in schizophrenic patients reflects a bilateral increase in ventricular volume, and corresponds to the displacement of the corpus callosum.

Some facts have been established about the neuropathology of schizophrenia. A 2006 meta-analysis of MRI studies found that whole brain and hippocampal volume are reduced and the ventricular volume is increased in schizophrenic patients with a first psychotic episode relative to healthy controls. The average volumetric changes in these studies are however close to the limit of detection by MRI methods, so it remains to be determined whether schizophrenia is a neurodegenerative process that begins at about the time of symptom onset, or whether it is better characterized as a neurodevelopmental process that produces abnormal brain volumes at an early age (Steen *et al.*, 2006).

A 2009 meta-analysis of diffusion tensor imaging studies identified two consistent locations of fractional anisotropy reduction in schizophrenia. One region, in the left frontal lobe, is traversed by white matter tracts interconnecting the frontal lobe, thalamus and cingulate gyrus. The second region in the temporal lobe, is traversed by white matter tracts interconnecting the frontal lobe, insula, hippocampus–amygdala, temporal and occipital lobe. It is suggested that two networks of white matter tracts may be affected in schizophrenia, with the potential for "disconnection" of the gray matter regions which they link (Ellison and Bullmore, 2009). During MRI studies, greater connectivity in the brain's default network and task-positive network has been observed in schizophrenic patients and may reflect excessive orientation of attention to introspection and to extrospection respectively. The greater anti-correlation between the two networks suggests excessive rivalry between the networks (Broyd *et al.*, 2008).

3.5.4 OBSTETRIC COMPLICATIONS

Many epidemiological studies have observed an association between obstetric complications during intrauterine life and schizophrenia. Dalman (1999) studied sets of risk factors representing three different etiological mechanisms that could lead to schizophrenia. These were (i) malnutrition during fetal life, (ii)extreme prematurity and (iii) hypoxia and ischemia. Malnutrition during fetal life could lead to a reduction in the supply of nutrients, such as oxygen, iodine, glucose and iron, which could impair development of the central nervous system (CNS). This may contribute to the development of schizophrenia. The study supports the theory of an association between obstetric complications and schizophrenia. There was evidence of increased

risk associated with all three etiological mechanisms. Pre-eclampsia was the strongest individual risk factor. Some of the factors that were looked at may not have been good indicators of the conditions they were defined as representing (i.e. small for gestational age to indicate malnutrition) therefore further studies need to be undertaken in this field.

Other researchers have also examined the relationship between obstetric complications and adult ventricular size. It has been found that obstetric complications appear to be predictive of increased ventricular size in adults, particularly in schizophrenia (Dalman, 1999). Other studies have reported earlier onset of schizophrenia in patients with a history of obstetric complications (Verdoux, 1997).

Obstetric insults do not equate with cerebral damage. Several variables probably interact in order for obstetric insults to lead to schizophrenia. These would include the site of any lesions, the timing of the injury and the presence of any genetic predisposition to schizophrenia.

3.5.5 VIRAL INFECTION

Evidence for the involvement of infectious diseases in the etiology of schizophrenia first came from the 'season of birth effect'. People born during the winter months appear to have a higher risk of developing schizophrenia. This is thought to be due to exposure to viruses during development. Epidemiological studies have shown high rates of schizophrenia among people whose mothers were exposed to the influenza virus while pregnant (Kirch,1993). The second trimester is a crucial period for the development of the CNS of the fetus. Disruption of brain development in this phase could cause the major structural deficits that are found in the brains of some people with schizophrenia. The mechanism of how a viral infection could lead to schizophrenia is unknown.

3.5.6 BRAIN DEVELOPMENT

There is a long latent period between early cerebral insults and the appearance of schizophrenia. The explanation for this could be due to the fact that brain

development continues throughout childhood and adolescence. Myelination continues into adolescence, and there is some evidence that the deleterious effects of damaged neurones may not become apparent until they myelinate (Bebbington and McGuffin, 1988). It has been suggested that synaptic elimination in adolescence may underlie the emergence of psychotic symptoms. Hormonal and sexual maturity during adolescence has also been thought to contribute to the onset of schizophrenia, but very few studies have been done in this respect.

All of these theories that tried to explain the long latency between cerebral insults and the appearance of schizophrenia, assumed that the structural abnormalities seen in the brains of schizophrenic people (Fig.6) can be regarded as 'vulnerability markers' which may lead to the development of schizophrenia during stress and the vulnerable time between puberty and old age (Harrison, 1999).

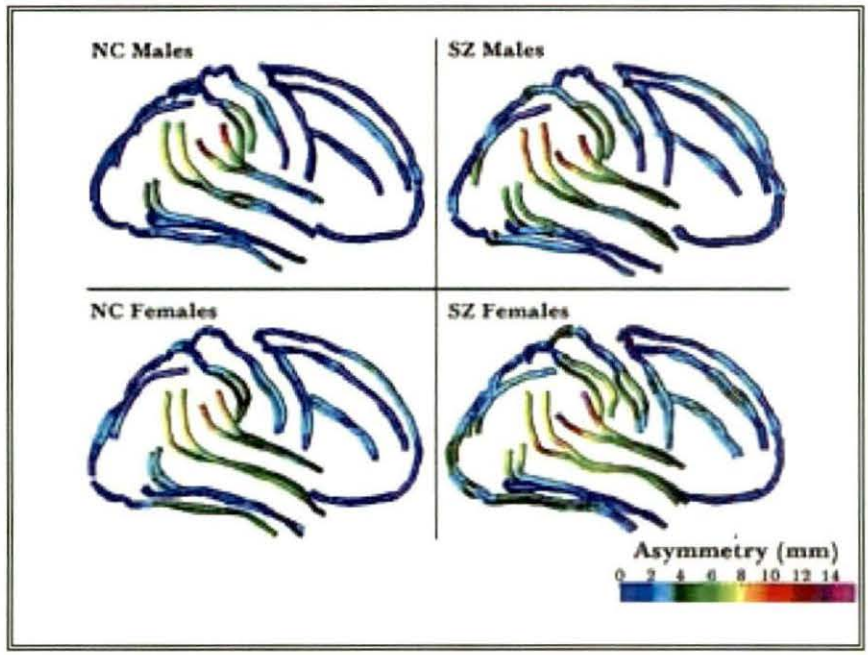


Figure 6: Asymmetry maps

Asymmetry maps were created in each group as defined by Sex and Diagnosis (NC = normal controls, SZ = schizophrenic patients). Sulcal mesh averages for each hemisphere were subtracted from a reflected version of the same structure in the other hemisphere to create displacement vectors. These maps represent in color the magnitude of average asymmetry in sulcal anatomy between the two hemispheres.

3.5.7 NEUROCHEMISTRY

The dysfunction of several neurotransmitter systems like dopamine, 5-hydroxytryptamine (5-HT) and glutamate are thought to play a part in schizophrenia.

3.5.7.1 Dopamine

Studies using neuropsychological tests and brain imaging technologies such as MRI and PET (Fig.7) to examine functional differences in brain activity have shown that differences seem to most commonly occur in the frontal lobes, hippocampus and temporal lobes (Kircher *et al.*, 2005). These differences have been linked to the neurocognitive deficits often associated with schizophrenia (Green, 2006).

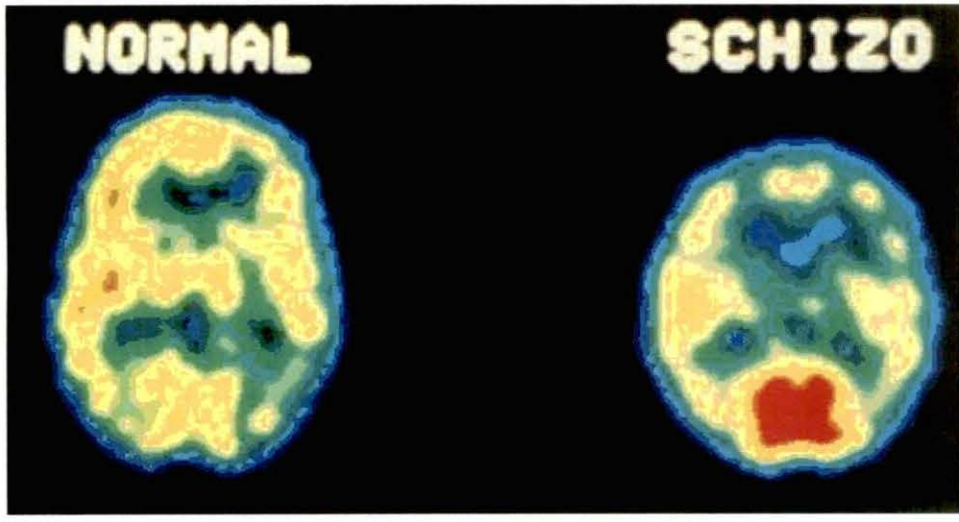


Figure 7: Positron Emission Tomography (PET) brain scans comparing a normal brain (left) with the brain of a schizophrenic (right).

Particular focus has been placed upon the function of dopamine in the mesolimbic pathway of the brain. This focus largely resulted from the accidental finding that a drug group which blocks dopamine function, known as the phenothiazines, could reduce psychotic symptoms. It is also supported by the fact that amphetamines which trigger the release of dopamine may exacerbate the psychotic symptoms in schizophrenia (Laruelle *et al.*, 1996). An influential theory, known as the Dopamine hypothesis of schizophrenia, proposed that excess activation of D2 receptors was the cause of the positive symptoms of schizophrenia. Although postulated for about 20

years based on the D2 blockade effect common to all antipsychotics, it was not until the mid-1990s that PET and SPET imaging studies provided supporting evidence. This theory is now thought to be overly simplistic as a complete explanation, partly because newer antipsychotic medication (called atypical antipsychotic medication) can be equally effective as older medication (called typical antipsychotic medication), but also affects serotonin function and may have slightly less of a dopamine blocking effect (Jones and Pilowsky, 2002).

3.5.7.2 5-hydroxytryptamine

5-hydroxytryptamine (5-HT) is thought to be involved in schizophrenia because the hallucinogen LSD is a 5-HT agonist. It has been found that in schizophrenia, there is a reduced number of 5-HT2A receptors and an increase in the number of 5-HT1A receptors in the frontal cortex (Harrison, 1999). Both of these changes were seen in the post-mortems of unmedicated patients. These changes were not seen in PET scans of younger unmedicated patients suggesting that these abnormalities may emerge during the course of the illness. Several hypothesis have been offered in order to explain the involvement of 5-HT in schizophrenia including, alterations in the trophic role of 5-HT in neurodevelopment and impaired interactions between 5-HT and dopamine.

3.5.7.3 Glutamate

Interest has also focused on the neurotransmitter glutamate and the reduced function of the N-methyl-D-asparate (NMDA) glutamate receptor in schizophrenia. The abnormally low levels of glutamate receptors found in the postmortem brain of schizophrenic patients (Konradi and Heckers, 2003). The discovery that the glutamate blocking drugs such as phencyclidine and ketamine which can mimic the symptoms and cognitive problems associated with the schizophrenic condition has added to the focus on glutamate (Lahti *et al.*, 2001). The reduced glutamate function is linked to poor performance on tests requiring frontal lobe and hippocampal function and that glutamate can affect dopamine function. This observation have suggested an important mediating (and possibly causal) role of glutamate pathways in schizophrenia (Coyle *et al.*, 2003). However other studies have observed that

glutamatergic medication failed to reduce the positive symptoms of schizophrenia (Tuominen *et al.*, 2005).

Clinically, schizophrenia is heterogeneous and this may point to heterogeneous etiology. It seems that genetics, neurodevelopmental problems, neurochemistry and abnormal connectivity, as well as psychosocial stressors probably all contribute to developing the typical clinical pictures of schizophrenia.

3.5.8 RISK FACTORS FOR SCHIZOPHRENIA

Although the twin studies have revealed the genetic predisposition to schizophrenia, the environmental factors also plays a vital role in the manifestation of the disorder. Figure 8 shows some of the schizophrenia environmental risk factors and odd ratio for each of the factors.

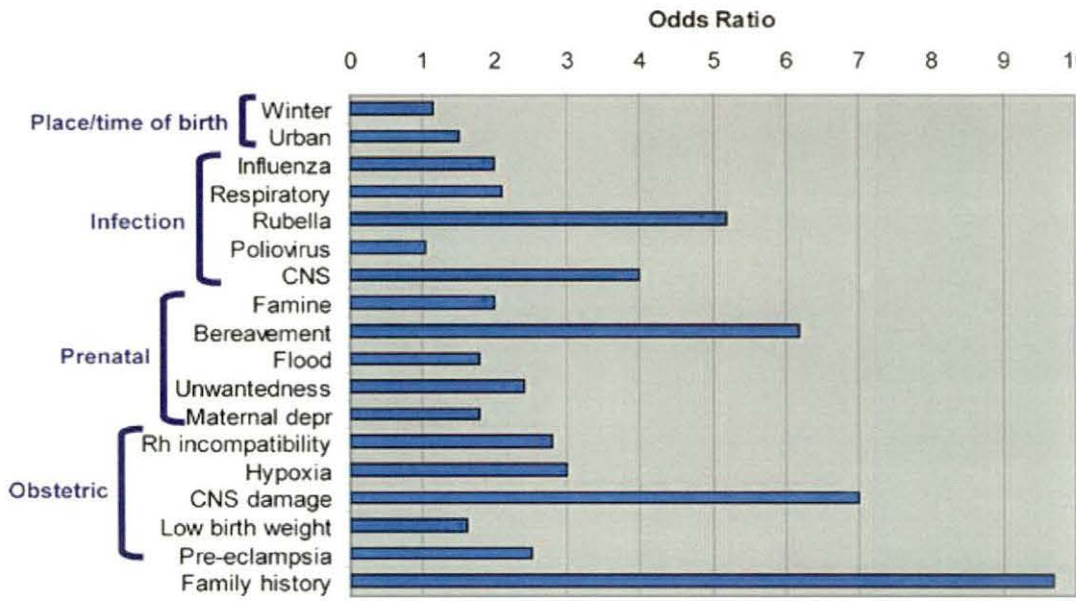


Figure 8: The risk factors for developing schizophrenia.

3.5.8.1 Season of birth

Over 40 studies have shown that individuals who later develop schizophrenia have a 5% to 15% excess of winter and spring births (Boyd *et al.*, 1986; Bradbury and Miller, 1985). Experiments in animals have shown that viruses are more likely to infect the central nervous system in conditions of cold. A time series analysis of New

York State data also reported a significant relationship between the seasonal birth patterns of schizophrenia and still births (Torrey *et al.*, 1993).

3.5.8.2 Urban birth

Two studies have shown that individuals who are born (Takei *et al.*, 1992) or who are raised (Lewis *et al.*, 1992) in cities have an increased risk for developing schizophrenia compared with those born or raised in rural areas. This is consistent with studies of psychiatric hospitalization for serious mental illnesses carried out between 1880 and 1962 that showed higher hospitalization rates for states with more urbanized populations (Torrey and Bowler, 1990).

3.5.8.3 Having older siblings

Sham *et al.* (1993) using data from a Swedish family study, reported that younger children in a family had a significantly increased risk of later developing schizophrenia if their siblings were 3 to 4 years older at the time the younger children were in utero. The researchers suggested explanation for this phenomenon was that older children are a source of viral infections, which they may transmit to their pregnant mothers, and these infections in turn may cause schizophrenia in the offspring.

3.5.8.4 Famine during pregnancy

Susser and Lin analyzed the incidence of schizophrenia among the offspring of women who were pregnant during the 1944 to 1945 war-induced severe famine in western Holland (Susser and Lin, 1992). They reported a statistically significant increase in schizophrenia among offspring who had been in the first trimester of development during the famine. Their original report found the increase for female offspring only, but subsequently research found it for both sexes (Susser and Lin, 1994). During the famine the researchers reported that "the strangest dishes were eaten," including cats and dogs. In addition to resulting in decreased intake of nutrients, famine conditions depress immune function and increase the spread of infectious diseases.

3.5.8.5 Household crowding

A study of psychiatric hospitalization rates for serious mental illnesses was carried out by Schweitzer and Su (Schweitzer and Su, 1977) in Brooklyn, New York. They utilized measures of persons per acre, buildings per acre, persons per household, and persons per room and concluded that "measures of household and family contact were found to be significantly correlated to rates of hospital utilization. If density does produce mental illness its likely mechanism of action will be routed through household contact." Similarly, King *et al.* (King *et al.*, 1982) in Northern Ireland found that prescriptions for antipsychotic medication were more frequent in areas with household crowding (persons per room). Household crowding was also common in the areas of northern Sweden (Book, 1978) and western Ireland (Torrey *et al.*, 1984) that had high reported schizophrenia prevalence rates. However, studies done to ascertain possible adult transmission of schizophrenia among siblings (Crow and Done,1986) or from psychiatric patients to psychiatric nurses (Cooper and King, 1987) have been negative. Therefore the household crowding is more likely to exert its effect in childhood than in adulthood.

3.5.8.6 Lower socioeconomic status

Studies of large cities have consistently found the prevalence rate of schizophrenia to be highest in the lowest socioeconomic class (Kohn, 1968). At least part of the explanation for this finding is that pre-schizophrenic individuals tend to drift downwards socio-economically. Other than this, it is unclear whether low socio-economical status per se is a risk factor for serious mental illnesses, or whether the correlation is due to urban birth and /or household crowding that often coexists with lower socioeconomic status.

3.5.8.7 Regional differences

Schizophrenia appears to be comparatively rare in most tropical countries and to increase in prevalence as one moves away from the equator, similar to the pattern seen in multiple sclerosis (Torrey, 1980). Areas of comparatively high prevalence have been described in diverse places such as northern Sweden (Book,1978), western

Ireland (Torrey *et al.*, 1984; Youssef *et al.*, 1991), western Croatia (Crocetti *et al.*, 1971) and some islands in Micronesia (Hezel and Wylie, 1992) and among West Indian immigrants in England (Harrison *et al.*, 1988; Wessely *et al.*, 1991). Schizophrenia prevalence rates vary from a high of 17.0 per 1,000 persons in northern Sweden (Book, 1978) to a low of 1.1 per 1,000 individuals among the rural Hutterites in the United States (Eaton and Weil, 1955). Although most studies report a prevalence in a range of 2 to 5 per 1,000 persons (Torrey, 1987). In addition to these regional differences in prevalence, a study in Ireland reported statistically significant space-time clusters of births of individuals who later developed schizophrenia (Youssef *et al.*, 1994).

3.5.8.8 Age and gender

Recent findings indicate a male excess in first-episode schizophrenia (Murray and Van Os, 1998), especially in populations with onset age 35 years (Iacono and Beiser, 1992; Jablensky, 1986; Jones *et al.*, 1998). It has been consistently reported that compared to females, males have a younger age of onset (Murray and Van Os, 1998) and are younger at the time of their first hospital admission. Larsen *et al.* (1996) also found, in a sample of 43 first-episode patients in Norway that the duration of untreated psychosis was significantly longer in males than in females, with 61% of males having duration longer than 1 year and 80% of females having duration shorter than 1 year. One variable, for example, that could contribute to the differences in age and gender patterns, at least in part, is a differential distribution of familial schizophrenia among the sample that have been studied. That is, some studies have shown that the gender difference in age of onset is not observed in familial schizophrenia but is more often found in "sporadic" cases (De Lisi *et al.*, 1994). Further examination of age and gender effects is important because differences in the timing of onset by age and/or gender might point to potential biologic clues about the etiology of schizophrenia.

3.6 SCHIZOPHRENIA AND AUTOIMMUNITY

The involvement of autoimmune process in schizophrenia is not a new concept and was first propagated by the German neuropsychiatrist Lehmann Facius in 1937 and

further popularized by Burch in the early 1960s (Burch, 1964). After analyzing the age-specific and sex-specific incidence rates and prevalence of several conditions presumed to be autoimmune in origin, Burch concluded that schizophrenia may also have an autoimmune basis. His conclusion was further strengthen by the age specific onset, sex differences and relapsing clinical course which matched well with disease such as rheumatoid arthritis. The autoimmune hypothesis was also strengthened by the finding of increased autoimmune disease in relatives of schizophrenic patients (Gilvarry *et al.*, 1996) and the inverse relationship of schizophrenia with rheumatoid arthritis (RA)(Gorwood *et al.*, 2004). While this latter association is somewhat counterintuitive, it has been hypothesized that they share a common immune etiology and that once an individual is affected by one of the diseases then they become relatively immune to the other (Torrey and Yolken, 2001). More specifically, several explanations have been proposed to explain this "negative association" at the immune system level. These explanations include factors of prostaglandin synthesis, T- and B-lymphocytes activity, serum interleukin receptor concentrations, IGF-II levels and HLA polymorphism differences (Gorwood *et al.*, 2004). The case for a dysfunction of the immunological system in schizophrenia has also been strengthened by the observation of abnormal lymphocytes in peripheral blood and bone marrow of schizophrenic patients and family members. More specifically, such lymphocytes have been described as having indented or lobulated nuclei and strong basophilic cytoplasm with perinuclear clear zones containing small vacuoles and lamellar structures (Fessel *et al.*, 1965; Hirata-Hibi and Fessel, 1964).

Witebsky and colleagues (Witebsky *et al.*, 1957) proposed criteria that could be used to determine whether a disease is actually autoimmune in origin, and these criteria were more recently refined by Rose and Bona. The criteria propose several levels of evidence such as (1) direct evidence, that is transmissibility by lymphoid cells or antibody of the characteristic lesions of the disease from human to human or human to animal or reproduction of the functional defects characteristic of the disease *in vitro*, (2) indirect evidence, that is reproduction of the autoimmune disease in experimental animals or isolation of autoantibodies or autoreactive T cells from the target organ, (3) circumstantial evidence, that is the presence of markers that are descriptive of autoimmune disease. The evidence provided in the schizophrenia literature for each level of the criteria has been described below.

3.6.1 DIRECT EVIDENCE

The most direct evidence for an autoimmune etiology of a disease is that direct transfer of T cells or antibody from a diseased to a healthy individual can induce the characteristic lesions of the disease. In the 1960s Health *et al.* (Heath *et al.*, 1967) isolated a protein that they termed 'taraxein' from the sera of actively psychotic people with schizophrenia. Taraxein was later identified as an immunoglobulin, but its specificity was not determined. When administered intravenously in monkeys, taraxein caused electroencephalographic (EEG) changes in the monkeys similar to those observed in people with schizophrenia. In addition, when taraxein was injected intravenously into healthy human volunteers it resulted in similar EEG changes and the induction of psychotic symptoms comparable to those observed in the active psychosis stage of schizophrenia.

3.6.2 INDIRECT EVIDENCE

In general, indirect evidence of autoimmunity includes such observations as the induction of autoimmunity in an animal model or the finding of autoimmune cells or antibodies in the target organ. No appropriate experiment to investigate this aspect of autoimmunity has been carried out for schizophrenia. Probably the best indirect evidence for an autoimmune basis for schizophrenia comes from studies examining systemic lupus erythematosus (SLE), a known autoimmune disease characterized by the presence of autoantibodies against double-stranded DNA. Between 14% and 75% of patients with SLE are estimated to experience neuropsychiatric symptoms, including mood and behavioural disturbances and psychotic symptoms (Kozora *et al.*, 1996; Hanly and Liang, 1997). Recently it has been shown that a subset of anti-DNA antibodies can cross–react with the NR2 subunit of the N-methyl-D-asparate (NMDA) glutamate receptor (DeGrorgio *et al.*, 2001). If the psychotic symptoms in SLE patients are the result of anti-DNA antibodies cross reacting with the glutamate receptor, then it is possible that the psychotic symptoms in some schizophrenic patients could be caused by similar reactions of antibodies with neurotransmitter receptors.

3.6.3 CIRCUMSTANTIAL EVIDENCE

Several features are common to many autoimmune diseases. These are (i) association with other autoimmune diseases in the same individual or the same family, (ii) the presence of immune cells in the affected organ, (iii) association with human leukocyte antigen (HLA), (iv) high serum level of autoantibodies, (v) alteration in the level of cytokines, (vi) deposition of antigen-antibody complexes in the affected organ and (vii) improvement of disease symptoms with immunosuppression. (Jones *et al.*, 2005).

3.6.3.1 Association with other autoimmune diseases

Epidemiological studies have shown that relatives of people with schizophrenia have an increased risk of developing several other autoimmune diseases, particularly type 1 diabetes mellitus and thyrotoxicosis (Wright *et al.*, 1996; Gilvarry *et al.*, 1996). SLE has an interesting association with schizophrenia because it arises relatively frequently in patients with schizophrenia, particularly as a result of treatment with phenothiazine or dibenzodiazepine antipsychotic agents (Gold and Sweeney, 1978; Goldman *et al.*, 1980; Wickert *et al.*, 1994). The reason for development of drug-induced SLE remains poorly understood, but it may indicate that some patients with schizophrenia have an underlying susceptibility to the development of autoimmunity.

3.6.3.2 Presence of immune cells in the target organ

Till to date there is no report of inflammatory infiltrate of mononuclear cells in the brains schizophrenic patients. There is however reports of an increased frequency of activated lymphocytes in the cerebrospinal fluid (CSF) of patients with acute schizophrenia (Nikkila *et al.*, 2001; Nikkila *et al.*, 1999). Only one study has investigated whether immunoglobulin can be detected in the brains of people with schizophrenia. Health and Krupp (1967) found that a fluorescein-tagged antihuman antibody labeled the nuclei of some neural cells in the brain tissues from 12 of 14 patients with schizophrenia, but not in the tissues from any of 19 non-schizophrenic controls.

3.6.3.3 Association with HLA

The first HLA association study of schizophrenia was reported by Cazzullo *et al.*, in 1974. More than 80 association studies have been reported since then (Bogacki *et al.*, 2005). In different ethnic population associations have been found for HLA-A*9 (Goudemand *et al.*, 1981), HLA-A*23 (Ivanyi *et al.*, 1983), HLA-A*24 (Asaka *et al.*, 1981;Wright *et al.*, 1995; Ivanyi *et al.*, 1976; Ivanyi *et al.*, 1978; Rosler *et al.*, 1980; Bogacki *et al.*, 2005), HLA-A28 (Wright *et al.*, 1995; Ivanyi *et al.*, 1976; Ivanyi *et al.*, 1978; Rosler *et al.*, 1980; Bogacki *et al.*, 2005) HLA-A*01 (Lahdelma *et al.*, 1998), HLA-A*2, HLA-A*03, HLA-A*11, HLA-B*17, HLA-B*27, HLA-B*8 and and Cw 2 (Rudduck *et al.*, 1984). However past association studies with various Class I alleles yielded inconsistent results (Nimgaonkar *et al.*, 1992) except HLA-A*9(now subdivided into A*23/A*24) (Mc.Guffin *et al.*, 1995). The reason for the inconsistencies include the differences in the diagnostic methods (Goudemand *et al.*,1981, Singer *et al.*, 1982) and typing method of HLA such as serological typing techniques (Joysey and Woolf, 1978), which have been found to be inaccurate, with 7-25% misassignment errors (Opelz *et al.*, 1991) compared with the DNA based techniques such as polymerase chain reaction (PCR) and sequence specific oligonucleotide probes (SSOP). Moreover the source of controls is not always described in sufficient detail to ensure that results are not simply due to population stratification. Significant results are not always corrected for the number of statistical tests performed (Hawi *et al.*, 1999).

In a study, chip-based mass spectrometry analysis for SNP within a 25Mb region on human chromosome 6p21 (which covers the MHC) found a significant increase in the frequency of a SNP in HLA-DOA in schizophrenia and a significant decrease in the frequency of a SNP in HLA-DRB1 (Herbon *et al.*,2003). Recently, The International Schizophrenia Consortium (2009) in the genome-wide association study also found association of schizophrenia in chromosome 6p. They have found more than 450 SNPs on chromosome 6p spanning the major histocompatibility complex. Further, the study revealed the best imputed SNP, which reached genome-wide significance was also in the MHC, 7 kilobases(kb) from NOTCH4, a gene with previously reported associations with schizophrenia (Wei and Hemmings, 2000).

However, the study didn't ascribe the association to a specific HLA allele, haplotype or region.

3.6.3.4 High serum levels of autoantibodies

In the early 1960s several investigators described a variety of antibrain antibodies in the sera of patients with schizophrenia (Heath and Krupp, 1967; Heath *et al.*, 1967; Fessel, 1962) but the consistency of these findings between different research groups has not been high. Increased level of autoantibodies in schizophrenic patients has also been observed against the brain or specific areas of the brain including the cerebrum (Shima *et al.*, 1991), septum (Heath *et al.*, 1989) and amygdale, frontal cortex, cingulated gyrus and septal area (Henneberg *et al.*,1994). However, there is no consistency in this finding. Several groups have reported significantly higher levels of antibodies to cerebral M1 colinergic muscarinic receptors (Borda *et al.*, 2002; Borda *et al.*, 2004; Tanaka *et al.*, 2003), Nicotinic acetylcholine receptors (Mukherjee *et al.*, 1994), Dopamine D2 receptors (Tanaka *et al.*, 2003; Chengappa *et al.*, 1993), astrocyte M1 and M2 muscarinic cholinergic receptors (Borda *et al.*, 2004), mu-opioid and serotonin (5-HT_{1A}) receptors (Tanaka *et al.*, 2003) in sera of schizophrenic patients. The results are suggestive of an autoimmune response directed against neurotransmitter receptors in at least some patients with schizophrenia. A number of studies have found a significantly higher frequency of non-specific autoantibodies such as circulating antinuclear antibodies in people with schizophrenia (Spivak *et al.*, 1995; Sirota *et al.*, 1993; Johnstone and Whaley, 1975). Autoantibodies to both single and double-stranded DNA have also been found to be significantly more common in people with schizophrenia compared to controls (Sirota *et al.*, 1993). This finding was not reproducible in the subsequent studies (Johnstone and Whaley, 1975).

Several possible reasons could account for the discrepancies in findings from different research groups. The heterogeneity of the disease, the medication status, the techniques used by different research groups and the diagnostic criteria used to classify patients could influence results.

3.6.3.5 Alteration in level of cytokines

The cytokine alterations in schizophrenia have been intensively investigated and reviewed by others (Gaughran, 2002; Rothermundt *et al.*, 1998, 2001; Schuld *et al.*, 2004). With regard to interleukin (IL-2), a significant negative correlation between the serum IL-2 levels and the Positive and Negative Syndrome Scale (PANSS) (Zhang *et al.*, 2002) and a positive correlation between the plasma IL-2 and homovanillic acid levels (Kim *et al.*, 2000) were observed in two studies. Additionally , Maes *et al.* (1995b) described a positive correlation between plasma soluble IL-2 receptor (sIL-2R) and transferring receptor (TfR), another marker of immune activation.

There is a discrepancy between the circulating levels of IL-2 and *ex vivo* production of this cytokine. To explain this phenomenon it was suggested that the reduced *ex vivo* IL-2 production may be a consequence of overproduction of IL-2 *in vivo* (Rothermundt *et al.*, 1998). Ganguli *et al.*, (1992) found that autoanibody-positive acutely ill schizophrenic patients had lower mitogen-stimulated IL-2 production than other patients. The only study investigating the levels of sIL-2R in the intrathecal compartment reported decreased values in the cerebrospinal fluid (CSF), but increased concentrations in serum of schizophrenic patients (Barak *et al.*, 1995). These findings indicate systemic immune activation in schizophrenia, however, more evidence in needed to support this conclusion.

Two studies demonstrated a positive association of the serum IL-6 levels with duration of illness (Ganguli *et al.*, 1994; Kim *et al.*, 2000). Another approach was presented in the study of Toyooka *et al.*, (2003) in which they measured protein and / or mRNA levels for IL-1β and IL-1 receptor antagonist (IL-1Ra) in the postmortem brain tissues of schizophrenic patients. They found decreased levels of both IL-1Ra protein and mRNA in the prefrontal cortex of the patients, whereas IL-1 levels were not altered. In the same study they found the increased serum levels of IL-1Ra in drug-free schizophrenic patients. It was suggested that the decreased IL-Ra levels in the CNS might enhance various IL-1 mediated actions in schizophrenic patients.

Interestingly, Inglot *et al.*, (1994) observed that the patients with high interferon (IFN) response to lipopolysaccharide (LPS) or phytohemagglutinin (PHA) stimulation had dominant positive symptoms of schizophrenia whereas in the patients with low IFN response, the negative symptoms prevailed. Moreover, a significant positive intercorrelation between the lowered production of IFN-γ and IL-2 were detected in the patients studied by Arolt *et al.*, (2000) during the 1-month treatment period. Preble and Torrey (1985) demonstrated that IFN-positive patients were more likely to have a recent onset or exacerbation of their illness than IFN-negative patients. Additionally, McAllister *et al.*, (1995) found that symptom exacerbation was associated with the increased CSF IL-2 levels.

3.6.3.6 Deposition of antigen-antibody complex in the affected organ

It has been proposed that schizophrenia may be caused by a covert immune complex-driven basal lamina disease of the choroids plexus (Rudin, 1980). This proposal was largely based on findings from patients with SLE and associated schizophreniform psychoses, in which immune complexes are deposited in the choroids plexus. However, in schizophrenia itself no studies have investigated whether or not such immune complex deposition occurs.

3.6.3.7 Improvement of disease symptoms with immunosuppression

Few studies have reported results of trials of immunosuppressive agents in schizophrenia. Levine and colleagues (Levine *et al.*, 1997) showed that short-term treatment with azathioprine improved the psychiatric symptomatology in a subgroup of patients with schizophrenia. Few other studies have set out to test well-defined immunosuppressive agents in schizophrenia. However, it is known that some of the antipsychotic drugs such as haloperidol and clozapine are highly immunosuppressive (Leykin *et al.*, 1997). If autoimmune responses are playing a role in the development of schizophrenia then treatment with some of the common antipsychotic drugs may act synergistically as direct antagonists of brain neurotransmitter receptors and also as inhibitors of autoimmune responses thus leading to amelioration of psychotic behaviour.

While the autoimmune hypothesis of schizophrenia remains interesting, it is still unclear precisely how the aberrant immune system observed in schizophrenic patients interferes with neuronal and glial function and how this precisely becomes expressed at the clinical level. In addition it remains incompletely understood what accounts for abnormal production of antibodies and cytokines at the molecular level. Since schizophrenia is well known to manifest as a heterogeneous illness, it may be proposed that some patients manifest aspects of autoimmune or immunological aberrations and others not. Further investigation including the newer areas of investigation of immune dysregulation may go a long way to shed the light in the etiology of this complex disorder.

3.7 MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (MHC) is a dense complex of genes with immunological and non-immunological functions and is present in all vertebrates. Its products play roles in intercellular recognition and in discrimination between self and non self. The MHC participates in the development of both humoral and cell mediated immune responses. While antibodies may react with antigens alone, most T cells recognize antigen only when it is combined with an MHC molecule. Furthermore, the particular set of MHC molecules expressed by an individual influences the repertoire of antigens to which that individual's T_H and T_c cells can respond because MHC molecules act as antigen-presenting structures. For this reason the MHC partly determines the response of an individual to antigens of infectious organisms and it has therefore been implicated in the susceptibility to disease and in the development of autoimmunity. The recent understanding that natural killer cells express receptors for MHC Class I antigens and the fact that the receptor-MHC interaction may lead to the inhibition or activation expands the known role of this gene family (Kuby, 2003).

3.7.1 BRIEF HISTORY OF HUMAN LEUKOCYTE ANTIGENS (HLA) - DISCOVERY AND CHARACTERIZATION

The MHC has been referred by different names in different vertebrates, such as HLA complex in humans and H-2 complex in mice. Human Leukocyte Antigens (HLA), were initially inferred to exist in 1954 when Dausset reported the observation that the

sera from 60 patients contained antibodies which agglutinated lymphocytes from certain individuals. He noted that 90% of these patients had received multiple transfusions. Dausset concluded that transfusion was responsible for creating antibodies against leukocytes as a result of an immune response towards the donor leukocytes (Dausset, 1954). In 1958 Payne and van Rood made separate observations that pregnant women formed antibodies against antigens present on foetal leukocytes. These antibodies were directed at antigens originating from the father of the foetus (Payne and Rolfs, 1958; Rood *et al.*, 1958). At the same time Dausset identified a leukocyte antigen which he named MAC, demonstrated to be present in 60% of the French population. He also showed that monozygotic twins exhibited identical agglutination patterns while dizygotic twins did not. This led him to hypothesize that leukocyte antigens are genetically controlled (Dausset, 1958). Family studies conducted by Payne and van Rood further corroborated this hypothesis (Payne and Rolfs, 1958; Rood *et al.*, 1959). In 1959 van Rood discovered additional antigens beside MAC which were designated antigens 2 and 3 (Rood *et al.*, 1959).

The complex nature of HLAs was realised early in these investigations. Researches were unable to find two antisera that would give identical agglutination patterns against a defined donor palette. In 1963 van Rood was able to define groups of antisera which gave similar but not identical agglutination patterns using 2 x 2 associations between each serum. He had defined two distinct groups of sera which he named 4a and 4b and described these as part of the system which was named Leukocyte Group FOUR (Rood and Leeuwen, 1963). In 1964 Payne *et al.*, discovered another group of antigens that differed from Group FOUR. These antigens were designated LA with two alleles LA1 and LA2 identified initially. The existence of an additional LA allele was inferred when it was observed that leukocytes of some individuals did not agglutinate with anti-LA1 nor with anti-LA2 (Payne *et al.*, 1964). The new allele LA3 was indeed identified two years later (Bodmer *et al.*, 1966). By the mid sixties, it was realized that the growing complexity of the leukocyte antigen system required a standardized approach to further investigations.

In 1965 the first HLA workshop was organized at Netherlands where it was possible for the researchers from different groups to compare their antisera on the same panel of individuals (Bruning *et al.*, 1965) (Table 7). It was discovered that antigens defined

by different groups were closely related but were not identical. It was at the second workshop held in Torino in 1967 that the many antisera from different research groups did for the first time produce identical agglutination patterns on the panel of selected individuals. The number of defined leukocyte antigens was starting to grow and it was realized that the naming of antigens required standardization, leading to the formation of the first nomenclature committee at the Torino workshop. The genes for "LA" and "FOUR" antigens were combined into a system named HL-A (human leukocyte-antigens) and newly discovered alleles that were well characterized in the workshop were given sequential numbers in the order of their discovery. The less well characterized alleles were given a provisional assignment containing the letter "w" (for workshop) followed by the appropriate allele number. Although this approach did bring some order into the naming of the antigens, it was soon realized that the nomenclature lacked clear discrimination between the two series of antigens LA and FOUR. This problem was resolved in 1975 when the HL-A became HLA (human leukocyte antigens) and locus LA was renamed A (HLA-A) and locus FOUR was renamed B (HLA-B). The provisional assignment of less well characterized alleles remained and the "w" appellation is still in the use.

Data from Torino workshop provided the first evidence that leukocyte antigens were the products of closely linked genes located on the same chromosome (Ceppellini *et al.*, 1967; Dausset *et al.*, 1967; Rood *et al.*, 1967). It was also observed that in children, both HLA-A and HLA-B loci were inherited together (Ceppellini *et al.*, 1967; Dausset *et al.*, 1970). Genetic segments containing two or more linked loci on one chromosome were called haplotypes (Ceppellini *et al.*, 1967). Although it was a feature frequently observed in family studies of HLA genes, haplotypic associations of two genes was not absolute and exceptions resulting from genetic recombination were observed (Kissmeyer-Nielsen *et al.*, 1969). Recombination results in a haplotype different from those observed in the parental chromosomes. By observing 40 recombinations in 4614 informative meioses Belvedere *et al.*, (1975) were able to estimate the physical distance between HLA-A and HLA-B loci to 0.87 centimorgans (cM). It was further observed that some HLA-A and HLA–B alleles associate more frequently then otherwise expected by chance and some associate less frequently. This phenomenon was named linkage disequilibrium.

In 1971 Lamm *et al.*, have demonstrated the linkage between HLA and phosphoglucomutase-3 (PGM3) genes and estimated the physical distance between the two genes to be approximately 20 cM (Lamm *et al.*, 1971). This finding was the starting point in the identification of the chromosome where HLA genes were located. Identification of the chromosome carrying HLA genes was achieved using hybrid mouse-human cell lines, a strategy in which the hybrid cell gradually looses human chromosomes. When several chromosomes remain and the hybrid cell is stabilized it is then cloned and the chromosomes are identified. The presence of a specific gene is tested by biochemical or immunological methods. This strategy was explored by Jongsma *et al.*, (1973) who demonstrated that PGM3 was contained in cells with human chromosome 6 and that PGM3 was absent in cells that did not contain this chromosome. More detailed information on localisation of the HLA genes was obtained from experiments with translocated chromosome 6. It was shown that HLA genes were contained on the short arm of chromosome 6, within the p21 banding region (Francke and Pellegrino, 1977).

In the 1970 Thorsby *et al.*, described an anti-serum designated anti-AJ which would react against particular A and B haplotypes in some individuals but not in other individuals with identical HLA-A and HLA-B (Thorsby *et al.*, 1970). It was hypothesized that a new locus, closely linked to HLA-A and HLA-B might exist. In 1973 this new locus -AJ was identified using the antigen capping method and shown to be expressed on leukocytes (Mayr *et al.*, 1973). This new locus was later designated HLA-C.

It was observed that leukocytes from HLA identical siblings did not stimulate each other in Mixed Leukocyte Culture (MLC) while leukocytes from HLA identical unrelated individuals did (Mempel *et al.*, 1973a; Sengar *et al.*, 1971). Yunis and Amos showed that in certain families HLA-A and HLA–B identical siblings produced a strong MLC, whereas siblings with non-identical HLA-A and HLA–B did not (Yunis and Amos, 1971). It was hypothesized that an unidentified HLA linked locus existed. The MLC test is based on the blast transformation property of leukocytes when mixed with leukocytes from different individuals. Cells marked to be stimulator cells are inactivated by radiation or mitomycin C. When mixed with the cells designated as responder cells they will stimulate them to go into blast transformation

and divide, indicating differences in HLA antigens present on the cell surface. Stimulator cells in the MLC reaction belong to the B lymphocyte population and only certain T cells may be stimulated and be responder cells (Simpson, 1976). With the MLC test it was possible to identify a "difference" between two individuals if it existed but the test could not actually identify the antigen types. This was resolved by the introduction of homozygous typing cells (HTC). These cells were known to be homozygous for determined HLA alleles and if those alleles were present on responder cells as well, no reaction should occur. Using this method it was possible to identify that novel specificities or MLC determinants do exist that stimulate the MLC reaction and were called D (Mempel *et al.*, 1973b). Primed lymphocyte typing (PLT) was also used to identify MLC determinants. This method was based on the observation that T lymphocytes, which were stimulated and then grown in culture for several days, have a much stronger secondary response when restimulated. This method developed by Sheehy *et al.*, (1975), can be directed against a single MLC determinant by using parents and children as stimulators and responders, so that only one MLC determinant in any direction is chosen in stimulation. It was later shown that determinants in classical MLC and PLT were virtually identical (Bach *et al.*, 1976).

A more efficient methodology however was required to type the D locus. This was aided by the observation that anti-HLA-A and anti-HLA-B antibodies can inhibit an MLC reaction (Grumet and Leventhal, 1970). The development of such serological methods required good controls to ensure that non D locus specific antibodies are not involved in MLC reaction inhibition. This was achieved by using HLA-A and HLA–B identical stimulator and responder cells which excluded the existence of anti-HLA-A or HLA–B antibodies. An experiment to detect D specific antibodies and confirm the existence of the D locus was performed by Leeuwen *et al.*, 1973. The study utilized the following design: a patient with a defined HLA–A and HLA–B haplotype had developed a cytotoxic antibody. This antibody was not specific to the HLA haplotype of the person that carries it therefore other individuals containing the same HLA haplotypes were selected and their cells used as stimulators and the patients cells as responders. The MLC tests were performed using normal control serum in which case all of the stimulator cells were able to stimulate the patient's responder cells. In another MLC test, the serum from the patient was used (containing the

cytotoxic antibody) and the MLC reaction was inhibited in half of the selected stimulator cells. These results were further investigated by incorporating an additional test to detect the antibody by introducing fluorescent conjugated anti-immunoglobulin which was designed to bind the anti-MLC antibody. Fluorescence was detected in about 17% of the inhibited lymphocytes which corresponded to the proportion of B lymphocytes to total lymphocytes. Serological methods were finally developed to type for the MLC determinants-D specificities which were named HLA-D antigens.

