STUDY OF THE ROLE OF HISTOCOMPATIBILITY ANTIGENS FOR IMMUNOMODULATING THE *HOST IMMUNE RESPONSIVENESS DURING* PULMONARY TUBERCULOSIS

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This is to certify that Ms Sreejata De Sarker, MSc. worked under our guidance and preceptorship for her dissertation on the topic entitled **"Study of the role of histocompatibility antigens for immunomodulating the host immune responsiveness during pulmonary tuberculosis"** for fulfilment of the requirement of the degree of Doctor of Philosophy of the University of North Bengal.

She is conversant with the techniques and literature cited in the dissertation and carried out the work thoroughly. It seems that the thesis is fit for submission for PhD and she is worthy for award of the degree.

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Dedicated to

My Parents

My Gratitudes

Gratitude is the memory of the heart, yet I wish to express my indebtness to those who helped me in carrying out this study.

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ABBREVIATIONS

AFB	- Acid fast bacillus
BE	- Bilateral extensive
BL	- Bilateral limited
HLA	- Human leucocyte antigen
Ir	- Immune response (genes)
Is.	- Immune suppression (genes)
Ig	- Immunoglobulin
kb	- Kilobase
MHC	- Major histocompatibility complex
PF	- Phenotype frequency
PTB	- Pulmonary tuberculosis
PBS	- Phosphate buffer saline
PBL	- Peripheral blood lymphocytes
rpm	- Revolution per minute
RR	- Relative risk
TNF	- Tumor necrosis factor
UE	- Unilateral extensive
UL	- Unilateral limited

CONTENTS

INTRODUCTION

Tuberculosis is a chronic bacterial infection caused by two species of bacterium *Mycobacterium tuberculosis* and rarely by *Mycobacterium bovis* characterised by the formation of granulomas in infected tissues and by florid cell-mediated hypersensitivity. In the absence of effective treatment, a chronic wasting course is usual and death ultimately supervenes in most cases.

In the 16th century, Frascatorices first postulated that tuberculosis was caused by small particle, the *Contagium vivum* which could be carried in the air from person to person. Not everyone, however, believed that tuberculosis was a communicable disease, and there was strong support for the alternative theory that heredity was the main causative factor. But subsequent generations of physicians from Galen onwards, confirmed that persons who had been in close contact with consumptive often developed the same illness. Finally Robert Koch applied himself to this task and in 1882 the elusive microbe was identified. No other disease has challenged and occupied the greatest minds in medicine and science from Hippocrates through Koch to an unprecedented degree. This disease currently remains the largest single infectious cause of death in the developing countries. Approximately one third of world's population is infected with *M. tuberculosis* and is at risk to develop the disease. The vast majority of people suffering from tuberculosis live in developing countries and it is the cause of almost one fifth of all deaths in adults, one fourth of all available deaths and approximately 7% of all deaths in developing countries.

M. tuberculosis is generally always initiated by inhalation of infected material, rarely by ingestion and more rarely by cutaneous inoculation (Prosector's wart). The number of bacilli excreted by most infected persons is no large; however, patients with laryngeal tuberculosis, endobronchial disease, recent transbronchial spread of tuberculosis and extensive cavitary pulmonary disease are often highly contagious. Infectiousness correlates with the number of organisms in the expectorated sputum, extent of pulmonary disease and frequency

of-caugh. Most-patients become noninfectious within two weeks after the institution of appropriate chemotherapy.

The usual infecting inoculum is not more than one to three organisms which reach the alveoli. They are then ingested by macrophages and transported to the regional lymph nodes which can be identified as pulmonary tuberculosis. It may be minimal infiltrate to extensive cavitation. If spread of the organism is not contained at the level of regional lymph nodes, then tubercle bacilli reach the blood stream and wide spread dissemination ensues. Disseminated tuberculosis may result in miliary or meningeal tuberculosis. Miliary tuberculosis is apt to be more culminating in children than in adults. Lesions develop synchronously throughout the body. The appearance of these lesions suggested millet seeds; hence the name miliary tuberculosis. The leptomeninges are relatively frequently seeded by organisms which disseminate during primary infection. Pleurisy with effusion results when the pleural space is seeded with *M. tuberculosis.* There are different types of extrapulmonary tuberculosis involving different organs like kidneys, bones, meninges etc.

There are two stages in which the infection may cause clinical disease

1. Primary tuberculosis in which the tubercle bacilli invade a host, having no specific immunity; at this stage the disease most commonly heals spontaneously, but it may progress to clinical disease if immune