Table 7: A summary of the events occurring at HLA workshops and chronicle of the milestones of achievement in HLA research (Roitt *et al.*, 1998; Thorsby, 2009).

1964- Acceptance of cytotoxicity over agglutination
1965- Allelism of HLA antigens proposed
1967- Segregation of alleles demonstrated in families
1970- Single locus now two – HLA-A, HLA-B
1972- 60 world populations typed by 75 laboratories.
1975- Third locus, HLA-C, demonstrated
1977- HLA-D defined by Homozygous Typing Cells
1977- The serum-detected, D-related, HLA-DR defined
1984- HLA and Disease associations explored
1984- Studies of gene structure
1984- Worldwide Renal Transplantation databases
1984- Definition of MB (later to be HLA-DQ)
1987- DNA techniques with serological, biochemical and cellular methods
1987- Definition of HLA-DP and HLA-DQ
1992- Use of Polymerase Chain Reaction- e.g. for SSOP.
1996- Molecular definition of HLA-Class I
1996- Roles of HLA-G, E, DM, Tap and LMP's better understood.
2002- Molecular characterization of HLA alleles and Non HLA genes
2003- Nomenclature of KIR genes better defined
2005- New data on KIR-HLA and applications, in particular in BMTs.
2008- New data on application of HLA in clinical medicine, anthropology etc.

3.7.2 HUMAN LEUKOCYTE ANTIGEN

The HLA genes are located on chromosome 6p 21.3. Based on the structure of the antigens produced and their function there are two classes of HLA antigens, HLA Class I and Class II (Fig.9). The overall size of the HLA gene is approximately 3.5 million base pairs. Within this the HLA Class I genes and the HLA Class II genes each spread over approximately one third of this length. The remaining section known as Class III contains loci responsible for complement, hormones, intracellular peptide processing and other developmental characteristics (Sanfilippo and Amos, 1986). Thus the Class III region is not actually a part of the HLA complex but is located within the HLA region. Its components are either related to the functions of HLA antigens or are under similar control mechanisms to the HLA genes. The common HLA antigens and their molecular types are presented in table 8.

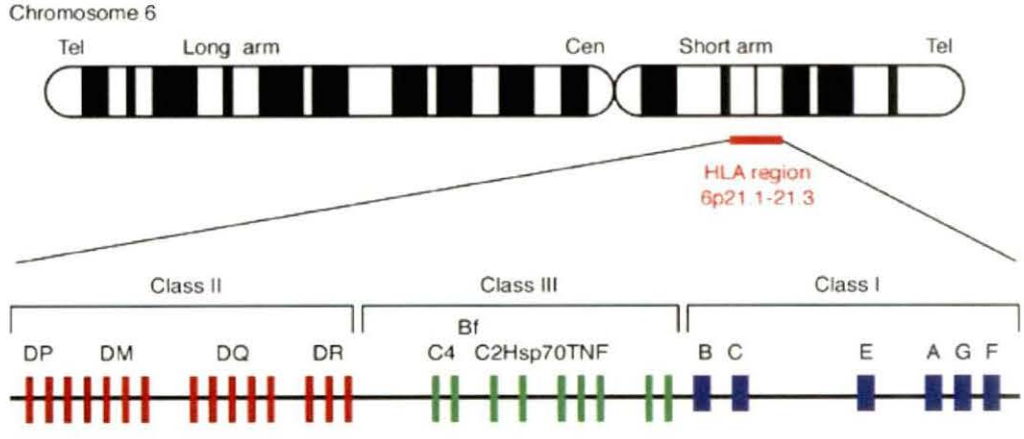


Figure 9: Gene map of the human leukocyte antigen (HLA) region.

3.7. 2.1 HLA Class I antigens

The cell surface glycopeptide antigens of the HLA-A, -B and –C series are called HLA Class I antigens (Roitt *et al.*, 1998). It is expressed on the surface of most nucleated cells of the body. Additionally, they are found in soluble form in plasma and are adsorbed onto the surface of platelets. Erythrocytes also adsorb HLA Class I antigens to varying degrees depending on the specificity (e.g. HLA-B7, A28 and B57 are recognizable on erythrocytes as so called "Bg" antigens). Immunological studies indicate that HLA-B (which is also the most polymorphic) is the most significant HLA Class I locus, followed HLA-A and HLA-C. There are other HLA Class I loci

(e.g. HLA-E,F,G,H,J,K and L) but most of these may not be important as loci for "peptide presenters".

Figure10 shows the structure of HLA Class I molecule. The HLA Class I antigens comprise a 45 Kilodalton (kD) glycopeptide heavy chain with three domains. It is non-covalently associated with β-2 microglobulin which plays an important role in the structural support of the heavy chain. The HLA Class I molecule is assembled inside the cell and ultimately sits on the cell surface with a section inserted into the lipid bilayer of the cell membrane and has a short cytoplasmic tail. The general structure of HLA Class I, HLA Class II and IgM molecules show such similarity of subunits that a common link between HLA and immunoglobulins back to some primordial cell surface receptor is likely. The full 3-dimentional structure of HLA-A Class I molecules has been determined from X-ray crystallography (Browning and Mc Michael, 1996). It has a cleft on its outermost surface which holds a peptide. If a cell becomes infected with a virus the virally induced proteins within the cell are broken down into small peptides and these are the peptides which are then inserted into this cleft during the synthesis of HLA Class I molecules. The role of HLA Class I molecules is to take these virally induced peptides to the surface of the cell and by linking to the T-Cell receptor of a Cytotoxic (CD8) T Cell (Fig.12) give clue for the presence of this virus to the immune system. The CD8 T Cell will now be "educated" and it will be able to initiate the process of killing cells which subsequently has that same viral protein/HLA Class I molecule on its surface. Due to its role in identifying cells which are changed (e.g. virally infected), HLA Class I needs to be present on all cells (Browning and Mc Michael, 1996).

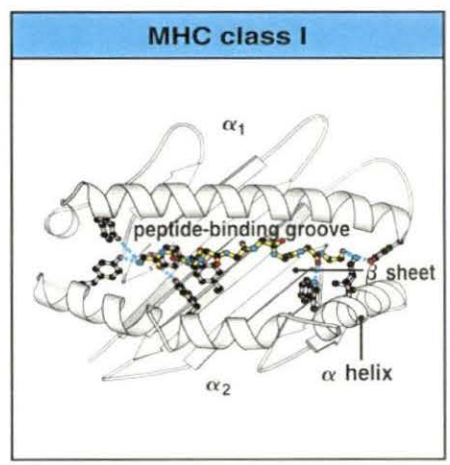


Figure 10: Detailed structure of HLA Class I molecule.

3.7.2.2 HLA Class II Antigens

Figure 11 shows the structure of HLA Class II molecule. The cell surface glycopeptide antigens of the HLA-DP, -DQ and –DR loci are termed HLA Class II (Sanfilippo and Amos, 1986). The tissue distribution of HLA Class II antigens is confined to the "immune competent" cells including B-lymphocytes, macrophages, endothelial cells and activated T-lymphocytes. The expression of HLA Class II on cells which would not normally express them is stimulated by cytokines like interferon γ and in a transplant this is associated with acute graft destruction. HLA Class II molecules consist of two chains each encoded by genes in the "HLA Complex" on Chromosome 6. HLA Class II molecule is a heterodimer consisting of two different polypeptide chains, an α chain of 33 kD and a β chain of 28 kD which associate with each other noncovalently. HLA Class II molecules are also membrane bound glycoprotein molecules like HLA Class I. HLA Class II also contains an external domain, a transmembrane segment and a cytoplasmic tail or anchor segment. The external domain contains α1 and α2 domains and bears sequence homology to the immunoglobulin-fold domain structure. Hence, HLA Class II molecules are classified as immunoglobulin superfamily. The peptide binding site of the HLA Class II molecule is composed of the α1 and β1 domains and forms the antigen binding cleft for processed antigen. The T Cells which link up to the HLA Class II molecules are Helper (CD4) T cells (Fig.12). Thus the "education" process which occurs from HLA Class II presentation involves the helper-function of setting up a general immune reaction and provides defense against the bacterial or other invasion. This role of HLA Class II in initiating a general immune response is the reason why they need only be present on "immunologically active" cells (B lymphocytes, macrophages, etc.) and not on all tissues (Browning and Mc Michael, 1996).

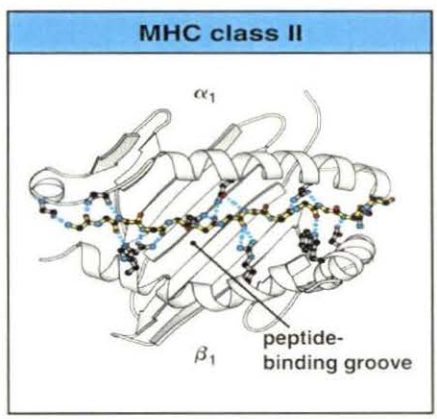


Figure 11: Detailed structure of HLA Class II molecule.

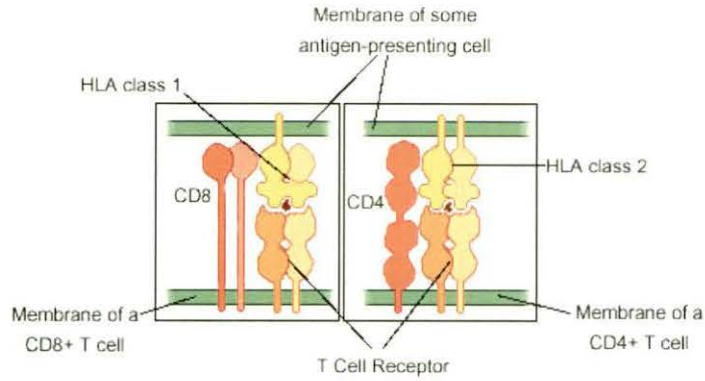


Figure 12: Class I HLA presents antigen peptide found within the cell, to CD8 cell surface protein (i.e. normally to cytotoxic T cells). Class II HLA presents antigen peptide found outside the cell, to CD4 cell surface protein (i.e. normally to helper T cells).

3.7. 2.3 HLA Class III antigens

The class III region of the MHC in humans and mice contains a heterogeneous collection of genes. These genes encode several complement components, two steroid 21-hydroxylases, two heat-shock proteins, and two cytokines (TNF-α and TNF- β). Some of these Class III gene products play a role in certain diseases. For example, mutations in the genes encoding 21-hydroxylase have been linked to congenital adrenal hyperplasia. Interestingly, the presence of a linked class III gene cluster with the HLA region is conserved in all species.

Table 8: The common HLA antigens and their molecular types expressed more frequently among the HLA-A, HLA-B, HLA-C, HLA-DR loci. (adapted from Shankarkumar, 2004)

HLA Antigens	Broad Group	No. of molecular types *	Most common alleles
HLA A			
A1		9	A*0101
A2		58	A*0201, A*0202
A3		9	A*0301
A11		13	A*1101
A23	A9	9	A*2301
A24	A9	36	A*2402
A25	A10	4	A*2501
A26	A10	18	A*2601
A29	A19	6	A*2901, A*2902
A30	A19	12	
A31	A19	8	A*3101
A32	A19	7	A*3201
A33	A19	6	A*3301

HLA Antigens	Broad Group	No. of molecular types *	Most common alleles
A34	A10	4	A*3401
A36		3	A*3601
A43		1	A*4301
A66	A10	4	A*6601
A68	A28	22	A*6801
A69	A28	1	A*6901
A74		8	A*7401
A80		1	A*8001
HLA B			
B7		31	B*0702
B8		16	B*0801
B13		10	B*1301
B14		6	B*1401, B*1402
B15		73	B*1501
B18		18	B*1801, B*1802
B27		24	B*2701, B*2702
B35		44	B*3501, B*3502
B37		5	B*3701
B38	B16	8	B*3801
B39	B16	26	B*3901
B40		44	B*4001
B41		6	B*4101
B42		4	B*4201
B44	B12	32	B*4402
B45	B12	6	B*4501
B46		2	B*4601
B47		4	B*4701
B48		7	B*4801
B49	B21	3	B*4901
B50	B21	3	B*5001
B51	B5	29	B*5101
B52	B5	4	B*5201
B53	B5	9	B*5301
B54	B22	2	B*5401
B55	B22	12	B*5501, B*5502
B56	B22	8	B*5601
B57	B17	9	B*5701
B58	B17	6	B*5801
B59		1	B*5901
B67		2	B*6701
B73		1	B*7301
B78		5	B*1517
B81		1	B*8101
B82		2	B*8201
B83		1	B*8301
HLA C			
Cw1		6	Cw*0101
Cw2		5	Cw*0202
Cw3		15	Cw*0303
Cw4		10	Cw*0401
Cw5		5	Cw*0501
Cw6		7	Cw*0602
Cw7		16	Cw*0701, Cw*0702
Cw8		9	Cw*0802
Cw12		8	Cw*1203
Cw14		5	Cw*1401
Cw15		11	Cw*1502

HLA Antigens	Broad Group	No. of molecular types *	Most common alleles
Cw16		3	Cw*1601
Cw17		3	Cw*1701
Cw18		2	Cw*1801
HLA DR			
DR1		8	DRB1*0101, 0103
DR15	DR2	13	DRB1*0501, 1502
DR16	DR2	8	DRB1*1601,1602
DR3		23	DRB1*0301
DR4		44	DRB1*0401,0404
DR11	DR5	43	DRB1*1101
DR12	DR5	8	DRB1*1201
DR13	DR6	52	DRB1*1301, 1302
DR14	DR6	43	DRB1*1401,1402
DR7		6	DRB1*0701
DR8		24	DRB1*0801,0802,0803
DR9		2	DRB1*0901
DR10		2	DRB1*1001

*The number of variants in approximate, as there will be more reported regularly

3.7.3 GENETICS OF HLA

There are a number of genetic characteristics of HLA antigens which have been described below.

3.7.3.1 Polymorphism

The polymorphism at the recognized HLA loci is extreme. It is likely that this extreme polymorphism has evolved as a mechanism for coping with all of the different peptides that the organism comes across. Therefore each HLA molecule differs slightly from each other in its amino acid sequence to gives rise to large array of HLA antigens. This difference causes a slightly different 3-dimentional structure in the peptide binding cleft. Since different peptides have different shapes and charge characteristics, it is important that the human race has a large array of different HLA antigens, each with different shaped peptide binding areas (clefts) to cope with all of these peptides. However this is not ubiquitous as the polymorphism is population specific. The frequent HLA antigens in different populations are clearly different. For example, HLA-A34, which is present in 78% of Australian Aborigines has a frequency of less than 1% in both Australian Caucasoid and Chinese. Several workers have reported HLA studies from various populations of World (Imanishi *et al.*, 1992; Clayton and Lonjou, 1997; Shankarkumar et al 1999a; Shankarkumar et al 1999b;

Mehra *et al.*,1986; Pitchappan *et al.*,1984). Thus HLA antigens are of great significance in anthropological studies. Populations with very similar HLA antigen frequencies are clearly derived from common stock. Conversely, from the point of view of transplantation it is very difficult to match HLA types between populations.

3.7.3.2 Inheritance of HLA

The normal way to present a tissue type is to list the HLA antigens as they have been detected. There is no attempt to show which parent has passed on which antigen. This way of presenting the HLA type is referred to as a Phenotype (Thomas *et al.*, 1998). When family data is available, it is possible to assign one each of the antigen at each locus to a specific grouping known as a haplotype. A haplotype is the set of HLA antigens inherited from one parent. For example a child is having HLA Phenotype HLA-A1, A3;B7, B8;Cw2, Cw4;DR15, DR4 the phenotype of his mother is HLA-A3, A69;B7, B45; Cw4, Cw9; DR15, DR17. Now it is evident from this example that the A3, B7, Cw4 and DR15 were all passed on from the mother to the child above. This group of antigens is a haplotype. Figure 13 shows the inheritance of HLA haplotypes. In the absence of genetic crossing over 2 siblings who inherit the same two HLA chromosomes (haplotypes) from their parents will be HLA identical. There is a one in four chance that this will occur and therefore in any family with more than four children at least two of them will be HLA identical. This is because there are only two possible haplotype in each parent.

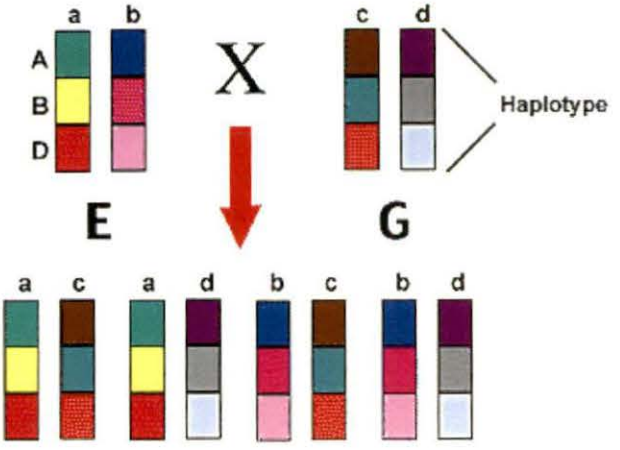


Figure 13: Inheritance of HLA haplotypes.

3.7.3.3 Linkage disequilibrium

Basic Mendelian genetics states that the frequency of alleles at one locus does not influence the frequency of alleles at another locus (Law of independent segregation). However in HLA genetics this is not true. There are a number of examples from within the HLA system that, alleles at different loci occurring together at very much higher frequencies than would be expected from their respective gene frequencies. This is termed linkage disequilibrium. The most extreme example is in Caucasians where the HLA-A1,B8,DR3 (DRB*0301), DQ2 (DQB1*0201) haplotype is so conserved that even the alleles at the complement genes (Class III) can be predicted with great accuracy. Similar haplotypes are observed in selected caste groups and tribal groups of India (Shankarkumar *et al.*, 1999a; Shankarkumar *et al.*, 1999b). Also, at HLA Class I, this phenomenon is to pronounced that the presence of specific HLA-DR alleles can be used to predict the HLA-DQ allele with a high degree of accuracy before testing. Because of linkage disequilibrium a certain combination of HLA Class I antigen, HLA Class II antigen and Class III products will be inherited together more frequently than would normally be expected. It is possible that these "sets" of alleles may be advantageous immunologically so that they have a positive selective advantage.

3.7.3.4 Cross-Reactivity

Cross-reactivity is the phenomenon whereby one antibody reacts with several different antigens, usually at the one locus (as opposed to a mixture of antibodies in the one serum) (Shankarkumar *et al.*, 1998). This is not a surprising event as it has been demonstrated that different HLA antigens share exactly the same amino acid sequence for most of their molecular structure. Antibodies bind to specific sites on these molecules and it would be expected that many different antigens would share a site (or epitope) for which a specific antibody will bind. Thus cross-reactivity is the sharing of epitopes between antigens.

The term CREG is often used to describe "Cross Reacting Groups" of antigens. It is useful to think in terms of CREG's when screening sera for antibodies as most sera found are "multi specific" and it is rare to find operationally monospecific sera. The

rarity of monospecific sera means that most serological tissue typing is done using sera detecting more than one specificity and a typing is deduced by subtraction. For example, a cell may react with a serum containing antibodies of HLA-A25, A26 and A34 and be negative for pure A26 and pure A25 antisera. In this case, HLA-A34 can be assigned, even in the absence of pure HLA-A34 antisera.

3.7.4 HLA AND DISEASE SUSCEPTIBILITY

In the 1960's, it was discovered that the mouse MHC (called H-2) controlled both the genetic susceptibility to certain leukemia's and the immune response to certain antigens. Since then innumerable reports have been published aimed to discovering the role of the HLA in the control of responsiveness and disease susceptibility (Tiwari and Terasaki, 1985).

The discovery of HLA associations with specific diseases implies that at least part of their genetic basis lies in the HLA and suggests that it may be possible to determine their etiology. Thus, in the case of insulin-dependent diabetes mellitus (IDDM) it is now known that alleles coding for an amino acid residue other than aspartate at position 57 of the DQβ chain are highly associated with IDDM (Dorman *et al.*, 1990; Khalil *et al.*, 1992; Pugliese *et al.*, 1995). However, two decades after the landmark discovery of association between HLA B27 and ankylosing spondylitis (Schlosstein *et al.*,1973; Brewerton *et al.*, 1973), the etiology of this disorder remains unknown. The same is true for Narcolepsy in that, despite finding association with HLA DR2 (Honda, 1988) the pathophysiology of this neuropsychiatric disorder is still obscure.

There are two general explanations for HLA and disease associations (Mc Devitt, 1985). Firstly, there may be a linkage disequilibrium between alleles at a particular disease associated locus and the HLA antigen associated with that disease. E.g., HLA-A*03 and Idiopathic Haemochromatosis.

Another possible explanation for these associations is that the HLA antigen itself plays a role in disease, by a method similar to one of the following models:-

i) By being a poor presenter of a certain viral or bacterial antigen.

ii) By providing a binding site on the surface of the cell for a disease provoking virus or bacterium.

iii) By providing a transport piece for the virus to allow it to enter the cell

iv) By having such a close molecular similarity to the pathogen, that the immune system fails to recognize the pathogen as foreign and so fails to mount an immune response against it.

It is most likely that all these mechanisms are involved but to a varying extent in different diseases (Throsby, 1977). In multiple sclerosis and ankylosing spondylitis cell mediated immunity is often depressed, not only in the patients but also in their parents and siblings (Kankonkar *et al.*, 2003; Shankarkumar *et al.*, 2002). Complement (C2) levels are known to be low in Systemic Lupus Erythematosus, Pulmonary Tuberclosis, Leprosy, a disease associated with HLA DR2 and DR3 (Shankarkumar *et al.*, 2003a; Shankarkumar *et al.*, 2003b; Rajalingham *et al.*, 1996; Shanmugalashmi and Pitchappan, 2002). In Gluten Enteropathy which shows a high association with HLA-DR3, a specific gene product is thought to act as an abnormal receptor for gliadin and present it as an immunogen to the body. Whatever the explanation for the long list of HLA and disease associations, it is clear that the HLA system, collaborating with other non-linked genes has an influence on our response to environmental factors which provoke the disease.

3.7.5 SCHIZOPHRENIA AND HLA

The first HLA association study of schizophrenia was reported by Cazzullo *et al.*, in 1974. More than 80 association studies have been reported since then. Table 9 shows the most frequently reported association between HLA Class I and schizophrenia. HLA and schizophrenia was first reviewed by McGuffin (1979), who commented that the MHC was a logical place in which to search for genetic markers for schizophrenia because schizophrenia was similar to diseases for which HLA association had been established in that it was familial, had a postulated autoimmune pathogenesis (Burch, 1964). These reasons, especially the autoimmune hypothesis (Wright *et al.*, 1993; Wright *et al.*, 1996), remain extant, but the following additional factors have provided the impetus for continuing investigations:

1. **Operationalised diagnostic criteria:** Several early studies did not use operationalised diagnostic criteria (Julien *et al.*, 1977; Perris *et al.*, 1979; Goudemand *et al.*, 1981; Ivanyi *et al.*, 1976, 1978) and those that did utilized several different diagnostic systems, making comparisons between studies very difficult. More recent studies have benefited from the application of DSM-IV-TR or ICD-10, and are therefore both diagnostically more reliable and more readily comparable to each other.

2. **Technical improvements in HLA serotyping:** Currently available alloantisera specify HLA antigens with a precision impossible in the 1980s, when the bulk of HLA association studies in schizophrenia were performed.

3. **The introduction of HLA genotyping:** This has effectively eliminated the inaccuracies associated with modern HLA serotyping (Mytilineos *et al.*,1990), has allowed rapid HLA typing of large numbers of samples and has revealed genetic polymorphism of the HLA to a complexity previously unimagined.

Inspite of tiring research it is evident that no common agreement regarding the association of HLA with of schizophrenia has been reached. Further research is awaited to throw light in the mechanism of association of HLA with schizophrenia. This will go a long way to find the etiology of this complex disorder.

Table 9: Most frequently reported association between HLA Class I and schizophrenia.

Investigator	Year	Ethnicity	Antigen
Eberhard *et al.*	1975	Caucasian	A9
Ivanyi *et al.*	1976	Caucasian	A28
Smeraldi *et al.*	1976	Caucasian	A10
Julien *et al.*	1977	Caucasian	A9
Ivanyi *et al.*	1977	Caucasian	Cw4
Ivanyi *et al.*	1977	Caucasian	B18
Ivanyi *et al.*	1978	Caucasian	A28
Crowe *et al.*	1979	Caucasian	Aw10 (A26 subtype)

Investigator	Year	Ethnicity	Antigen
Luchins *et al.*	1980	Caucasian	A2
Gattaz and Beckmann	1980	Caucasian	B27
Asaka *et al.*	1981	Japanese	A9 (Aw24 subtype)
Asaka *et al.*	1981	Japanese	A10 (A26 subtype)
Rosler *et al.*	1980	Caucasian	A28
Wright *et al.*	1995	Caucasian	A9 A24 (sub specificity of A9)
Blackwood *et al.*	1996	Caucasian	B35 Cw5
Bogacki *et al.*	2005	Caucasian	A24, A28
Debnath *et al.*	2005	Indian (Bengali)	A3

3.8 CYTOKINES

The development of an effective immune response involves lymphoid cells, inflammatory cells and hematopoietic cells. The complex interactions among these cells are mediated by a group of proteins collectively designated cytokines to denote their role in cell-to-cell communication (cyto-, "cell", from the Greek kinein, "to move"). Cytokines are low-molecular-weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to a number of stimuli. These proteins assists in regulating the development of immune effector cells and some cytokines possess direct effector functions of their own.

The term cytokine encompasses broad category of protein factors, those secreted by lymphocytes are called lymphokines and those secreted by monocytes and macrophages are called monokines. There are some low molecular weight cytokines, which are specifically called as chemokines, which play an important role in inflammation. Both lymphocyte and mononuclear phagocytes produce cytokines such as colony-stimulating factors (CSFs), which stimulate the growth and differentiation of immature leukocytes in the bone marrow, providing a source of additional leukocytes to replace the cells that are consumed during inflammatory reactions. Many of the cytokines are made by certain populations of blood leukocytes (e.g., T

cells or monocytes) and act on other leukocyte populations (e.g., monocytes , neutrophils or eosinophils), these molecules are called interleukins (IL). This term should not be construed to imply that cytokines are only synthesized by or only act upon white blood cells. However, the term "interleukin" has been useful because as new cytokines are molecularly characterized they may be assigned a designated interleukin number (e.g., IL-1, IL-2) to assure that there is an unambiguous shared nomenclature among investigators. Some cytokines and their functions have been mentioned the table 10.

Table 10: Selected immune cytokines and their activities.

Cytokine	Producing Cell	Target Cell	Function**
GM-CSF	Th cells	progenitor cells	growth and differentiation of monocytes and DC
IL-1α IL-1β	monocytes macrophages B cells DC	Th cells	co-stimulation
		B cells	maturation and proliferation
		NK cells	activation
		various	inflammation, acute phase response, fever
IL-2	Th1 cells	activated T and B cells, NK cells	growth, proliferation, activation
IL-3	Th cells NK cells	stem cells	growth and differentiation
		mast cells	growth and histamine release
IL-4	Th2 cells	activated B cells	proliferation and differentiation IgG_1 and IgE synthesis
		macrophages	MHC Class II
		T cells	proliferation
IL-5	Th2 cells	activated B cells	proliferation and differentiation IgA synthesis
IL-6	monocytes macrophages Th2 cells stromal cells	activated B cells	differentiation into plasma cells
		plasma cells	antibody secretion
		stem cells	differentiation
		various	acute phase response
IL-7	marrow stroma thymus stroma	stem cells	differentiation into progenitor B and T cells
IL-8	macrophages endothelial cells	neutrophils	chemotaxis

Cytokine	Producing Cell	Target Cell	Function**
IL-10	Th2 cells	macrophages	*cytokine production*
		B cells	activation
IL-12	Macrophages B cells	activated Tc cells	differentiation into CTL (with IL-2)
		NK cells	activation
IFN-α	leukocytes	various	*viral replication* MHC I expression
IFN-β	fibroblasts	various	*viral replication* MHC I expression
IFN-γ	Th1 cells, Tc cells, NK cells	various	*Viral replication*
		macrophages	MHC expression
		activated B cells	Ig class switch to IgG_{2a}
		Th2 cells	*proliferation*
		macrophages	pathogen elimination
MIP-1α	macrophages	monocytes, T cells	chemotaxis
MIP-1β	lymphocytes	monocytes, T cells	chemotaxis
TGF-β	T cells, monocytes	monocytes, macrophages	chemotaxis
		activated macrophages	IL-1 synthesis
		activated B cells	IgA synthesis
		various	*proliferation*
TNFα	macrophages, mast cells, NK cells	macrophages	CAM and cytokine expression
		tumor cells	cell death
TNF-β	Th1 and Tc cells	phagocytes	phagocytosis, NO production
		tumor cells	cell death

*** CTL: cytotoxic T lymphocytes; DC: dendritic cells; GM-CSF: Granulocyte-Monocyte Colony Stimulating Factor; IL: interleukin; IFN: Interferon; TGF: Tumor Growth Factor; TNF: Tumor Necrosis Factor.**

**** Italicized activities are inhibited.**

3.8.1 TH1/TH2 SYSTEMS

Th1- and Th2-system are originally defined on the basis of their cytokine profiles and effector functions. They are effective against intracellular and extra-cellular pathogens (Mosmann and Coffman, 1989). The balance between both Th-subsets is thought to be pivotal in determining the outcome of an immune response towards an infectious organism (Breytenbach *et al.*, 2001) and is therefore critical for host defense and the

pathogenesis of immune-mediated diseases (Agnello *et al.*, 2003; McGuirk and Mills, 2002).

Th1 cells mainly produce IFN-γ, IL-2, TNF-α and IL-12 while Th2 lymphocytes predominantly release IL-4, IL-6, IL-10 and IL-13. However, both TNF-α and IL-10 can be secreted by Th1 and Th2 cells (Romagnani, 1999; Katsikis *et al.*, 1995). The development of Th1 and Th2 cells from a common undifferentiated precursor is regulated by the interactions of peptide antigen with the T cell receptor (TCR), cytokine signaling, actions of co-stimulatory molecules, induction of transcription factors and antigen dose (Agnello *et al.*, 2003; Rothoeft *et al.*, 2003; Farrar *et al.*, 2002; Ben Sasson *et al.*, 2001; Murphy *et al.*, 2000; Ausubel *et al.*, 1997; Carballido *et al.*, 1997; Kuchroo *et al.*, 1995; Prabhu Das *et al.*, 1995). IL-4 activates the Janus kinase 1 (JAK1) and JAK3, leading to activation of the signal transducer and activator of transcription 6 (STAT6), whereas IL-12 activates JAK1 and TYK2 conducting to STAT4 activation (Santana and Rosenstein, 2003). STAT6 and STAT4 are essential for the development of Th1 and Th2 correspondingly (Anderson *et al.*, 2003). IL-4 drives the development of the Th2-system. IL- 12 is in most cases not necessary for maintaining Th1 responses once Th1 responses are induced (Gazzinelli *et al.*, 1994). Instead, IFN-γR signaling is required for Th1 further differentiation (Tau *et al.*, 2000). Binding of IFN-γ to its receptor IFN-γR activates JAK1 and JAK2, leading to phosphrylation of STAT1 (Bach *et al.*, 1997). In addition to STAT4 and STAT6, there are some other transcription factors specific for Th1/Th2 systems. They are GATA3 and c-Maf in Th2 cells as well as ERM and T-bet in Th1 cells (Murphy *et al.*, 2000). IFN-γ and IL-4 were characterized as the key cytokines of the Th1 and Th2 system due to their roles in the differentiation and development of the Th1/Th2 system. The ratio between both major cytokines is thought to implicate the balance between both Th1/Th2 systems (Giannakoulas *et al.*, 2004; Li *et al.*, 2003; Sakami *et al.*, 2002). Recently the IFN-γ/IL-10 ratio is regarded as an indicator of Th1/Th2 balance in various viral infections (Avery and Hoover, 2004; McElhaney *et al.*, 2004; Zhang *et al.*, 2000).

The Th1 system induces cell-mediated immune responses and is associated with inflammation and tissue destruction that leads to organ-specific autoimmune diseases

(Chen *et al.*, 2000; Golding and Scott, 1995). On the contrary, the Th2 system promotes humoral immune responses, allergic reactions to environmental antigens as well as anti-inflammatory activities (Chen *et al.*, 2000; Mosmann and Sad, 1996). Activation of Th2 cells may inhibit the central nervous system (CNS) inflammation and limit the noxious effects of Th1-mediated immunity (Chen *et al.*, 2000; Racke *et al.*, 1994).

3.8.2 INTERLEUKIN-2 (IL-2) AND INTERLEUKIN-6 (IL-6) AND THEIR PRINCIPAL BIOLOGICAL FUNCTIONS

3.8.2.1 IL-2 (T cell growth factor)

IL-2 was first described as "T-cell growth factor". It is a protein of 133 amino acids with a molecular weight of 15 kDa (Malek, 2003). The main secretory source of IL-2 is the T-helper cell, particularly naïve T cells and Th1 cells. The human IL-2 gene contains four exons and maps to human chromosome 4q26-28 (Sykora *et al.*, 1984). Figure 14 shows the crystal structure of human IL-2.

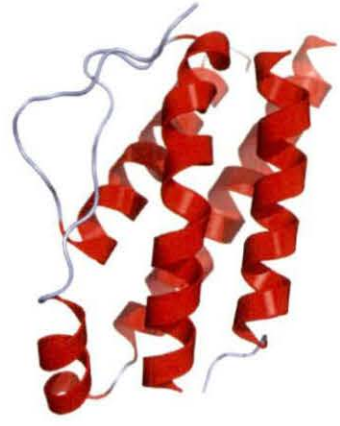


Figure 14: Crystal structure of human interleukin-2 (IL-2).

3.8.2.1.1 Interleukin-2 Receptor (IL-2R)

Three different types of IL-2Rs with high, intermediate and low affinity are distinguished. They are expressed differentially and independently. The high-affinity IL-2R consists of subunits IL-2Rα (p55), IL-2Rβ (p75) and a γ chain (64 kDa). The

intermediate-affinity IL- 2R comprises IL-2Rβ and γ chain, while the low-affinity IL-2R contains solely IL-2Rα. IL-2Rα functions as a T-cell activation (TAC) antigen, IL-2Rβ as the ligand binding domains and γ chain as a signaling component. The γ-subunit is required for the generation of high and intermediate affinity IL-2R, but does not bind IL-2 by itself (Minami *et al.*, 1993). The genes encoding these three subunits map to human chromosome 10p14-15, 22q11.2-12 and Xq13 respectively. Besides, activated lymphocytes continuously secrete a 42 kDa/55 kDa fragment of the TAC antigen, a soluble IL-2 receptor (sIL-2R), which circulates in the serum and plasma (Miska and Mahmoud, 1993; Pizzolo *et al.*, 1992). Brain IL-2Rs are enriched in the hippocampal formation. This area critical for the acquisition and consolidation of spatial learning and memory (Petitto *et al.*, 1999).

3.8.2.1.2 Interleukin-2 secreting cells

IL-2 is produced mainly by activation of CD4+ T-cells (de Waal *et al.*, 1993; Ferrer *et al.*,1992). Resting cells do not produce IL-2. There are detectable levels of IL-2-like material in the hippocampus, striatum and frontal cortex. However, specific IL-2 binding sites were observed only in the hippocampus (de Waal *et al.*, 1993; Ferrer *et al.*, 1992; Araujo *et al.*,1989). IL-2 is a growth factor for all subpopulations of T-lymphocytes (Abbas, 2003). It is an antigen-nonspecific proliferation factor for T-cells that induces cell cycle progression in resting cells and thus allows clonal expansion of activated T-lymphocytes (Malek, 2003). This effect is modulated by hormones such as prolactin (Moreno *et al.*, 1998). In addition, IL-2 mediates multiple biological processes including growth and differentiation of B cells, generation of lymphokine-activated killer cells and augmentation of NK cells (Wustrow, 1991). In the CNS, IL-2 stimulates the growth of oligodendroglial cells *in vitro* (Benveniste and Merrill, 1986), modulates N-methyl-D-aspartate receptors (NMDA-R) of native mesolimbic neurons (Ye *et al.*, 2001) and influences mesocorticolimbic dopamine release (Ye *et al.*, 2001). IL-2 damages the blood-brain-barrier (BBB) and the integrity of the endothelium of brain vessel (Ellison *et al.*, 1987). However, it does not cross the BBB via a saturable transport system.

3.8.2.2 IL-6 (neuro-endo-immunological mediator)

IL-6, also called IFN-β2/B-cell stimulatory factor 2/hepatocyte stimulating factor (Ferguson-Smith *et al.*, 1988) is a 26 kDa protein with 185 amino acids (Conti *et al.*, 2002). The human IL-6 gene contains five exons and maps to human chromosome 7p15-21 (Ferguson-Smith *et al.*, 1988). The IL-6R is a protein of 80 kDa (Fujisawa *et al.*, 2002). IL-6R consists of 2 chains, IL-6Rα and IL-6Rβ. The IL-6/IL-6R complex associates with a 130-kDa23 transmembrane glycoprotein (gp130). Glycoprotein 130 is involved in signal transduction.The IL-6R is expressed on various cell types such as lymphocytes, monocytes, fibroblasts,vascular endothelial cells and pituitary cells (Barton, 1997). In addition, soluble IL6 receptor (sIL-6R) enhances the effect of IL-6 (Schobitz *et al.*, 1995). Figure 15 shows the crystal structure of human Interleukin-6.

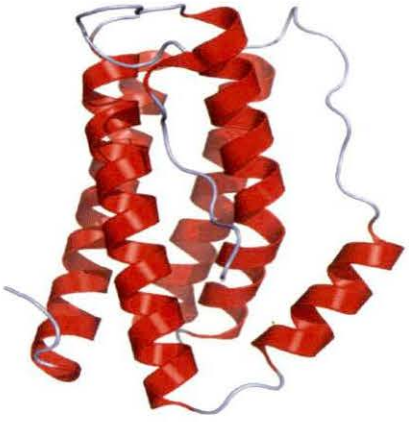


Figure 15: Crystal structure of human Interleukin-6.

3.8.2.2.1 IL-6 secreting cells

Many different cell types produce IL-6. The main sources *in vivo* are stimulated monocytes/macrophages, fibroblasts and endothelial cells (Coil *et al.*, 2004; Dalal *et al.*, 2003; Ng *et al.*, 2003; Soderquist *et al.*, 1998; Yachie *et al.*, 1990). Additionally, T-cells, B lymphocytes, eosinophils, mast cells, astrocytes and microglia also produce IL-6 after stimulation (Azzolina *et al.*, 2003; Delgado *et al.*, 2003; Inoue, 2002; Diehl and Rincon, 2002; Hoenstein *et al.*, 2001; Lorentz *et al.*, 2000; Frei *et al.*, 1989). IL-6 mRNA was found to be generally low in the brain (Schobitz *et al.*, 1993). It is present in the hippocampal formation with highest signal in the dentate gyrus, habenular nucleus, piriform cortex, hypothalamus and striatum (Chen *et al.*, 2003; Gadient and Otten, 1994; Schobitz *et al.*, 1992).

3.8.2.2.2 Biological activities of IL-6

IL-6 is involved in regulating a wide variety of immune functions, such as B- and cytotoxic T-cell differentiation, induction of IL-2 production and IL-2R expression in T cells, T cell growth, acute-phase reactions and hematopoiesis (Hirano, 1998; Taga and Kishimoto, 1997).

Recently, Diehl and Rincón (Diehl and Rincon, 2002) suggested that APC IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization through IL-12 independent molecular mechanisms. IL-6 activates transcription mediated by the transcription factor Nuclear Factor of Activated T cells (NFAT), leading to IL-4 production by naïve CD4+ T cells and their differentiation into effector Th2 cells. The induction of Th2 differentiation by IL-6 is dependent upon endogenous IL-4. In addition, IL-6 binds to IL-6Rα, leading to the dimerization of gp130/IL-6Rβ (Brakenhoff *et al.*, 1995). Dimerization of gp130 by IL-6 causes the activation of two signaling pathways: (1) the JAK/STAT pathway and (2) the CCAAT/enhancer binding protein (C/EBP) pathway (Weihua *et al.*, 2000; Heinrich *et al.*, 1998). IL-6 inhibits Th1 differentiation via the JAK/STAT1 pathway by inducing the suppressor of cytokine signaling 1 (SOCS1) expression (Siewert *et al.*, 1999). IL-6 upregulates SOCS1 expression in activated CD4+ T cells, thereby interfering with signal transducer and activator of transcription 1 (STAT1) phosphorylation induced by IFN-γ. Inhibition of IFN-γR-mediated signals by IL-6 prevents auto-regulation of IFN-γ gene expression by IFN-γ during CD4+ T cell activation, thus preventing Th1 differentiation. This pathway is IL-4- and IL-12-independent (Diehl and Rincon, 2002). Furthermore, IL-6 exerts distinct effects on the CNS such as activation of the hypothalamic-pituitary-adrenal axis (HPA), reduction of food intake, induction of fever and neuronal growth (Godbout and Johnson, 2004; Path *et al.*, 2000). IL-6 induces nerve growth factor (NGF) in astrocytes, enhances NGF-stimulated astrocyte proliferation (Levison *et al.*, 2000; Marz *et al.*, 1999; Schafer *et al.*, 1999; Kossmann *et al.*, 1996), promotes survival of the mesencephalic catecholaminergic and septal cholinergic neurons *in vitro* (Kushima and Hatanaka, 1992; Hama *et al.*, 1991) and attenuates the neurotoxic effects of NMDA onstriatal cholinergic neurons (Toulmond *et al.*, 1992).

3.8.3 CYTOKINES AND SCHIZOPHRENIA

The cytokine alterations in schizophrenia have been intensively investigated and reviewed by others (Gaughran, 2002; Rothermundt *et al.*, 1998; Rothermundt *et al.*, 2001; Schuld *et al.*, 2004). The most momentous findings in this respect are summarized in table 11. Additionally, some interesting observations concerning cytokines and schizophrenia are described below.

With regard to interleukin (IL)-2, a significant negative correlation between the serum IL-2 levels and the positive subscale P of the Positive and Negative Syndrome Scale (PANSS) (Zhang *et al.*, 2002) and a positive correlation between the plasma IL-2 and homovanillic acid levels (Kim *et al.*, 2000) were observed in two studies. Additionally, Maes *et al.*, (1995b) described a positive correlation between plasma soluble IL-2 receptor (sIL-2R) and transferring receptor (TfR), another marker of immune activation.

As has been observed, there is a discrepancy between the circulating levels of IL-2 and its *ex vivo* production. To explain this phenomenon, it was suggested that the reduced *ex vivo* IL-2 production may be a consequence of overproduction of IL-2 *in vivo* (Rothermundt *et al.*, 1998). Ganguli *et al.* (1992) found that autoantibody-positive acutely ill schizophrenic patients had lower mitogen-stimulated IL-2 production than other patients.

The only study investigating the levels of sIL-2R in the intrathecal compartment reported decreased values in the cerebrospinal fluid (CSF), but increased concentrations in serum of schizophrenic patients (Barak *et al.*, 1995). These findings indicate systemic immune activation in schizophrenia. However, more evidence is needed to support this conclusion.

Two studies demonstrated a positive association of the serum IL-6 levels with duration of illness (Ganguli *et al.*, 1994; Kim *et al.*, 2000). Another approach was presented in the study by Toyooka *et al.*, (2003) in which they measured protein and /or mRNA levels for IL-1β and IL-1 receptor antagonist (Il-1Ra) in the postmortem brain tissues of schizophrenic patients. They found decreased levels of both IL-1Ra protein and mRNA in the prefrontal cortex of the patients, whereas IL-1 levels were

not altered. In the same study they found the increased serum levels of IL-1Ra in drug-free schizophrenic patients. It was suggested that the decreased IL-1Ra levels in the CNS might enhance various IL-1 mediated actions in schizophrenic patients.

Interestingly, Inglot *et al.*, (1994) observed that the patients with high interferon (IFN) response to lipopolysaccharide (LPS) or phytohemagglutinin (PHA) stimulation had dominant positive symptoms of schizophrenia whereas in the patients with low IFN response, the negative symptoms prevailed. Moreover, a significant positive inter-correlation between the lowered production of INF-γ and IL-2 were detected in the patients studied by Arolt *et al.*, (2000) during the 1-month treatment period. Preble and Torrey (1985) demonstrated that IFN-positive were more likely than IFN-negative patients to have a recent onset or exacerbation of their illness and to be on low-dose or no medication. Additionally, McAllister *et al.*, (1995) found that symptom exacerbation was associated with the increased CSF IL-2 levels.

Table 11: Cytokine profiles of schizophrenic patients as reported by the various authors.

Cytokine	Level	Plasma / serum level	Ex vivo production	CSF
IL-2	Increase	Zhang *et al.* (2002), McAllister *et al.* (1995), Kim et al. (1998)	Cazzullo *et al.* (2001), Cazzullo *et al.* (1998), O'Donnel *et al.* (1996)	McAllister *et al.* (1995), Licinio *et al.* (1993)
	Decrease	Theodoropoulou *et al.* (2001)	Xu *et al.* (1994) Kim *et al* (1998) Rothermundt et al. (1998), Ganguli *et al.* (1992), Hornberg *et al.* (1995), Bessler *et al.* (1995), Bessler *et al.* (1995), Arolt *et al.* (2000), Ganguli *et al.* (1995), Ganguli *et al.* (1989), Villemain *et al.* (1987), Yang *et al.* (1994), Villemain *et al.* (1989)	Not reported
	Normal	Barak *et al.* (1995),Gattaz *et al.* (1992), Xu *et al.* (1994)	Rothermundt *et al.* (2000)	Barak *et al.* (1995), Rapaport *et al.* (1997), el-Mallakh *et al.* (1993)

Cytokine	Level	Plasma / serum level	Ex vivo production	CSF
sIL-2R	Increase	Barak *et al.* (1995), Maes *et al.* (1994), Akiyama (1999), Rapaport and Lohr (1994), Gaughran *et al.* (1998), Rapaport *et al.* (1989)	Not investigated	Not reported
	Decrease	Not reported	Not reported	Barak *et al.* (1995)
	Normal	Haack *et al.* (1999), Muller *et al.* (1997), Erbagci *et al.* (2001)	Not reported	Not reported
IL-6	Increase	Zhang *et al.* (2002), Maes *et al.* (1994), Akiyama (1999), Naudin *et al.* (1996), Frommberger *et al.* (1997), Ganguli *et al.* (1994), van Kammen *et al.* (1999), Maes *et al.* (1995a)	Not reported	Not reported
	Decrease	Not reported	Not reported	Not reported
	Normal	Haack *et al.* (1999), Erbagci *et al.* (2001), Baker *et al.* (1996), Katila *et al.* (1994a), Cazzullo *et al.* (2001)	Kim *et al.* (1998), Hornberg *et al.* (1995)	van Kammen *et al.* (1999), Katila *et al.* (1994b)
sIL-6R	Increase	Maes *et al.* (1997)	Not investigated	Not investigated
	Decrease	Maes *et al.* (1994)	Not reported	Not reported
	Normal	Muller *et al.* (1997)	Not reported	Not reported
IL-1	Increase	Theodoropoulou *et al.* (2001), Katila *et al.* (1994a)	Sirota *et al.* (1995)	Not reported
	Decrease	Barak et al. (1995) Rothermundt *et al.*	Not reported	Barak *et al.* (1995)
	Normal	(2000),Erbagci *et al.* (2001), Kim *et al.* (1998), Baker *et al.* (1996), Katila *et al.* (1994b)	Bessler *et al.* (1995), Kim *et al.* (1998)	el-Mallakh *et al.* (1993)
IL-1Ra	Increase	Akiyama (1999),Maes et al. (2000) Maes *et al.* (1997),Toyooka *et al.* (2003)	Not investigated	Not investigated

Cytokine	Level	Plasma / serum level	Ex vivo production	CSF
	Decrease	Not reported	Not reported	Not reported
	Normal	Haack *et al.* (1999)	Not reported	Not reported
IFN	Increase	Preble and Torrey (1985), Kim *et al.* (2004)	Cazzullo *et al.* (2001), Cazzullo *et al.* (2002)	Not investigated
	Decrease	Not reported	Rothermundt *et al.* (1998), Rothermundt *et al.* (2000), Arolt *et al.* (1997), Katila *et al.* (1989), Moises *et al.* (1985), Naidenova *et al.* (1988), Wilke *et al.* (1996), Inglot *et al.* (1994)	Not reported
	Normal	Gattaz *et al.* (1992), Rimon *et al.* (1985), Becker *et al.* (1990)	Not reported	Not reported
TNF-α	Increase	Theodoropoulou *et al.* (2001)	Not reported	Not investigated
	Decrease	Not reported	Not reported	Not reported
	Normal	Haack *et al.* (1999), Erbagci *et al.* (2001), Baker *et al.* (1996), Kudoh *et al.* (2001), Schattner *et al.* (1996)	Schattner *et al.* (1996)	Not reported
IL-10	Increase	Maes *et al.* (2002)	Not reported	Not investigated
	Decrease	Not reported	Not reported	Not reported
	Normal	Not reported	Rothermundt *et al.* (1998), Cazzullo *et al.* (2001), Cazzullo *et al.* (1998)	Not reported

3.8.3.1 Th2 shift hypothesis in schizophrenia

In vivo and *in vitro* studies have shown that schizophrenia may be associated with an imbalance in cytokines network, suggesting suppression of some immune functions and activation of others. To explain the phenomenon of immunosuppression and immune activation in schizophrenic patients, Muller *et al.*, (1999) put forward 'Th2 hypothesis', which states that the Th1-Th2 balance is shifted to Th2 in schizophrenia

(Muller *et al.*, 1999). The key characteristics of the Th-1 system are the production of interleukin-2 and interferon-γ, which have been reported to be decreased *in vitro* (Ganguli *et al.*,1989; Ganguli *et al.*,1995; Villemain *et al.*,1989; Bessler *et al.*,1995; Arolt *et al.*, 2000) and increased *in vivo* (Kim *et al.*, 2000).On the contrary, Th-2 system in schizophrenia is characterized by activation , which is characterized by increased IL-6, sIL-6R (Maes *et al.*, 1995a; Ganguli *et al.*,1995; Frommberger *et al.*, 1997; Lin *et al.*, 1998; Muller *et al.*, 1997; Van Kammen *et al.*, 1999; Na and Kim, 2007) and IL-10 levels (Cazzullo *et al.*, 1998a; Cazzullo *et al.*, 1998b), as well as increase IL-4 levels in the CSF of juvenile schizophrenic patients (Mittleman *et al.*,1997; Na and Kim, 2007) . Empirical evidence in support of the Th1/Th2 imbalance has been inconsistent. Some authors could not replicate the previously reported findings (Baker, 1996), or the findings are in conflict with each other, for example, increased *in vitro* IL-2 or interferon (IFN)-γ production or decreased IL-2 serum levels was recently found in schizophrenia (O'Donnell *et al.*, 1996; Cazzullo *et al.*, 2001; Theodoropoulou *et al.*, 2001). Recently Potvin *et al.*, 2008 in their quantitative review refuted the current hypothesis of a Th2 slant in schizophrenia.

3.9 C-REACTIVE PROTEIN

Human C-reactive protein (CRP) can be classified under two partially overlapping groups of proteins. On the basis of its best known biological property i.e., the striking increase of its plasma concentration during infection and inflammation, CRP is categorized as an acute-phase protein. On the basis of its structure and Ca++ dependent binding specificities, CRP is classified as pentraxin (Pepys and Hirschfield, 2003) . The name CRP is derived from the fact that this protein has the capacity to precipitate the somatic C-carbohydrate of *pneumococcus*. Acute phase proteins constitute a heterogeneous group of proteins of hepatic origin (Pepys and Hirschfield, 2003) that share the property of increased plasma concentration during infection and/or tissue injury. CRP is the most characteristic human acute-protein, since its plasma concentration raises by several hundred folds within 24-48 hours from tissue injury. These high levels persist for the duration of the acute-phase response, returning to the normal low concentrations with restoration of tissue structure and function.

3.9.1 HISTORY

Tillett and Francis first discovered C-reactive protein in 1930 as a substance in the serum of patients with acute inflammation that reacted with the C polysaccharide of *pneumococcus* (Tillett and Francis, 1930). Initially, CRP was thought to be a pathogenic secretion, as it was elevated in people with a variety of illnesses, including cancer. Discovery of hepatic synthesis and secretion of CRP closed that debate. It is thought to bind to phosphocholine, thus initiating recognition and phagocytosis of damaged cells. (Pepys and Hirschfield, 2003).

3.9.2 BIOCHEMISTRY

CRP is a normal alpha globulin composed of 224-residue protein with a monomer molar mass of 25106 Da (Pepys and Hirschfield, 2003). It has an annular pentameric disc in shape and a member of the small pentraxins family (Fig.16). Native CRP is a bit different, as it has 10 subunits making two pentameric discs, with an overall molecular mass of 25106 Da.

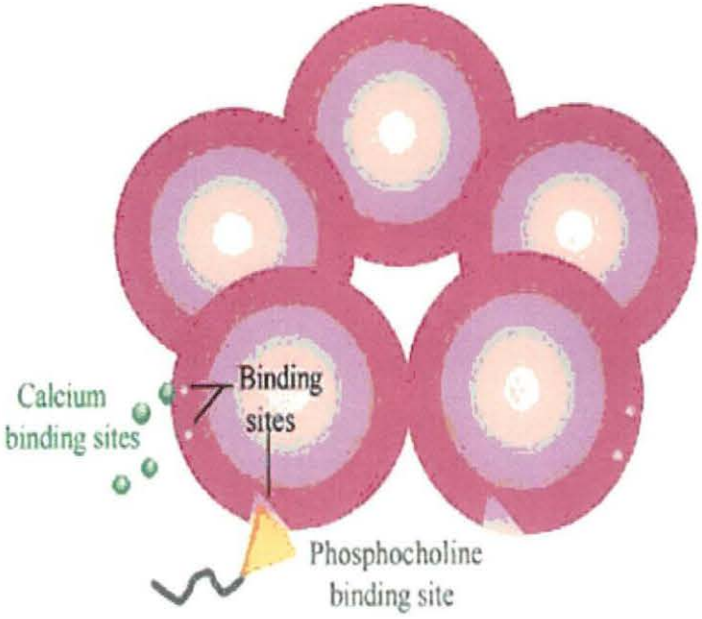


Figure 16: Pentamer structure of CRP, including calcium and phosphocholine binding sites. These sites enable CRP to recognize and bind to a variety of microorganisms, cellular debris, and nuclear material from damaged cells.

3.9.3 FUNCTION

The level of CRP rises dramatically during inflammatory process rising upto 50,000 fold in acute inflammation, such as infection (Fig.17). It rises above normal limits within 6 hours and peaks at 48hours. This increment is due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages as well as adipocytes (Pepys and Hirschfield, 2003; Lau *et al.*, 2005). CRP binds to phosphocholine on microbes. It is thought to assist in complement binding to foreign and damaged cells and enhances phagocytosis by macrophages which express a receptor for CRP. It is also believed to play another important role in innate immunity, as an early defense system against infections. The half-life of CRP is constant and therefore its level is mainly determined by the rate of production (and hence the severity of the precipitating cause). Serum amyloid A is a related acute-phase marker that responds rapidly in similar circumstances (Pepys and Hirschfield, 2003).

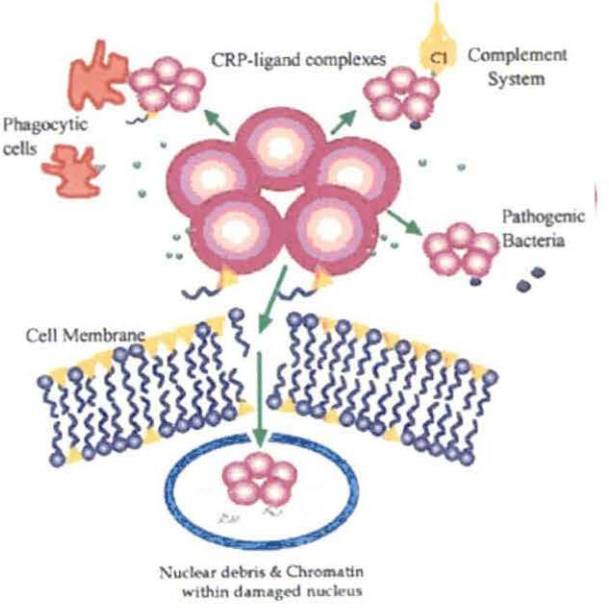


Figure 17: ***Key functions of CRP within the innate immune system include the ability to (1) recognize and bind to phosphocholine exposed in damaged cell walls and found in many bacteria, fungi, and parasites; (2) act like an opsonin, marking bacteria, damaged cell walls, and nuclear debris for phagocytosis; (3) bind to C1, the first component of the classical pathway of the complement system that triggers phagocytic activity; and (4) bind to polymorphonuclear leukocytes (PMNs) and monocytes, which stimulate the production of inflammatory cytokines.***

Elevated CRP levels are usually observed in a variety of infections and inflammatory conditions where there is tissue destruction (Fig.18). Elevated CRP is known to be the risk factor for the cardiovascular diseases, diabetes and other metabolic dysfunctions (Bassuk *et al.*, 2004; Pfutzner and Forst, 2006). In addition, it is also known to be associated with the depression (Ford and Erlinger, 2004) and cognitive impairment (Yaffe *et al.*, 2003).CRP is also useful in helping diagnose autoimmune conditions such as vasculitis, systemic lupus erythematosus, inflammatory bowel disease and rheumatoid arthritis. However, because autoimmune disorders tend to wax and wane the level CRP will not be elevated if patients with these disorders are not in an active disease state (Szalai *et al.*, 1999).The CRP test is also a generalized test that doesn't indicate the cause or site of inflammation. In chronic autoimmune disorders the CRP level is helpful in gauging a patient's response to therapy or to tell if disease flares are present. However, in terms of diagnosing autoimmune conditions a low CRP level can't be used to rule out specific diseases (Szalai *et al.*,1999).

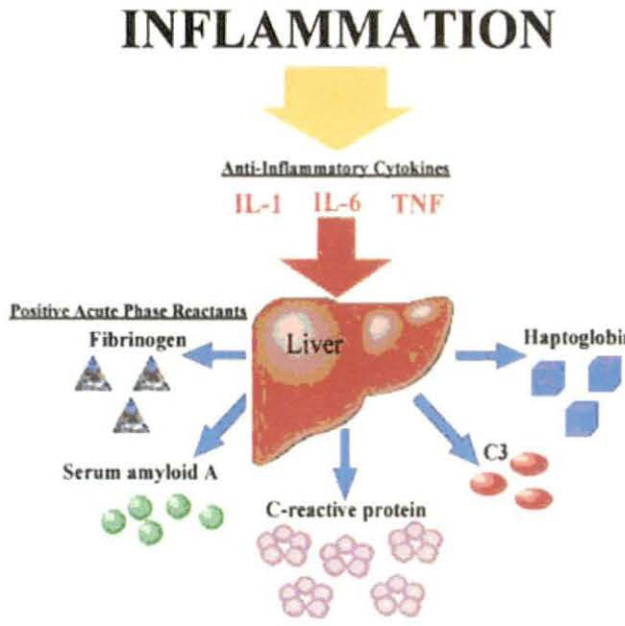


Figure 18: Stimulation and synthesis of positive acute-phase reactants during inflammation. Inflammation caused by infection or tissue damage stimulates the circulating inflammation-associated cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α. These cytokines stimulate hepatocytes to increase the synthesis and release of positive acute-phase proteins, including CRP. IL-6 is the major cytokine stimulus for CRP production.

3.9.4 C-REACTIVE PROTEIN AND SCHIZOPHRENIA

As schizophrenia is thought to be manifested by some inflammatory process CRP study is implicated in this respect. Few studies have been carried out to investigate the association of CRP and schizophrenia. In one study, elevated serum levels of CRP was found in patients who showed more severe clinical symptoms of schizophrenia as reflected by the PANSS total score (Fan *et al.*, 2007). In another study, the elevated serum levels of C-reactive protein in schizophrenia are found to be associated with the severity of cognitive impairment but not of psychiatric symptoms (Dickerson *et al.*, 2007). Further studies are awaited to throw the light in the role of inflammatory process in the manifestation of schizophrenia.

3.10 LYMPHOCYTES

T cells belong to a group of white blood cells known as lymphocytes which plays a central role in cell-mediated immunity They can be distinguished from other lymphocyte types (e.g., B cells and natural killer cells) by the presence of a special receptor on their cell surface called T cell receptors (TCR). There are two defined types of TCR, one is a heterodimer of two disulphide-linked polypeptides (α and β), the other is structurally similar but consists of γ and δ polypeptides and CD3 complex Together they form the T-cell receptor complex (TCR-CD3 complex). Approximately 90-95% of blood T cells are αβ T cells and the remaining 5-10% are γδ T cells. αβ T cells are further distinguished by their expression of CD4 or CD8 marker.

3.10.1 CD4+ AND CD8+ CELLS

αβ T cells are subdivided into two distinct non-overlapping populations. A subset which carries the CD4 marker and mainly 'helps' or 'induces' immune response (TH) and a subset which carries the CD8 marker and is predominantly cytotoxic (Tc). CD4+ T cells recognize their specific antigens in association with the major histocompatibility complex (MHC) class II molecules. On the other hand CD8+ T cells recognize antigens in association with MHC class I molecules. Thus, the presence of CD4 or CD8 limits the type of cell with which the T cell can interact. A small proportion of αβ T cells express neither CD4 nor CD8 and these 'double

negative' T cells might have a regulatory function. Similarly, most circulating γδ cells are 'double negative', although a few of them are CD8+. By contrast, most γδ T cells in tissue express CD8.

3.10.2 FUNCTION OF CD4+ AND CD8+ CELLS

CD4+ T cells bind an epitope consisting of an antigen fragment lying in the groove of a class II histocompatibility molecule (Fig.19). CD4+ T cells are essential for both the cell-mediated and antibody-mediated branches of the immune system. The CD4+ cells bind to antigen presented by antigen-presenting cells (APCs) like phagocytic macrophages and dendritic cells. The T cells then release lymphokines that attract other cells to the area. The result is inflammation that is the accumulation of cells and molecules that attempt to wall off and destroy the antigenic material (an abscess is one example, the rash following exposure to poison ivy is another).The CD4+ cells, called helper T cells bind to antigen presented by B cells. The result is the development of clones of plasma cells secreting antibodies against the antigenic material.

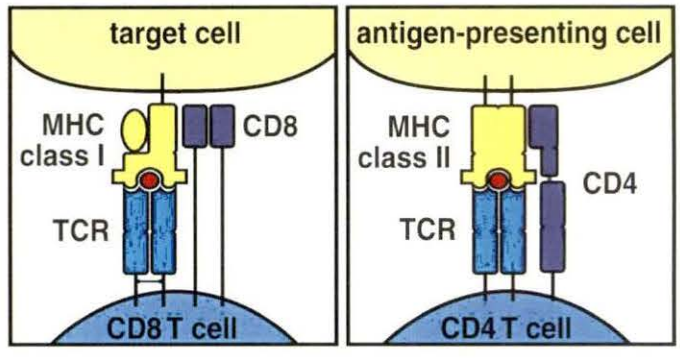


Figure 19: Binding interactions between CD8 T cells and target (virus-infected) cells and between CD4 T cells and professional antigen presenting cell.

The best understood $CD8^+$ T cells are cytotoxic T lymphocytes (CTLs). They secrete molecules that destroy the cell to which they have bound. This is a very useful function if the target cell is infected with a virus because the cell is usually destroyed before it can release a fresh crop of viruses that are able to infect other cells. In general, the role of the $CD8^+$ T cells is to monitor all the cells of the body, ready to destroy any that express foreign antigen fragments in their class I molecules.

Changes in T helper/inducer (CD4) and T cytotoxic/suppressor (CD8) cells are related to a variety of illnesses (Riddell *et al.*, 1993). CD4 cells facilitate both humoral and cell-mediated immune processes. In AIDS patients, large declines in CD4 cell counts usually result in both a general decline in immune function and a vulnerability to opportunistic infections. Indeed, higher CD4 cell counts are thought to be consistent with good health. In contrast, CD8 cells act to shut off CD4 cell activity when sufficient antibodies have been produced. The low CD4/CD8 ratios are associated with immunodeficiency (Reinherz and Schlossman, 1980). T-lymphocyte subset ratio supply valuable information about the state of the cellular immune system. An increase in CD8 lymphocytes suppresses cellular immunity, whereas a decrease could cause excessive functioning. The appropriate CD4/CD8 lymphocyte ratio is expected to be 2:1. Ratios below 1:1 indicate serious disorder of the immune system (Kouttab *et al.*, 1989). It has also been suggested that changes in the T-lymphocyte ratio reflect changes in the metabolism of central nervous system cells and that they could be used as neural markers in the analysis of psychiatric disorders (Gladkevich *et al.*, 2004).

3.10.3 CD4+ AND CD8+ CELLS AND SCHIZOPHRENIA

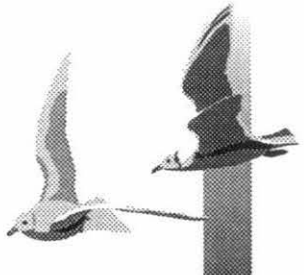
Advances in immunologic techniques as well as a deepening understanding of lymphocyte function have opened the way towards the quantitation of specific, functionally distinct lymphocyte subsets. Initially, these studies focused on thymus-derived (T lymphocyte) subsets. Reductions in T-cell percentage or number in schizophrenia were found by several investigators using a resetting procedure (Nyland *et al.*, 1980; Coffey *et al.*, 1983). With the development of automated cell counting methods using fluorescent-labeled monoclonal antibodies, elevations in total T lymphocytes as well as CD4 (helper) cells were observed by several investigators (DeLisi *et al.*, 1982; Henneberg *et al.*, 1990; Muller *et al.*, 1993; Masserini *et al.*, 1990). DeLisi *et al.*, (1982) and Cazullo *et al.*, (1998a) also documented an increase in the CD8 (suppressor) subset. In contrast to these results, Villemain *et al.*, (1989) observed no differences in the percentages of either CD4 or CD8 lymphocytes between unmedicated schizophrenic subjects and normal control subjects. Recently Zhang *et al.*, (2002) have found the lower CD4 cell percentage in Chinese patients. Using flow-cytometry, Mazzarello *et al.*, (2004) found lower CD8 lymphocyte level in schizophrenia patients than in controls. Rudolf *et al.*, (2004) on the other hand did

not find any differences in the T-lymphocyte subset ratios of schizophrenic patients and controls. This finding also corroborated with the recent finding by Craddock *et al.*, (2007). Moreover, some studies observed an increase in total T-lymphocytes (De Lisi *et al.*, 1982). Pirildar *et al.*, (2001) reported that there were no differences between the schizophrenic patients and the control in terms of CD4 and CD8 lymphocyte percentage.

It has been suggested that T-lymphocyte level and T-lymphocyte subset ratios could be laboratory markers for treatment response in schizophrenia (Muller *et al.*, 1993). Zhang *et al.*, (2006) have shown that post-treatment clinical improvement in schizophrenia is associated with an increase in the CD4-lymphocyte level. Despite of these findings there is no general agreement regarding the role of T-lymphocytes in schizophrenia and awaits further research.

Section - 4

Materials and Methods



4. MATERIALS AND METHODS

4.1 SUBJECT

A total number of 136 schizophrenic patients were included in this study. Out of 136 schizophrenic patients, 118 were paranoid, 9 were disorganised, 1 was catatonic, 5 were undifferentiated and 3 were residual. All the subjects were India born schizophrenic patients of West Bengal referred to the outpatient department (OPD) of Psychiatry, North Bengal Medical College and Hospital, Siliguri. Three major selection criteria were considered for the screening of schizophrenic group: - (i) unrelatedness of individuals from each other, (ii) resident of the state of West Bengal and Bengalee by ethnicity, (iii) subjects satisfying DSM-IV-TR diagnostic criteria for schizophrenia (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, text revision, 2002). The exclusion criteria followed in the present study include:- (i) history of smoking or substance abuse, (ii) presence of personality disorder, (iii) presence of dementia and mental retardation (iv) general medical condition. The patients group comprise of 87 Bengali, 9 Nepali, 11 Bihari, 17 Tribal and 12 Rajbanshi individual ranging from 16-55 years of age. The patients were diagnosed by psychiatrist using Structured Clinical Interview (First, 1996) and according to the standard diagnostic criteria of DSM-IV-TR and assessed by the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham,1962). Some of the demographic variables are also considered for the study.

A total number of 150 unrelated, ethnically matched healthy individuals were considered as controls. The criteria for the selection of controls include :- (i) same ethnic group as the patients,(ii) sex and age matched with patients,(iii) absence of family history of autoimmune and psychiatric disorder,(iv) recent history of intercurrent infection and allergies,(v) no history of smoking or any substance abuse and (vi) no history of general medical condition. All the participants provided their written consent for giving the blood sample after the study procedures were explained. The study has been carried out according to the principles of the Declaration of Helsinki (1964) and approved by the ethical committee of the institution.

The schizophrenic and control subjects were divided into 4 different groups for various studies such as :- (i) HLA association (if any) (ii) serum IL-2 and IL-6, (iii) CRP and (iv) CD4+ and CD8+ percentage.

4.2 TYPING OF HLA CLASS-I

In the present study the subjects were typed for HLA-Class I antigens by serological method (i.e. two-stage microlymphocytotoxicity assay) as well as by molecular method (ARMS-PCR SSP typing).

4.2.1 SEROLOGICAL TYPING OF HLA CLASS I ANTIGEN (Microlymphocytotoxicity assay)

Serological typing of HLA-ClassI antigens were carried out in all the subjects by complement mediated two-stage microlymphocytotoxicity assay (Terasaki & McClelland, 1964). In the two-stage test, the first step employs the sensitization step of HLA antigen with antibody. The second step is the 'specificity test', achieved by the addition of rabbit complement.

The principle behind this technique is the binding of anti-HLA antibody to a specific HLA Class I antigen expressed on the lymphocytes. A suspension of lymphocytes mixed with a specific antiserum results in the formation of antigen-antibody complex. In the presence of complement, if the antibody used has the potential for complement activation, cells carrying the appropriate antigen are damaged resulting in increased permeability. The damaged cells are not completely lysed but suffer membrane damage to allow uptake of stain such as Eosin. Where the antigens on the cell surface do not react with antibody, the lymphocytes are not stained (Negative Reaction). Microscopic identification of the stained cells indicate the presence of a specific HLA antibody. Viable cells appear bright and refractory while the dead cells look dark and distinctly larger.

4.2.1.1 Isolation and preparation of lymphocyte cell suspension

1. Approximately 5ml of blood sample was collected from each individual with the help of disposable syringes and was added 100-150 IU preservative free Lithium Heparin in a clean test tube and mixed well.

2. The blood was diluted with equal volume of PBS and mixed thoroughly by gentle shaking.
3. The diluted blood was layered over lymphoprep carefully with a Pasteur pipette in a ratio of 2:1 respectively.
4. The tubes were centrifuged at 2000 rpm for 20 minutes at room temperature.
5. Using a clean Pasteur pipette, the interface (white-foggy) layer of mononuclear cells was transferred into a clean test tube.
6. The cells were washed 2-3 times with PBS (pH 7.2) by centrifuging at 1000 rpm for 10 minutes at room temperature and lymphocyte cell suspension was prepared.
7. Finally, the lymphocyte cell suspension was counted in a Neubauer Haemocytometer and adjusted to a final concentration of $2x10^6$ cells/ml using PBS.
8. Viability test was done using 1% trypan blue and observing under phase contrast microscope. A drop of cell suspension was mixed with a drop of trypan blue on a clean glass slide and allowed to stand for 5 min. Dead cells became dark blue. At least 85% viability was required.

4.2.1.2 Method of HLA class I typing

Two sets of well defined HLA antisera (one from BAG, Germany and another obtained from 12^{th} International Histocompatibility Workshop and Conference) were used. The HLA class I antisera used for the present study included 17 A-locus alleles and 25 B-locus alleles. Terasaki plates made up of nontoxic disposable polysterene material (NUNC, Denmark) containing 60-72 wells were used for testing of class I antigens. The typing trays were thawed before use at room temperature. 1µl of lymphocyte suspension ($2x10^6$ cells/ml) was dispensed carefully into each well and the cells and sera were mixed well. The trays were incubated at 22-24°C for 30minutes. 5µl nontoxic rabbit complement was added to each well with Hamilton six-needle dispenser (250µl capacity). The trays were further incubated for a period of 60minutes. 4µl of 5% Eosin-Y dye (centrifuged before use) was added to each well. After 5 minutes 4µl of 40% formalin (centrifuged before use) was added to each well.

The trays were capped tightly and kept in the refrigerator for reading the results after a gap of at least 2 hours.

Trays were read using inverted phase contrast Microscope (Leitz, West Germany) with 10x objective and 10x eyepiece. Live or viable cells that excluded dye were small, refractile and unstained, while dead cells looked dull, larger in size and stained red with Eosin. Scoring was done by estimating the percentage of cell lysis as adapted in the 8th International Histocompatibility Workshop (Table 12). Typing trays were scored on a 'subjective scale' taking into consideration the amount of 'background' (dead cells) in the negative control well.

Table 12: Scoring system as adapted in the 8th International Histocompatibility Workshop.

SCORE	INTERPRETATION	INCREASE IN CELL DEATH OVER THE NEGATIVE CONTROL-%
1	Negative (-ve)	0-19% kill
2	Doubtful weak positive (+)	20-29% kill
4	Positive (+ +)	30-39% kill
6	Strong Positive (+ + +)	50-79% kill
8	Very Strong Positive (+ + + +)	80-100% kill
0	Invalid	Not readable

4.2.2 MOLECULAR TYPING OF HLA CLASS I GENES (ARMS-PCR SSP TYPING) Amplification refractory mutation system-polymerase chain reaction- sequence specific primer

4.2.2.1 DNA Extraction (Phenol Chloroform Method)

DNA was isolated from frozen peripheral blood samples using phenol chloroform extraction method with slight modifications. The procedure is as follows :-

1. Blood samples were thawed first.
2. 500µl blood sample was taken and washed with 1ml of 1X SSC, mixed gently and centrifuged at 5000rpm for 5 minutes.

3. The supernatant was removed and 1.2 ml of 1X SSC was added and mixed gently and centrifuged at 5000rpm for 2 minutes.
4. The supernatant was removed and 1.2ml of 50 mM KCl was added and mixed gently and then centrifuged.
5. Again the supernatant was removed and 375µl of High Salt Lysis Buffer was added.
6. 25µl of 10% SDS and 12.5µl of 8mg/ml proteinase K stock was added and incubated for 1 hour at 56°C.
7. The proteinase K digested suspension was transferred into a new microfuge tube and an equal amount of Phenol-Chloroform (PCl) was added and spinned at 12000 rpm for 20 minutes.
8. The DNA was recovered and rinsed with 1ml of 100% C_2H_5OH .
9. The DNA was rinsed with 500µl of 70% C_2H_5OH, three times.
10. The DNA was dried and dissolved in 50µl of TE.

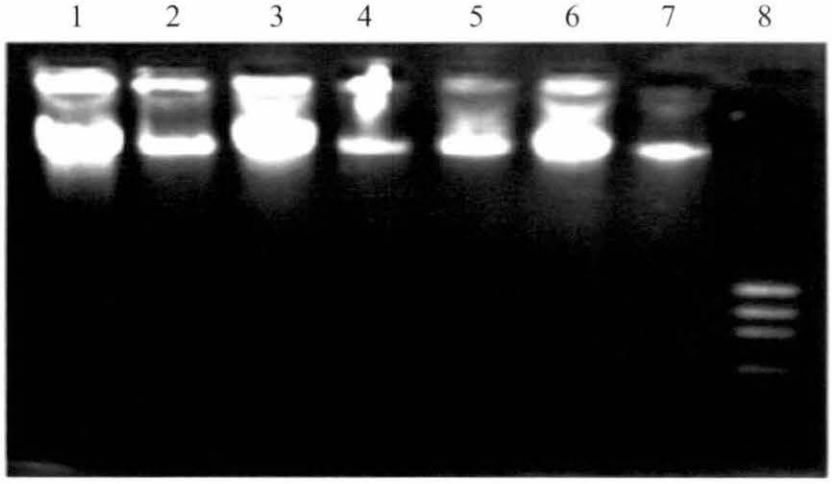


Figure 20: Electrophoregram showing the electrophoresis of DNA Lane 1-7 DNA, lane 8 ladder.

4.2.2.2 Quantification of DNA

10µl of dissolved DNA was diluted to 1.5ml using deionized water and mixed properly. OD of the diluted DNA was measured at wavelengths of 260 nm and 280 nm using a UV spectrophotometer. Protein-free DNA samples gave 1.7-2.0 reading at 260/280. The concentration of DNA was calculated using the formula - OD at

260Xdilution factorX50 (1 OD = 50µg of double stranded DNA). The DNA were also visualized in the agarose gel (Fig. 20).

4.2.2.3 PCR Amplification

PCR was carried out in thermostable PCR-tubes with 50µl of PCR reaction mixture consisting of PCR buffer, $MgCl_2$, primer set, deoxynucleotide triphosphate mix and Taq DNA polymerase along with the template DNA. The methodology and sequence information were taken from Bunce *et al.*, 1995 as well as from the 12th IHW protocols. The nucleotide sequences of primers used for amplification of various HLA- and HLA-B locus alleles in this study are listed in table2 & 3

4.2.2.3.1 Preparation of PCR reaction mixture

PCR amplification was performed in thermostable sterile PCR tubes on a DNA thermal cycler (Perkin Elmer, USA) containing 50 µl reaction mixture of the following combination:-

Reagents		Reaction Mix (50µl)
Initial conc. Of stock solution	Final conc. Of stock solution	For 1 test
dH_2O	----	36.8µl
10X PCR Buffer	1X	5 µl
dNTPs	2.5 mM	3.2 µl
P1	10 pm	1 µl
P2	10 pm	1 µl
Target DNA	50µg	2 µl
Taq polymerase	3 unit	1 µl

Table 13: List of primers used for HLA-A locus typing.

Name of the allele	Sequence	nucleotide Length
A*01		
P1	5′-GGA CCA GGA GAC ACG GAA TA-3′	20
P2	5′-AGG TAT CTG CGG AGC CCG-3′	18
A*02		
P1	5′-TCC TCG TCC CCA GGC TCT-3′	18
P2	5′-GTG GCC CCT GGT ACC CGT-3′	18
A*03		
P1	5′-AGC GAC GCC GCG AGC CA-3′	17
P2	5′-CAC TCC ACG CAC GTG CCA-3′	18
A*23		
P1	5′-GGC CGG AGT ATT GGG ACG A-3′	19
P2	5′-CCT CCA GGT AGG CTC TCA A-3′	19
A*24		
P1	5′-GGC CGG AGT ATT GGG ACG A-3′	19
P2	5′-CCT CCA GGT AGG CTC TCT G-3′	19
A*25		
P1	5′-TCA CAG ACT GAC CGA GAG AG-3′	20
P2	5′-ATG TAA TCC TTG CCG TCG TAA-3′	21
A*26/4301		
P1	5′-ACT CAC AGA CTG ACC GAG C-3′	19
P2	5′-ATG TAA TCC TTG CCG TCG TAA-3′	21
A*11		
P1	5′-ACG GAA TGT GAA GGC CCA G-3′	19
P2	5-CTC TCT GCT GCT CCG CCG-3	18
A*29		
P1	5′-AGG ATG GAG CCG CGG GCA-3′	18
P2	5′-AGC GCA GGT CCT AGT TCA A-3′	19
A*30		
P1	5′-CCC GGC CCG GCA GTG GA-3′	17
P2	5′-CCG TCG TAG GCG TGC TGT-3′	18
A*31		
P1	5′-GAT AGA GCA GGA GAG GCC T-3′	19
P2	5′-AGC GCA GGT CCT AGT TCA A-3′	19

Table 14: List of primers used for HLA-B locus typing.

Name of the allele	Sequence	nucleotide Length
B*07		
P1	5′-GGA GTA TTG GGA CCG GAA C-3′	19
P2	5-TAC CAG CGC GCT CCA GCT-3	18
B*08		
P1	5′-GAC CGG AAC ACA CAG ATC TT-3′	20
P2	5′-CCG CGC GCT CCA GCG TG-3′	17
B*1801		
P1	5′-GGC GCC GTG GAT AGA GCA A-3′	19
P2	5′-GCC GCG GTC CAG GAG CT-3′	17
B*27/7301		
P1	5′-ACC GGG AGA CAC AGA TCT G-3′	19
P2	5′-GAG CCA CTC CAC GCA CTC-3′	18
B*3701		
P1	5′-GCC GCG AGT CCG AGG AC-3′	17
P2	5′-CCT CCA GGT AGG CTC TGT C-3′	19
B*4001		
P1	5′-CCA CTC CAT GAG GTA TTT CC-3′	20
P2	5′-CCG CGC GCT CCA GCG TG-3′	17
B*4201		
P1	5′-GAC GAC ACC CAG TTC GTG A-3′	19
P2	5′-CCG CGC GCT CCA GCG TG-3′	17
B*44		
P1	5′-TAC CGA GAG AAC CTG CGC-3′	18
P2	5′-CCA GGT ATC TGC GGA GCG-3′	18
B*44/4501/1514		
P1	5′-ACC GGG AGA CAC AGA TCT C-3′	19
P2	5′-CCA GGT ATC TGC GGA GCG-3′	18
B*5101-5105		
P1	5′-GGA GTA TTG GGA CCG GAA C-3′	19
P2	5′-CGT TCA GGG CGA TGT AAT CT-3′	20
Positive internal Control		
P1	5′-ATG ATG TTG ACC TTT CCA GGG-3′	21
P2	5′-ATT CTG TAA CTT TTC ATC AGT TGC-3′	24

4.2.2.3.2 Amplification Procedure

50 µl of reaction mix was dispensed into PCR tubes. The tubes were spun for 1min for mixing and arranged in the heat block of thermal cycler. The primer for the hemoglobin gene was used as positive internal control. We used touch down method and following conditions were adopted.

No. of Cycle	Time	Temperature
1 Cycle of denaturation	1 min	96°C
5 cycles of	25 secs	96°C
	45 secs	70°C
	30 secs	72°C
21 cycles of	25 secs	96°C
	45 secs	65°C
	30 secs	72°C
4 cycles of	25 secs	96°C
	60 secs	55°C
	120 secs	72°C
1 cycle of Hold at 15°C	10 mins	72°C

4.2.2.3.3 Amplification check by agarose gel electrophoresis

We used mini gel electrophoresis apparatus (BIOTECH, India) for rapid separation of amplified PCR products. PCR products were separated on 2% submerging agarose gel (low EEO)(Sisco Research Laboratories, India) containing 0.5 µg/ml ethidium bromide (Boehringer Mannheim, Germany) in TBE buffer to check efficiency and specificity of the reaction. ϕX174 DNA marker (Bangalore Genei, India) providing even banding patterns of uniform intensity (1353bp, 1078bp, 872bp, 603bp, 310bp, 281/271bp, 234bp, 194bp,118bp and 72bp) was loaded in one of the well. Figure 21 shows the electrophoregram of the HLA gene.

Procedure

1) The agarose gel was prepared in 1X TBE buffer. The mixture was mixed properly and gently till the agarose was dissolved by using the microwave oven.

2) The content was cooled to 45°C and ethidium bromide to a final concentration of 0.5 mg/ml was added in the dissolved gel and mixed it by swirling.

3) The gel was then poured on the gel tray to make a 2mm thick gel and comb was placed.

4) The gel tray was left for 30 minute to solidify the gel.

5) The comb was then removed carefully from the solidified gel.

6) The gel was then placed in the electrophoresis tank filled with TBE buffer.

7) A mixture of 5µl gel loading buffer and 15µl amplified product was loaded in the well of the gel with the help of micropipette.

8) ϕX174 DNA was used as size marker.

9) The electrophoresis was then carried out at 80volt and a current of 40mAmp. The gel was removed when the tracking dye traveled 2/3rd of the gel.

4.2.2.3.4 Documentation and Interpretation

The gel was visualized under the UV-transilluminator (Vilber Laurmat, France) and the photographic documentation of the gel was done by using Polaroid camera and analyzed by using Bio1D Analysis Software (Vilber Lourmat, France). Allele assignment and interpretation were done manually by referring the protocols of 12th IHW.

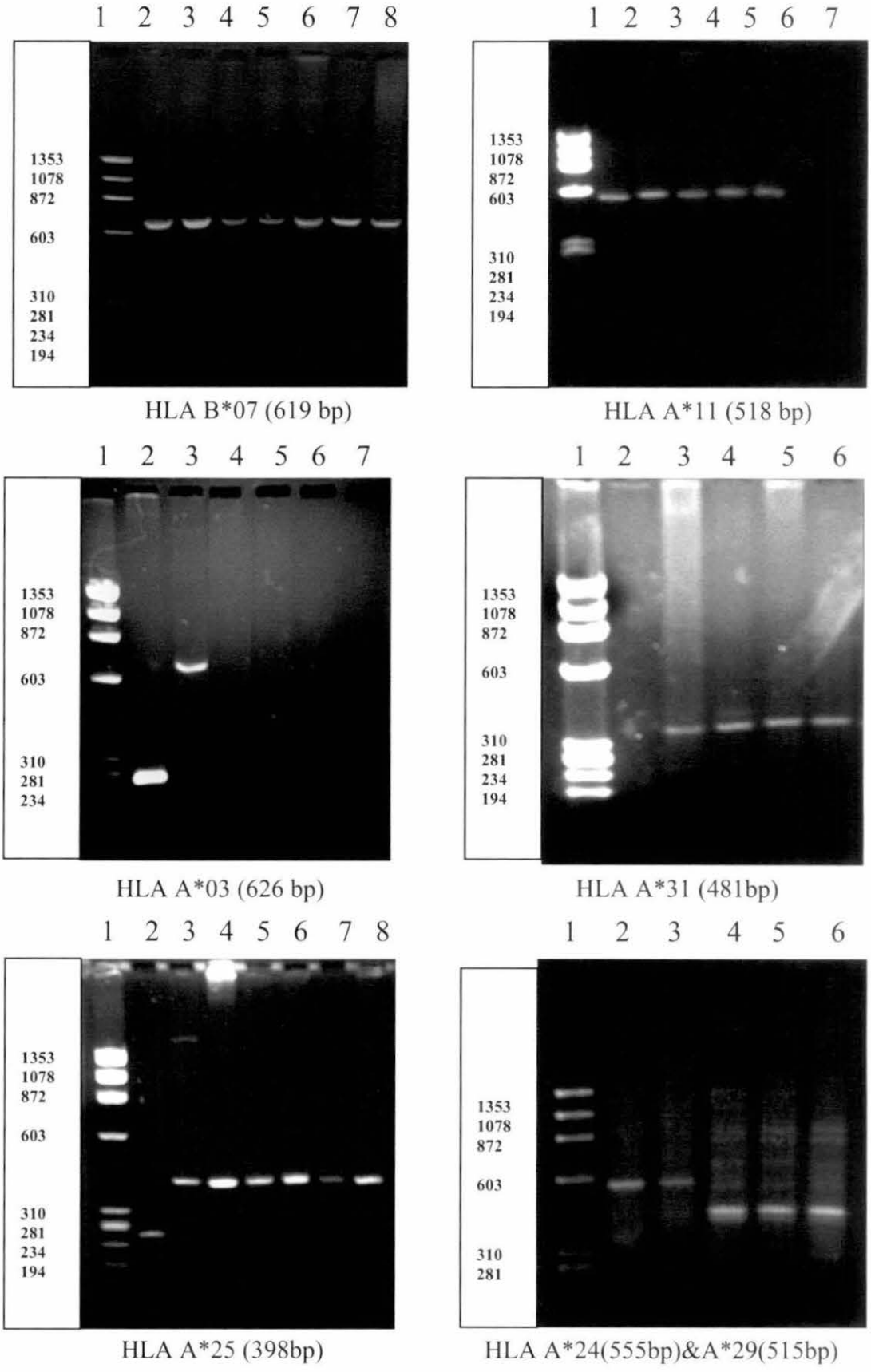


Figure 21: Electrophoregram showing the result of PCR-SSP typing of various HLA genes.

Lane 1, ϕX174 marker

4.2.3 STATISTICAL ANALYSIS

4.2.3.1 Estimation of phenotype frequency

Phenotype frequencies of antigens/ alleles at various loci of MHC region in patients and controls were estimated by direct counting. The percent phenotype frequencies were calculated by using the following formula:

$$f_A = \frac{nA}{n}$$

Where, f_A = frequency of allele
n =number of subjects studied
nA=number of times an allele is present

4.2.3.2 Estimation of significance of difference

Significance of difference in the frequency of different MHC alleles between patients and controls was calculated using χ^2 analysis:

	Antigens	
	+	-
Patient	a	b
Control	c	d

$a + b = n1$
$c + d = n2$
$a + c = n3$
$b + d = n4$
$n1+n2+n3+n4 = N$

$$\text{Chi-Square } (\chi^2) = \frac{(ad-bc)^2 \times N}{n1 \times n2 \times n3 \times n4}$$

4.2.3.3 Probability (P) Value and Bonferroni Correction

The level of significance is expressed in terms of probability (p) value. Since each individual is tested for several HLA alleles and the same data used to compare the frequency of all the detected alleles, it is probable that one of these alleles will by chance deviate significantly. To overcome this, Fisher's exact test was done.

4.2.3.4 Estimation of Relative Risk (RR)

Relative risk was estimated by using Haldane's method (1956) using the following formula:

$$\text{Relative Risk (RR)} = \frac{(2a+1)(2d+1)}{(2b+1)(2c+1)}$$

4.2.3.5 Calculation of Haplotype frequencies and linkage disequilibrium

The haplotype frequency was estimated by the equation derived from Cavalli-Sforza and Bodmer (1971) and linkage disequilibrium (delta values, D) for two locus model was calculated by the Chi Square test.

$$\text{Delta} = \sqrt{\frac{d}{n}} - \sqrt{\frac{(b+d)}{n} \times \frac{(c+d)}{n}}$$

n = total no. of sample

$$\text{HF} = 1 - \sqrt{\frac{b+d}{n}} - \sqrt{\frac{c+d}{n}} + \sqrt{\frac{d}{n}}$$

		B	
		+	–
A	+	a	b
	–	c	d

4.3 ENZYME LINKED IMMUNO SORBENT ASSAY (ELISA) FOR INTERLEUKIN-2 AND INTERLEUKIN-6 ESTIMATION

The serum level of interleukins (ILs) was measured on the 50 of the schizophrenic patients and control. They were further classified into two groups, 20 schizophrenic patients who had stopped taking antipsychotic drugs for at least 6 weeks were considered as psychotropic medication free, rest 30 schizophrenic group were under antipsychotic medication. A total number of 30 unrelated, age, sex and ethnically matched healthy individuals were considered as controls.

4.3.1 PREPARATION OF SERUM

The blood samples from the patients and controls were collected between 10 am-12 noon by vein puncture method and allowed to clot at room temperature for 2-3 hrs. Blood clot was cut and centrifuged for separating the serum. The separated serum was aliquoted and stored at – 70^0C before use.

4.3.2 MEASUREMENT OF INTERLEUKIN-2 AND INTERLEUKIN-6 CONCENTRATION

Serum IL-2 and IL-6 levels were measured by enzyme linked immunosorbent assay kit (Endogen Human IL kit, Pierce Biotechnology, Inc. Rockford). The sensitivities for IL-2 and IL-6 were <6pg/ml and <1pg/ml,respectively, with inter and intra assay coefficient of variation of <10% in both the cases. Absorbance was measured by a microtiter plate reader set at 450 nm. Each assay was carried out by the same investigator.

4.3.3 ASSAY PROCEDURE

(i) 50µl of reconstituted standards and samples were added to each microtiter well.

(ii) 50µl of Biotinylated Antibody Reagent was added to each well. It is mixed by gently tapping the plate several times.

(iii) The plate was covered with an adhesive microtiter plate cover, ensuring all edges and strips were sealed and incubated for three hours at room temperature, 20-25°C.

(iv) The adhesive plate cover was removed and the plate was washed.

(v) 100µl of prepared streptavidin-HRP solution was added to each well.

(vi) Again a new adhesive plate cover was attached, ensuring all edges and strips were sealed tightly. The plate was incubated for 30minutes at room temperature, 20-25°C.

(vii) The plate cover was removed and the plate contents were discarded. Then the plate was washed again.

(viii) 100µl of TMB substrate solution was added into each well.

(ix) The plate was allowed to develop enzymatic color reaction at room temperature in the dark for 30 minutes.

(x) After 30 minutes, the reaction was stopped by adding 100µl of stop solution to each well. The substrate reaction yielded a blue solution that turned yellow when stop solution was added.

(xi) The absorbance was measured on an ELISA plate reader set at 450nm.

(xii) The obtained OD was converted to actual value (pg/ml) with the help of the software ORIGIN lab 6.1.

4.3.4 STATISTICAL ANALYSIS

For variables:- (i) mean and standard deviations were calculated and (ii) t-tests was done to test the equality of means of patient and control subjects. For attributes: (i) percentages were calculated and (ii) χ^2- tests were done to test the equality of percentages. The p-values of <0.05 were considered statistically significant.

4.4 ESTIMATION OF SERUM C-REACTIVE PROTEIN

After diagnosis, 64 schizophrenic patients were included for CRP study. The CRP level in the serum was measured by latex agglutination slide test (Ranbaxy Fine Chemicals Ltd., HP, India).

4.4.1 PREPARATION OF SERUM

About 5ml. of blood samples were collected from each patient by vein puncture method. The samples were allowed to coagulate at room temperature for 2-3 hours. Blood clot was cut and centrifuged for separating the serum.

4.4.2 MEASUREMENT OF C-REACTIVE PROTEIN BY LATEX AGGLUTINATION TEST

The freshly separated serum sample was used for the latex agglutination slide test (Fig. 22). The limitation of detection of serum CRP level was less than 6mg/L. CRP was treated as categorical variable: undetectable or normal(<6mg/L) and detectable or elevated (≥6mg/L).

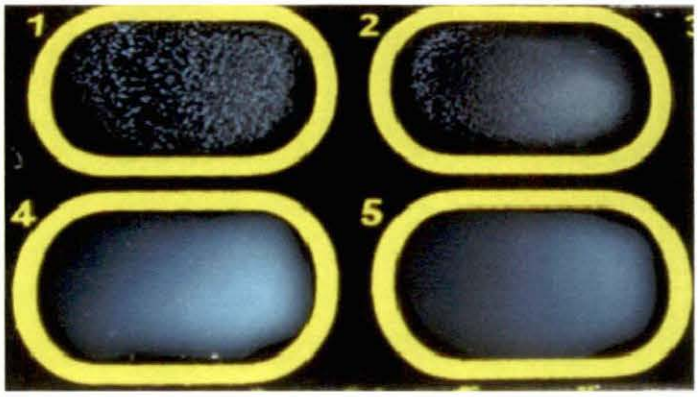


Figure 22: Latex agglutination test for C-reactive protein. 1 and 2 positive reaction from the patients and 4 and 5 negative reaction from the patients.

4.4.3 STATISTICAL ANALYSIS

Statistical analysis was performed for the bivariate associations between elevated CRP groups versus normal group by employing one-way analysis of variance. The association between CRP groups and deficit was examined by Chi-square analysis. The association between CRP group and other clinical/demographic variables were also examined by utilizing one way analysis for continuous variables and Chi-square tests for dichotomous variables.

4.5 ESTIMATION OF CD4+ AND CD8+ CELLS

4.5.1 COLLECTION OF BLOOD

The whole blood was collected by vein puncture method in heparinized and EDTA tubes between 10 am to12 noon.

4.5.2 METHOD

The collected blood samples were transported immediately to Suraksha Diagnostics, The Siliguri Clinic, Sevoke Road, Siliguri for the estimation of the lymphocyte subsets. The lymphocyte subsets were evaluated using a flow cytometer (Becton Dicknson) by the technician.

4.5.3 STATISTICAL ANALYSIS

Significance of difference in the percentage of CD4 and CD8 cells between the patients and controls were calculated using χ^2 analysis.

Section - 5

Results and Discussion

5. RESULTS AND DISCUSSION

This section has been divided into four major parts. Apart from describing the results, we have compared and evaluated our findings with the recently available findings in the discussion part. Also the analysis of the demographic characteristics of the population included in each of the study had been done. This has also been evaluated in the discussion part. Furthermore, a comprehensive analysis of the demographic characteristics of the individuals included in the study have been done and correlated with the available hypothesis and findings in schizophrenia.

5.1 HLA CLASS I TYPING

5.1.1 RESULTS

The incidence and frequency of HLA Class I genes among patients and controls have been presented in table 15 and 16. As the patient groups consist of mostly paranoid subtypes and the number of patients of the different subtypes were few (Table 25), we have considered the schizophrenic patients as a whole. The result of the HLA study showed a significantly higher frequency of HLA-A*03 (χ^2=77.519, p<0.001) in patients than the control groups. On the other hand A*31 (χ^2=34.160, p<0.001) and B*51 (χ^2=31.083, p<0.001) showed significantly lower value even after the Bonferroni corrections (Fig.23).

Table 15: Allele frequency, Chi square, relative risk (RR) values and probability of HLA-A loci alleles in the patients with schizophrenia and healthy controls.

	Patients (N=136)	Control (N=150)			
Allele	Allele Frequency %	Allele Frequency %	Chi square	RR	P value
A1	11.8	12.3	0.050	0.941	0.466
A2	15.4	17	0.316	0.869	0.332
A3	39.7	13.7	77.519	10.045	<0.001 *
A11	18	18	0.000	1.002	0.449
A23	7.7	6.3	0.456	1.255	0.434
A24	11.8	14.3	0.973	0.769	0.197
A25	10.3	15	3.324	0.609	0.045
A26	11.4	15	1.898	0.692	0.107
A29	12.1	8.3	2.547	1.593	0.271
A30	12.1	10	0.755	1.279	0.438
A31	0.7	13.3	34.160	0.051	<0.001 *

* significant after the Bonferroni's correction, Bonferroni's probability is 0.0025

Table 16: Allele frequency, Chi square, relative risk (RR) values and probability of HLA- B loci alleles in the patients with schizophrenia and healthy controls.

	Patients (N=136)	Control (N=150)			
Allele	**Allele Frequency %**	**Allele Frequency %**	**Chi square**	**RR**	**P value**
B7	17.3	13	2.485	1.498	0.357
B8	15.8	16.7	0.096	0.926	0.428
B18	9.2	11.3	0.799	0.772	0.228
B37	11.4	13.3	0.573	0.815	0.268
B40	6.9	8.3	0.398	0.817	0.321
B42	10.7	9	0.500	1.232	0.397
B44	4.4	2.7	1.336	1.683	0.321
B49	8.5	5.7	1.845	1.580	0.303
B51	0.5	11.7	31.083	0.036	<0.001*

*significant after the Bonferroni's correction, Bonferroni's probability is 0.0025

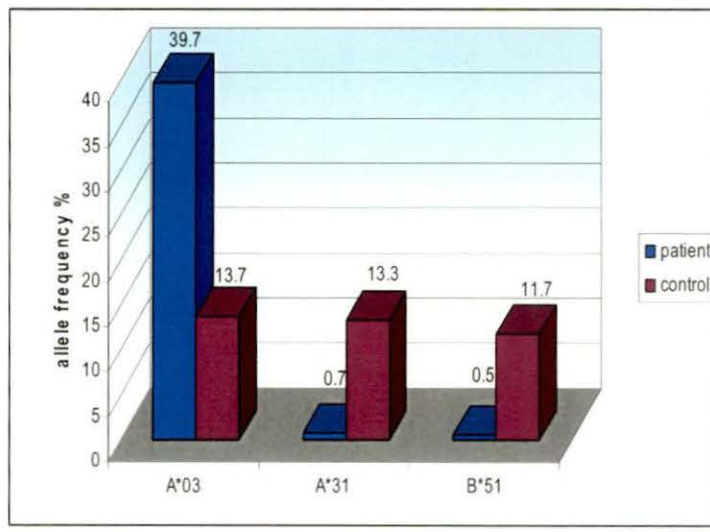


Figure 23: Bar diagram showing the frequency of some of the alleles in the patients and controls that showed the association with schizophrenia.

Significant HLA-A and B haplotypes among the schizophrenic patients and healthy control subjects are presented in the table 17 and 18. Some of the prominent haplotypes observed in the patients were not seen amongst the healthy subjects. The haplotype A1-B8 (χ^2 = 12.216) and A23-B49 (χ^2 = 11.444) were found to have the higher χ^2 value among the patient group, whereas among the control A26-B51 (χ^2 = 8.365) A01-B37(χ^2 =6.861) were found to have the higher χ^2 value.

Table 17: Significant haplotypes and delta values per 1000 among schizophrenic patients.

Haplotype	HF	Delta	χ^2
A1-B8	126.448	71.864	12.216***
A11-B8	163.800	73.719	8.616**
A11-B49	95.555	49.789	6.749**
A23-B49	58.634	42.041	11.444***
A24-B40	63.539	42.203	9.242**
A26-B49	57.987	40.505	10.101**

* = significant
Note: *= p<0.05, **=p<0.01, ***=p<0.001
HF=Haplotype frequency per 1000.

Table 18: Significant haplotypes and delta values per 1000 in normal individuals.

Haplotype	HF	Delta	χ^2
A01-B37	499.343	96.328	6.861**
A01-B40	346.586	75.501	5.270*
A25-B51	133.304	55.997	6.090*
A26-B51	184.790	73.245	8.365**
A29-B40	366.394	77.904	4.237*
A30-B51	179.267	58.369	4.904*
A31-B40	317.260	81.835	6.614*

* = significant
Note: *= p<0.05, **=p<0.01, ***=p<0.001
HF=Haplotype frequency per 1000.

5.1.2 DISCUSSION

Several researchers have the opinion that the likely schizophrenia locus is on chromosome 6p close to the human leukocyte antigen (HLA) region by linkage analysis (Moises *et al.*, 1995; Schwab, 1995; Wang, 1995; Straub, 1995; Antonarakis *et al.*, 1995; Schizophrenia Linkage Collaborative Group, 1996; Levinson *et al.*, 1996; Schwab *et al.*, 1998; Lindholm *et al.*, 1999; Li *et al.*, 2001; Schwab *et al.*, 2002). Since the first study done by Cazzolo *et al.*, 1974 , more than 80 schizophrenia HLA

association studies have been reported till to date (Bogacki, 2005). A recent study also found the significant increase in the frequency of a SNP in HLA-DOA in schizophrenia and a significant decrease in the frequency of a SNP in HLA-DRB1 region (Herbon, 2003). Furthermore genome-wide association study also found the association of schizophrenia in chromosome 6p (The International Schizophrenia Consortium, 2009).

The major findings of the present study is the increased frequency of HLA-A*03 and decreased frequency of HLA A*31 and B*51 among the schizophrenic patients. Thus, our results also strengthen the possibility of likely schizophrenia locus on chromosome 6p close to HLA region. A significant higher frequency of HLA-A*03 observed in the present study is in accordance with the previous study reported by Debnath *et al.,* (2005) but unlike the present study only paranoid schzophrenics were considered for the study. Moreover their sample size consisted of only fifty patients. The result of the present study is also in accordance with the study of Rudduck *et al.*, (1984) in Swedish population. On the other hand the lower frequency of HLA A*31 and B*51 observed in the patient group is the new findings of the present study than the previous study. Moreover the frequency of HLA-A*25 was also found to be lower among the patient groups but were not statistically significant. The increased frequency of A*11 found in the previous study by Debnath *et al.*, (2005) was not reproducible in the present study. However we have not found any association between HLA-A*23 and A*24, the most consistent association reported so far in schizophrenia (Ivanyi *et al.*,1983; Asaka *et al.*, 1981).

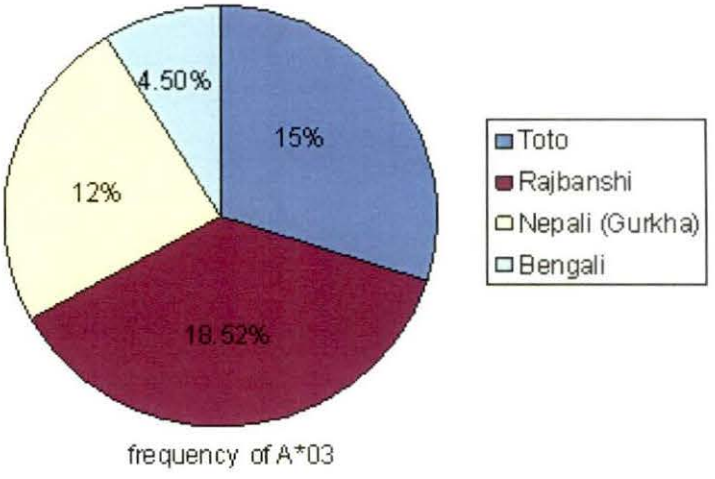


Figure 24: Frequency of HLA A*03 alleles in some of the population of North Bengal.

It is also to be noted that utmost care have been taken to match the case and control subjects in this study and our results could not be an artifact arising from the inadvertent ethnic

mismatching of cases and controls, as there is no ethnic group known in this region for which the HLA-A*03 frequency is higher than about 19%. The pattern of HLA-A*03 distribution in some of the population of India, especially in West Bengal is as follows: Indian tribe 6.0%(Imanishi *et al.*, 1992), Rajbanshi population 18.52% (Mandal *et al.*, 2000), Nepali (Gurkha)12%(Chaudhuri *et al.*, 1995) and Bengali 4.5% (Singh *et al.*, 2009) (Fig. 24).

Significant HLA-A and B haplotype among the schizophrenic patients and healthy control subjects are shown in the Table 3 and 4. The most significant haplotype HLA A1-B8 observed among the patient is the most common Caucasian haplotype, associated with the several autoimmune diseases such as type 1 diabetes (Degli-Esposti *et al.*, 1992; Cheong *et al.*, 2001) celiac disease (Sollid and Thorsby, 1993), common variable immunodeficiency (Schroeder *et al.*, 1998) , myasthenia gravis (Vandiedonck *et al.*, 2004) and systemic lupus erythematosus (Christiansen *et al..*, 1991). Thus our results hint towards the autoimmunological background of schizophrenia.

The exact nature of the mechanism underlying the empirically observed association of the HLA-A*03 gene with schizophrenia remains unknown. However, several genetic and environmental factors may be involved with such an association. HLA generally plays a critical role in the control of infectious and other immune functions. Many microbial factors have been implicated in the pathogenesis of schizophrenia, but so far each microbial factor has been identified in a relatively small subgroup of patients (Bechter, 1998; Karlsson, 2001). The heterogeneity of these microbial factors are also reflected by the associations with different HLA loci and their alleles. The set of inherited HLA alleles determine the susceptibility or resistance to particular microbes (Laumbacher, 2003). Also, it is widely accepted that a disturbance in neurodevelopment may be related to the development of schizophrenia. Results have also shown that an interaction between HLA and a perinatal or prenatal infection, which can affect neurodevelopment, may be associated with schizophrenia (Narita, 2000).

The analysis of the demographic data suggests that the present study comprises more number of paranoid schizophrenic patients (Table 25). On the other hand the present schizophrenic population comprises more number of Bengali populations. However, as mentioned earlier they were strictly matched according to their ethnicity, age and sex with the controls. The study comprised more number of male schizophrenic patients and duration of illness was found to be longer in them. On the other hand the study comprises more number of married schizophrenic patients.

The present study further strengthens the earlier findings regarding the association of HLA-A*03 with schizophrenia along with the negative association of HLA A*31 and B*51. Nevertheless, it is too early to speculate the exact mechanisms of the association. However, the study suggests that an immunological mechanism may contribute in the etiology of schizophrenia. In the present study the sample size of the different subtypes of schizophrenic patients were small for detecting the real differences in the HLA distribution among them. This is the limitation of the present study. Taking this into account, the present study suggests the possible existence of a susceptible locus for schizophrenia within the HLA region.

5.2 INTERLEUKIN ASSAY

5.2.1 RESULTS

The results of the IL-2 and IL-6 assay are summarized in table 19 and 20 and figure 25 and 26 and the absolute value of IL-2 and IL-6 are plotted in the dot scatter diagram in figure 27 and 28 respectively. The serum IL-2 level in antipsychotic medicating patients were found to be significantly lower (34.54±22.09 pg/ml) than the control subjects (56.04±18.82 pg/ml) ($p<0.001$). The serum IL-2 level in psychotropic medication free patients (38.76±27.23 pg/ml) were also found to be significantly lower than the normal controls (56.04±18.82 pg/ml) ($p<0.05$).

Table 19: Comparison of serum IL-2 levels between psychotropic medication free, antipsychotic medicating schizophrenic patients with the normal controls.

Variables	Psychotropic medication free schizophrenic group (n=20)	Control (n=30)	P value	
IL-2 (pg/ml)	Mean= 38.76 SD=27.23	Mean=56.04 SD=18.82	P<0.05 *	t= - 2.656 d.f.=48

Variables	Antipsychotic medicating schizophrenic group (n=30)	Control (n=30)	P value	
IL-2 (pg/ml)	Mean=34.54 SD=22.09	Mean=56.04 SD=18.82	P<0.001*	t= - 4.058 d.f.=58

* = significant

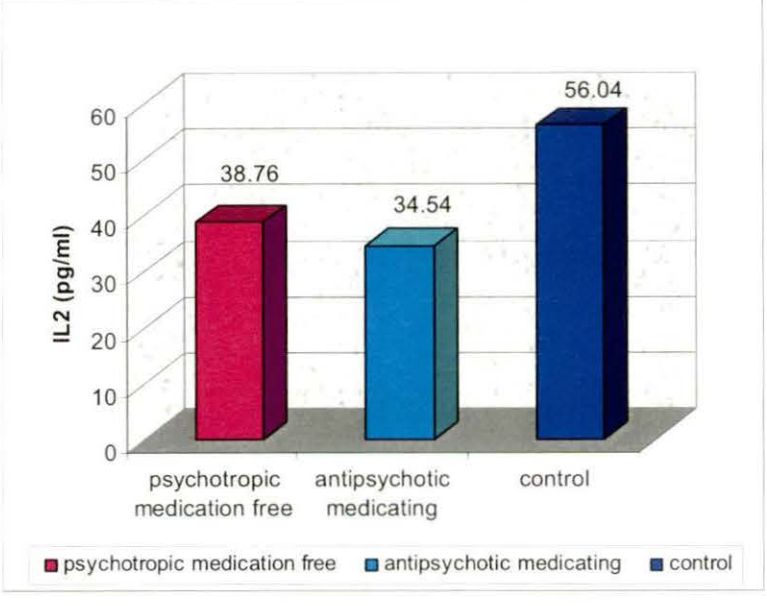


Figure 25: Comparison of serum level of IL-2 between psychotropic medication free, antipsychotic medicating patients and normal controls.

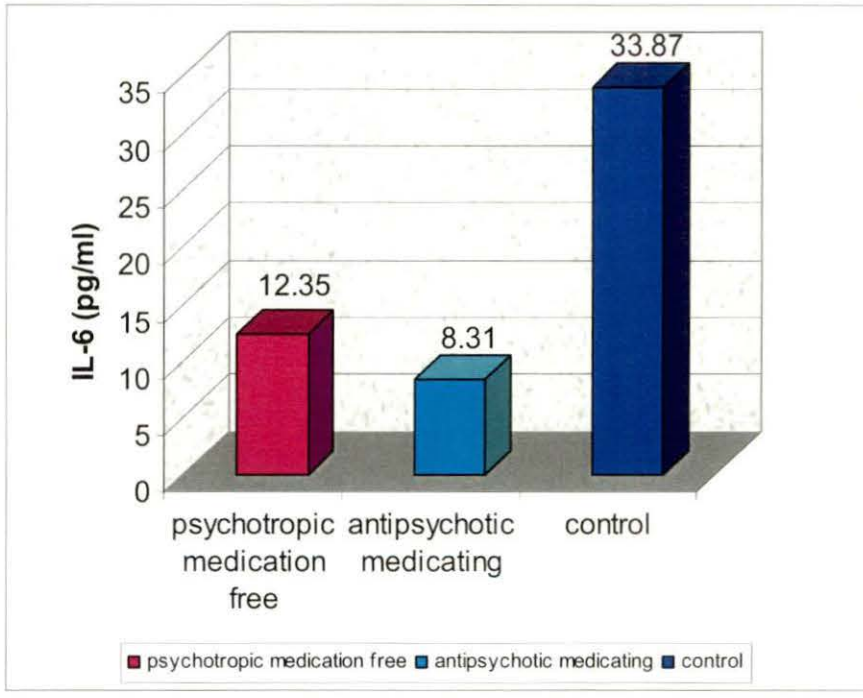


Figure 26: Comparison of serum level of IL-6 between psychotropic medication free, antipsychotic medicating patients and normal controls.

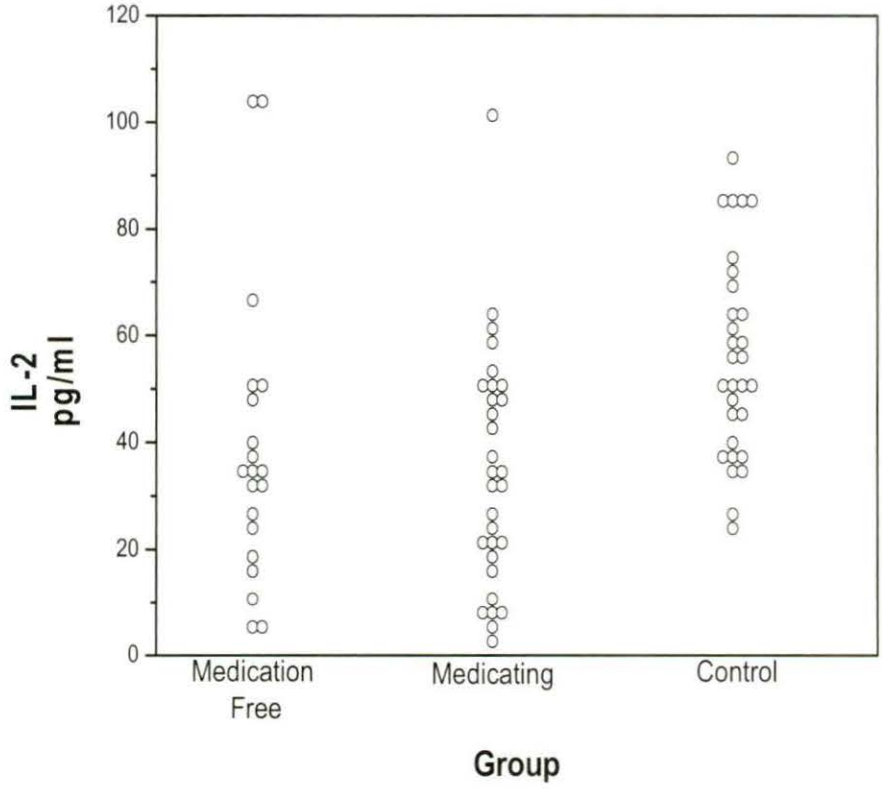


Figure 27: Dot scatter diagram of serum IL-2 level (pg/ml) between medication free, medicating schizophrenic patients and control.

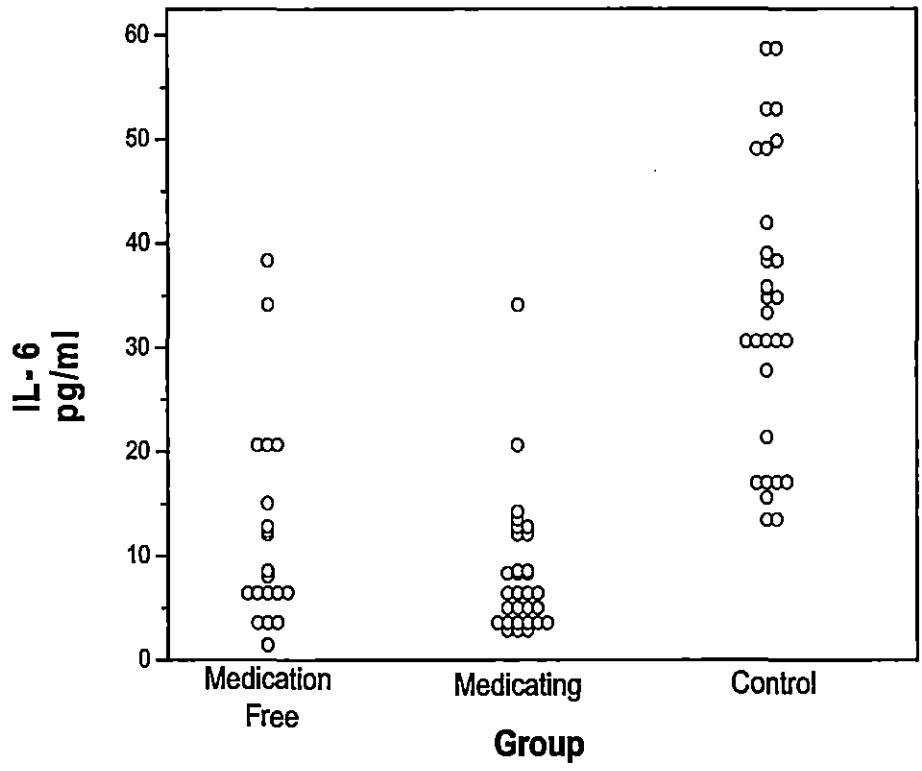


Figure 28: Dot scatter diagram of serum IL-6 level (pg/ml) between medication free, medicating schizophrenic patients and control.

Table 21: BPRS score between medication free and medicating schizophrenic patients.

Medication free BPRS (N=53)	Mean=42 SD=2.402	P<0.001***	t=5.505 d.f.=134
Medicating BPRS (N=83)	Mean=40 SD=1.821		

*=p<0.05, **=p<0.01,***p=<0.001
* = significant

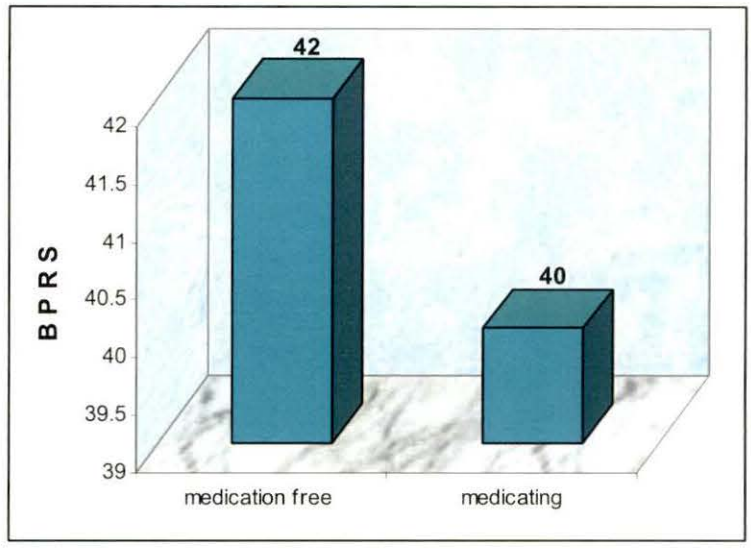


Figure 29: BPRS score depicted in the bar diagram in medication free and medicating patients.

Table 22: Demographic characteristics of the schizophrenic patients and the control subjects.

	Schizophrenia N=50	**Mean**	**standard deviation (SD)**		**Control N=30**	**Mean**	**standard deviation (SD)**
Sex, Male Female	 30 (60%) 20 (40%)				 18 (60%) 12 (40%)		
Age		32.36	10.50			33.87	11.35
Duration of illness		5.57	5.47				
Duration of treatment in years (N=30)		3.33	4.19				
Age of onset		26.18	9.58				
Subtypes of schizophrenia Paranoid Residual	 44 (88%) 1 (2%)			χ^2 =106.32			

	Schizophrenia N=50	Mean	standard deviation (SD)		Control N=30	Mean	standard deviation (SD)
Disorganized Catatonic	4 (8%) 1 (2%)			(d.f.=3) p<0.001 *			
Medication status of patients Psychotropic medication free Antipsychotic medicating	20 (40%) 30 (60%)			χ^2=2.00 (d.f. =1)			
Body mass index (kg/m^2)		22.496	1.426			22.657	1.514 df=78 P<0.635
Smoking status	0				0		
Antipsychotic types and dose Olanzapine (10-20 mg/day) Quetiapine (300mg-600mg/day) Clozapine (100mg-300mg/day) Risperidone (2mg-8mg/day)	(N=30) 8 8 4 10						
BPRS score Psychotropic medication free Antipsychotic medicating		42 40	2.714 1.702	t = 3.207 df = 48 p<0.01 *			

Abbreviation: IL= Interleukin, BPRS=Brief psychiatric rating scale

* = significant

5.2.2 DISCUSSION

To the best of our knowledge this is the first attempt to study the role of interleukins in the Indian schizophrenic subjects. The lower level of IL-2, observed among the schizophrenic patients in the present study is in agreement to Theodoropoulou *et al.*, (2001). However our findings are in contrast to the previous findings of Ebrinc *et al.*, 2002 and Zhang *et al.*, (2002, 2005) who have found elevated level of IL-2 in their subjects. Also this finding is in contrast to the studies of Kim *et al.*, (1998, 2000) who found an increase of serum IL-2 level in Korean schizophrenic patients.

The unique finding of the present study is the significantly lower level of IL-6 in the schizophrenic subjects. To our knowledge this is the first report of lower level of IL-6 in the schizophrenic patients and nowhere else have been reported previously. The finding is in contrast as reported by Zhang et al., 2002, who have found the elevated level of IL-6 in the schizophrenic group than the normal control. On the other hand some other groups have reported no significant differences between the patients and the controls (Baker *et al.*,1996).

The non agreement of some of the previous and present findings may be due to the different assay methods, differences in test materials (serum vs plasma), sampling of patients in different stages of disease progression (acute vs chronic or active phase vs remission), exposure to a variety and duration of neuroleptic treatments and different disease progressions. Much of the current research works are directed towards the heterogeneity of schizophrenia (Graver *et al.*, 2003). Pulver (2000) makes this point by describing schizophrenia as a syndrome with 'genetic heterogeneity' having susceptibility loci at several different chromosomal regions. Kirkpatrick and Carpenter have proposed strong evidence in support of a dichotomy, 'deficit' and 'nondeficit' schizophrenia (Kirkpatrick *et al.*, 2001). Garver *et al.*, (1988) also delineated and subsequently replicated three distinct clusters or 'endophenotypes' within the group of patients that meet conventional criteria for the DSM-IV schizophrenia syndrome (Garver *et al.*, 1999; Garver *et al.*, 2000). If such etiologically distinct endophenotypes exist, it should be suspected that central immune activation may be a component of one, but not all of the endophenotypes. On the other hand statistically significant ethnicity-based variability in the allelic

frequency and genotype inheritance patterns for IL-2 and IL-6 has been reported (Hoffmann *et al.*, 2002) suggesting the polymorphisms within these cytokine genes may be responsible for the ethnic-based differences in IL-2 or IL-6 levels (Zhang *et al.*, 2005). This may be one of the reasons for the difference in the opinions regarding the results.

The immunosuppressive and cytokine modulating effects of the antipsychotic drugs have been found by different studies (Maes *et al.*, 1995; Song *et al.*, 2000). Atypical antipsychotics such as risperidone and clozapine also appear to have anti-inflammatory activities (Song *et al.*, 2000; Leykin *et al.*, 1997; Maes *et al.*, 2000). In the present study only atypical antipsychotics were prescribed to the patients (Table 22). Therefore the lower levels of IL-2 and IL-6 observed among the medicating patients in the present study suggest the cytokine modulating activity of the atypical antipsychotics. Thus, our findings support the earlier findings that treatment with antipsychotic drugs affect the cytokine network (Pollmacher *et al.*,1996; Schuld *et al.*, 2004).

The results of our findings do not agree with the exhaustion theory of schizophrenia which is characterized by decreased production of IL-2 *in vitro* and increase of IL-2 in serum (Ganguli *et al.*,1989; Ganguli *et al.*,1995; Villemain *et al.*, 1989; Arolt *et al.*, 2000) and increase in IL-6 *in vitro* (Maes *et al.*,1995; Muller *et al.*, 1997, Van Kammen *et al.*, 1999). Also, our findings do not fit well into the Th1/Th2 paradigm or with the Th2 shift hypothesis. Until recently, IL-2 and IL-6 were classified as Th1 and Th2 type cytokines respectively (Mosmann *et al.*, 1986; Romagnani, 1995; Lucy *et al.*, 1996).This probably contributed to the formulation of the hypothesis of a shift from Th1 to Th2 cytokines on the basis of studies showing decreased *in vitro* IL-2 secretion and increased *in vivo* circulating sIL-2R and IL-6 levels in schizophrenia (Schwarz *et al.*, 2001). However, classification of cytokines is being re-examined, because new CD4+ T cell subsets, such as Th17 and Treg (Dong, 2006 ;Tato *et al.*, 2006) are emerging. The data on Th17 and Treg defining cytokines in schizophrenia are still lacking or insufficient at this point of time because of the lack of novelty of the findings and awaits further research (Potvin *et al.*, 2008).

Table 22 shows the demographic characteristics of the patients and normal controls. No significant relationships between age, sex, BMI and serum IL-2 and IL-6 were observed. Age of onset and duration of illness did not significantly correlate with the IL-2 and IL-6 levels in the patient groups. The patient group consists mostly of Paranoid schizophrenics (88%).

The limitations of the present study include the successive follow-up, which could not be done on the same patients to understand the effect of antipsychotics on the serum level of interleukins. This is because the same patients did not turn up in the OPD to follow up the treatment. However, it is evident from our study that antipsychotics downregulate the serum level of interleukin.

To conclude, the results of our present findings strengthens the earlier findings of the immune system dysregulation in schizophrenia which may be one of the etiological factors for the disorder. However our finding does not fit well to the autoimmune hypothesis of schizophrenia. Further studies involving other cytokines and *in vitro* cytokine production may throw light in this respect. Additional studies are invited further to unfold the effect of antipsychotics in the immune system which may help in future drug development.

5.3 C-REACTIVE PROTEIN TEST

5.3.1 RESULTS

Sera levels of CRP were measured for 64 schizophrenic patients. Among them the elevated level of CRP (≥6 mg/L) was observed in 3 patients and 61 patients were found to have the normal CRP (<6mg/L). All the three elevated cases were found to be of paranoid type. No differences were found in CRP levels among the different subgroups of schizophrenia. Further, when the level of CRP was compared to the other demographic variables as shown in table 23, only the drug naïve status of the patients showed statistically significant value ($\chi^2 = 16.997$, p value$<3.75 \times 10^5$).

Table 23: Comparison of demographic and clinical characteristics between the normal / elevated CRP groups.

	Elevated CRP N=3		Normal CRP N=61			
	Mean or N%	Standard deviation	Mean or N%	Standard deviation	Statistic (Z)	*P* value
Age	37.67	21.13	34.69	9.64	F[2,60]=0.24	>0.62
Gender Men Women	33.33%		70.49%		X^2 [1]=1.84	>0.17
Drug naïve patients Patients under antipsychotic medication	100%		11.48%		X^2 [1]=17.00	<0.001 Significant
Substance abuse Yes No	33.33%		55.74%		X^2[1]=0.58	>0.44
First child Yes No	33%		18.03%		X^2[1]=0.44	>0.50
Autoimmune disease in patients or in family Yes No	0%		24.59%		X^2[1]=0.97	>0.32

5.3.2 DISCUSSION

This preliminary first hand study provides further evidence of the involvement of inflammatory processes behind the etiopathology of schizophrenia. The elevated level of CRP in our study is in accordance to the findings of Fan *et al.*, 2007 and Dikerson *et al.*, 2008. But unlike previously reported findings, we have considered the CRP level of patients with their medication status, which showed significantly higher value. In the study by Fan *et al.*, 2007 the level of CRP was found to be higher in the patiens, who were experiencing psychotic symptoms. In the follow up study of non-psychotic state, the level of CRP was found to be normal. In this respect, the present study suggests that the antipsychotic drug may perhaps down regulate the inflammatory process, which in turn brings the CRP level to the normal state.

Thus, these findings further suggest that the inflammation may be another possible mechanism in the etiopathology of schizophrenia. It is, however, not clearly understood whether the elevation of the level of CRP is the by-product of the pathophysiology of schizophrenia or directly contributes to the clinical manifestation of the disorder (Fan *et al.*, 2007).

Until now, it is not clearly understood regarding the mechanism of inflammation in schizophrenia. It is suggested that the vascular-structural brain abnormalities may be one of the factors in the etiology of schizophrenia like psychoses (Howard *et al.*, 2001; Bachneff, 1996; Shinba, 2004). It is proposed that chronic inflammation might damage the micro-vascular system in the brain and cerebral blood flow (Hanson, 2005). Further, scientific evidence suggests that an increase in the stress hormone like norepinephrine may activate the inflammatory arm of the immune system and triggers the expression of genes that cause chronic, low-grade inflammation. This inflammation is characterized by the degree of the levels of CRP (Boyle *et al.*. 2007).

This is possibly the first reported study of the association between CRP and the schizophrenia in the Indian scenario. The psychopathology measures of the patients were not considered unlike the previous studies. This is the limitations of the present study. In contrary to the studies conducted by Fan and Dickerson, the higher cut off value (6mg/L) was used for the CRP levels. The sample size of the present study was small. The patients were recruited from the OPD which has limited the follow-up study. Nevertheless, taking this limitation in account the present study provides further evidence that some kind of inflammation may play a role in the etiopathology of schizophrenia. The study further reveals the immunomodulatory effect of the antipsychotic drugs in the patients.

5.4 CD4+ AND CD8+ COUNT

5.4.1 RESULT

The results of the CD4+ and CD8+ cell count have been presented in table 24 and summarized in figure 30. Although the mean percentage of CD4+ cells was found to be little higher (36.10± 4.59) in the patients. It was not significantly higher than the control groups (34.50±6.62). Also the mean percentage of CD8+ cells is not found to

be significantly deviated in patients (29.7 ± 6.72) from the control (28.1±6.38) groups. On the other hand the CD4+ and CD8+ subset ratio did not show any significant deviation between the patients (1.34 vs 1.29) and the control groups. Further, there was no significant correlation between the percentage of CD4+ cells and the serum IL-2 level.

Table 24: The result of the CD4+ and CD8+ count.

	CD4%		CD8%		CD4-CD8 ratio	
	Patient (N=20)	Control (N=20)	Patient (N=20)	Control (N=20)	Patient (N=20)	Control (N=20)
Mean	36.1	34.5	29.7	28.1	1.34	1.29
SD	4.59	6.62	6.72	6.38	0.46	0.39
t-value	- 0.887 df=38		-0.772 df=38		-0.427 df=38	
p-value	0.381		0.445		0.67	
Mann Whitney U test	171.00		163.5		190.5	
P value	0.432		0.322		0.797	

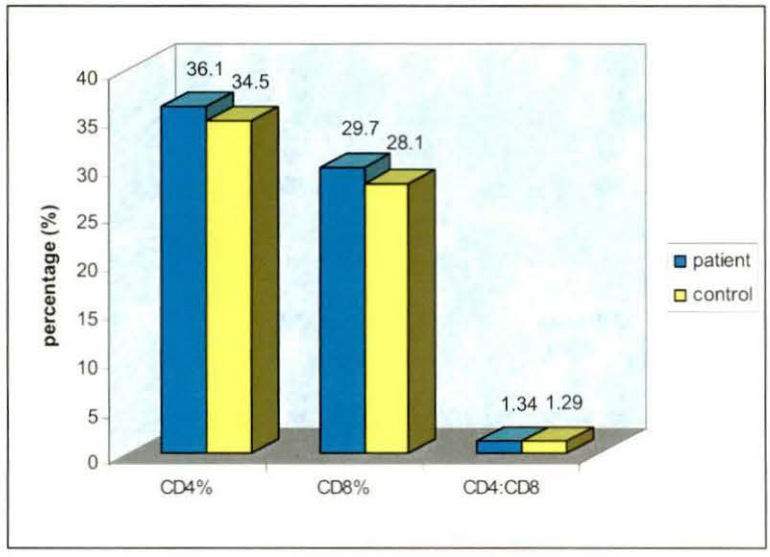


Figure 30: Showing bar diagram of CD4+ and CD8+ cell percentage and CD4-CD8 ratio in patients and controls.

5.4.2 DISCUSSION

The results of our findings regarding the CD4+ cells reflected the findings of Villemain *et al.*, (1989), Achirion *et al.*, (1994) and Baskak *et al.*, (2008), who also did not find any difference among CD4+ cells. However our finding is in contrast to the findings of some other workers who have reported decreased number of CD4+ cells (Zhang *et al.*, 2002; Cosentino *et al.*, 1996). Moreover, some workers have also reported the increased number of CD4+ cells (Ganguli *et al.*, 1987; Henneberg *et al.*, 1990; Muller *et al.*, 1993; Cazzullo *et al.*, 1998a; Sperner-Unterweger *et al.*, 1999). On the other hand our result on CD8+ cells are in agreement with the Sperner-Unterweger *et al.*, (1999) who also found no significant difference, but not in agreement with the Cazzullo *et al.*, (1998a) who have found increase of CD8+ cells. Villemein *et al.*, 1989 on the other hand found significant decrease in CD8+ cells in schizophrenic patients which is in agreement to the work of Achiron *et al.*, 1994 and Baskak *et al.*, (2008). Our findings on CD4+ and CD8+ ratio is not in agreement to the findings of Sperner-Unterweger *et al.*, (1999) who have found higher CD4/CD8 ratio than healthy controls.

For CD4 T cells, the Th1 and Th2 T cells are categorized by the cytokines that they produce. IL-2 is released into the circulation at high levels from CD4+ (Th1) cells upon activation. One of the most frequently reported immunological alterations in schizophrenia is an imbalance in T helper 1 (Th1) and T helper 2(Th2) cytokine profile production with increase Th2 cytokine secretion *in vivo* and decrease *in vitro* (Muller *et al.*, 1999; Muller *et al.*, 2000), suggest diminished pro-inflammatory Th1 responses in schizophrenia. Lower number of CD4+ cells and hence IL-2 production have also been reported in schizophrenia (Zhang *et al.*, 2002), although Muller described the increased CD4+ cell number (Muller *et al.*, 1991) in addition to the altered plasma and serum levels of other cytokines such as IL6 (Frommberger *et al.*, 1997). With respect to the above mentioned studies, our findings are of complete contrast i.e., low level of IL-2 but normal percentage of CD4 cells. This suggests that the abnormal production of ILs is not due to the abnormal number of CD4 cells but it may be due to some abnormalities in the CD4 cells. However given the size of our sample the results should be interpreted cautiously. It is a well known fact that IL- 2 is not solely secreted by the CD4 cells, hence there can be other factors for this

contrasting results. Moreover the knowledge about the new CD4 T cell subsets such as Th17 and Treg are emerging. It is now evident that the Treg cells are capable of suppressing the function of the other T cells. Treg cells have been shown to play a role in regulation a variety of immune responses from autoimmunity to viral infection (Levings *et al.*, 2006).The exact mechanism about these cells triggers autoimmunity has not been revealed yet (Miyara and Sakaguchi, 2007). As the knowledge about these cells will grow, the role of CD4+ and CD8+ T cells in the etiopathology of schizophrenia will become understandable.

5.5 DEMOGRAPHIC STUDIES

5.5.1 RESULT AND DISCUSSION

The comprehensive demographic data of all the patients and controls included in this study have been presented in table 25 which shows the study comprises more number of paranoid (118) schizophrenic patients. On the other hand, most of the patients included in the study belong to Bengalee community (63.97%). However, utmost care was taken to strictly match the patients and controls according to their ethnicity, age and sex. The present study comprises of more number of male schizophrenics and they have long duration of illness compared to females. The data hints the higher vulnerability of men to schizophrenia at least in this region. A comprehensive study involving large number of samples should be done in order to shed light in this respect. The higher number of married patients in this study hints the strict social customs and strong social bondage of the Indian society but again an elaborate study awaits before making any conclusion in this respect. Although it has been hypothesized that the onset of schizophrenia is earlier in males but our result did not show any significant difference in the age of onset between male and females. When the family history of the psychiatric and autoimmune disorder was analyzed we did not found any association, rather we found the significantly higher number of patients who did not have family history of psychiatric and autoimmune disorders. Regarding the educational status it has been noticed that most of the patients did not continue their study beyond class X and there were more number of patients whose educational status was less than VIIIth standard. On the other hand the study also comprises a good number of illiterate patients. Studies have found the increase incidence of

smoking among schizophrenics, but this finding was not reproducible in the present study. One of the interesting observations of the present study is the significant association of schizophrenia with the patients who are not the first child. Our finding is in accordance to the study of Sham *et al.*, (1993) which suggests that in addition to the genetic predisposition, some environmental factors such as viral infection might play a pivotal role on the onset of the disorder and the first child may be the source of infection. Thus the present study strengthens the hypothesis of "younger children in a family has a significantly increased risk of later developing schizophrenia."

Table 25: Comprehensive demographic characteristics of the schizophrenic patients and controls.

	Patients N=136			**Control N=150**		
Gender						
Male	85			92	X^2=0.041	
Female	51			58		
Ethnicity						
Bengali	87	X^2=165.618				
Nepali	9	P<0.001 ***				
Bihari	11					
Tribal	17					
Rajbanshi	12					
Subtypes						
Paranoid	118					
Disorganised	9	X^2= 380.176				
Catatonic	1	P<0.001				
Undifferentiated	5					
Resudal	3					
Marital status						
Married	79	X^2= 66.211				
Unmarried	54	P<0.001***				
Left/divorcee	3					
Age						
Male (N=85)		Mean=33.788 SD= 10.638		Male (N=92)	Mean=33.467 SD=10.524	t=0.2016 d.f.=175
Female(51)		Mean=32.098 SD=10.092		Female (N=58)	Mean=32.190 SD=8.914	t=-0.050 d.f.=107

	Patients N=136			Control N=150		
Duration of illness						
Male (N=85)		Mean=6.827 SD=6.659	t=2.333 d.f.=134			
Female (N=51)		Mean =4.386 SD=4.358	p=0.021 p<0.05*			
Age of onset						
Male (N=85)		Mean=26.145 SD=9.579	t= – 1.054 d.f.=134			
Female (N=51)		Mean=27.944 SD=9.626				
Education						
Nil	24	X^2_5=51.112				
<class VIII	46	P<0.001***				
<Class X	33					
Class X pass	19					
Higher secondary	8					
Graduation	6					
Family history of:-						
Psychiatric disorder	17	X^2_2=134.610 P<0.001***				
Autoimmune disorder	10					
No family history	109					
Substance abuse						
Smoking	37	X^2_2=11.561				
Paan, gutka and alcohol	35	P<0.01**				
Nil	64					
HLA A*3 Positive patients (N=108)						
First child	30	X^2_1=21.333				
Not first child	78	P<0.001***				
Taken together N=136						
First child	32	X^2_1=38.118				
No first child	104	P<0.001***				

*=p<0.05, **=p<0.01,***p=<0.001
* = significant

Over 40 studies have shown that the individuals who later develop schizophrenia have a 5 to 15% excess of winter and spring births (Boyd et al., 1986;Bradbury and Miller, 1985). Thus in our study we analyzed the seasonal birth pattern in schizophrenic patients. 62 patients for whom authentic date of birth were available have been included in this study and compared with 100 controls (Table 26). For this study we have divided the season of birth of the patients and controls into three categories, winter (Nov-Feb), summer (March-June) and monsoon (July-Oct) (Fig.31). Interestingly, there was an increase incidence of winter birth among the patients but when compared and analyzed statistically with the normal control, it was not found to be significant.

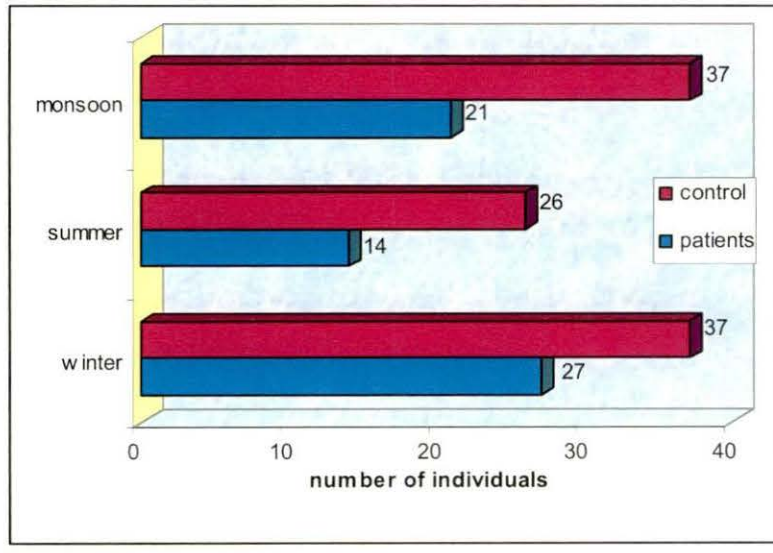


Figure 31: Showing bar diagram of season of birth among schizophrenic patients and normal individuals according to the season.

Again the birth pattern was studied according to months, which revealed the increase number of birth during February among the patients but still the finding was not significant (Fig.32). Thus our results do not corroborate with the winter birth hypothesis of schizophrenia. Even though the present study hinted the winter birth access in schizophrenics, small number of sample size greatly limited the study. More comprehensive study including large number of the samples may throw light in this respect.

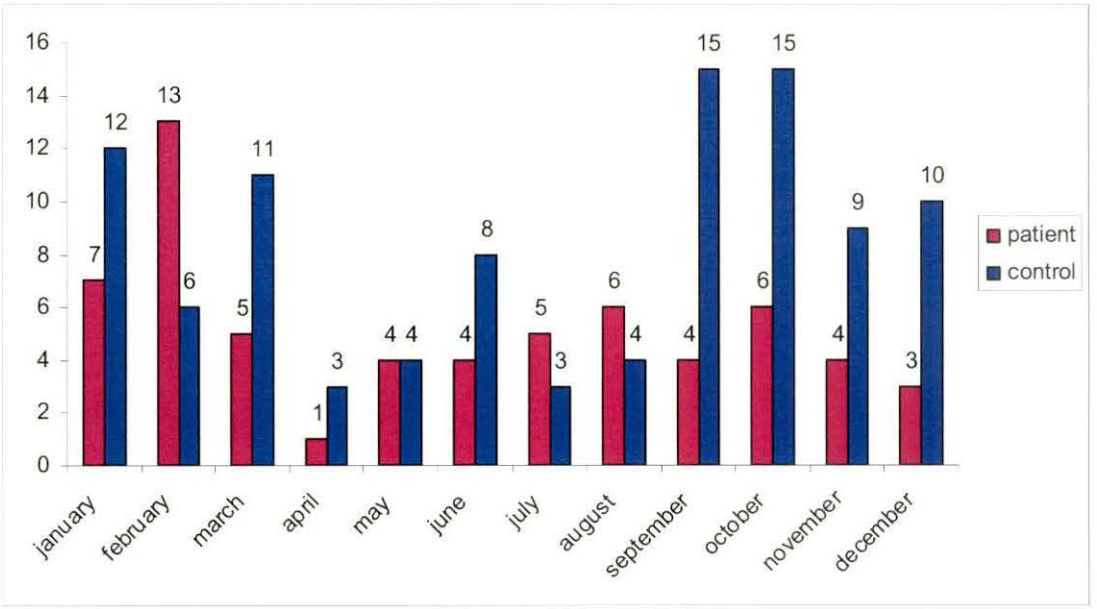


Figure 32: Showing bar diagram of season of birth among schizophrenic patients and normal individuals according to months.

Table 26: Season of birth among schizophrenic patients and normal individuals.

	Patients N=62		Control N=100		Chi square
Season of birth					
Winter (Nov-Feb)	27	$X^2_2 = 4.097$	37	$X^2_2=2.418$	$X^2_2=0.0701$
Summer (March-June)	14		26		
Monsoon (July-Oct)	21		37		
Season of birth					
Jan	7		12		
Feb	13		6		
March	5	$X^2_{10}=12.877$	11	$X^2_{10}=17.700$	$X^2_{10}=16.631$
April	1		3		
May	4		4		
June	4		8		
July	5		3		
Aug	6		4		
Sept	4		15		
Oct	6		15		
Nov	4		9		
Dec	3		10		

*=p<0.05, **=p<0.01,***p=<0.001
* = significant

Section - 6

Comprehensive Discussion

6. COMPREHENSIVE DISCUSSION

Chronic diseases of the central nervous system including schizophrenia are suspected of having genetic, immunological and viral etiology by many investigators (Michels and Marzuk, 1993).

The argument in favour of an autoimmune basis for schizophrenia was popularized by Burch in the early 1960s. After analyzing the age-specific and sex-specific incidence rates, relapsing clinical course and prevalence of several conditions presumed to be autoimmune in origin, Burch concluded that schizophrenia may also have autoimmune basis. However, these evidences were not sufficient for establishing schizophrenia as an autoimmune disease. In 1950s, Witebsky and his colleagues proposed the criteria that could be used to determine whether a disease is actually autoimmune in origin. These criteria were more recently refined by Rose and Bona (1993). They proposed several levels of evidences for the autoimmunity: (i) direct evidence, that is transmissibility by lymphoid cells or antibody of the characteristic lesions of the disease from human to human or human to animal or reproduction of the functional defects characteristic of the disease *in vitro*, (ii) indirect evidence, that is reproduction of the autoimmune disease in experimental animals or isolation of autoantibodies or autoreactive T cells from the target organ and (iii) circumstantial evidence, that is the presence of markers that are descriptive of all autoimmune disease.

The present study has been carried out to investigate the circumstantial evidence of autoimmunity in schizophrenia. Several features are common to many autoimmune diseases which can be taken together as the circumstantial evidence of autoimmunity. These include association with other autoimmune diseases in the same individual or the same family, the presence of immune cells in the affected organ, association with particular MHC haplotype, high serum levels of autoantibodies, deposition of antigen-antibody complexes in the affected organ and improvement of disease symptoms with immunosuppression. In some cases of autoimmune diseases the alterations in the level of cytokines have also been observed.

The parameters we have taken for the present investigation includes HLA genes, Th-1 and Th-2 cytokine IL-2 and IL-6 respectively, CD4+ and CD+ cells and C- reactive

protein to study the immunological alteration in schizophrenia, if any. In addition, demographic characteristics were studied to investigate the role of environmental factors in the etiopathology of the disorder. The effort was also been given to shed light in the autoimmune basis of schizophrenia.

Study on the immunogenetic aspect of the disease is most useful in identifying not only the mode of inheritance of a particular disease process but also in understanding the immunopathogenic mechanisms underlying in it. The discovery of HLA associations with the specific disease implies that at least part of their genetic basis lies in the HLA region which suggests the possibility of determining the etiology. Incidentally, most diseases that show strong HLA associations are having unknown etiology and mode of inheritance, e.g. various autoimmune and rheumatological diseases.

Two general explanations for HLA and disease associations given by McDevitt (1985) is that there may be linkage disequilibrium between alleles at a particular disease associated loci and the HLA antigen associated with that disease; secondly the HLA antigen itself plays a role in disease manifestation by: (i) being a poor presenter of a certain viral or bacterial antigen , (ii) providing a binding site on the surface of the cell for a disease provoking virus or bacterium, (iii) providing a transport piece for the virus to allow it to enter the cell ,(iv) having such a close molecular similarity to the pathogen. It is most likely that all these mechanisms are involved, but to a varying extent in different diseases (Thorsby, 1977).

The first HLA association study was reported in schizophrenia by Cazzullo *et al.*, in 1974. More than 80 association studies have been reported since then. HLA and schizophrenia was first reviewed by McGuffin (1979), who commented that the MHC was a logical place to search for genetic markers for schizophrenia. This is because schizophrenia is similar to the diseases for which HLA association had been established as it was familial, had an imperfectly understood etiology, and had a postulated autoimmune pathogenesis (Burch, 1964).

Linkage analysis have also found the likely schizophrenia locus on chromosome 6p close to the human leukocyte antigen (Moises *et al.*, 1995; Schwab, 1995; Wang, 1995; Straub, 1995; Antonarakis *et al.*, 1995; Schizophrenia Linkage Collaborative

Group, 1996; Levinson *et al.*, 1996; Schwab *et al.*, 1998; Lindholm *et al.*, 1999; Li *et al.*, 2001; Schwab *et al.*, 2002). A recent study also found a significant increase in the frequency of a SNP in HLA-DOA in schizophrenia and a significant decrease in the frequency of a SNP in HLA-DRB1 region (Herbon, 2003). Furthermore genome-wide association study also found association of schizophrenia in chromosome 6p (The International Schizophrenia Consortium, 2009).

In our study, initially we have reported an association of HLA-A*03 with the sample size of 50 schizophrenic patients (Singh *et al.*, 2008). In the same study we have also reported the negative association of some of the HLA genes such as A*25, A*31 and B*51. Subsequently we have analyzed our findings in the large cohort of patient and control sample where we got the significantly higher frequency of HLA-A*03 genes in patient group. Our results also showed the significantly lower frequency of HLA A*31 and B*51 but not A*25 among the patient group. The association of HLA-A*03 found in the present investigation was in accordance with the previously reported study of our laboratory by Debnath *et al.*, 2005. However the sample size of the previous study was small and the study comprised only of the paranoid schizophrenics. Both the present findings and the findings of Debnath *et al.*, (2005) corroborated with the study by Rudduck *et al.*, (1984). On the other hand, in the present study the frequency of HLA-A*31 and B*51 are significantly lower which is the new findings of the present study. The most significant haplotype HLA A1-B8 observed among the patient is associated with the several autoimmune diseases such as celiac disease, autoimmune active chronic hepatitis, myasthenia gravis, adrenocortical hyperfunction-cushing's syndrome and systemic lupus erythematosus (Dorman *et al.*, 1990; Khalil *et al.*, 1992; Pugliese *et al.*, 1995; Brewerton *et al.*, 1973). Thus our results hint towards the autoimmunological background of the disorder. At this moment it is difficult to propose the mechanism of association of HLA with schizophrenia. Correlations with several other factors are also need to be established such low birth weight, viral infections, prenatal infections etc. which are at least common in Indian population to further shed light in this respect. Our result may be considered preliminary as the results had so far not been correlated with these factors.

Nevertheless, our result suggests the possible existence of a susceptibility locus for schizophrenia within the HLA region. The study further strengthens our earlier

finding of association of HLA-A*03 with schizophrenia along with the negative association of HLA A*31 and B*51. The study further suggests the susceptibility locus to schizophrenia in chromosome 6 close to the HLA locus.

Several immunological findings also suggest that immunological dysfunctions may have relevant implications for the etiology of schizophrenia. Accumulating evidence suggests that in some cases, schizophrenia is accompanied by changes in the immune system, such as the presence of anti brain antibodies in serum (Henneberg *et al.*, 1994), an altered distribution of T-cell subsets (Muller *et al.*, 1993), reduced mitogen-induced lymphocyte production of interleukin-2 (IL-2) (Bessler *et al.*, 1995, Ganguli and Gubbi, 1997), alteration in serum levels of interlukin-2 soluble receptor α (IL-2sRα) (Hornberg *et al.*, 1995), interleukin 2 (Theodoropoulou *et al.*, 2001; Zhang *et al.*, 2002 ; Zhang *et al.*, 2005) and interleukin 6 (IL-6) (Shintani *et al.*, 1991; Ganguli *et al.*, 1994; Frommberger *et al.*, 1997). These findings indicate that aberrant immune function in schizophrenia may be associated with the manifestation of the clinical phenotype and disease processes (McAllister *et al.*, 1997).

In the present study we have studied the serum level of Th-1 and Th-2 cytokines IL-2 and IL-6 respectively in schizophrenic patients and compared with the matched controls. The purpose of our study was to: (i) study the immunological alteration in schizophrenia, (ii) investigate the Th-2 shift hypothesis and (iii) investigate the immunomodulatory effect of the antipsychotic medicine. For this, we have divided the patients into psychotropic medication free and medicating groups. The serum level of IL-2 and IL-6 was measured by ELISA method. In the result we have observed the decreased level of IL-2 and IL-6 in both the groups. Further, we found the lower level of both the interleukins in the medicating patients than the psychotropic medicine free patients. Our results were in agreement with Theodoropoulou *et al.*, (2001) but in contrast to Ebrinc *et al.*, (2002), Zhang *et al.*, (2005) and Kim *et al.*,(2000). Taking this into account our results suggest some kind of immunological abnormalities in the schizophrenic patients and further hints the heterogeneity of schizophrenia which has also been suggested by Graver *et al.*, (2003). The unique finding of the present study is the lower level of IL-6 in the serum of the patients which we have reported first time in the world (Singh *et al.*, 2009). On the other hand our findings are not in agreement of the Th-2 shift hypothesis in schizophrenia. The lower level of

interleukins in the antipsychotic medicating patients suggests the immunomodulatory affect of the antipsychotic drugs.

The result of our present findings strengthens the hypothesis of immune system dysregulation in schizophrenia which may be one of the etiological factors for the disorder. Further, studies are needed to throw light on the exact mechanism of the changes in the serum level of IL-2 and IL-6 in schizophrenia.

Several researchers have suggested some kind of inflammatory process involved in schizophrenia (Rapaport and Lohr, 1994; Sirota, *et al.*, 2005). Therefore, in the present investigation we have studied a well known inflammatory marker C-reactive protein (CRP) in the schizophrenic subjects. Simple latex agglutination test was followed for determining the elevated level of CRP. CRP was treated as categorical variable, undetectable or normal (<6mg/L) and detectable or elevated (≥6mg/L). The results showed the elevated level of CRP in the drug naïve patients. The elevated level of CRP in this study provides further evidence of the involvement of inflammatory processes behind the etiopathology of schizophrenia. The elevated level of CRP in our study is in accordance to the findings of Fan (2007) and Dikerson (2007). In the study by Ohaeri *et al.*, 1993, the elevated level of CRP was found in the patient who was experiencing psychotic symptoms. In the follow up study of non-psychotic state, the level of CRP was found to be normal. In this respect the present study suggests that the antipsychotic drug may perhaps down regulate the inflammatory process which in turn brings the CRP level to the normal state. It is however not clearly understood whether the elevation of the level of CRP is the by-product of the pathophysiology of schizophrenia or directly contributes to the clinical manifestations of the disorder. Moreover our findings suggest that the inflammation may be another possible mechanism in the etiopathology of schizophrenia. Additional studies using the highly sensitive techniques like ELISA with longitudinal follow up studies in the large cohort of samples would be required to further strengthen the present study.

Another important marker which can highlight the immunological state of an individual is the T-lymphocyte. It has been suggested that the changes in the T-lymphocyte ratio reflects the changes in the metabolism of central nervous system cells and could be used as neural markers in the analysis of psychiatric disorders

(Gladkevich *et al.*, 2004). The appropriate CD4/CD8 lymphocyte ratio is expected to be 2:1. Ratio below 1:1 indicates serious disorder of the immune system (Kouttab *et al.*, 1989). Therefore, we investigated the percentage of CD4+ and CD8+ cells in the schizophrenic patients and compared with the normal controls. The result of our study did not show any abnormality in the percentage of CD4+ and CD8+ cells among the patients when compared with the control. On the other hand the CD4+ and CD8+ subset ratio also did not show any significant deviation from the control groups. The result of our findings corroborate with the work of Villemain *et al.*, (1989), Achirion *et al.*, (1994), Baskak *et al.*, (2008) and Sperner-Unterweger *et al.*, (1999) who also failed to find any differences in T lymphocyte subsets. However our result is in contrast to the findings of Zhang *et al.*, (1996) (2002), Cosentino *et al.*, (1996), Ganguli *et al.*, (1987) Henneberg *et al.*, (1990), Muller *et al.*, (1993), Cazzullo *et al.*, (1998), Cazzullo *et al.*, (1998) and Villemein *et al.*, (1999). Our findings on CD4+ and CD8+ ratio is not in agreement to Sperner-Unterweger *et al.*, (1999) who have found higher CD4/CD8 ratio than healthy controls.

When we compared our results with our own findings on IL-2 and IL-6. It was complete contrast and unlike the findings of Zhang *et al.*, 2002 who reported lower number of CD4+ cells and lower IL-2. In our study we found lower level of IL-2 and normal CD4+ cell percentage. Our result suggests the abnormal production of ILs is not due to the abnormal number of CD4+ cells but it may by due to some abnormality in the CD4+ cells or else there may have some other factors responsible for this phenomenon. Moreover knowledge about Th17 cells and Treg is growing day by day and it may help to understand the role of CD4+ and CD8+ in schizophrenia.

The demographic data show the study comprises of more number of male schizophrenics and they have long duration of illness compared to females which hints the higher vulnerability of men to this disorder, at least in this region. A comprehensive study should be done in order to shed light in this respect. Most of the patients included in the study did not have any family history of the psychiatric and autoimmune disorder. As far as smoking and other substance abuse is concerned, there is decrease incidence of smoking and other substance abuse among the patients. The present findings of significant association of schizophrenia with the patients who are not the first child was in accordance to the study of Sham *et al.*, (1993). The study suggests that in addition to the genetic predisposition some environmental factors

such as viral infection may play a pivotal role on the onset of the disorder and the older children in the family may act as a source of viral infection for developing fetus in the mother. Thus the present study strengthens the hypothesis "younger children in a family has a significantly increased risk of later developing schizophrenia". On the other hand the analysis of the season of birth among the patients and the control subjects did not show any correlation between them.

From our study it is still too early to speculate the autoimmune etiopathology of schizophrenia but the study definitely strengthens the hypothesis of immune dysregulation in schizophrenia which may be one of the etiological factors for the disorder. Our study also supports the hypothesis of increase risk of developing the disorder among the younger children of the family. Additional studies are needed to reveal the mechanism of the changes in the immune system in schizophrenia.

There are several strengths in this study. First, clinical assessment was rigorously conducted. The most refined assessment procedure was used for diagnosis. The assessment of psychotic symptoms was performed on the basis of interview data and supplementary data from family information as well as data from medical records. Great care was taken for selecting the control subjects. Modern molecular methods were used for the studies which are most reliable. To evaluate the significance of the results, the calculations were corrected for the fact that we performed multiple tests.

To conclude with, the present study provides suggestive, but not conclusive evidence for autoimmune basis of schizophrenia. Moreover our study suggests the immunological dysfunction in schizophrenia which may be one of the etiological factors for the disorder. The findings which have been reported in this study should be regarded as preliminary since they are based on only a small number of individuals and awaits further research.

Section - 7

Summary and Conclusions

7. SUMMARY AND CONCLUSIONS

Schizophrenia is a devastating mental illness characterized by debilitating hallucinations, paranoia and delusions, that affect one percent of the world's population. Current researches primarily focus on the neurochemical and biological pathology of schizophrenia. But a subset of research has taken a decidedly different approach. This research postulates that, in at least some cases the immune system causes the disastrous psychotic symptoms of schizophrenia. This autoimmune hypothesis describes that somehow the immune system is triggered to attack the brain producing neurodegeneration and inflammation. For nearly a century ago the autoimmune basis for the onset of schizophrenia and progression have been proposed. This hypothesis continues to grow stronger as more markers of immune dysfunction are linked to schizophrenia.

Schizophrenia as an autoimmune disease was first theorized based on observed commonalities between the onset and progression of well-known autoimmune diseases. In this study we have investigated the circumstantial evidence of autoimmunity in schizophrenia. For this purpose we have selected certain parameters of the immune system like HLA system, serum IL , C-reactive protein and CD4 and CD8 cells. Along with this, we have studied the demographic characteristics and tried to correlate it with the disorder.

HLA study

HLA system comprises polymorphic class I and class II loci. The peculiarity of these regions is the high degree of polymorphism of most loci, that is, these locus encode a multitude of alleles which have evolved to fight a multitude of microbial factors. In the present investigation HLA-Class I genes were studied because HLA-A loci is juxtaposed to a non-classical HLA locus i.e., HLA-G which is involved in the protection of the tropoblast. Besides, many of the proven autoimmune disorder have shown association with HLA.

In the present investigation 136 schizophrenic patients and 150 unrelated controls were included. We have studied the frequency HLA Class I genes in all the cases. Among them the patients comprising of different subtypes were as follows: 118

Paranoid, 9 Disorganised, 1 Catatonic, 5 undifferentiated and 3 Residual. All the subjects were India born schizophrenic patients of West Bengal and recruited from the outpatient department (OPD) of Psychiatry, North Bengal Medical College and Hospital, Siliguri. The typing of the HLA was done with the help of serological as well as PCR SSP method.

The findings of the HLA Class I study yielded the interesting results. The result showed a significantly higher frequency of HLA-A*03 in patients than the control groups. On the other hand HLA-A*31 and HLA-B*51 showed the decreased frequency in the patient groups. At this moment it is difficult to propose the mechanism of association of HLA with schizophrenia. Correlations with several other factors are also need to be established such as low birth weight, viral infections, prenatal infections etc. which are at least common in Indian population to further shed light in this respect. Nevertheless, our result provides the evidence for the possible existence of a susceptibility locus for schizophrenia within the HLA region.

Interleukin study

Several circulating cytokines have been identified that mediate immune response. Cytokines have multiple roles including induction of an antiviral state. Abnormalities in cytokine concentrations many reflect the presence of an infectious or modulation in the immune process. To date, the most frequently studied cytokines in schizophrenia are IL-2 and IL-6. IL-2 is a T-cell growth factor and has been shown to modulate some neurotransmitter systems including dopamine metabolism within the central nervous system. More interestingly, a range of psychiatric manifestations including delusions, delirium, paranoia, hallucinations and lethargy have been observed in patients receiving IL-2 immunotherapeutically. These findings suggest that IL-2 may contribute to the pathophysiology of schizophrenia. IL-6 exerts trophic effects on glial cells, including oligodendroglia themselves, producing increased expression of glial fibrillary-acidic protein. Paradoxically, IL-6 increases intracellular calcium levels during N-methyl-D-aspartate receptor (NMDA-receptor) activation, enhancing neurotoxicity and cell death in granular neurons. Thus IL-6 can have both neurotrophic and neurotoxic effects in different neuronal types and at different developmental stages. This dual role that IL-6 appears to play in the CNS may explain the wide range of psychiatric disorders. Therefore, the present preliminary study was

undertaken to investigate the serum levels of IL-2 and IL-6 in the Indian schizophrenic patients.

For the study of serum level of Interleukins, 50 schizophrenic patients were considered for the present study. They were further classified into two groups, 20 schizophrenic patients who had stopped taking antipsychotic drugs for at least 6 weeks were considered as psychotropic medication free, rest 30 schizophrenic group were under antipsychotic medication. The study comprises of 44 Paranoid, 1 Residual, 4 Disorganized and 1 Catatonic schizophrenic patient. A total number of 30 unrelated, ethnically matched healthy individuals were considered as controls. The assay was carried out with the help of ELISA kit.

The result of the interleukin assay showed the lower level of IL-2 and IL-6 in the patient group. The unique finding of the present study is the significantly lower level of IL-6 in the schizophrenic subjects. To our knowledge this is the first report of lower level of IL-6 in the schizophrenic patients and nowhere else have been reported previously. The immunosuppressive and cytokine modulating effects of the antipsychotic drugs have been found by different studies. In the present study only atypical antipsychotics were prescribed to the patients. Therefore the lower levels of IL-2 and IL-6 observed among the medicating patients in the present study suggest the cytokine modulating activity of atypical antipsychotics. Thus, our finding supports the earlier findings that treatment with antipsychotic drugs affects the cytokine network. On the other hand our findings are not in agreement with the exhaustion theory of schizophrenia, also our findings do not fit well into the Th1/Th2 paradigm or with the Th2 shift hypothesis. Moreover the present findings strengthen the previously reported studies of immune system dysregulation in schizophrenia which may be one of the etiological factors for the disorder.

C- reactive protein study

The roles of immune dysfunction and inflammation have been described in schizophrenia. One of the well known inflammatory marker is C-reactive protein (CRP). We have studied the CRP as it has been hypothesized that some kind of inflammatory process is involved in schizophrenia.

Sera level of CRP were measured for 64 schizophrenic patients. Out of them, 57 were paranoid, 2 residual, 3 undifferentiated and 2 were disorganized type. Latex agglutination test was followed to detect the serum level of CRP.

The elevated level of CRP was observed in 3 patients and 61 patients were found to have normal CRP. All the three elevated cases were found to be of paranoid type. No differences were found in CRP levels among different subgroups of schizophrenia. Further, when the level of CRP was compared to the other demographic variables, only the drug naïve status of the patients showed statistically significant value. The study provides further evidence that some kind of inflammatory process may play a role in the etiopathology of schizophrenia.

CD4+ and CD8+ study

Investigations of lymphocytes in patients with schizophrenia started as early as 1900 AD. Advances in immunologic techniques, as well as a deepening understanding of lymphocyte function have opened the way towards the quantitation of specific, functionally distinct lymphocyte subsets. Initially, these studies focused on T lymphocytes such as CD4 and CD8. The CD4+ T lymphocytes, also known as T helper cells facilitate both humoral and cell-mediated immune processes. In contrast, CD8 cells act to shut off CD4 cell activity when sufficient antibodies have been produced. Changes in T helper/inducer (CD4) and T cytotoxic/ suppressor (CD8) cells are related to a variety of illnesses. The appropriate CD4/CD8-lymphocyte ratio is expected to be 2:1. Ratios below 1:1 indicate serious disorder of the immune system. It has also been suggested that changes in the T-lymphocyte ratio reflects changes in the metabolism of central nervous system cells and it can be used as neural markers in the analysis of psychiatric disorders.

The CD4+ and CD8+ ratio in the blood were estimated by flow cytometry in 20 patients and compared with the same number of control. Although the mean percentage of CD4+ cells were found to be little higher in the patients, it was not significantly higher than the control groups. Also the mean percentage of CD8+ cells is not found to be significantly deviated in patients and control groups. On the other hand the CD4+ and CD8+ subset ratio was found to be normal in both patients and the control. When we compare our results with our own findings on IL-2 and IL-6 it is

a complete contrast in the sense that the lower level of IL-2 and normal CD4+ cell percentage, which is unlike the findings by Zhang *et al.*, 2002 who reported lower number of CD4 cells and lower IL-2. Our results suggest the abnormal production of ILs is not due to the abnormal number of CD4 cells but it may by due to some abnormality in the CD4 cells or else there are other factors responsible for this phenomenon. Moreover, the knowledge about Th17 cells and Treg are emerging. As more and more knowledge about the mechanism of their function would become available, the role of CD4+ and CD8+ in schizophrenia will be understandable in a better way.

Demographic study

During the three year period of study, it was observed that more number of male patients were attending the OPD than the females. Therefore the present study consists of more number of male schizophrenics and they were found to have long duration of illness compared to females. Although the observation hinted the higher vulnerability of men to this disorder in this region, a more comprehensive study is needed in this respect before concluding any remarks. One of the interesting observations of the present study is that, the majority of the schizophrenic patients were not the first child of the family. This finding corroborated with of the study of Sham *et al.*, (1993). Thus the present study strengthens the hypothesis "younger children in a family has a significantly increased risk of later developing schizophrenia". The study also suggests that in addition to the genetic predisposition some environmental factors such as viral infection may play a pivotal role on the onset of the disorder and the older children in the family may act as a source of viral infection for developing fetus in the mother. These infections may alter the neurodevelopmental process leading to schizophrenia to the unborn child in the later years.

Suggestive conclusion

At this moment it is not clearly understood whether the changes in the immune system is the byproduct of the pathophysiology of schizophrenia or directly contributes to the clinical manifestations of the disorder. Moreover it is too early to speculate the autoimmune hypothesis of schizophrenia. On the other hand the results of our present

investigation definitely suggest some kind of immune dysregulation in schizophrenia which may be one of the etiological factors for the disorder. Overall, from our study we can make the following specific concluding remarks:-

1. HLA-A*03 gene may contribute to the risk of the disease or else that there might be a separate gene in strong linkage disequilibrium with HLA-A*03 gene.

2. HLA-A*03 gene may be used as genetic marker for schizophrenia.

3. HLA-A*31, B*51 may act as the protective markers for schizophrenia.

4. The older children in the family may be the source of viral infections, which they transmit to their pregnant mothers and these infections may alter the neurodevelopmental processes leading to schizophrenia.

5. The inflammatory process may play a role in the etiopathology of schizophrenia.

6. The study suggests that some kind of dysfunction in immune system may be involved with schizophrenia.

Section - 8

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Section - 9

Annexure

ANNEXURE – I

INFORMATION SHEET

Cellular Immunology Laboratory

Department of Psychiatry

Serial No.- ..... Date: .....

SCHIZOPHRENIA

Sub-type: .....

Name: .....

Address:.....

DEMOGRAPHIC DETAILS

Date of birth: ..... Age: .....

Sex: M/F: ..... Cast: .....

Occupation: .....

Education:<class VIII/Class VIII/Madhyamik/H.S./Graduation/Masters/other:

Marital status: Marrid/Unmarrid/Widow/Divorced

Monthly income: .....

Age of onset: .....

Taking medicine from: .....

Any sort of abuse: .....

Migration (if any): .....

Any sort of other infection during the period of illness: .....

Complication during birth: .....

Any other autoimmune disease in the patient or in the family: .....

Any sort of substance abuse: .....

Pedigree:

GENERAL PHYSICAL EXAMINATION

MENTAL STATUS EXAMINATION

LABORATORY INVESTIGATION AND REPORT

HLA-Class I typing :

IL-2 and IL-6 level :

C-reactive protein :

CD4 and CD8 count :

CLINICAL DIAGNOSIS

ANTIPSYCHOTIC DRUGS PRESCRIBED

OTHER INVESTIGATIONS

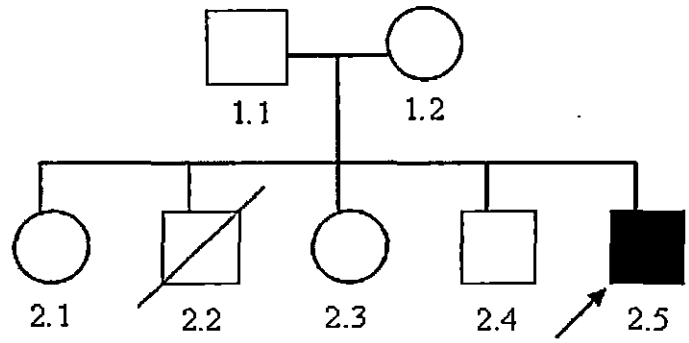
ANNEXURE – II

FAMILY PEDIGREE

Symbol Used

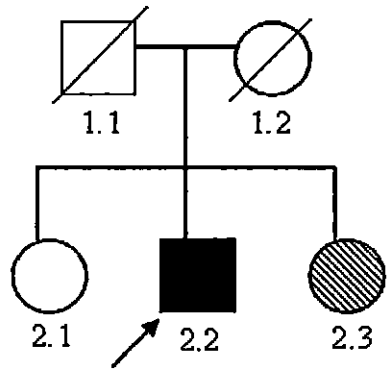
A	:	Aunt	N	:	Normal
B	:	Brother	P	:	Patient
F	:	Father	S	:	Sister
GF	:	Grand Father	So	:	Son
GM	:	Grand mother	PF	:	Paranoid Feature
M	:	Mother	U	:	Uncle
US	:	Undifferentiated Schizophrenia	PS	:	Paranoid schizophrenia

FAMILY – 1



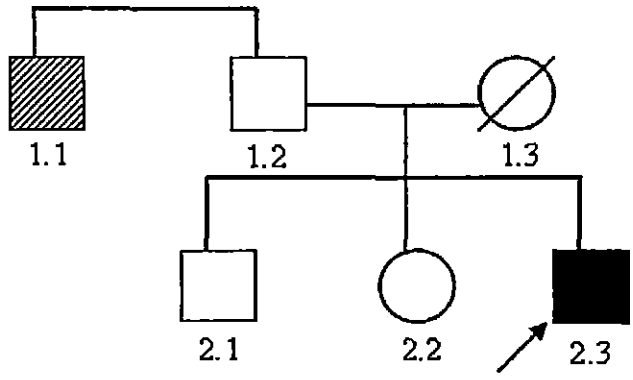
Name	Age/Sex	Relation	Diagnosis
1.1	80/male	F	N
1.2	70/female	M	N
2.1	50/female	S	N
2.2	45/male	B	N
2.3	45/female	S	N
2.4	35/male	B	N
2.5	30/male	P	PS

FAMILY – 2



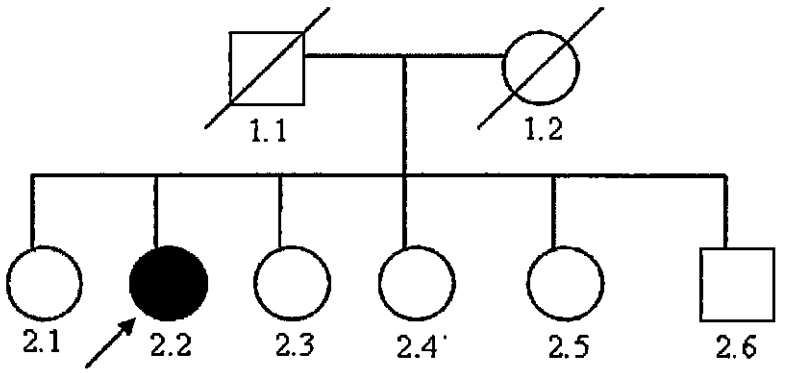
Name	Age/Sex	Relation	Diagnosis
1.1	**69**	**F**	**N**
1.2	**61**	**M**	**N**
2.1	**58/female**	**S**	**N**
2.2	**53/male**	**P**	**PS**
2.3	**50/female**	**S**	**PF**

FAMILY – 3



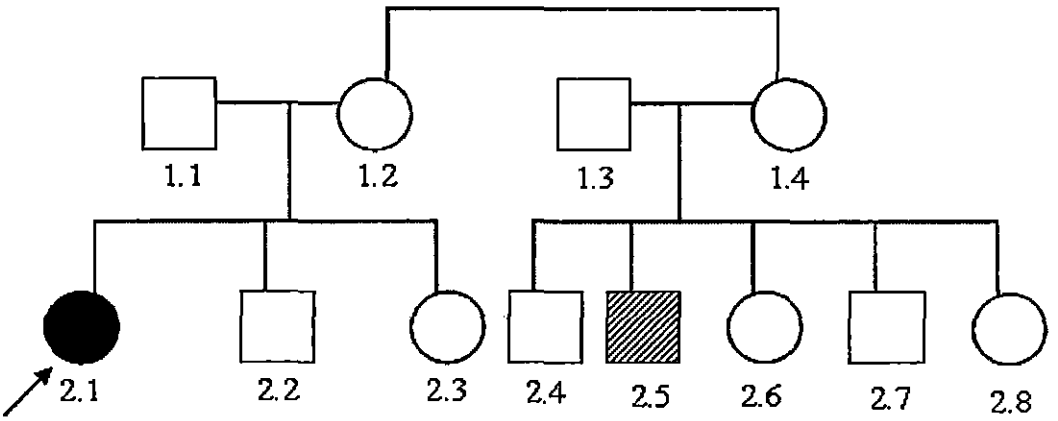
Name	Age/Sex	Relation	Diagnosis
1.1	75/male	U	PF
1.2	73/male	F	N
1.3	57/F	M	N
2.1	51/male	B	N
2.2	47/female	S	N
2.3	45/male	P	PS

FAMILY - 4



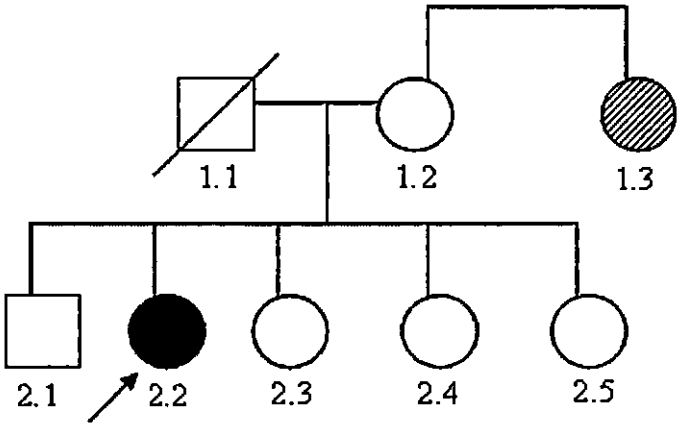
Name	Age/Sex	Relation	Diagnosis
1.1	57/male	F	N
1.2	60/female	M	N
2.1	32/female	S	N
2.2	30/Female	P	US
2.3	27/female	S	N
2.4	25/female	S	N
2.5	21/female	S	N
2.6	18/brother	B	N

FAMILY – 5



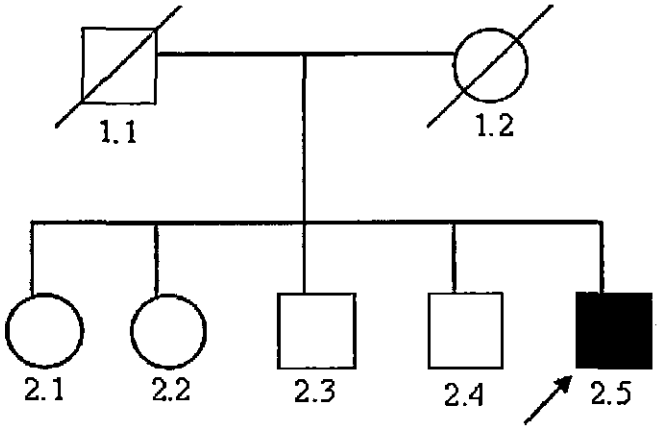
Name	Age/Sex	Relation	Diagnosis
1.1	48/male	F	N
1.2	40/female	M	N
1.3	45/male	U	N
1.4	37/female	A	N
2.1	22/female	P	DS
2.2	17/male	B	N
2.3	13/female	S	N
2.4	20male	B	N
2.5	19/male	B	PF
2.6	17female	S	N
2.7	15/male	B	N
2.8	13/female	S	N

FAMILY - 6



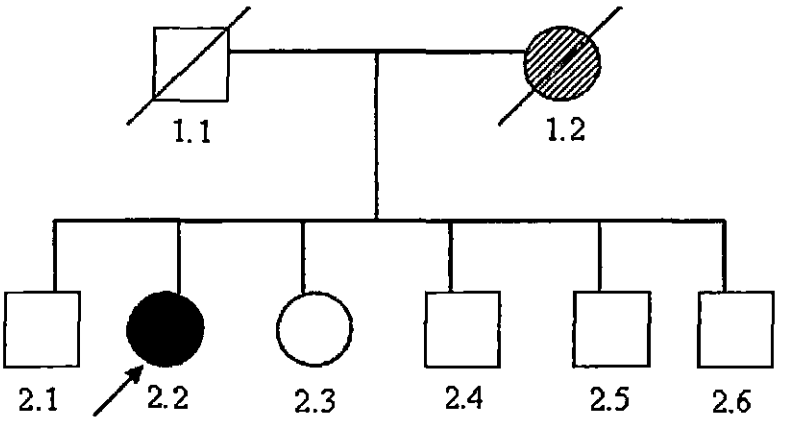
Name	Age/Sex	Relation	Diagnosis
1.1	71 /male	F	N
1.2	60/female	M	N
1.3	57/female	A	PF
2.1	41/male	B	N
2.2	38/female	P	PS
2.3	35/female	S	N
2.4	31/female	S	N
2.5	29/female	S	N

FAMILY – 7



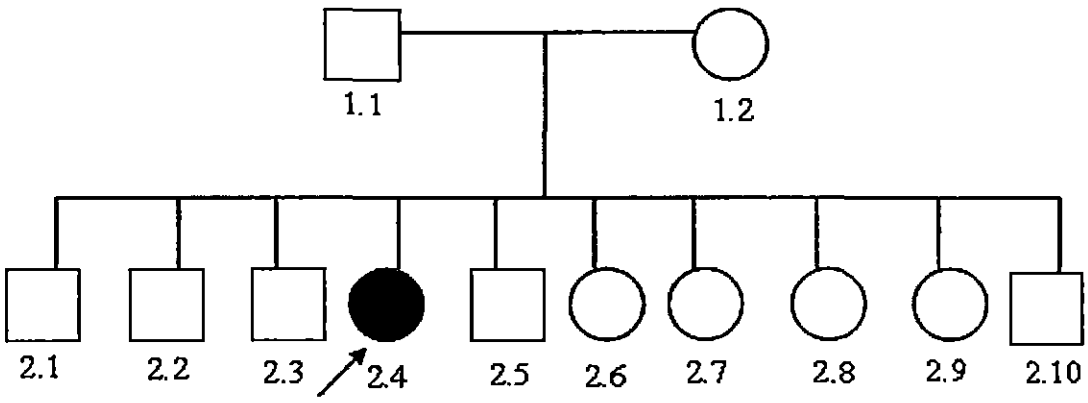
Name	Age/Sex	Relation	Diagnosis
1.1	59/male	F	N
1.2	45/female	M	N
2.1	49/female	S	N
2.2	43/female	S	N
2.3	40/male	B	N
2.4	37/male	B	N
2.5	35/male	P	PS

FAMILY - 8



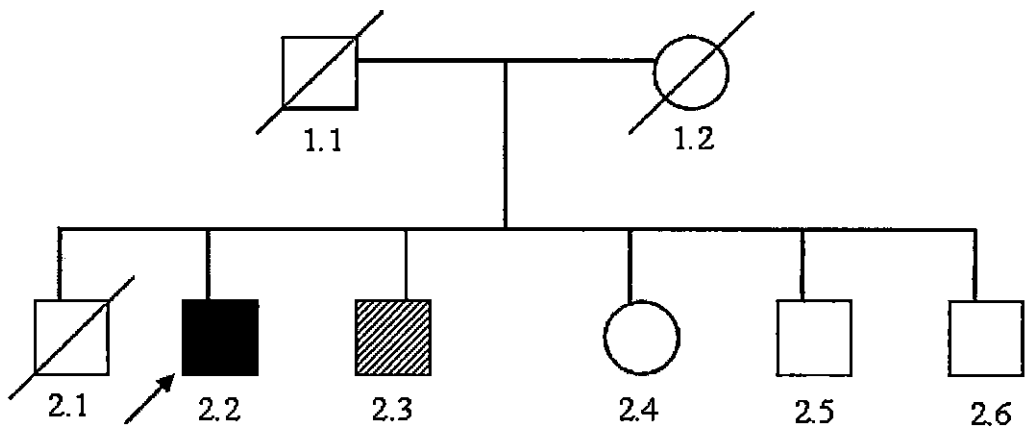
Name	Age/Sex	Relation	Diagnosis
1.1	77/male	F	N
1.2	75/female	M	N
2.1	51/male	B	N
2.2	48/female	P	PS
2.3	43/female	S	N
2.4	40/male	B	N
2.5	38/male	B	N
2.6	35/male	B	N

FAMILY – 9



Name	Age/Sex	Relation	Diagnosis
1.1	80/male	F	N
1.2	77/female	M	N
2.1	61/male	B	N
2.2	58/male	B	N
2.3	57/male	B	N
2.4	55/female	P	PS
2.5	53/male	B	N
2.6	51/female	S	N
2.7	49/female	S	N
2.8	45/female	S	N
2.9	42/female	S	N
2.10	39/male	B	N

FAMILY – 10



Name	Age/Sex	Relation	Diagnosis
1.1	57/male	F	N
1.2	65/female	M	N
2.1	54/male	B	N
2.2	52/male	P	PS
2.3	50/male	B	PF
2.4	42/female	S	N
2.5	39/male	B	N
2.6	35/male	B	N

ANNEXURE – III

SOLUTIONS AND REAGENTS

1. Heparin (Preservative free) (Centron Research Laboratory, Bombay)

- 500 I.U. per ml.

2. Lymphoprep, specific Gravity, 1.077 (Sigma Diagnostic, USA)

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

4. Tris NH_4Cl

TRIS - 20.6 gm/L dH_2O

NH_4Cl - 0.83 gm/100ml H_2O

5. Rabbit Complement (M/S Pel Freeze Biological Inc.) and prepared locally.

-Aliquot and store at -70°C

6. 1% Trypan Blue stock solution

Filter and store at 4°C

Working solution -0.3% prepared in buffer

7. Eosin Y (Qualigens, India)

5% in dH_2O

Filter and store at room temperature

8. Formalin (Sisco, India)

-Add 2ml of 0.5% phenol red to 500 ml formaldehyde and concentrated KOH to bring pH to 7.2-7.4

-Store at room temperature

9. 20 X SSC (pH 8.0)

NaCl – 175.3 gm

Na-citrate- 88.2 gm

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

10. HSB (High Salt Buffer) pH 7.6

10mM Tris-Cl

10mM KCl

10mM $MgCl_2$

0.4 M NaCl

2mM EDTA

11. Proteinase – K solution

-dissolve 100mg Proteinase-K in 10mL of dH_2O

-aliquote into 200µl in microfuge tubes and store in -20°C

12. 10% SDS

At the final concentration of 0.5%, i.e. 5mg/ml of sample.

13. 50mM KCl

3.728 gm of KCl in 1000ml. of dH_2O.

14. 4M NaCl

116.9 gm in 500 ml of dH_2O

15. PC (Phenol Chloroform)

4 part Phenol + 1 part chloroform. pH of phenol should be adjusted to 8.5-9.0 by adding Tris-HCl.

16. Deoxyribonucleotide Triphosphates (dNTPs) (Bangalore Genei, India)

The dNTPs are the monomers for DNA polymer consisting of dATP, dCTP, dGTP and dTTP. The dNTPs are used at saturing concentration in PCR amplification.

17. PCR buffer with Magnesium Chloride (Bangalore Genei, India)

The PCR buffer if optimized for use in PCR experiments. Generally, the PCR buffer is supplied along with Taq polymerase by the commercial companies. We use PCR buffer from Bangalore Genei, India which has 10mM Tris-HCl (pH 9.0), 1.5mM $MgCl_2$, 50mM KCl and 0.01% gelatin.

18. Ethidium Bromide (10mg/ml)

Added 100mg of ethidium bromide to 10ml of DDW water, stirred on a magnetic stirrer for several hours to ensure that dye was fully dissolved. Wrapped the container in aluminum foil or transferred the solution to a dark bottle and stored at room temperature.

19. Gel Loading Solution

0.05% Bromophenol blue	50mg
4.0% Sucrose	20.0g

ANNEXURE - IV

SCID-I (DSM – IV) version 2.0 (Feb. 1996 final) screening questions

SCID SCREENING MODULE (OPTIONAL)

Now I want to ask you some more specific questions about problems you may have had. We'll go into more detail about them later.

RESPOND TO POSITIVE RESPONSES WITH, we'll talk more about that later.

		1	2 3
1.	Has there been any time in your life when you had five or more drinks (beer, wine or liquor) on one occasion ?	CIRCLE "NO" ON E.1	CIRCLE "YES" ON E.1
2.	Have you ever used street drugs?	1 CIRCLE "NO" ON E.10	2 3 CIRCLE "YES" ON E.10
3.	Have you ever gotten 'hooked' on a prescribed medicine or taken a lot more of it than you were supposed to?	1 CIRCLE "NO" ON E.10	2 3 CIRCLE "YES" ON E.10
4.	Have you ever had a panic attack when you suddenly felt frightened or anxious or suddenly developed a lot of physical symptoms ?	1 CIRCLE "NO" ON F.1	2 3 CIRCLE "YES" ON F.1
5.	Were you ever afraid of going out of the house alone, being in crowds, standing in a line or traveling on buses or trains?	1 CIRCLE "NO" ON F.7	2 3 CIRCLE "YES" ON F.7

6. Is there anything that you have been afraid to do or felt uncomfortable doing in front of other people, like speaking, eating or writing?

1 | 2 3

CIRCLE "NO" ON F.11 | CIRCLE "YES" ON F.11

7. Are there any other things that you have been especially afraid of, like flying, seeing blood, getting a shot, heights, closed places, or certain kinds of animals or insects?

1 | 2 3

CIRCLE "NO" ON F.16 | CIRCLE "YES" ON F.16

8. Have you ever been bothered by thoughts that didn't make any sense and kept coming back to you even when you tried not to have them?

1 | 2 3

CIRCLE "NO" ON F.20 | CIRCLE "YES" ON F.20

9. Was there ever anything that you had to do over and over again and couldn't resist doing, like washing your hands again and again, counting up to a certain number or checking something several times to make sure that you'd done it right?

1 | 2 3

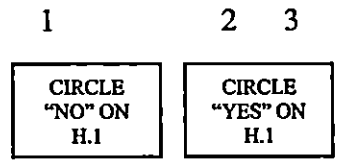
CIRCLE "NO" ON F.21 | CIRCLE "YES" ON F.21

10. In the last six months, have you been particularly nervous or anxious?

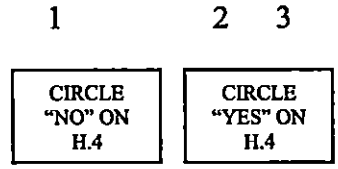
1 | 2 3

CIRCLE "NO" ON F.31 | CIRCLE "YES" ON F.31

11. Have you ever had a time when you weighed much less than other people thought you ought to weigh?



12. Have you often had times when your eating was out of control?



1= absent or false 2= subthreshold 3 = threshold or true

ANNEXURE- V

BRIEF PSYCHIATRIC RATING SCALE

Symptoms	Initial	7th week	13th week
Somatic Anxiety			
Anxiety (Psychic)			
Emotional withdrawal			
Conceptual Disorganisation			
Self-depression & guilt feelings			
Anxiety (Somatic)			
Specific Motor Disturbances			
Exaggerated Self-esteem			
Lowered Mood			
Hostility			
Suspiciousness			
Hallucinatory Behaviour			
Decreased Psychomotor Activity			
Uncooperativeness			
Unusual Thought Content			
Blunted or Inappropriated Affect			
Increased Psychomotor Activity			
Disorientation and Confusion			

Corrigendum

I would like to thank the expert for his constructive criticism on the thesis. I have considered all the raised by the expert and corrected the thesis as suggested. The answer to the comments are given be

My comments and some suggested small corrections are given below:-

1. Usually a supervisor and co-supervisor may be there for a thesis. Are two supervisors allow your University?
   I would like to draw the kind attention of the examiner that Prof. T.K.Chaudhuri and Dr.N.K. acted as Chief Supervisor and Co-supervisor respectively. The same has been mentioned in the "Certificate" given by the supervisors and as well as in "Statement by the candidate". This is al in the University,otherwise the permission would not have been granted for the registration of degree.

2. In materials and methods, page 86, it appears that written consent was not taken from contro subjects. If that is not true, it should be mentioned properly.
   Yes, we have taken the written consent from both patients and controls. Therefore, we used the common term "participants" for both patients and controls. So, the term "All participants" was used. The sentence reads "All the participants provided their written consent for giving the blood sample after the study procedures were explained".

3. Page 89, what is ARMS-PCR-SSP? It is not described.
   The full form of ARMS-PCR-SSP is Amplification refractory mutation system-polyme chain reaction-sequence specific primer. We have followed this method of typing HLA genes and it is described in the text.

4. Page 90, 8th point, how DNA was recovered and then rinsed with absolute ethanol.
   After proteinase K digested suspension is recovered it is put in another vile containing absolute ethanol and it is centrifuge at 12000 rpm. By centrifugation the DNA sticks to base of the vile and we can throw out the supernatant and rinse the DNA again by agai putting the alcohol and discarding the supernatant.

5. Page 90, fig 20 why such marker is used which does not run with your DNA. What purpose that serve?
   The main purpose of running the extracted high molecular weight DNA is to see the approximat quantity and purity of DNA. The marker we used is for the HLA not for the high molecular wei DNA as it does not have the high bp therefore it did not match with the crude DNA but it can gi approximate knowledge of presence of any impurity. The gel shown below in Fig1 have some impurity and in figure 2 in lane 5 there is no DNA or it is so low in quantity it is not visible in th

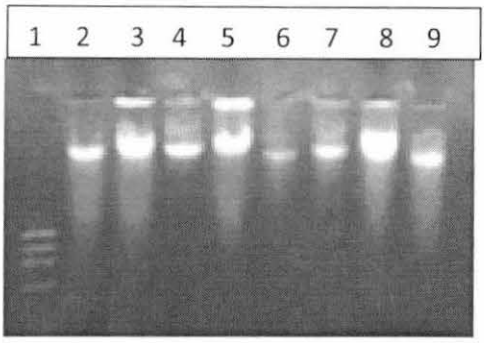


Fig 1

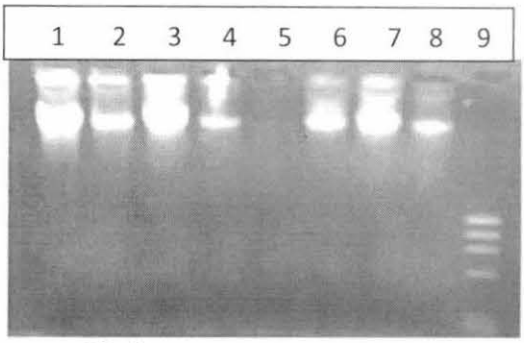


Fig 2

6. Page 91, section 4.2.2.3.1, what are thermostable PCR tubes?
   Page 92, primer length is given in 'mer'. There is no base pair in primers.
   We have used Eppendorf PCR Tubes which are made up of thin-walled polypropylene fo efficient, homogeneous heat transfer of samples. It can resist the high heat therefore it is called thermostable PCR tubes. We used 94°C as the denaturing temperature. Therefore thermostable PCR tubes were used in thermal cycler which can resist the high temperatu

   Now BP is replaced by neucleotide length.

7. Page 95, line 1. How did your mixed agarose properly and gently till agarose was dissolve insi a microwave oven?
   It is convenient to heat the agarose for 45 sec-1min to melt the agarose in the microwave oven. I used to swirl it after 45 sec to allow the agarose to mix properly. Utmost care was taken not to allow it to boil.

8. It is always useful to elaborate the formulae used (page 97).
   I hope the expert is referring to the formula given below, it is now elaborated

$$f_A = \frac{nA}{n}$$

Where, f_A = frequency of allele
n =number of subjects studied
nA=number of times an allele is present

9. Page 98. Which antipsychotic drug was used. Same page last but one line comma may be put after unrelated, age, sex.....
   Yes, we have not mentioned the names of the antipsychotics in this page, but I would like draw your kind attention that it is mentioned in page 114 in table no.22. Comma has been put.

10. Page 99. A comma required before respectively (on line 10).
Comma has been put.

11. Page 100. Delete "the" from last para first line.
I hope the expert is referring to this line "About 5ml. of blood samples were collected from the each patient by vein puncture method" and he wants me to de "the" from "the each patient". It has now been deleted.

12. Page 101, figure legend "negative" is duplicated.
One "negative" has been omitted.

13. Page 103. Why chi-square value for both A31 and B51 is high? It has not been explained.
"On the other hand A*31 (χ_2=34.160, p<0.001) and B*51 (χ_2=31.083, p<0.001) showed significantly lower value even after the Bonferroni corrections." I hope t expert is referring to the line mentioned above. But I would like to mention here that it is too early to speculate why the frequency of A31 and B51 are higher in the cont subjects. At this moment we are unable to explain the exact mechanism involved in it. It mentioned in the discussion section in page 108 and also in the summary and conclusion page 135. Moreover, various hypothesis given by the workers regarding the HLA and disease susceptibility has been mentioned in page 63 under the heading "HLA AND DISEASE SUSCEPTIBILITY". Therefore, at present, we have only mentioned that i may act as protective marker for schizophrenia (heading "suggestive conclusion" page 1 point no. 3). Further studies are needed to throw light to this question.

14. Page 127, third para, "The present study has been carried out" not "is been". In last line a con is required before respectively.
The correction has been done.
15. Page 134. Place a comma after i.e., (in third para)
The correction has been done.

Signature of the candidate

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Impact of HLA Antigens and Other Risk Factors on the Etiopathology of Schizophrenia

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ABSTRACT

Schizophrenia is the paradigmatic illness of psychiatry. The involvement of immunological and immunopathological mechanisms along with the environmental factors in the etiopathogenesis of schizophrenia has been a matter of research. We investigated the incidence of HLA Class I antigens to understand the role of HLA genes in schizophrenia. We further studied the birth of the siblings in the family and age of onset to understand the role of environmental factors for triggering the disorder. Some of the HLA antigens are associated with schizophrenia and significant increase was found for HLA-A3 antigen along with the significant decrease in HLA-A25, A31 and HLA-B51.Further, significant value was found after $\times 2$ test between the first child patient and the patient who are not the first child. The study provides the evidence for the possible existence of susceptibility locus for schizophrenia within the HLA region. The study further suggests that in addition to genetic predisposition some environmental factors such as viral infection might play a pivotal role on the onset of the disorder. This preliminary observation may help to understand the etiological basis of this disorder.

Key words: Schizophrenia, etiology, HLA antigens, association

INTRODUCTION

Schizophrenia is the paradigmatic psychiatric illness. Inspite of its one percent worldwide incidence, it has become a leading public health problem. The etiological process or processes by which causal agents causes the physiopathology is not yet understood. With recent progress in the understanding of the immune system, autoimmune etiology of schizophrenia has become a major focus of research (Ganguli *et al.*, 1993; Knight,1984). Susceptibility to almost all autoimmune diseases is influenced by genes encoded by the human leukocyte antigen (HLA) region, particularly the Class I (A,B and C) and Class II (DR,DQ,DP) antigens. The HLA gene complex is located on the short arm of chromosome 6 (p21.3). It is one of the most gene-rich and polymorphic regions in human genome and traditionally, investigators compare phenotypic or genotypic frequencies between unrelated cases and unaffected controls.

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There have been numerous association studies of HLA antigen and schizophrenia (Mc Guffin and Sturt,1986; Hawi *et al.*,1999). Past association studies with various Class I and Class II alleles yielded inconsistent results (Nimgaonkar *et al.*,1992) except with HLA-A9 (now subdivided into A23 and 24) (Mc.Guffin *et al.*, 1995). In different ethnic populations associations of schizophrenia has been found with HLA-A9 (Goudemand and Goudemand, 1981), HLA-A23 (Ivanyi *et al.*, 1983),HLA-24(Asaka *et al.*,1981) , HLA-A1(Lahdelma *et al.*,1998), HLA-A2, HLA-A3, HLA-A11, HLA-B17, HLA-B27, HLA-B8 and Cw2 (Rudduck *et al.*,1984), HLA-A3(Debnath *et al.*, 2005), DRB1-04(Wright *et al.*, 1996) , DR1 (Sasaki *et al.*,1999) alleles.

Along with genetic factors there are other risk factors for schizophrenia which have been identified. It has been proposed that maternal infection during pregnancy increases the risk of the offspring developing schizophrenia and other developmental neuro-disorders (Wright *et al.*, 1995). Sham *et al.*(1993), using data from a Swedish family study , reported that younger children in a family had a significantly increased risk of later developing schizophrenia if their siblings were 3 to 4 years older at the time when they were in uterus. The researchers suggest that older children can be a source of viral infections to their mother and also to the developing fetus, which they may transmit to their pregnant mothers, and these infections in turn may cause schizophrenia in the offspring.

The present study aimed to investigate the role of HLA antigens in the etiopathology of schizophrenia and evaluate the data with reference to the hypothesis "younger children in a family have a significantly increased risk of later developing schizophrenia."

MATERIALS AND METHODS

We studied 50 India-born schizophrenic patients residing in and around Siliguri Subdivision of West Bengal, who attended the outpatient department (OPD) of Psychiatry, North Bengal Medical College and Hospital. The patients considered for the present study belonged to Bengali, Nepali, Bihari and some tribal communities. They were diagnosed independently by two psychiatrists with the help of standard diagnostic criteria of DSM IV and assessed by the Brief Psychiatric Rating Scale (BPRS). The age was within the range of 17-58 and male to female ratio was 2.8: 1 were studied. Further, the schizophrenic patients and the family members were made to answer a questionnaire. The questionnaire included self-reported age, caste, sex, medical history, age of onset, month of birth, marital status, education, substance abuse, incidence of any autoimmune disease among patients or in family members etc. A complete pedigree for each of the patient was also drawn.

A total number of 50 healthy individuals, which matched the age, sex and ethnicity of schizophrenic patients, were considered as controls. All control subjects were screened for a recent history of intercurrent infections and allergies. Those with a past history of autoimmune or psychiatric disorders were excluded. Written consent from all the participants were taken prior to collection of blood samples from them.

Methodology: The blood was drawn by the vein puncture method and collected in EDTA anticoagulant. DNA was collected from peripheral mononuclear cells of the blood by the Phenol Chloroform method. The typing of HLA Class I was performed by PCR-SSP technique. The primers, Taq polymerase, nucleotides etc. were obtained from Bangalore Genei, India and the typing and sequence information of primers were taken from Bunce *et al.* (1995).

Statistical Analysis: The phenotype frequencies were calculated by direct count. Chi-square test was done to compare the frequency of each antigen in the patient and control groups and it was followed by Fisher's exact test. Since testing for a large number of antigens can reveal at least one positive association where none really exists, the p values from each Fisher's exact test had to be less than the Bonferroni p [0.05 divided by the number of antigens tested minus two degrees of freedom (one for each of the two loci examined), which equals to 0.0014] to be called stastically significant. Relative risk was estimated as recommended by Svejgard (1974). Furthermore, Chi-square test was employed for birth of the siblings and Welch's t test for age at onset.

RESULTS

As summarized in the Table I, there was a significantly higher frequency ($\chi2=11.45$, $p<0.001$) of HLA-A3 in patients than the in the control group. On the other hand HLA A25 (Fig. 1) ($\chi2=13.619$,$p<0.001$), A31 ($\chi2=22.56$,$p<0.001$),B51 ($\chi2=42.85$, $p<0.001$) showed a significantly lower value after the Bonferroni correction. Besides A2 ($\chi2=6.05$,$p<0.008$)showed lower frequency and B7 ($\chi2=4.069$, $p<0.02$) and B42 ($\chi2=5.47$,$p<0.033$) showed higher frequency but they were not found to be significant after the Bonferroni correction.
Further, as shown in Table II, when the Chi- square test was performed between the first child patients and the patients who were not the first child, the test showed a significant diffrence, on the other hand the age of onset did not show a significant difference between the two groups. It is worth mentioning here that both the groups of the patients were HLA-A3 positive.

Table I: Phenotype frequency, Chi square, relative risk (RR) values and probability of HLA-A and B loci alleles in the patients with schizophrenia and healthy controls.

Antigen	Patients (N=50)	Control (N=50)	Chi square	RR	P value	
A*02	24	27	6.0529	0.32	<0.008	*
A*03	50	38	11.458	0.00	<0.001	**
A*11	36	31	11.723	1.58	<0.288	
A*23	21	26	0.6423	0.67	<0.317	
A*24	32	26	1.026	1.64	<0.224	
A*25	10	29	13.619	0.18	<0.001	
A*26	21	17	0.382	1.41	<0.410	
A*29	33	32	0.000	1.09	<0.833	
A*30	33	25	2.0114	1.94	<0.105	
A*31	0	20	22.522	0.00	<0.001	**
B*07	47	39	4.069	4.42	<0.02	*
B*21	36	38	0.0519	0.81	<0.825	
B*4001	17	13	0.428	1.47	<0.513	
B*4201	39	28	0.522	2.79	<0.033	*
B*44	14	10	0.493	1.59	<0.349	
B*5101-5105	0	30	40.047	0.00	<0.001	**

* Significant; ** Significant after the Bonferroni correction Bonferroni's probability is 0.003571

Table II: Comparison of demographic characteristics between the first schizophrenic child and the other schizophrenic siblings in different families.

	N=50		Analysis	
	patients who are first child	patients who not the first child	Statistic	P value
1. Number of patients	11	39	$\chi 2$ =15.68 (df=1)	<0.001 *
2. Age of Onset (years)				
Mean	24.45	28.03		= 0.30
Standard deviation (SD)	12.84	8. 97	t = -1.056	

*** significant**

DISCUSSION

A significantly higher frequency of HLA-A3 obtained in the present study is in accordance with the previously reported study by Debnath *et al.* (2005). Although the association of HLA-A3 antigen and schizophrenia is found to be significantly higher, the present studying does not corroborate a very strong association. This might be due to the small size of control population the present study.

On the other hand, the present study A25, A31 and B51 shows a significantly low negative value which is the unique finding of the present study. The alleles A31 and B51 show strong negative association (RR=0.000). The increased frequency of A11 found in the previous study has not been reproduced in the present study. Apart form this, several other alleles like B7 and B42 show higher values but are not statistically significant. We have also observed a negative but insignificant association of A2 which corroborates the findings of Debnath *et al.* (2005).

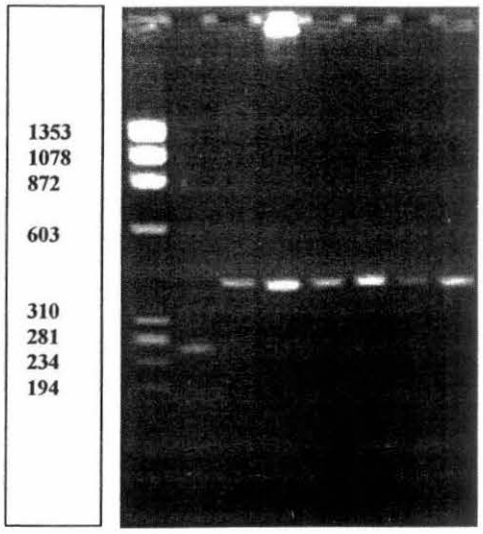

Fig.1: Electrophoregram showing the results of HLA-A*25

Lane:
1. Marker : Phi X174; 2. Control : 256 bp
3-8. A*25 : 398 bp

The findings in the present study paves the way for the possible association of HLA antigen with schizophrenia but the exact mechanism of the association of HLA with schizophrenia is still obscure. However, the presence of the disease in only a small fraction of the people carrying the marker HLA allele can be explained as the following : (i) Genetic factors not linked with HLA may contribute toward the disease susceptibility; (ii) the disease may be heterogeneous and may have more than one etiology; the observed HLA association is only with a subset of affected individuals; (iii)environmental

factors, such as exposure to viruses, may determine whether individuals with disease susceptibility will manifest the disease clinically; and (iv) if the association is due to a thus-far undetected allele at a closely linked locus, then this allele may occur at a much lower frequency than the one detected at the presently known A,B,C and D/DR loci.(Mc Devitt and Bodmer,1974; Bodmer and Bodmer, 1978).

The present finding of significant association of schizophrenia with the patients who are not the first child was in accordance to the study of Sham *et al.* (1993).It is needless to mention here that all the patients were HLA-A3 positive. Therefore, the present study suggests that in addition to genetic predisposition some environmental factors such as viral infection might play a pivotal role on the onset of the disorder. However, the present study strengthens the hypothesis "younger children in a family had a significantly increased risk of later developing schizophrenia".

The present study supports earlier finding of association of HLA-A3 with schizophrenia along with the negative association of some more alleles, which is the new finding of the present study. However, it is too early to speculate the exact mechanism of association. The result is preliminary and can not be reliably correlated with the birth status, viral infections, prenatal infections and so on. However, this study provides an evidence for the possible existence of a susceptibility locus for schizophrenia within the HLA region. The study also presupposes the idea of requirement of environmental factors along with the genetic predisposition for triggering the disorder. Further study needs to be carried out on larger sample size to decipher the etiopathology of the disorder.

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ORIGINAL ARTICLE

Analysis of the role of human leukocyte antigen class-I genes to understand the etiopathology of schizophrenia

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ABSTRACT

Background: Schizophrenia is the paradigmatic illness of psychiatry. The involvement of immunological and immunopathological mechanisms in the etiopathogenesis of schizophrenia has been a matter of research, with recently increasing effort.
Aims: In this study, we investigated the incidence of human leukocyte antigen (HLA) Class I antigens to understand the role of HLA genes in schizophrenia.
Materials and Methods: India born schizophrenic patients in and around Siliguri who attended outpatient department (OPD) of Department of Psychiatry, North Bengal Medical College and Hospital were considered for the present study. After the longitudinal follow up, 50 patients were enrolled for the study. The same number of age, sex and ethnically matched healthy subjects were considered as control. Low resolution polymerase chain reaction-sequence specific primer method was applied for typing the HLA antigens.
Statistics: The phenotype frequencies were calculated by direct count. χ^2 test was done to compare the frequency of each antigen among the patients and control group and it was followed by Fisher's exact test. Relative risk was estimated by using Haldane's method.
Results: The result showed that some of the HLA antigens are associated with the schizophrenia and significant increase were observed for HLA A*03 antigen along with the significant decrease for HLA A*25, A*31 and HLA B*51.
Conclusions: The study provides the evidence for the possible existence of susceptibility locus for schizophrenia within the HLA region. This preliminary observation may help to understand the etiological basis of this disorder and the study may further strengthen the HLA antigens as the marker for schizophrenia.

Key words: Etiology, human leukocyte antigen, schizophrenia

INTRODUCTION

Schizophrenia is a severely debilitating neuropsychiatric disorder characterized by "disturbances of thought, auditory hallucinations and multiple delusions".[1] It affects 1% of the worldwide population.[2] The essential biological pathology of schizophrenia ia partially understood till to date.[3] However, there is substantial evidence to indicate a major genetic component.[4][5] Different chromosomes have been pinpointed as harbouring genes involved in the pathogenesis of schizophrenia.[6] A susceptibility locus has been identified on chromosome 6.[7][8] Several researches have also found evidence for schizophrenia vulnerability genes on chromosome 6p close to the HLA genetic region by linkage analysis.[9] HLA and schizophrenia was first reviewed by Mc Guffin (1979),[10] who commented that the MHC was a logical place in which to search for genetic markers for schizophrenia because schizophrenia was similar to diseases for which HLA association had been established in that it was familial, had an imperfectly understood etiology, and had a postulated autoimmune pathogenesis.[11]

The first HLA association study of schizophrenia was

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reported by Cazzullo *et al.*, in 1974.[12] More than 60 association studies have been reported since then.[13] The details of past association studies is given in Table 1.

Past association studies with various Class I and Class II alleles yielded inconsistent results[14] except HLA-A*9 (now subdivided into A*23/A*24).[15] In different ethnic population, associations have been found for HLA-A9,[16] HLA-A23,[17] HLA-A*24,[18] HLA-A*1,[19] HLA-A*2, HLA-A*3, HLA-A*11, HLA-B*17, HLA-B*27, HLA-B*8 and Cw 2,[20] HLA-A*3.[21] The reason for the inconsistencies include the diagnostic methods, particularly in early studies , are imprecise and vary greatly.[22] The majority of the previous association studies were carried out using serological typing techniques [microlymphocytotoxicity testing][23], which have been found to be inaccurate, with 7-25% misassignment errors[24] compared with the DNA based techniques (polymerase chain reaction (PCR) and sequence specific oligonucleotide probes (SSOP). The source of controls is not always described in sufficient detail to ensure that results are not simply due to population stratification. Significant results are not always corrected for the number of statistical tests performed.[25]

The present study has been carried out to investigate the association of HLA Class I alleles in Schizophrenia with the help of DNA-based typing method in well-characterized sample of ethnically matched patients and controls. The study may help to identify disease-specific susceptibility (risk) and protective markers that can be used in immunogenetic profiling, risk assessment and therapeutic decisions. Further, the study may refine already known associations in the light of modern DNA based HLA typing method.

MATERIALS AND METHODS

We studied 50 India-born schizophrenic patients residing in and around Siliguri subdivision of West Bengal, referred to the psychiatric outpatient department (OPD) of Psychiatry, North Bengal Medical College and Hospital. Three major selection criteria were considered for selection of schizophrenic group; (i) unrelatedness of individuals from each other, (ii) resident of the state of West Bengal and (iii) subjects satisfying DSM IV[3] diagnostic criteria for schizophrenia . The exclusion criteria followed in the present study include; (i) history of substance abuse, (ii) presence personality disorder, (iii) presence of dementia and mental retardation. The patients considered for the present study were belonging to the Bengali, Nepali, Bihari and some tribal community. They were diagnosed independently by two psychiatrists using Structured Clinical Interview[26] and according to the standard diagnostic criteria of DSM IV and assessed by the Brief Psychiatric Rating Scale (BPRS).[27] The present study comprise of 45 Paranoid, 2 Residual, 2 Undifferentiated and 1 disorganised schizophrenic patients. Considering the small number of different subtypes of schizophrenic patients in this study (except Paranoid), we have considered schizophrenic patients as a whole. Some of the demographic variables which have been studied in the patient group are given in the table 2.

A total number of 50 ethnically matched healthy individuals were considered as controls. To avoid the spurious associations resulting from population stratification great care was taken. The following criteria were strictly followed for the selection of controls, (i) same ethnic group as the patients, (ii) sex and age matched with patients, (iii) absence of family history of autoimmune or psychiatric disorder, (iv) recent history of intercurrent infection and allergies, (v) unrelatedness of individuals from one another, (vi) no history of any substance abuse. All the participants provided their written consent for giving the blood sample after the study procedures were explained.

Methodology

The blood was drawn by vein puncture method and EDTA was added as anticoagulant. DNA was extracted from peripheral mononuclear cells of the blood by the Phenol Chloroform method. The typing of HLA Class I was performed by PCR-SSP technique. The typing and sequence information of primers were taken from Bunce *et al.*, (1995).[28] The primers, Taq polymerase, nucleotides etc. were obtained from Bangalore Genei, India. In general 25µl of reaction mixture include 1x PCR buffer, 200µM of each of dNTP, 1.5mM MgCl2, 0.4µM of forward and reverse primers, 100ng of genomic DNA and 1unit of Taq polymerase. The amplifications were accomplished on a thermal cycler (Perkin Elmer, USA). PCR reaction are subjected to 30cycles, each consisted of 94°C for 30s, 60°C for 1min. and 72°C for 1min. with initial denaturation step of 2min and final extension of 2min.

Statistical analysis

The phenotype frequencies were calculated by direct count.

Table 2: Psycho-socio-demographic characteristics of the schizophrenic patients

		Standard deviation	
Gender			
Male	P= 0.78 (78%)		Z=6.49
Female	p<0.001		
Age			
Mean	34.06	9.46	
Disease duration			
(in years) mean	5.77	6.37	
Substance abuse			
Yes	P=0%		
No			
Marital status			
Married	P=0.64 (64%)		Z=2.06
Unmarried	p<0.05		
Ethnicity			
Bengali	68%		X^2=51.28
Nepali	10%		(d.f.=3)
Tribal	18%		p<0.001
Bihari	4%		

Table 1: HLA association studies of schizophrenia- Class I (A, B and C) antigens*†

Investigator, year‡	Ethnicity Diagnosis (number)	Patient subjects Origin (number)	Comparison subjects	Result (comment)
Cazzullo *et al.*, 1974	Caucasian	Feignner (52)	Population (386)	no association
Eberhard *et al.*, 1975	Caucasian	Bleuler (47)	-(1263)	A9 (RR=2.9)
Ivanyi *et al.*, 1976	Caucasian	–(148)	Population (1200)	A28(RR=3.4)
Smeraldi *et al.*, 1976a	Caucasian	Feighner (70)	Popualtion (386)	No association
Smeraldi *et al.*, 1976b	Caucasian	Feighner (144)	Population (386)	A10 (RR=0.4)
Julien *et al.*, 1977	Caucasian	-(65)	Population (250)	A9 (RR=2.5)
Ivanyi *et al.*, 1977	Caucasian	-(40)	Population (438)	Cw4 with paranoid schizophrenia (RR=3.7)
			Population (1200)	B18 with paranoid schizophrenia (RR=3.4)
Bennahum *et al.*,1977	Caucasian	Feighner (38)	-(102)	No association
Kyner *et al.*,1978	Caucasian	Feighner (20)	-(67)	No association
Ivanyi *et al.*,1978	Caucasian	-(200)	Population (1200)	A28(RR=3.0)
Mc Guffin *et al.*, 1978	Caucasian	ICD-9 (80)	Blood donors(458)	No association
Perris *et al.*, 1979	Caucasian	-(50)	Blood donors (449)	No association
Crowe *et al.*, 1979	Caucasian	Feighner (45)	Population(1263)	Aw 10 (A26 subtype) with hebephrenia (RR=6.6)
Luchins *et al.*, 1980	Caucasian	RDC(38)	Published data (743)	No association
	African-USA	RDC(92)	Published data (563)	A2 (RR=2.3)
Gattaz and Beckmann, 1980	Caucasian	Feighner (100)	–(472)	B27 with poor prognosis patients
Mendlewicz *et al.*,1981	Caucasian	Feighner (64)	Blood donors (113)	No association
Asaka *et al.*, 1981	Japanese	-(136)	Blood donors (187)	A9(Aw24 subtype) (RR=2.0) A10(A26 subtype) (RR=1.9)
Goudemand *et al.*,1981	Caucasian	-(51)	Blood donors (94)	No association
Singer *et al.*, 1982	Caucasian	-(75)	Blood donors (184)	No association
Ivanyi *et al.*, 1983	Caucasian	Feighner (62)	-(1018)	No association
Rosler *et al.*, 1980	Caucasian	Feighner (107)	Blood donors (600)	A28 (RR=3.1)
Miyanaga *et al.*,1984	Japanese	DSM-III (77)	Blood donors (1252)	No association
Rudduck *et al.*1984a	Caucasian	DSM-III (100)	Blood donors (919)	No association
Rudduck *et al.*1984b	Caucasian	DSM-III (116)	Blood donors (919)	No association
Adler *et al.*,1985	Caucasian	RDC (14)	-(365)	No association
Amar *et al.*, 1988	Jewish	-(32)	-(151)	No association
Metzer *et al.*, 1988	Caucasian	DSM-III (53)	Blood donors (114)	No association
Alexander *et al.*, 1990	Caucasian	DSM-III (55)	Published data (1029)	No association
DiMichele *et al.*1990	Caucasian	DSM-III (36)	- (500)	No association
Campion *et al.*, 1991	Caucasian	DSM-III (107)	Relatives (174)	No association
Wright *et al.*, 1995	Caucasian	DSM-III-R (93)	Screened controls (141)	A9 (RR=1.94) A24 subspecificity Of A9 (RR=2.76)
Blackwood *et al.*, 1996	Caucasian	RDC&DSM-III-R (107)	-(133)	B35(corrected P=0.004, RR=0.06) Cw5 (corrected P=0.05, RR=0.38)
		Blood donors (264)	B35	
Ozcan *et al.*,1996	Caucasian	-(75)	-(3731)	No association
Jocobsen *et al.*, 1998	Caucasian	DSM-IV(28 children)	Population controls (51)	No association
Gibson *et al.*, 1999	Caucasian	DSM-III-R (256)	Blood donors (261)	No association
Debnath *et al.*,2005	Indian (Bengalee)	DSM-IV-TR (50)	Blood donors (100)	A3 (RR=5.66)
Total studies	*Total patients per ethnic group*	*Total controls per group§*	*Associations reported more than once*	
35 serotyping studies	Caucasian 3146 (including 28 children	7802 unknown	4 studies:A9 or A24 subspecificity of A9	
1genotyping study	Japanese 213	4895 blood donors	3 studies: A28	
	African-USA 92	4788 population	3 studies: A10	
	Jewish 32	2335 published data		
	174 relatives			
	141 screened controls			

* Table based on data from Nimgaonkar *et al.*,(1992), Hawi *et al.*, (1999), Index Medicus, MEDLINE and EMBASE searches from 1974 to 2000, and personal communications, † RR=relative risk when significant association remains after correction for multiple comparisons; n=number of schizophrenic patients or number of controls; diagnostic criteria utilized in the above studies are those of Feighner *et al.*, (1972) and Bleuler (1950), the International Classification of Diseases 9 (WHO,1978), the Diagnostic and Statistical Manual III, III-R and IV (American Psychiatric Association, 1980, 1987, 1994) and the Research Diagnostic Criteria of Spitzer *et al.*, 1978), ‡ All studies utilized HLA serotyping, except that of Gibson *et al.*, (1999) which utilized genotyping, § Total controls per group is not equal to total number of controls, because the same control groups were used by some investigators, [This table has been reproduced (with slight modification) by seeking permission from the Review , by Padraig Wright *et al.*, title "Schizophrenia and HLA: a review", Volume 47, pg no.4-5, Copyright Elsevier ,2001.]

χ^2 test was performed to compare the frequency of each antigen among the patient and control group followed by Fisher's exact test. Since testing for a large number of antigens can reveal at least one positive association where none really exists, the *p* values from each Fisher's exact test had to be less than the Bonferroni *p* [0.05 divided by the number of antigens tested which equals to 0.003125] to be called statistically significant.[21] Relative risk was estimated by using Haldane's method (1956).[29]

RESULTS AND DISCUSSION

The incidence and frequency of HLA Class I antigens among patients and control has been presented in table 3. There was a significantly higher frequency of HLA-A*03 (χ^2=11.458, *p*=1.155e-4) in patients than the control groups. On the other hand HLA-A*25 (χ^2=13.619, *p*=9.185e-5), A*31 (χ^2=22.562, *p*=8.793e-8) and B*51 (χ^2=40.047, *p*=1.604e-12) showed lower value significantly even after the Bonferroni correction. Though A*02 (χ^2=6.052, *p*=6.699e-3)showed lower frequency and B*07 (χ^2=4.069, *p*=2.035e-2) and B*42 (χ^2=4.522, *p*=1.632e-2) showed higher frequency they were not found to be significant after the Bonferroni correction.

A significant higher frequency of HLA-A*03 observed in the present study is in accordance with the previously reported study by Debnath *et al.*[21] which is also in accordance with the study of Rudduck *et al.*, (1984a, 1984b)[30] in Swedish population. Although the association was found to be significantly higher, the present study did not reveal very strong association as it has been reported earlier.

On the other hand, in the present study A*25, A*31 and B*51 showed significantly lower negative value which is the unique finding of the present study. Among these alleles, A*31 and B*51 showed strong negative associations (RR=0.014 and RR=0.006 respectively). The increased frequency of A*11 found in the previous study was not reproducible in the present study. Apart from this, several other alleles like B*7 and B*42 showed higher value but were not statistically significant. We also observed a negative association of A*2 but the association was not found to be significant which was in accordance with the findings of Debnath *et al.*[21] But the finding was unlike the previous findings by Luchins *et al.*, (1980)[31] which showed positive association of A*02 with schizophrenia in African-USA population. However, we have not found association between HLA-A*23 and A*24 in our study as has been reported by previous studies.

Many microbial factors have been implicated in the pathogenesis of schizophrenia, but so far each microbial factor has been identified in a relatively small subgroup of patients.[32][33] The heterogeneity of these microbial factors is also reflected by the associations with different HLA loci and their alleles.[34] Polymorphic HLA molecules process, select and present degraded microbial proteins.[35][36] The set of inherited HLA alleles determines susceptibility or resistance to particular microbes.[34]

Table 3: Phenotype frequency, Chi square, relative risk (RR) values and probability of HLA-A and B loci alleles in the patients with schizophrenia and healthy controls

Antigen	Patients (N=50)	Control (N=50)	Chi-square	Chi square (y)	RR	*P* value
A*02	24	37	7.103	6.052	0.332	6.699e-3*
A*03	50	38	13.636	11.458	32.792	1.155e-4†
A*11	36	31	1.130	0.723	1.558	1.975e-1
A*23	21	26	1.003	0.642	0.673	2.115e-1
A*24	32	26	1.477	1.026	1.624	1.555e-1
A*25	10	29	15.174	13.619	0.188	9.185e-5†
A*26	21	17	0.679	0.382	1.395	2.684e-1
A*29	33	32	0.043	0.000	1.089	5.000e-1
A*30	33	25	2.627	2.011	1.914	7.787e-2
A*31	0	20	25.000	22.562	0.014	8.793e-8†
B*07	47	39	5.315	4.069	3.951	2.035e-2*
B*21	36	38	0.207	0.051	0.817	4.099e-1
B*4001	17	13	0.761	0.428	1.451	2.565e-1
B*4201	39	28	5.472	4.522	2.711	1.632e-2*
B*44	14	10	0.877	0.493	1.532	2.414e-1
B*5101-5105	0	30	42.857	40.047	0.006	1.604e-12†

y= Yates's correction, * Significant, † Significant after the Bonferroni correction Bonferroni's probability is 0.003125, Note= The abbreviation 1.156 e-4 means 1.156 x 10-4, like wise the other values may be interpreted.

The analysis of the demographic variables suggests the present schizophrenic population is not in equal composition for the different ethnic group. The study comprises more number of Bengali populations. However, as mentioned earlier they were strictly matched according to their ethnicity, age and sex with the patients. The study comprise of higher number of male schizophrenics, which suggest the higher vulnerability of men to this disorder, at least in this region. The higher number of married patients in this study may be due to the strict social customs and strong social bondage of the Indian society.

The present study supports earlier finding of association of HLA-A*03 with schizophrenia along with the negative association of some more alleles, which is the new finding of the present study. However, it is too early to speculate the exact mechanism of the association. The result is preliminary and so far not correlated with the parameters like birth status, viral infections, prenatal infections, etc. However, this study provides the evidence for the possible existence of a susceptibility locus for schizophrenia within the HLA region. Given the size of our sample the result of our finding should be interpreted with caution. The present study needs to be replicated in the large sample size to strengthen our hypothesis of genetic association of HLA Class I antigen with schizophrenia.

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ELEVATED LEVEL OF C-REACTIVE PROTEIN IN DRUG NAÏVE PATIENTS WITH SCHIZOPHRENIA

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ABSTRACT

The well known inflammatory marker C-reactive protein (CRP), was investigated in the drug naïve and antipsychotic medicating patients to understand the role of inflammation in the etiopathology of schizophrenia.

The level of serum CRP was investigated among 64 schizophrenic patients, diagnosed with DSM IV criteria and categorized into different subgroup of schizophrenia. Latex agglutination test was performed to measure the level of CRP. The limitation of detection of serum CRP was less than 6 mg/L. CRP was treated as categorical variable: normal (6 mg/L) and elevated ($\geq$ 6mg/L). Further, patients were made to answer a questionnaire, which included self-reported age, sex, medical history, age of onset, substance abuse etc. All subjects came from an India-born Bengali population.

3 Paranoid patients showed the elevated level of CRP ($\geq$ 6mg/L) whereas rest of the patients had normal CRP ($<$ 6mg/L). When the findings were compared to the demographic variables, the results showed a significant value for the elevated level of CRP and drug naïve status.

The study suggests that some kind of inflammatory process may be one of the etiological factors for schizophrenia and the antipsychotic drug might play an important role in down regulating this inflammatory process and thereby bringing the level of CRP to the normal state.

Key words: Schizophrenia, C-Reactive protein, Inflammation, Drug naïve

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INTRODUCTION

Schizophrenia is the paradigmatic illness of psychiatry. Although the worldwide prevalence of schizophrenia is about 1%, but it has become a leading public health problem now-a-days and exerts enormous personal and economic costs worldwide. Inspite of tireless research efforts, the etiological process or processes by which a causal agent creates the pathophysiology of schizophrenia is not yet clearly understood. However, a good deal is known about risk factors for developing schizophrenia, which leads to direct inferences regarding possible etiopathophysiologies[1]. A large number of studies have also shown the association between the HLA genes and schizophrenia. In such a study, we have reported the association of HLA A*03 allele with paranoid schizophrenia in Indian Bangalee population[2,3].

The roles of immune dysfunction and inflammation have been described in schizophrenia[4,5]. In the past a number of attempts have been made to identify the inflammatory markers for schizophrenia but with conflicting findings. The inconsistent results in the literature might be explained by the heterogeneity of schizophrenia, difference in illness state (acute versus chronic) and effects of antipsychotic medication[6].

One of the well known inflammatory markers is C-reactive protein (CRP). CRP is a normal alpha globulin, which increases in inflammatory processes. The name CRP is derived from the fact that this protein has the capacity to precipitate the somatic C-carbohydrate of Pneumococcus. Elevated CRP levels are usually observed in a variety of infections and inflammatory conditions where there is tissue destruction. Elevated CRP is known to be the risk factor for the cardiovascular diseases, diabetes and other metabolic dysfunction[7,8]. In addition, it is also known to be associated with the depression[9] and cognitive impairment[10].

However, very few studies have been carried out to investigate the association of CRP and schizophrenia. In one study, elevated serum levels of CRP was found in patients who showed more severe clinical symptoms of schizophrenia as reflected by the PANSS total score[11]. In another study, the elevated serum levels of C-reactive protein in schizophrenia are found to be associated with the severity of cognitive impairment but not of psychiatric symptoms[12].

In the present preliminary first hand study, we have investigated the level of CRP in serum of the patients with schizophrenia and its relation with other demographic variables.

EXPERIMENTAL

Materials and methods

India-born Bengali population referred to the psychiatric outpatient Department (OPD) of Psychiatry, North Bengal Medical College and Hospital were considered for the present study. Patients were diagnosed independently by two psychiatrists according to the standard diagnostic criteria of DSM IV and assessed by the Brief Psychiatric Rating Scale (BPRS). After diagnosis, 64 schizophrenic patients were included for the study.

Further, the schizophrenic patients and the family members were made to answer a questionnaire. The questionnaire included self-reported age, cast, sex, medical history, age of onset, month of birth, marital status, education, substance abuse, incidence of any autoimmune disease among patients or in family members etc. All the participants provided their written consent for giving the blood sample after the study procedures were explained.

Procedure

About 5 mL. of blood samples were collected from the each patient. The samples were allowed to coagulate at the room temperature for 2-3 hrs. Blood clot was cut and centrifuged for separating the serum. The CRP level in the serum was measured by latex agglutination slide test (Ranbaxy Fine Chemicals Ltd., HP, India). The limitation of detection of serum CRP level was less than 6 mg/L. CRP was treated as categorical variable: undetectable or normal(< 6 mg/L) and detectable or elevated (≥ 6 mg/L).

Statistical analysis

Statistical analysis was performed for the bivariate associations between elevated CRP groups versus normal group by employing one-way analysis of variance. The association between CRP groups and deficit was examined by Chi-square analysis. The association between CRP group and other clinical/demographic variables were also examined by utilizing one way analysis for continuous variables and Chi-square tests for dichotomous variables.

RESULTS AND DISCUSSION

Sera levels of CRP were measured for 64 schizophrenic patients. Out of them, 57 were paranoid, 2 residual, 3 undifferentiated and 2 were disorganised type. The elevated level of CRP (≥ 6 mg/L) was observed in 3 patients and 61 patients were found to have

normal CRP (< 6 mg/L). All the three elevated cases were found to be of paranoid type. No differences were found in CRP level among different subgroups of schizophrenia.

Further, when the level of CRP was compared to the other demographic variables as shown in the Table 1, only the drug naïve status of the patients showed statistically significant value ($\chi^2 = 16.997$, P value $< 3.75 \times 10^5$).

Table 1. Comparison of demographic and clinical characteristics between the normal/elevated CRP groups

	Elevated CRP N = 3		Normal CRP N = 61		Statistic (Z)	P value
	Mean or N%	S. D.	Mean or N%	S. D		
Age	37.67	21.13	34.69	9.64	F[2,60] = 0.24	>0.62
Gender Men v/s Women	33.33%		70.49%		χ^2 [1] = 1.84	>0.17
Drug naïve patients v/s Patients under antipsychotic medication	100%		11.48%		χ^2 [1] = 17.00	<0.001 Significant
Substance abuse Yes v/s No	33.33%		55.74%		χ^2 [1] = 0.58	>0.44
First child Yes v/s No	33%		18.03%		X2[1] = 0.44	>0.50
Autoimmune disease in patients or in family Yes v/s No	0%		24.59%		χ^2 [1] = 0.97	>0.32

This preliminary first hand study provides further evidence of the involvement of inflammatory processes behind the etiopathology of schizophrenia. The elevated level of CRP in our study is in accordance to the findings of Fan et al.[11] and Dikerson et al.[12]. But unlike previously reported findings, we have considered the CRP level of patients with

their medication status, which showed significantly higher value. In one study, the level of CRP was found to be higher in the patient, who was experiencing psychotic symptoms, in the follow up study in the non-psychotic state, the level of CRP was found to be normal[13]. In this respect, the present study suggests that the antipsychotic drug may perhaps down regulate the inflammatory process, which in turn brings the CRP level to the normal state.

Thus, these findings further suggest that the inflammation may be another possible mechanism in the etiopathology of schizophrenia. It is, however, not clearly understood whether the elevation of the level of CRP is the by-product of the pathophysiology of schizophrenia or directly contributes to the clinical manifestations of the disorder[11].

Until now, it is not clearly understood regarding the mechanism of inflammation in schizophrenia. It is suggested that the vascular-structural brain abnormalities may be one of the factors in the etiology of schizophrenia, like psychoses[14-16]. It is proposed that chronic inflammation might damage the micro-vascular system in the brain and cerebral blood flow[17]. Further, scientific evidence suggests that an increase in the stress hormone like norepinephrine may activate the inflammatory arm of the immune system and triggers the expression of genes that cause chronic, low-grade inflammation. This inflammation is characterized by the degree of the levels of CRP[18].

This is possibly the first reported study of association between CRP and schizophrenia in the Indian scenario. The limitations of the present preliminary study are that the psychopathology measures were not considered for the patients, unlike the previous studies. In contrary to the studies conducted by (Fan et al.[11] Dickerson et al.[12](5 mg/μL), the higher cut off value (6 mg/L) was used for the CRP levels. The sample size of the study is small and the patients were attended in the OPD, which has limited the follow up study.

The study provides further evidence that some kind of inflammation may play a role in the etiopathology of schizophrenia. The study further reveals the immunomodulatory effect of the antipsychotic drugs in the patients.

Additional studies with the highly sensitive techniques like ELISA with longitudinal follow up studies in the large sample size would be required to further strengthen the present study.

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Contributors -

Bisu Singh designed the study and wrote protocol and final manuscript; Sikta Banerjee contributed to the design of the study and recruitment of participants; Nirmal K. Bera is a psychiatrist and contributed to the diagnosis of patients; Chitta R Nayak provided the laboratory evaluations and the statistical analysis; Tapas K Chaudhuri contributed substantially in the design of the study and writing the final manuscript.

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Analysis of the Role of Dopamine Receptor Genes in the Susceptibility of Delusional Disorder

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ABSTRACT

Delusional disorder is characterized by monosymptomatic paranoid symptoms comprises of an uncommon and probably heterogenous group of illness. The underlying etiological mechanism is poorly understood, though involvement of biological factors has long been suspected. Several investigators have suggested that delusional disorder is a naturally occurring model psychosis based on abnormalities of the dopaminergic temperolimbic system. In the present study, we examined the incidence of dopamine receptor genes in patients with delusional disorder. Significant negative association was found for D2 long chain variants. This preliminary study of allelic association of dopamine receptor genes with delusional disorder may lead to develop future strategies to understand the neurogenetic basis of this disorder.

Key words: Delusional disorder, dopamine receptor genes, association, polymorphism.

INTRODUCTION

Delusional disorder is characterized by monosymptomatic paranoid symptoms, and in contemporary classifications of mental disorders, delusions are considered as cornerstone symptoms for the diagnosis of psychotic disorders. Since the beginning of psychiatry, delusional disorder has been a central subject of attention and continues to engender controversy right up to now. Delusion formation is a fascinating and enigmatic psychic process that has been the subject of numerous scientific debates and theoretical models; however, surprisingly few empirical studies have been done (Butler & Braff ,1991; Berrios, 1991).

Delusions are understood to mean intersubjectively disconcerting convictions, with a tendency toward subjective certainty, that lose their disconcerting character when made the object of psychiatric analysis. Delusions involve thought contents and, as such, tend to be idiosyncratic and richly varied. Delusional disorder comprises an uncommon and probably heterogeneous group of illnesses; it is complicated by more than 100 conditions and agents, including neurologic disorders, metabolic and endocrine disorders, infections, pharmacologic agents, alcohol and other substances, and psychiatric disorders (Manchreck, 1999). Although its prevalence is low, delusional disorder is not rare (Manchreck, 1996). Recent studies have revealed that delusional disorder is underdiagnosed, which results in poor anticipation of its

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implications (American Psychiatric Association. 1994).The underlying etiologic mechanism and the pathophysiology of delusional disorder are poorly understood. However, surprisingly very few empirical studies have been done (Butler & Braff,1991; Berrios, 1991). Involvement of biological factors has long been suspected (Manchreck, 1999). Recent epidemiological and clinical studies suggest that certain risk factors like advanced age, sensory impairment, personality features, family history etc. may be relevant to etiology (Miller *et al.*,1988). Genetic or family studies lead to convincing data like increased prevalence of delusional disorder and related personality traits (e.g. suspiciousness, jealousy and secretiveness) in the relatives of delusional disorder probands (Munro,1994) and also indicate possible specific family transmission of delusional disorder. Several attempts have been made to identify genetic markers associated with delusional disorder. Since delusional disorder is characterized by monosymptomatic paranoid symptoms, several investigators have suggested that delusional disorder is a naturally occurring model psychosis based on abnormalities of the dopaminergic temperolimbic system(Kaplan *et al.*, 1994).

A recent study of genetic variation in the DNA sequence coding for Dopamine type 4 (DRD4) Exon 3 strongly suggests the involvement of the relevant gene in conferring susceptibility to delusional disorder (Serretti *et al.*, 2001). However, these studies are not uniformly consistent and needs to be replicated on a large sample size to confirm the tentative results of dopaminergic mechanisms responsible for paranoid symptoms (Kendler & Hays,1981).

In the present study, we examined the incidence of dopamine receptor genes in patients with delusional disorder and in healthy control subjects to investigate if dopamine receptor genes could be used as a genetic marker and to explore a possible neurological etiology for delusional disorder.

MATERIALS & METHODS

Subjects: Subjects were recruited from an India-born Bengali population referred to the psychiatric outpatient department at North Bengal Medical College and Hospital. On an average, 1500 new patients with various psychiatric illnesses and about 4000 recurrent follow-up cases attend the outpatient department every year. 150 unrelated patients were recruited (82 women and 68 men) with the symptom of delusions and studied them for 5 years. All subjects were screened independently by two psychiatrists using the Structured Clinical Interview for DSM-IV (SCID) to determine a diagnosis of delusional disorder (American Psychiatric Association. 1994). After longitudinal follow-up, 100 patients represented genuine cases of delusional disorder of various subtypes. We compared delusional disorder patients with normal healthy individuals of no previous history of psychotic illness. The distribution of delusional disorder subtypes, as defined by DSM-IV criteria, were as follows: 62.5% persecutory, 12.5% mixed, 12.5% jealous, 6.25% somatic, 3.75% erotomanic, and 2.5% grandiose. Most patients were clustered between the ages of 25 and 55 years. Of the initial 150 patients, we excluded from the patient group the 50 patients suffering from psychiatric conditions other than delusional disorder. Hundred healthy individuals belonging to the same ethnic group as the patients were used as control subjects. Control subjects were mainly selected from the university as well as from hospital employees. Written consent from all the patients were taken prior to the collection of blood samples from them. All the patients and control subjects were matched for sex, age, and other socio-economic variables. The subjects were mostly from middle-class urban and semirural society and belonged to a nuclear family. The above-mentioned medical college is one of the rural medical institutes in India.

PCR analysis: Approximately 5ml of venous blood was taken from each individual. Molecular typing was done only on the final 100 patients with delusional disorder and the equal number of healthy controls. DNA was extracted from the peripheral mononuclear cells using a salting out procedure (Miller *et al.*,1988). Molecular typing was carried out by using polymerase chain reaction sequence-specific primer (PCR-SSP) technique to detect dopamine receptor genes (Figure 1). The primers, Taq polymerase, nucleotides, and other reagents were used as per the standard method and recommended by Svejgaard *et al.* (1974).

Statistical Analysis: The phenotype frequencies were calculated by direct count. The frequency of each allele among the patients was compared with that of the control population, using the chi-square test followed by Fisher's exact test. Testing for a large number of alleles can reveal at least one positive association where none really exists; to be statistically significant, therefore, the *P* values from each Fisher's exact test had to be less than the Bonferroni *P* (0.05 divided by the number of alleles tested [$n = 37$] minus 2 degrees of freedom [1 for each of the 2 loci examined], which equals 0.0014).We estimated relative risk (RR) as recommended by Svejgaard *et al.* (1974).

RESULT

Molecular typing was done for 100 patients with delusional disorder. The data are shown in Table-1. The results demonstrate a marked down regulation of the long chain variant of D2 receptor in patients with delusional disorder, compared with healthy control subjects. The *P* value after Bonferroni correction was significant.

Table1: Shows phenotype frequency (%), chi square, relative risk(RR) and p values dopamine receptor genes and their alleles in patients compared with healthy controls.

Dopamine receptor genes	Phenotype frequency (%)		Chi-square	RR	*p*	Remark
	Patients	Controls				
D1	75	100	2.28571	0.00000	2.33333e-1	NS
D2(L)	0	50	5.33333	0.00000	3.84615e-2*	Significant
D5	8.75	100	1.06667	0.00000	5.00000e-1	NS

*Significant after Bonferoni correction.

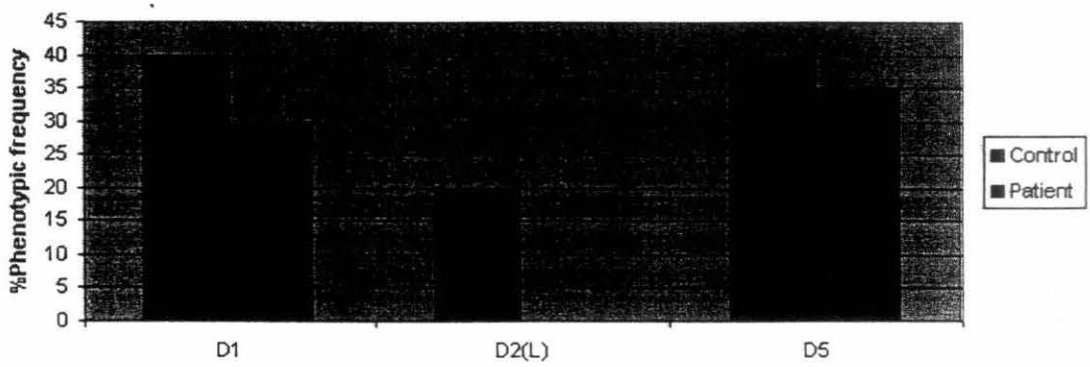
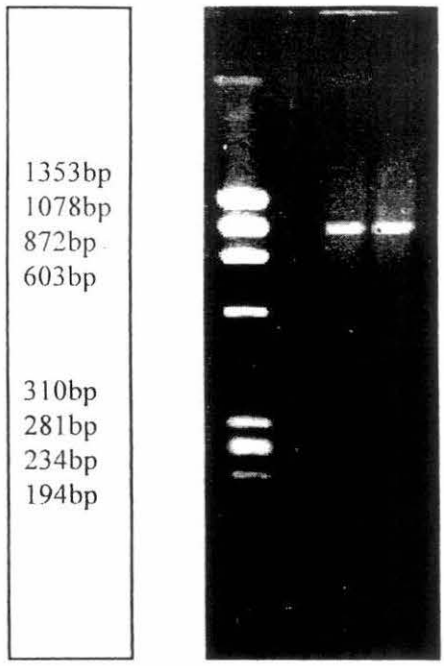


Fig: Phenotype frequency(%) of D1,D2(L) and D5 dopamine receptor genes in patients with delusional disorder and the healthy controls.

DISCUSSION

A strong negative association has been observed between delusional disorder and dopamine D2 receptor long chain variant (D2L) in the present study. When the strength of negative association is measured by cross product ratio or relative risk of developing a disease, D2L showed RR=0.0000, thus reflecting a negative association. A higher frequency of incidence of D2L in the controls along with total absence of the allele in the patients group,($c^2 = 5.33333$) suggest a strong negative association of that particular allele to delusional disorder. However, the exact nature of the mechanism underlying the empirically observed negative association between D2Land the delusional disorder is not fully understood.

At this point though it is difficult to predict that the D2L is the sole determinant of delusional disorder but this significant negative association might contribute to the disease risk. A comprehensive study is presently going on to understand the pattern of D2L inheritance in the affected families, which may help to determine the validity and specificity of the absence of D2Lgene as genetic marker of delusional disorder.



LANE:
1:Marker : φX174
2:D2L(patient)
3:D2L(control-1): 1000bp
4:D2L(control-2): 1000bp

Fig.2: Gel photograph of D2L dopamine receptor gene in patients with delusional disorder and the normal healthy controls.

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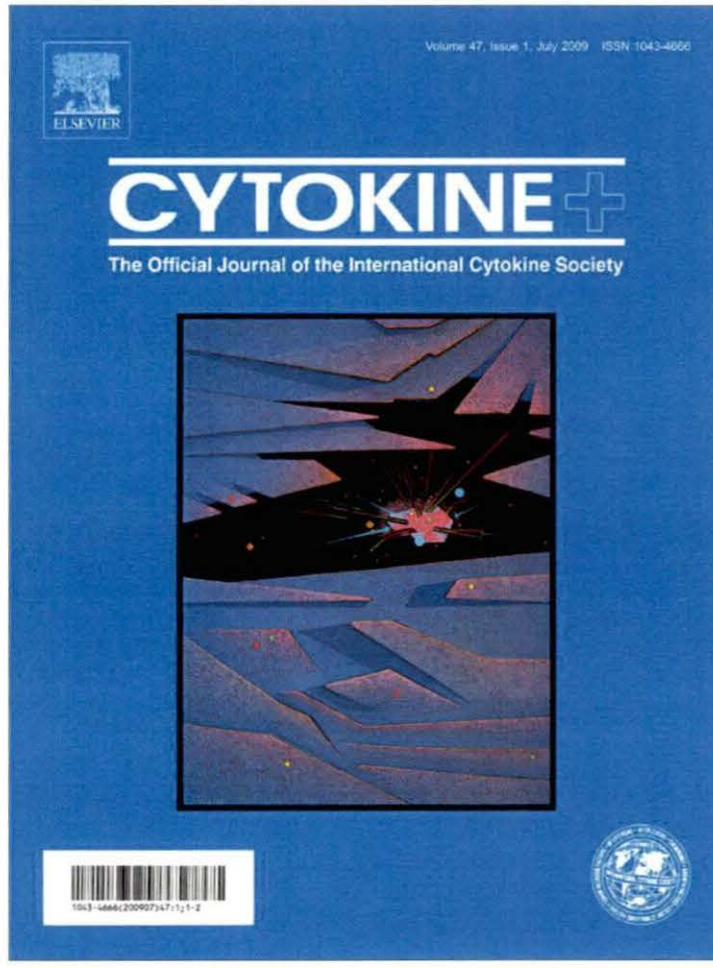

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Short Communication

Decreased serum levels of interleukin-2 and interleukin-6 in Indian Bengalee schizophrenic patients

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ABSTRACT

Background and purpose: Autoimmune process is thought to be involved in the pathophysiology in some cases of schizophrenia. Alteration in interleukin (IL) regulation is regarded as additional proof of autoimmunological background in schizophrenia. Most of the research in interleukin activity in schizophrenia has been in Caucasian and some Mongoloid patients. We have studied the serum IL-2 and IL-6 level in psychotropic medication free and antipsychotic medicating schizophrenic patients who are Indian Bengalee by ethnicity. *Method:* Twenty psychotropic medication free and 30 antipsychotic medicating schizophrenic patients who fulfilled DSM-IV-TR criteria and 30 of the same age and sex matched controls were recruited. Serum level of IL-2 and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). *Result:* There was a significant decrease of IL-2 and IL-6 in both antipsychotic medicating and psychotropic medication free patients. Further the medicating patients showed lower level of IL-2 and IL-6 than the psychotropic medication free patients. *Conclusion:* This is the first study to describe a decrease serum level of IL-6 in schizophrenic patients. The study provides the evidence that some kind of immune dysregulation is involved in pathophysiology of schizophrenia. The study also provides the evidence for the immunosuppressive effect of antipsychotic drugs.

1. Introduction

Many general immune abnormalities have been reported in schizophrenia, among them the most widely studied is cytokines [1]. Growing evidence suggests that, in addition to providing communication between immune cells, specific cytokines play a role in signaling the brain to produce neurochemical, neuroendocrine, neuroimmune and behavioral changes [2,3].

In vivo and *in vitro* studies have shown that schizophrenia may be associated with an imbalance in cytokines network, suggesting suppression of some immune functions and activation of others. To explain the phenomenon of immunosuppression and immune activation in schizophrenic patients, Muller et al. [4] put forward 'Th2 hypothesis', which states that the Th1–Th2 balance is shifted to Th2 in schizophrenia. The key characteristics of the Th1 system are the production of interleukin-2 and interferon-γ, which have been reported to be decreased *in vitro* [5–9] and increased *in vivo* [10]. On the contrary, Th2 system in schizophrenia is characterized by activation, which is shown as increase IL-6, sIL-6R [6,11–16] and increase IL-10 levels [17,18], as well as increase IL-4 levels in the CSF of juvenile schizophrenic patients [16,19]. Empirical evidence in support of the Th1/Th2 imbalance has been inconsistent [20]. Recently Potvin et al. [21] in their quantitative review refuted the current hypothesis of a Th2 slant in schizophrenia.

There are numerous studies reporting that treatment with antipsychotic drugs affects the cytokine network [22]. For example, the typical antipsychotic drug haloperidol normalizes the initially increased serum IL-6 and IL-6R [10,23] whereas repeated administration of atypical antipsychotics, i.e., clozapine and risperidone, significantly increases plasma concentrations of IL-2R, IL-6, and TNF-α [22,23]. Drzyzga et al. [24] in his review suggested the antipsychotic drugs suppress the activity of the IL-2 system. However, some studies have demonstrated that serum or plasma concentration of IL-6, sIL-2R, IL-1RA, or TNF-α, in schizophrenia patients did not vary after treatment with antipsychotic drugs [25–27]. Therefore further investigation needs to be carried out to throw light in this important issue of whether the neuroleptic drugs influence cytokine levels in schizophrenia or not.

To date, the most frequently studied cytokines in schizophrenia have been IL-2 and IL-6 [21]. IL-2 is a T-cell growth factor and has been shown to modulate some neurotransmitter systems including dopamine metabolism within the central nervous system [28]. More interestingly a range of psychiatric manifestations, including

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delusions, delirium, paranoia, hallucinations and lethargy have been observed in patients receiving IL-2 immunotherapeutically [29]. These findings suggest that IL-2 may contribute to the pathophysiology of schizophrenia. IL-6 exerts trophic effects on glial cells, including oligodendroglia themselves, producing increased expression of glial fibrillary-acidic protein [30]. Paradoxically, IL-6 increases intracellular calcium levels during *N*-methyl-D-aspartate receptor (NMDA-receptor) activation, enhancing neurotoxicity and cell death in granular neurons [31]. Thus IL-6 can have both neurotrophic and neurotoxic effects in different neuronal types and at different developmental stages. This dual role that IL-6 appears to play in the CNS may explain the wide range of psychiatric disorders [32].

Therefore, the present preliminary study has been undertaken to investigate: (i) the serum levels of IL-2 and IL-6 in the Indian Bengalee schizophrenic patients and correlate our finding with the previously reported studies; (ii) if there is any heterogeneity of interleukin level in schizophrenic patients of different ethnic group; (iii) the cytokine-modulating activity of antipsychotic drugs; and (iv) the validity of Th2 hypothesis in the Bengalee schizophrenic subjects.

2. Materials and methods

Fifty India born schizophrenic patients of West Bengal referred to the outpatient department (OPD) of Psychiatry, North Bengal Medical College and Hospital, Siliguri were considered for the present study. Three major selection criteria were considered for selection of schizophrenic group: (i) Unrelatedness of individuals from each other, (ii) resident of the state of West Bengal and Bengalee by ethnicity, and (iii) subjects satisfying DSM-IV-TR diagnostic criteria for schizophrenia [33]. The exclusion criteria followed in the present study include: (i) History of smoking or substance abuse, (ii) presence of personality disorder, (iii) presence of dementia and mental retardation, and (iv) general medical condition. The patients considered for the present study were Bengalee by ethnicity and ranged in age from 16 to 55 years. They were further classified into two groups, 20 schizophrenic patients who had stopped taking antipsychotic drugs for at least 6 weeks were considered as psychotropic medication free, rest 30 schizophrenic group were under antipsychotic medication. The patients were diagnosed by psychiatrist using Structured Clinical Interview [34] and according to the standard diagnostic criteria of DSM-IV-TR [33] and assessed by the Brief Psychiatric Rating Scale (BPRS) [35]. The present study comprise of 44 Paranoid, 1 Residual, 4 Disorganized and 1 Catatonic schizophrenic patient. Some of the demographic variables are also considered for the study.

A total number of 30 unrelated, ethnically matched healthy individuals were considered as controls. The criteria for the selection of controls include: (i) Same ethnic group as the patients, (ii) sex and age matched with patients, (iii) absence of family history of autoimmune and psychiatric disorder, (iv) recent history of intercurrent infection and allergies, (v) no history of smoking or any substance abuse, and (vi) no history of general medical condition. All the participants provided their written consent for giving the blood sample after the study procedures were explained. The study has been carried out according to the principles of the Declaration of Helsinki (1964).

2.1. Biochemical measurements

The blood samples from the patients and controls were collected between 10 am and 12 noon by veinpuncture method and allowed to clot at room temperature for 2–3 h. Blood clot was cut and centrifuged for separating the serum. The separated serum was aliquoted and stored at −70 °C before use.

2.2. Measurement of IL-2 and IL-6 concentration in serum

Serum IL-2 and IL-6 levels were measured by enzyme-linked immunosorbent assay kit (Endogen Human IL kit, Pierce Biotechnology, Inc., Rockford). The sensitivities for IL-2 and IL-6 were <6 pg/ml and <1 pg/ml, respectively, with inter and intra-assay coefficient of variation of <10% in both the cases. Absorbance was measured by a microtiter plate reader set at 450 nm. Each assay was carried out by the same investigator.

2.3. Statistical analysis

For variables: (i) mean and standard deviations have been calculated and (ii) *t*-tests have been done to test the equality of means of patient and control subjects. For attributes: (i) percentages have been calculated and (ii) χ^2-tests have been done to test the equality of percentages. The *p*-values of <0.05 were considered statistically significant.

3. Results

Table 1 shows the demographic characteristics of the patients and normal controls. No significant relationships between age, sex, BMI and serum IL-2 and IL-6 observed. Age of onset and duration of illness did not significantly correlate with IL-2 and IL-6 levels in the patient groups. The patient group consists mostly of Paranoid schizophrenics (88%). The BPRS score of antipsychotic medicating patients were lower than the psychotropic medication free schizophrenic patients.

The results of the IL-2 and IL-6 assay are summarized in Figs. 1 and 2, respectively. The serum IL-2 level in antipsychotic medicating patients were found to be significantly lower (34.54 ± 22.09 pg/ml) than the control subjects (56.04 ± 18.82 pg/ml) ($p < 0.001$). The serum IL-2 level in psychotropic medication free patients (38.76 ± 27.23 pg/ml) were also found to be significantly lower than the normal controls (56.04 ± 18.82 pg/ml) ($p < 0.05$).

The serum IL-6 level in antipsychotic medicating patients were found to be significantly lower (8.31 ± 6.52 pg/ml) than the control subjects (33.87 ± 13.60 pg/ml) ($p < 0.001$). On the other hand the level of IL-6 in psychotropic medication free patients (12.35 ± 10.06 pg/ml) was also found to be significantly lower than control groups (33.87 ± 13.60 pg/ml) ($p < 0.01$). The serum IL-2 and IL-6 level was found to be lower in antipsychotic medicating patients then the psychotropic medication free patients (Figs. 1 and 2).

4. Discussion

To our knowledge this is the first attempt to study the role of ILs in the Indian Bengalee schizophrenic subjects. The lower level of IL-2, observed among the schizophrenic patients in the present study is in agreement to Theodoropoulou et al. [36]. However our finding is in contrast to previous findings by Ebrinc et al. [37], Zhang et al. [38,39] who have found elevated level of IL-2 in their subjects. Also the finding is in contrast to the studies by Kim et al. [40,10] who found an increase of serum level of IL-2 in Korean schizophrenic patients.

The unique finding of the present study is the significantly lower level of IL-6 in the schizophrenic subjects. To our knowledge this is the first report of lower level of IL-6 in the schizophrenic patients and nowhere else have been reported previously. The finding is in contrast as reported by Zhang et al. [38], who have found the elevated level of IL-6 in the schizophrenic group than the normal control. On the other hand some other groups have reported no significant differences between patients and controls [20].

Table 1
Demographic characteristics of the schizophrenic patients and the control subjects.

	Schizophrenia $N = 50$	Mean	Standard deviation (SD)		Control $N = 30$	Mean	Standard deviation (SD)
Sex							
Male	30 (60%)				18 (60%)		
Female	20 (40%)				12 (40%)		
Age		32.36	10.50			33.87	11.35
Duration of illness		5.57	5.47				
Duration of treatment in years ($N = 30$)		3.33	4.19				
Age of onset		26.18	9.58				
Subtypes of schizophrenia							
Paranoid	44 (88%)			$\chi 2 = 106.32$ (df = 3)			
Residual	1 (2%)						
Disorganized	4 (8%)						
Catatonic	1 (2%)			$p < 0.001^*$			
Medication status of patients							
Psychotropic medication free	20 (40%)			$\chi 2 = 2.00$ (df = 1)			
Antipsychotic medicating	30 (60%)						
Body mass index (kg/m2)		22.496	1.426			22.657	1.514, df = 78, $p < 0.635$
Smoking status	0				0		
Antipsychotic types and dose	($N = 30$)						
Olanzapine (10–20 mg/day)	8						
Quetiapine (300 mg–600 mg/day)	8						
Clozapine (100 mg–300 mg/day)	4						
Risperidone (2 mg–8 mg/day)	10						
BPRS score							
Psychotropic medication free		42	2.714	$t = 3.207$, df = 48			
Antipsychotic medicating		40	1.702	$p < 0.01^*$			

Abbreviation: IL, interleukin; BPRS, brief psychiatric rating scale.
* Significant.

The nonagreement of the some of the previous and present finding may be due to the different essay method, differences in tested material (serum vs plasma), sampling of patients in different stages of disease progression (acute vs chronic or active phase vs remission), exposure to a variety and duration of neuroleptic treatments and different disease progression. In our study, we have used sandwich ELISA kit whose sensitivity was <6 pg/ml for IL-2 and <1 pg/ml for IL-6. This highly sensitive assay kit may be the reason we were able to detect ILs in schizophrenic patients and the normal controls. In addition the inconsistent results may be due to the fact that schizophrenia is generally treated as though it were a single disease process, instead of several etiologically distinct disorders. Much of the current research points to the heterogeneity of schizophrenia [32]. Pulver [41] makes this point by describing schizophrenia as a syndrome with 'genetic heterogeneity' having susceptibility loci at several different chromosomal regions. Kirkpatrick and Carpenter have proposed strong evidence in support of a dichotomy: 'deficit' and 'nondeficit' schizophrenia [42]. Garver et al. [43] also delineated and subsequently replicated three distinct clusters or 'endophenotypes' within the group of patients that meet conventional criteria for the DSM-IV schizophrenia syndrome [44,45]. If such etiologically distinct endophenotypes exist, it should be suspected that central immune activation may be a component of one, but not all of the endophenotypes. On the other hand statistically significant ethnic-

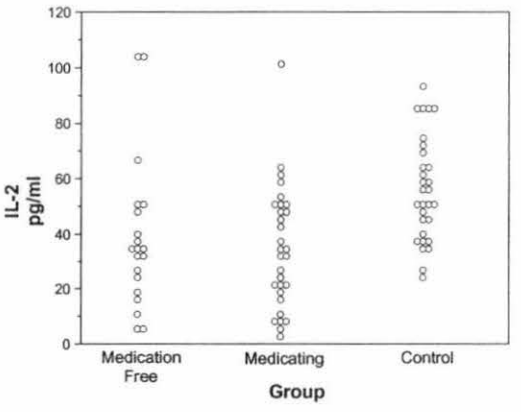

Fig. 1. Comparison of serum IL-2 level (pg/ml) between medication free, medicating schizophrenic patients and control.

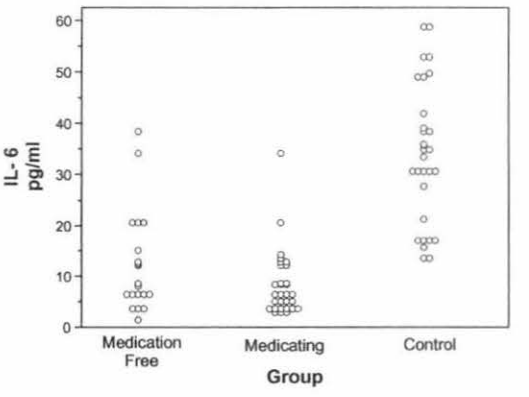

Fig. 2. Comparison of serum IL-6 level (pg/ml) between medication free, medicating schizophrenic patients and control.

ity-based variability in the allelic frequency and genotype inheritance patterns for IL-2 and IL-6 has been reported [46] suggesting that polymorphisms within these cytokine genes may be responsible for the ethnic-based differences in IL-2 or IL-6 levels [39]. This may be another reason for the nonagreement of our result.

The immunosuppressive and cytokine modulating effects of the antipsychotic drugs have been found by different studies [47,48]. Atypical antipsychotics such as risperidone and clozapine also appear to have anti-inflammatory activities [48–50]. In the present study only atypical antipsychotics were prescribed to the patients (Table 1). Therefore the lower levels of IL-2 and IL-6 observed among the medicating patients in the present study suggest the cytokine-modulating activity of atypical antipsychotics. Thus, our finding supports the earlier findings that treatment with antipsychotic drugs affects the cytokine network [22,51].

The results of our findings are not in agreement with exhaustion theory of schizophrenia, which is characterized by decrease IL-2 *in vitro* production and increase of IL-2 in serum [5–7,9] and increase in IL-6 *in vitro* [11,14,15], also our finding does not fit well into the Th1/Th2 paradigm or with the Th2 shift hypothesis. Until recently, IL-2 and IL-6 were classified as Th1 and Th2 type cytokines, respectively [52–54]. This probably contributed to the formulation of the hypothesis of a shift from Th1 to Th2 cytokines on the basis of studies showing decreased *in vitro* IL-2 secretion and increased *in vivo* circulating sIL-2R and IL-6 levels in schizophrenia [55]. However, classification of cytokines is being re-examined, because new CD4+ T cell subsets, such as Th17 and Treg [56,57] are emerging. Unfortunately and because of the novelty of the findings, the data on Th17 and Treg defining cytokines in schizophrenia are still lacking or insufficient at this time point and awaits further research [21].

The limitations of the present study include that the successive follow-up study was not undertaken on the same patients to understand the effect of antipsychotics on the serum level of interleukins. However, it is evident from our study that antipsychotics downregulate the serum level of interleukin.

To conclude, the result of our present findings strengthens the earlier findings of immune system dysregulation in schizophrenia which may be one of the etiological factors for the disorder. However our finding does not fit well to the autoimmune hypothesis of schizophrenia. Further, studies involving other cytokines and *in vitro* cytokine production may throw light in this respect. Additional studies are invited to further unfold the effect of antipsychotics in immune system, this may help in future drug development.

Acknowledgments

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Study of Selected HLA-A and -B Antigens by PCR-SSP Method in Bengali Population of Siliguri and Adjoining Areas of West Bengal

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KEYWORDS Human Leukocyte Antigen. Polymorphism. Linkage. Haplotype. West Bengal

ABSTRACT Human leukocyte antigen is a highly polymorphic gene cluster which has made it a valuable tool in the population genetic studies. In this study one hundred individuals belonging to Bengali community of Siliguri sub-division of West Bengal were studied for 20 of the HLA-A and B loci. The HLA alleles were analyzed by using sequence specific primers for polymerase chain reaction (PCR-SSP). The result showed the increase frequency of HLA-A*02, -A*11, -A*24, -A*31, - B*07, -B*08, and -B*37 amongst the tested alleles. The notable observation of this study is the higher incidence of HLA-B*37 and -B*08 which is observed to be the highest amongst the Indian populations. The two-locus haplotype analysis revealed significant positive linkage disequilibrium for A*01-B*37, A*01-B*40, A*29-B*40, A*30-B*51, A*31-B*40. The study provides the HLA data of the Bengali population of this region.

INTRODUCTION

West Bengal is on the eastern bottleneck of India, stretching from the Himalayas in the north to the Bay of Bengal in the south and occupies only 2.7% of the India's land area, and supports over 7.8% of Indian population (West Bengal Human Development Report 2004). West Bengal lies between 85° 50' and 89° 50' east longitude, and 21° 10' and 27° 38' north latitude. The majority of the population of the state consists of Bengalis (http://www.infobengal.com).

Bengali race is a mixed breed of population broadly of Dravidians, Mongols and Aryans. There is some amount of admixture of aboriginals like Mundari and Santhals (Mazumdar 1998).Thus it may be stated that Bengali as a community is not too homogeneous (Raha 1975).

Siliguri is a cosmopolitan city of the sub himalayan region of West Bengal, consisting of Marwari, Punjabi, Bihari, Gorkha and Bengali communities, with Bengalis being the most prominent community here. Siliguri has seen waves of massive immigration over the years. The present Bengali population consists mostly of immigrants from Bangladesh and Assam (Kumar 2006).

Due to its high polymorphism, tight linkage among the loci and non-random association of alleles, Human leukocyte antigen (HLA) has become interesting from the perspective of population genetics. All the regions of HLA are known to be highly polymorphic, consisting of large number of closely linked genes that can be further split into many allelic types. Therefore the importance of this system in the study of polymorphism and their significance in population selection and survival and in providing clues to mechanism of generation as well as maintenance of this variability within the populations is immense (Srivastava 2007a).

HLA profiles of various populations are available from various parts of India (Mehra et al. 1986; Selvakumar et al. 1988; Balakrishanan et al. 1996; Agarwal et al.1999; Chhaya and Shankarkumar 2001). The HLA data are available for some of the ethnic and tribal populations of West Bengal (Debnath et al. 2006a; Debnath et al. 2006b; Srivastava 2007b; Agrawal 2008). To our knowledge only one study has been carried

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out to study the HLA profile in Bengalis by Raha (1975). This study has been carried out long before the advent of the modern molecular typing methods. On the other hand from our literature review it is obvious that HLA study has not been carried out in the Bengali population of Siliguri. Moreover, very recently Ali et al. (2008) have reported the HLA allele frequencies in Bangladeshi Bengalis. Therefore, the present study has been carried out to study the HLA Class I profile of Indian Bengali population residing in Siliguri, West Bengal, with the help of modern PCR based molecular typing technique.

MATERIALS AND METHOD

Samples: Blood samples were collected from 100 unrelated healthy Bengalis residing in and around Siliguri sub-division of West Bengal. Three-generation pedigree charts were prepared to assure that the samples were unrelated. All the participants provided their written consent for giving the blood samples after the study procedure were explained. The study has been carried out according to the principles of the Declaration of Helsinki (1964).

***HLA Molecular Typing*:** In this study 20 of the HLA alleles from HLA-A and -B were selected for the molecular typing in all individuals who were included in the study. DNA was extracted from peripheral mononuclear cells of the blood by the Phenol Chloroform method as described by Comey et al., 1994. Low-resolution molecular typing using polymerase chain reaction-based sequence-specific primer technique was performed for detecting HLA-Class I alleles. The typing and sequence information of primers were taken from Bunce et al. (1995) and 12th IHWC. The primers, Taq polymerase, nucleotide etc were obtained from Bangalore Genei, India. The sequence of the some of the primers which was used for genotyping HLA is as follows:- HLA-A*11; forward primer (5'ACGGAATGTGAA GGCCCAG3), and reverse primer (5'CTCTCTG CTGCTCCGCCG3'), HLA-B*08 forward primer (5'GACCGGAACACACAGATCTT3') and reverse primer (5'CCGCGCGCTCCAGCGTG3'). In general 25µl of reaction mixture in 1x PCR buffer, 200µM of each of dNTP, 1.5mM $MgCl_2$, 0.4µM of forward and reverse primers, 100ng of genomic DNA and 1unit of Taq polymerase. The amplifications were accomplished on a thermal cycler (Perkin Elmer, USA). PCR reactions are subjected to 30 cycles, each consisted of 94°C for 30sec., 60°C for 1min. and 72°C for 1min. with initial denaturation step of 2min. and final extension of 2min.

Statistical Analysis: Phenotype frequencies were calculated by direct count. Gene frequency was calculated by the formula $P=1-\sqrt{1-F}$, where F=frequency of allele. SPSS 15.0 software was used for calculating frequencies of different alleles. Haplotype frequencies were calculated by direct counting. The linkage disequilibrium was calculated from a 2x2 table and indicated as a delta value. The significance of this value was tested using the χ^2 test.

RESULTS

Allele frequencies of HLA-A and HLA-B loci of Bengali population studied are presented in table 1. The frequency of the alleles A*02, A*11, A*24, A*31, B*07, B*08, B*18 and B*37 were found to be highest among all the alleles tested.

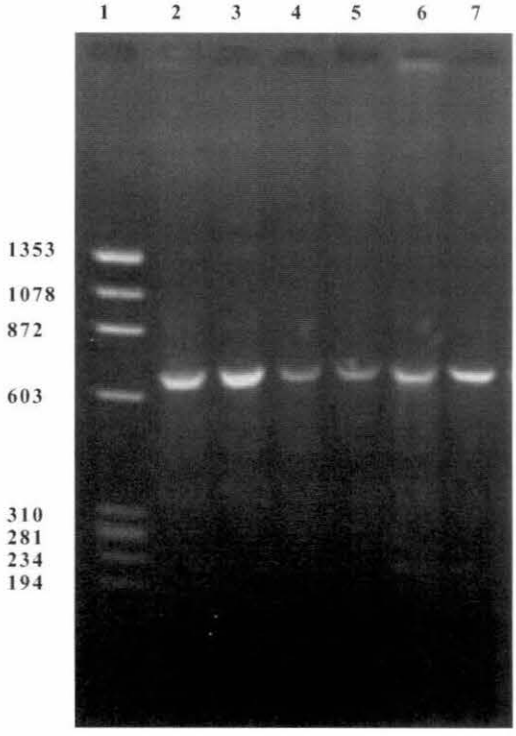

Fig. 1. Electrophoregram showing the result of HLA-B*07 (619 bp)
Lane 1, fX174 marker
Lane 2-7, amplified HLA gene

Among HLA-A group HLA-A*11 with frequency of 24% is found to be the most frequent allele followed by A*02 (20%), A*24(17.50%), A*31(18.50%) and among HLA-B alleles B*08 (22%),B*07 (17%) (Fig. 1), B*18 (14.50 %), B*37 (17 %) were found to have increased frequency.

Most common haplotype with significant positive linkage disequilibrium are shown in table 2. The two locus haplotype analysis revealed significant positive disequilibrium for A*01-B*37($p<0.01$), A*01-B*40, A*29-B*40, A*30-B*51, A*31-B*40($p<0.05$).

DISCUSSION

In the history of the Indian subcontinent, the most important invasion was by the West Asian semi nomadic tribes around 2000 BC. Some of these tribes migrated west and populated parts of Europe, whereas others migrated east towards Persia and over the Hindukush Mountains into the Indian subcontinent. Here they subjugated the dark skinned pre-Aryan (Dravidian) inhabitants of the Indus Valley civilization (Wolpert 2000). Since then, repeated invasions over the centuries by the Persian, Greeks, Turks, Arabs and Mongols have added to the genetic and cultural diversity of the Indo-Pak sub-continent and have a large amount of genetic admixture thus there may be certain level of shared ancestry (Mohyuddin et al. 2002).

In the present investigation frequency distribution of HLA-A and –B loci is studied among the Bengali population of Siliguri. Table 3 and 4 present the comparison of frequency distribution of HLA antigen among different populations. It has been observed that the frequency of A*02 and A*11 is uniformly high in all the populations like Sikh (Babita and Usha 2004), Gujarathi, Maharastrian (Kankonkar et al. 2004), different caste group of western India (Shankarkumar et al. 2001) and North India (Mehra et al.1997). Our previous study on Gurkha population also revealed the higher allele frequency of A*02 and A*11 (Debnath et al. 2006a). However, when the frequency profile of Bengalis were compared with other major world populations it was found that HLA-A*02 and A*11 were also higher in Greeks (Pachoula-Papasteriadis et al.1989).The higher frequency of A*24 observed in the present study is also observed in the majority of the South Indian (Thomas and Banerjee 2005), Northern Indian populations (Rajalingam et al. 2002) and Bangladeshi Bengali population (Ali et al. 2008). The higher frequency of A*31 is also observed in some of the South Indian populations (Vettriselvi et al. 2006).

Among the HLA-B alleles the higher frequencies of B*07 and B*08 is also observed in Sikhs (Babita and Usha 2004).In the North Indian population HLA-B*07 is the third highest frequency allele and is present at highest frequency in the Western European populations (Thomas and Banerjee 2005). HLA-B*37 is also found in Adiya, Ezhava of Kerala India (Thomas and Banerjee 2005) but its frequency is not as high as has been observed in the present investigation. The observed frequency of B*37 (17%) in this study is perhaps the highest among all the populations studied so far.

Table 1: Gene frequencies of HLA-A and-B antigens in Bengali population from Siliguri, West Bengal.

Allele	*Gene frequency*	*Gene frequency %*	*Standard error of gene frequency*
A*01	0.0808	8.0761	2.7247
A*02	0.1056	10.5573	3.0729
A*03	0.0228	2.2759	1.4913
A*11	0.1282	12.8220	3.3433
A*23	0.0356	3.5635	1.8538
A*24	0.0917	9.1705	2.8861
A*25	0.0408	4.0834	1.9791
A*26	0.0808	8.0761	2.7247
A*29	0.0540	5.3956	2.2593
A*30	0.0673	6.7262	2.5048
A*31	0.0972	9.7227	2.9627
B*07	0.0890	8.8957	2.8468
B*08	0.1197	11.9659	3.2456
B*18	0.0753	7.5338	2.6394
B*21	0.0151	1.5114	1.2201
B*37	0.0890	8.8957	2.8468
B*40	0.0540	5.3956	2.2593
B*42	0.0050	0.5013	0.7062
B*44	0.0025	0.2503	0.4997
B*51	0.0566	5.6602	2.3108

The frequent alleles such as A*02 and A*11 and A*24 observed in the present investigation were also found to be in higher incidence amongst the Bangladeshi Bengalis. Among HLA-B locus alleles the most common alleles of Bangladeshis such as B*44, B*40, B*51 were not found to be in higher frequencies amongst the Bengalis of the present study. Although the past history suggests the Bengalis of Bangladesh and West Bengal are closely related the differences seen here may be attributed to founder effect and genetic drift.

Table 2: Haplotypes showing significant significant linkage disequilibrium in Bengali population from Siliguri, West Bengal

Haplotype	*HF/1000*	*1000"x*	χ^2
A1-B37	499	96	6.861**
A1-B40	347	76	5.270*
A29-B40	366	78	4.237*
A30-B51	179	58	4.904*
A31-B40	17	82	6.614*

Note: *= p<0.05, **=p<0.01
HF=Haplotype frequency per 1000.
"=Delta

Table 2 presents the observed two locus hyplotype frequencies among Bengalis. The most frequent haplotype A1-B37, observed in the present investigation is also observed among Mumbai Marathas (Shankarkumar et al. 2001) and among the world population it is observed in Korean (Lee et al. 2005), Northern Ireland (Williams et al. 1999), North Western Islands (Spinola et al. 2002) and Azores (Spinola et al. 2005).

To our knowledge this study is the first of its kind to study the HLA status of the Bengali population of Siliguri, West Bengal using modern PCR based molecular typing method. The study presents preliminary HLA data which may be valuable to bone marrow transplantation registries and are useful in the study of molecular anthropology.

CONCLUSION

The distribution of HLA alleles among the Bengali population of Siliguri showed similarity with the other Indian populations as well as to Bangladeshi Bengalis. The most striking observation of this investigation is the high incidence of HLA-B*08 and B*37 which are observed to be highest when compared with other Indian populations.

RECOMMENDATION

Large amount of data involving both HLA Class I and Class II will be required to throw light to the phylogenetic history of Bengali population of this region.

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Table 3: Comparison of gene frequencies of HLA-A with the other populations

	Present study N=100	*Toto N=40*	*Gurkha N=50*	*Marathas N=289*	*Sikhs N=404*	*Bangladeshi Bengali N=141*
HLA						
A*01	0.0808	0.0253	0.01	0.1434	0.1212	0.0491
A*02	0.1056	0.2254	0.14	0.1569	0.142	0.0756
A*03	0.0228	0.0780	0.062	0.0430	0.1212	0.0179
A*11	0.1282	0.0917	0.1633	0.1227	0.1058	0.0813
A*23	0.0356	0.0646	0.01	0.0016		0.0036
A*24	0.0917	0.0382	0.02	0.0583		0.0852
A*25	0.0408	0.0513	0.0202			0.0197
A*26	0.0808	0.0780	0.04	0.0130		0.0161
A*29	0.0540	0.0382	0.000	0.0146		0.0071
A*30	0.0673	0.0513	0.01	0.0016		0.0036
A*31	0.0972	0.0382	0.0513	0.0081		0.0233

Table 4: Comparison of gene frequencies of HLA-B with the other populations

	Present study N=100	*Toto N=40*	*Gurkha N=50*	*Marathas N=289*	*Sikhs N=404*	*Bangladeshi Bengali N=141*
HLA						
B*07	0.0890	0.1197	0.041	0.1221	0.0626	0.0251
B*08	0.1197	0.0780	0.0304	0.0303	0.0706	0.0036
B*18	0.0753	0.0780	0.073	0.0117	0.0137	0.0071
B*21	0.0151			0.0218	0.0213	
B*37	0.0890	0.0126	0.041	0.0252	0.0175	0.0161
B*40	0.0540	0.0513		0.1033	0.0600	0.0341
B*42	0.0050					
B*44	0.0025	0.0646	0.073	0.0424		0.0472
B*51	0.0566	0.0646	0.0945	0.0235		0.0306

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Immune Dysregulation:Can it Be an Etiological Factor for Schizophrenia?

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ABSTRACT

In the present investigation the incidence of HLA Class I antigens, C-reactive protein (CRP), cytokines were studied along with the demographic variables in the schizophrenic patients of Siliguri sub-division and adjacent areas to enlighten the immune dysregulation and autoimmune etiopathology of schizophrenia in the Indian context. Low resolution PCR-SSP method was applied for typing the HLA antigens. The level of serum CRP was investigated by latex agglutination test. Serum level of cytokines were measured by ELISA method. Further the patients and family members were made to answer a questionnaire to assess the demographic variables. The result showed the significant increase for HLA A*03 antigen along with the significant decrease for HLA-A*25, A*31 and HLA-B*51. Further, the drug naïve patients showed elevated level of CRP, cytokines such as IL-2 showed decrease level in the schizophrenic group. The demographic variables showed significantly increase frequency of schizophrenia among the patients who are not the first child. The results suggest the possible association of HLA antigen with schizophrenia like most of the other autoimmune disorders. The findings are suggestive of immune dysregulation in schizophrenia. The study also supports the hypothesis of increase risk of developing the disorder among the younger children of the family.

Key words: Autoimmunity, schizophrenia, HLA, CRP, cytokine,

INTRODUCTION

Schizophrenia is a debilitating, often chronic, mental disorder characterized by disturbances in thinking, perception, emotion, and social relationships. The term schizophrenia, which literally means "split mind", was first applied by Swiss psychiatrist Eugen Bleuler in 1911.(Calkins and Iacono, 2003). Several etiological theories have been proposed for schizophrenia , including developmental (Horning *et al.*, 2002) or neurodegenerative processes (Lieberman, 1999), neurotransmitter abnormalities (Aghajanian and Muck, 2000), viral infection and immune dysfunction or autoimmune mechanisms (Noy *et al.*, 1994). In addition, there is substantial evidence for a genetic predisposition to schizophrenia (Mueser and MeGurk, 2004). However, none of the current etiological theories can fully explain the varied symptoms observed in different patients. Several lines of evidence indicate that immunological dysfunction may have

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relevant implication for the etiology of schizophrenia. It was suggested that viral infections and/or autoimmune reactions against central nervous structures may play an important role in the pathogenesis of the disease (Kirch,1993)

With recent advances in technology and an increased understanding of the immune system, autoimmune theories of schizophrenia have once again become a major focus of research. Many general immune abnormalities have been reported in schizophrenia. These include increase (Masserini *et al.*, 1990), decrease (Nyland *et al.*, 1980, Coffey *et al.*, 1983) or unchanged (Printz *et al.*, 1999) lymphocyte population, morphological changes in lymphocytes (Fessel and Hirala Hibi1963), altered levels of CD4+, CD45RA+ T cells, CD8+ T cells(Cazzullo *et al.*,1998), CD5+ B cells (Printz *et al.*, 1999), altered levels of IL-2 (Interleukin-2)(Becker *et al.*, 1996, IL-6 (Ganguli *et al.*, 1994, Akiyama ,1999), IFNγ (Interferon γ) (Arolt *et al.*, 2000) and increased levels of antiviral antibodies (Kaufmann *et al* ,1983).In other study elevated level of C-reactive protein (CRP) is also found in schizophrenic patients (Fan *et al.*, 2006).

Since the HLA (Human leukocyte antigens) system governs the immune responses, and many of proven autoimmune disorder had shown association with HLA, this system were implicated in the etiology of schizophrenia. A large number of studies have also shown the association between the class I HLA antigens and schizophrenia viz. HLA-A9. HLA-A23 (Ivany *et al.*,1983) and HLA-A24 (Asaka *et al.*,1981). Recently HLA-A*03 showed positive association among paranoid schizophrenics in the Bengali population of Siliguri (Debnath et al., 2005). Association of HLA-A1 and schizophrenia have been reported by Lahdelma *et al.*, (1998). In a similar research conducted in south Sweden the significant increases were found for A2, A3, B17, B27 and CW2 and decrease of A1, A11 and B8 among the schizophrenic patients (Rudduck *et al.*,1984). Past association studies with various HLA Class I alleles yielded inconsistent results (Nimgaonkar *et al.*, 1992), except HLA-A9.

The present study has been undertaken to study whether there is any immune dysregulation is schizophrenia and to investigate the autoimmune basis of schizophrenia in the Indian perspective.

MATERIALS AND METHODS

India-born Bengali psychiatric patient referred to the psychiatric outpatient Department (OPD), North Bengal Medical College and Hospital were considered for the present study. Patients were diagnosed independently by two psychiatrist according to the standard diagnostic criteria of DSM IV and assessed by the Brief Psychiatric Rating Scale (BPRS). After diagnosis, 64 schizophrenic patients were included for the study. Further, the schizophrenic patients and the family members were made to answer a questionnaire containing self-reported age, cast, sex, medical history, age of onset, month of birth, marital status, education, substance abuse, incidence of any autoimmune disease among patients or in family members etc. A total number of 50 unrelated, ethnically matched healthy individuals were considered as controls. All the participants provided their written consent for giving the blood sample after the study procedures were explained.

Five ml blood was drawn by vein puncture method out of which 2 ml was collected in EDTA, an anticoagulant for the DNA extraction. 3 ml blood was allowed to coagulate at the room temperature for 2-3 hrs. Blood clot was cut and centrifuged for separating the serum. The serum samples were aliquoted and kept at -70 refrigerator. DNA was extracted from peripheral mononuclear cells of the blood by the Phenol Chloroform method.fifty number of

the patients and controls were typed for the HLA Class I antigens by PCR-SSP technique. The typing and sequence information of primers were taken from Bunce *et al.*, (1995). The primers, Taq polymerase, nucleotides etc. were obtained from Bangalore Genei, India. The CRP level in the serum was measured by latex agglutination slide test (Ranbaxy Fine Chemicals Ltd., HP, India). Serum IL-2 levels were measured by enzyme linked immunosorbent assay kit (Endogen Human IL kit, Pierce Biotechnology, Inc. Rockford). The sensitivities were <6pg/ml and <1pg/ml respectively, with inter and intra assay coefficient of variation of <10%. Absorbance was measured by a microtiter plate reader set at 450 nm. For the IL-2 study the patients were grouped into two categories, one under antipsychotic medication and the second without antipsychotic medication i.e. neuroleptic-free, under this category the patients who have not taken antipsychotic for minimum period of 1month were considered.

RESULTS

The incidence and frequency of HLA Class I antigens among patients and control has been presented in Table I. There was a significantly higher frequency of HLA-A*03 (X^2=11.458, p=1.155e-4) in patients than the control groups. On the other hand HLA-A*25 (X^2=13.619, p=9.185e-5), A*31 (X^2=22.562, p=8.793e-8) and B*51 (X^2=40.047, p=1.604e-12) showed lower

Table I: Phenotype frequency, Chi square, relative risk (RR) values and probability of HLA-A and B loci alleles in the patients with schizophrenia and healthy controls.

Antigen	Patients (N=50)	Control (N=50)	Chi-square	Chi square (y)	RR	P value	
A*02	24	37	7.103	6.052	0.332	6.699e-3	*
A*03	50	38	13.636	11.458	32.792	1.155e-4	†
A*11	36	31	1.130	0.723	1.558	1.975e-1	
A*23	21	26	1.003	0.642	0.673	2.115e-1	
A*24	32	26	1.477	1.026	1.624	1.555e-1	
A*25	10	29	15.174	13.619	0.188	9.185e-5	†
A*26	21	17	0.679	0.382	1.395	2.684e-1	
A*29	33	32	0.043	0.000	1.089	5.000e-1	
A*30	33	25	2.627	2.011	1.914	7.787e-2	
A*31	0	20	25.000	22.562	0.014	8.793e-8	†
B*07	47	39	5.315	4.069	3.951	2.035e-2	*
B*21	36	38	0.207	0.051	0.817	4.099e-1	
B*4001	17	13	0.761	0.428	1.451	2.565e-1	
B*4201	39	28	5.472	4.522	2.711	1.632e-2	*
B*44	14	10	0.877	0.493	1.532	2.414e-1	
B*5101-5105	0	30	42.857	40.047	0.006	1.604e-12	†

y= Yates's correction

* Significant

† Significant after the Bonferroni correction

Bonferroni's probability is 0.003125

(Note: The abbreviation 1.156 e-4 means 1.156 x 10^{-4}, like wise the other values may be interpreted)

Table II. Comparison of demographic and clinical characteristics between the normal / elevated CRP groups.

	Elevated CRP N=3		Normal CRP N=61			
	Mean or N%	**Standard deviation**	**Mean or N%**	**Standard deviation**	**Statistic (Z)**	***P* value**
Age	37.67	21.13	34.69	9.64	F[2,60]=0.24	>0.62
Gender Men Women	33.33%		70.49%		X^2 [1]=1.84	>0.17
Drug naïve patients Patients under antipsychotic medication	100%		11.48%		X^2 [1]=17.00	<0.001 Significant
Substance abuse Yes No	33.33%		55.74%		X^2[1]=0.58	>0.44
First child Yes No	33%		18.03%		X^2[1]=0.44	>0.50
Autoimmune disease in patients or in family Yes No	0%		24.59%		X^2[1]=0.97	>0.32

TableIII: Comparison of serum IL-2 levels between psychotropic medication free, antipsychotic medicating schizophrenic patients with the normal controls.

Variables	Psychotropic medication free schizophrenic group (n=20)	Control (n=30)	P value	
IL-2 (pg/ml)	Mean= 38.76 SD=27.23	Mean=56.04 SD=18.82	P<0.05 *	t= - 2.656 d.f.=48

Variables	Antipsychotic medicating schizophrenic group (n=30)	Control (n=30)	P value	
IL-2 (pg/ml)	Mean=34.54 SD=22.09	Mean=56.04 SD=18.82	P<0.001*	t= - 4.058 d.f.=58

* = significant

Table IV. Comparison of demographic characteristics between the first schizophrenic child and the other schizophrenic siblings in different families.

	N=50		Analysis	
	patients who are first child	patients who not the first child	Statistic	P value
1. Number of patients	11	39	$\chi 2$ =15.68 (df=1)	<0.001 *
2. Age of Onset (years)				
Mean	24.45	28.03	t = -1.056	= 0.30
Standard deviation (SD)	12.84	8. 97		

value significantly even after the Bonferroni correction. Though A*02 (X^2=6.052,p=6.699e-3) showed lower frequency and B*07 (X^2=4.069, p=2.035e-2) and B*42 (X^2=4.522, p=1.632e-2) showed higher frequency they were not found to be significant after the Bonferroni correction.

Table II shows the comparison of demographic and clinical characteristics between the normal / elevated CRP groups. Sera levels of CRP were measured for 64 schizophrenic patients. The elevated level of CRP (e"6mg/l) was observed in 3 patients and 61 patients were found to have normal CRP (<6mg/l). All the three elevated cases were found to be of paranoid type. Further, when the level of CRP was compared to the other demographic variables as shown in the Table I, only the drug naïve status of the patients showed statistically significant value (X^2 = 16.997, *P* value <3.75x 10^5).

The results of IL-2 assay is summarized in Table III. The serum IL-2 level in antipsychotic medicating patients were found to be significantly lower (34.54±22.09 pg/ml) than the control subjects (56.04±18.82,t= -4.058; df=58; p<=0.00015). Whereas the serum IL-2 level in drug naïve patients (45.69±51.49pg/ml) and controls (56.04±18.81pg/ml) were found to have no significant difference with each other.

Further, as shown in Table IV, when the Chi- squire test was performed between the first child patients and the patients who were not the first child, the test showed a significantly high incidence of schizophrenia among the patients who are not the first child. It is worth mentioning here that both the groups of the patients were HLA-A3 positive.

DISCUSSION

A significantly higher frequency of HLA-A3 obtained in the present study is in accordance with the previously reported study by Debnath *et al.* (2005) which is also in accordance with the study of Rudduck *et al.*(1984). Although the association of HLA-A3 antigen and schizophrenia is found to be significantly higher, the present studies do not reveal a very strong association as has been reported earlier.

On the other hand, in the present study A*25, A*31 and B*51 showed significantly lower negative value which is the unique finding of the present study. Among these alleles, A*31 and B*51 showed strong negative associations (RR=0.014 and RR=0.006 respectively). The increased frequency of A*11 found in the previous study was not reproducible in the present

study. Apart from this, several other alleles like B*07 and B*42 showed higher value but were not statistically significant. We also observed a negative association of A*02 but the association was not found to be significant which was in accordance with the findings of Debnath *et al.*, (2005).But the finding was unlike the previous findings by Luchins et al., (1980) which showed positive association of A*02 with schizophrenia in African-USA population. However, we have not found association between HLA-A*23 and A*24 in our study as has been reported by previous studies.

The elevation of CRP in this study provides further evidence of the involvement of inflammatory processes behind the etiopathology of schizophrenia. The elevated level of CRP in our study is in accordance to the findings of Fan and Dikerson (2007). But unlike previously reported findings, we have considered the CRP level of patients with their medication status, which showed significantly higher value. In one study, the level of CRP was found to be higher in the patient who was experiencing psychotic symptoms, in the follow up study in the non-psychotic state, the level of CRP was found to be normal (Ohaeri *et al.*, 1993). In this respect the present study suggests that the antipsychotic drug may perhaps down regulate the inflammatory process which in turn brings the CRP level to the normal state. Thus, these findings further suggest that the inflammation may be another possible mechanism in the etiopathology of schizophrenia. It is however not clearly understood whether the elevation of the level of CRP is the by-product of the pathophysiology of schizophrenia or directly contributes to the clinical manifestations of the disorder (Fan *et al.*, 2007).

To our knowledge this is the first attempt to study the role of IL-2 in the Indian Bengalee schizophrenic subjects. The major findings of the present study are the decreased level of IL-2 in the schizophrenic group who were under antipsychotic medication. The finding is new for the lower level of IL-2 in the antipsychotic medicating patients. Our finding is in contrast to previous findings by Ebrinc et al., (2002) and Zhang *et al.*, (2005) who have found elevated level of IL-2. Also the finding is in contrast to the studies by Kim et al.,(2000) who found an increase of IL-2 serum level in Korean schizophrenic patients. On the other hand our finding is in agreement to Theodoropoulou *et al.*, (2001) who found that IL-2 serum levels were significantly lower in both medicated and non-medicated schizophrenic patients.

At this moment it is too early to speculate the autoimmune etiopathology of schizophrenia but the result of our present investigation definitely strengthens the hypothesis of immune dysregulation in schizophrenia which may be one of the etiological factor for the disorder. Our study also supports the hypothesis of increase risk of developing the disorder among the younger children of the family. Further, studies are needed to reveal the mechanism of the changes in the immune system in the disorder.

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Dopamine Receptors and the Dopamine Hypothesis in Schizophrenia

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ABSTRACT

Alteration in dopaminergic activity may play an important role in the pathogenesis of schizophrenia. In the present study we investigated the incidence of dopamine receptor genes in the schizophrenic patients of Siliguri sub-division and adjacent areas to understand the dopamine hypothesis of schizophrenia in the Indian context. We used a nested case-control study design.Low resolution PCR-SSP method was applied for typing the dopamine receptor genes. The result showed the significant increase of dopamine D1 receptor gene along with the significant decrease of dopamine D3 receptor gene. The results suggest the possible association of dopamine D1 receptor gene with the schizophrenia.

Key words: dopamine, schizophrenia, dopamine receptor gene, etiopathology.

INTRODUCTION

The term schizophrenia, which literally means "split mind", was first applied by a Swiss psychiatrist, Eugen Bleuler, in 1911(Calkins and Iacono, 2003). It is a severely debilitating neuropsychiatric disorder characterized by 'disturbances of thought', auditory hallucinations and multiple delusions (Shaw et al., 1998). Although the worldwide prevalence of schizophrenia is about 1%, (Sawa and Syndar, 2000), it has become a leading public health problem now-a-days and exerts enormous personal and economic costs worldwide. In spite of tireless research efforts, the etiological process or processes by which a causal agent creates the pathophysiology of schizophrenia is not yet clearly understood (Buchanan and Carpenter, 2000).

Several etiological theories have been proposed for schizophrenia like developmental (Horning *et al.*, 2002) or neurodegenerative processes, neurotransmitter abnormalities (Aghajanian et al., 2000), viral infection and immune dysfunction or autoimmune mechanisms. In addition, there is substantial evidence for genetic predisposition in case of schizophrenia (Mueser and Mc Gurk, 2004). Different chromosomes have been pinpointed as harboring genes involved in the pathogenesis of schizophrenia (Barondes *et al.*, 1997).

In recent studies dopamine hypothesis continues to provide the principal conceptual gateway into the mysteries of schizophrenia. Dopamine hypothesis of schizophrenia was first suggested by Van Rossum in 1966 (Seeman, 2004). The central effects of dopamine are mediated by at least five G protein-coupled receptors, DI, D2, D3, D4 and D5. Molecular genetic evidence for this dopamine hypothesis has been supported by several studies. However, past association studies mainly with dopamine receptor D1, D2 and D3 yielded inconsistent results. A large number of studies have also shown the association between the dopamine D3

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and D2 receptor genes and schizophrenia in Chinese (Liao *et al.*, 2001) and Swedish population (Jönsson et al., 2003), while in other studies neither any linkage of D2 receptor has been observed among Italian (grassi et al, 1996), Swedish and Californian populations (Moises *et al.*, 1991) nor any significant association has been found with dopamine D3 receptor gene (Yang *et al.*, 2005). the study of Jensen *et al.*, (1993) suggested that dopamine D1 receptor gene is unlikely to be associated with schizophrenia. The reason may be due to the diagnostic method which particularly in earlier studies, are imprecise and vary greatly (Singer *et al*, 1982). The source of controls is not always described in sufficient details to ensure that results are not simply due to population stratification. Significant results are not always corrected for the number of statistical tests performed (Hawi *et al.*, 1999).

Considering the above mentioned inconsistency of specific dopamine gene association with schizophrenia, we were stimulated to examine the possible association of dopamine receptor gene with the disease in the Indian perspective.

MATERIALS AND METHODS

Subjects: India-born Bengali psychiatric patient & referred to the psychiatric outpatient Department (OPD) of Psychiatry, North Bengal Medical College and Hospital were considered for the present study. Patients were diagnosed independently by two psychiatrist according to the standard diagnostic criteria of DSM IV and assessed by the Brief Psychiatric Rating Scale (BPRS). After diagnosis, 30 schizophrenic patients were included for the study. Further, the schizophrenic patients and the family members were made to answer a questionnaire containing self-reported age, cast, sex, medical history, age of onset, month of birth, marital status, education, substance abuse, incidence of any autoimmune disease among patients or in family members etc. A total number of 30 unrelated, ethnically matched healthy individuals were considered as controls. All the participants provided their written consent for giving the blood sample after the study procedures were explained.

***DNA isolation*:** 5ml blood was drawn by vein puncture method and was collected in EDTA, an anticoagulant for the DNA extraction. DNA was extracted from peripheral mononuclear cells of the blood by the Phenol Chloroform method.

***PCR amplification with Sequence Specific Primer (SSP)*:** Polymerase chain reaction was performed for all the dopamine receptor genes using 10ng of DNA, 250mM dNTP, 1.5mM MgCl2, 5µl 10X buffer (Sigma-Aldrich Pvt Ltd), 1.5 units of Taq polymerase (Sigma-Aldrich Pvt Ltd) and 0.1mM each of the primer (Sigma-Aldrich Pvt Ltd) in a total volume of 50ml.

Statistical analysis: Phenotypic frequency was calculated by direct count. The frequency of the alleles of all the dopamine receptor genes is compared in the patient group as a whole with that of the control population using c^2 test. Relative risk was also been estimated.

RESULTS

Table 1I shows the allele profile of the dopamine receptor genes for the patients with schizophrenia, and the healthy control subjects. The results demonstrate a marked elevation in the frequency of dopamine D1 receptor gene in patients with schizophrenia, compared with control subjects (c^2=17.55, $p<0.01$). Statistically, this was highly significant even after Bonferroni and Yates correction. In addition, we also noticed the significantly decreased frequency of dopamine D3 receptor gene among the patients.

Table I: Percentage of allele frequency, chi square, RR values, and probability of the alleles of dopamine receptor genes in the patients and healthy control subjects

Dopamine receptors	Phenotypic frequency of the patients	Genotypic frequency of the patients	χ^2 with Bonferonni correction	χ^2 with Yates correction	RR	*p*
D1	0.867	0.635	19.817	17.5542	15.1666	Significant
D2	0.033	0.017	1.0714	0.2678	0.3103	NS
D3	0.000	0.000	4.2857	2.4107	0.0000	NS
D4	0.233	0.124	0.7387	0.3283	0.6086	NS

DISCUSSION

In this study we found a strong association between the dopamine D1 receptor gene and schizophrenia in Indian individuals. Though this finding is at variance with the reports of no association of dopamine D1 receptor gene in schizophrenic patients (Cichon *et al,* 1994, Liu *et al,* 1995), but our work suggest that the dopamine D1 receptor gene may play a significant role in conferring the susceptibility of schizophrenia.

Schizophrenia has been considered a heterogeneous group of disease and more homogeneous phenotype definitions may increase the power of association (Serretti *et al,* 1996). There is the possibility that a single gene may underlie susceptibility to psychopathology traits of schizophrenia. Again, this study corroborates with the findings of Kojima *et al,* (1999) for the disorganized type.

In conclusion, further studies will be needed with larger samples of patients to prove or disprove the initial positive association between the schizophrenia and the genotypes of the D1 dopamine receptor gene.

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PLASMA HOMOVANILLIC ACID IN DELUSIONAL DISORDER : IMPLICATIONS FOR DOPAMINE DYSFUNCTION

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ABSTRACT

Plasma concentrations of the major dopamine (DA) metabolite homovanillic acid (HVA) are useful indicators of brain DA activity[1] in clinical research[2,3]. It is the potential index of central dopamine turnover[4] and is the most suitable instrument currently available to assess DA activity under relatively natural behavioral conditions i. e., without any pharmacological manipulation or inducing stress from study conditions[5].

In this study, We compared the plasma homovanillic acid (pHVA) concentration in the delusional disorder patients as well as in the healthy controls to investigate if the abnormalities of the central dopaminergic transmission may involve in the expression of the delusional symptoms in the patients. 30 Delusional disordered patients have been considered for this study. 30 age and sex matched healthy individuals were considered as the control. A significant increase has been found in pHVA concentration of delusion disorder patient than the healthy controls.

The finding suggests that there is a positive correlation between pHVA concentration and delusional disorder, which further provides the evidence for hyperdopaminergic activity in the brain of delusional disorder patients.

Key words : Plasma homovanillic acid, Dopamine, HPLC, Delusional disorder.

INTRODUCTION

The dopamine hypothesis continues to provide the principal conceptual gateway

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into the mysteries of many psychotic disorders such as schizophrenia, attention deficit hyperactivity (ADHD), addictive disorder, alcoholism etc. Very recently, dopamine hypothesis of delusional disorder has been supported by a few studies on D2 receptor variation[6] and D4 receptor Exon 3 variation[7]. Therefore, the measurement and dynamic assessment of HVA, the major metabolite of dopamine, offer opportunities for obtaining substantial understanding of dopamine's role in delusional disorder. Plasma concentrations of the major dopamine (DA) metabolite homovanillic acid (HVA) are useful indicators of brain DA activity[1] in clinical research[2, 3]. It is the potential index of central dopamine turnover[4] and is the most suitable instrument currently available to assess DA activity under relatively natural behavioral conditions i. e., without any pharmacological manipulation or inducing stress from study conditions[5].

Recent evidence from the study of delusional misidentification syndrome indicates that delusions of very specific type may arise in association with certain well-defined brain insults. Delusional disorder is characterized by monosymptomatic paranoid symptoms. In the field of psychiatry it is the only disease where delusion is recorded as the discrete symptom. Since delusional disorder is characterized by mono-symptomatic paranoid symptoms, several investigators have suggested that delusional disorder is a naturally occurring model psychosis based on abnormalities of the dopaminergic temporolimbic system[9].

In the present investigation, We have studied the concentration of plasma homovanillic acid (pHVA) to see if the abnormalities of the central dopaminergic transmission is involved in the expression of the delusional symptoms in delusional disorder patients and if it can be used as the state marker of this disorder.

EXPERIMENTAL

Materials and methods

Subjects

30 patients with delusional disorder, classified according to DSM-IV and ICD-10 criteria, attended the psychiatric out patient department (OPD) of North Bengal Medical College and Hospital were studied. On an average of 1500 new patients with various psychiatric illness and about 4000 recurrent follow-up cases attend the OPD every year. Initially, about 50 unrelated patients were recruited with the symptoms of delusions. All subjects were screened independently by two psychiatrists using the structured clinical interview (SCID) for DSM-IV to determine the diagnosis of delusional disorder (American

Psychiatric Association, 1994). After longitudinal follow-up, 20 patients represented genuine cases of delusional disorder of various types have been considered. Most patients were clustered between the ages of 25-55 years. The male/female ratio was 1 : 2.The patients having any one of the following conditions were excluded from the study like : (i) substance abuse during the past year; (ii) any history of other general medical illness or treatment with anti-inflammatory or immunosuppressive medication; (iii) any past history of psychotic illness and (iv) any history of co morbidity. Further, the delusional patients and the family members were made to answer a questionnaire included self reported age, sex, caste, medical history, age of onset, age of severity, pedigree, disease duration among patients. Written informed consent was obtained from each subject after a complete description of the study. The study was conducted as per the norms of the "World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects". 20 age and sex matched healthy individuals with no past history of psychiatric illness belonging to the same ethnic group as the patients were used as control subjects. The subjects were mostly from middle-class urban and semirural society and belonged to a nuclear family.

Isolation of plasma

Blood samples were collected in heparinized tube from all 40 individuals. None of the participating subjects took psychotropic medication. Each sample was obtained by vein puncture. The blood was centrifuged for 10 mins at 4000 xg, within 30 mins and the plasma was collected and stored at -80°C until use. All the participants provided their written consent for giving the blood sample after the study procedure was explained.

Determination of homovanillic acid in plasma

Homovanillic acid was measured using HPLC method according to Zumarraga *et al.*, 1987[10].

Sample preparation : Plasma (0.5 mL) was mixed with 50 µmol of EDTA, 1.82mmol of heptanesulfonic acid, 0.02 mol/lit citric acid and 0.02mol/lit disodium phosphate (pH 3.1). Further, plasma proteins were precipitated by adding 20 µL of concentrated perchloric acid and vortex for several seconds. Then the samples were centrifuged for 10 mins at 42000xg. From 1.2 mL of supernatants HVA was extracted, three times with 1.5 mL of ethyl acetate. The combined organic phases were evaporated to dryness under a stream of nitrogen and the residue was dissolved in 0.4 mL of 1 mmol Na_2-EDTA. The extracts were stable for upto 3 days when frozen in liquid nitrogen and stored at -80°C.

Chromatographic system : HPLC analysis was carried out in a liquid chromatographic system consisted of a Model 6000A solvent delivery system (Waters Associates, Milford, MA); a Waters WISP 7101 automatic injector and Waters 464 pulsed ECD with glassy carbon electrode. The detector was operated at 0.6V potential between the working electrode and the Ag/AgCl reference electrode at a sensitivity of 2nA full scale deflection. The column was a 150 x 3.9 mm Novapack C_{18}, 5-μm (Waters, USA). Chromatograms were recorded with Model 023 recorder (Perkin-Emer Corp., Norwalk, CT operated at 10mV full scale. A Waters guard column containing C_{18} Corasil was used. All separations were achieved isocratically at room temperature. Flow rate 1 mL/min. and injection volume was 20 μL.

Quantification of HVA in plasma : The linearity of the detector response was estimated by injecting increasing amounts of standard HVA. Known amount of authentic HVA (Sigma-Aldrich Pvt. Ltd., USA) were added to study the linearity of the purification procedure and the analytical recovery. The standard addition calibration curve was constructed for each plasma sample. The calibration curve was extrapolated to zero concentration of added HVA. The samples were assayed on the basis of peak heights relative to those of external standards.

Statistical analysis

Statistical analysis was performed for the bivariate associations between delusional disorder patients versus normal group by employing one-way analysis of variance. Student's t-test was performed to determine the significance of differences of means among the patients and the control group.

RESULTS AND DISCUSSION

The mean value, standard deviation and coefficient of variance in both the patient group and normal controls are presented in the Table 1. In the present study, the pHVA concentrations in delusional disorder patients were found to be significantly higher (28.014 ng/mL) than in normal controls (7.437 ng/mL) ($p<0.001$) (Fig. 1).

Several studies have been done on the association between pHVA and schizophrenia but all the studies were failed to obtain consistent results [11]. This inconsistency were suggested to be due to the following reasons (i) pHVA is correlated positively with the severity of the psychotic symptoms[12,13,14]; (ii) in good responders pHVA decreases along with neuroleptic treatment[13,15,16,17,18];and (iii) pHVA is lower in

deficit-type than in non-deficit-type[14, 19, 20].

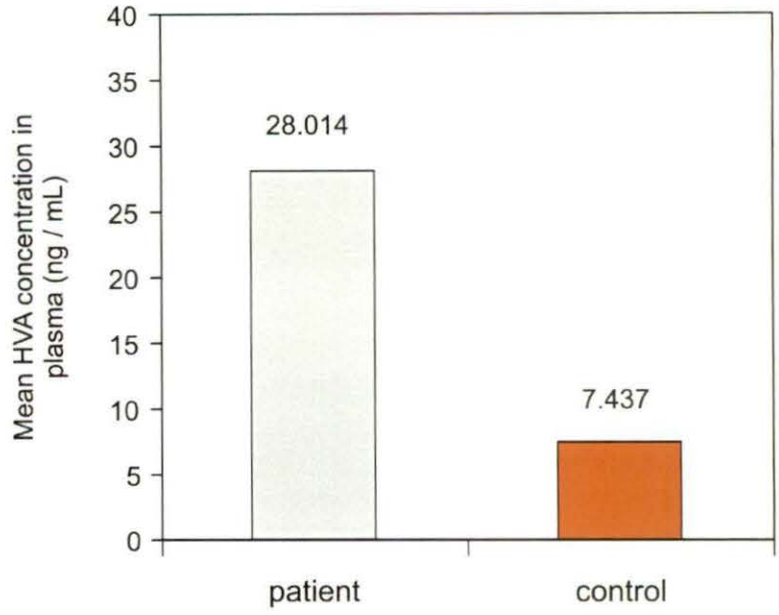

Fig. 1 : Comparison of mean HVA concentrations (ng/mL) in the plasma of both the controls and the patients

Table 1 : Plasma HVA levels (ng/mL) in both the patients and the controls

Characteristics	Patients (n = 30)	Controls (n = 30)
Range of pHVA (ng/mL)	9-95	2-20
Mean (ng/mL)	28.014	7.437
SD	19.363	5.091
Coefficient of variation	69.118%	68.448%

In contrast to these complicated results for pHVA in schizophrenia, more consistent results have been observed in delusional disorder[11]. It is suggested from earlier studies on the pHVA levels and psychosis, that consistent results has been observed in psychoses with a good prognosis, which is unrelated to their conventional diagnosis[21, 22, 23, 24]. Garver et al. (1997) have suggested that the higher pHVA psychosis is a dopamine psychosis that may have a familial origin. Our findings on pHVA in delusional disorder are

in agreement with the results of Garver et al. (1997) and Morimoto et. al (2002).

Present study had some limitations. Time of blood sampling was not strictly controlled, since OPD were included in the study. Despite the limitations of the present study the higher level of pHVA in the present finding suggests that the hyperfunction of the dopamine system, which may be at least partly responsible for the brain mechanisms underlying its delusional symptoms and could be used as state marker for the delusional disorder.

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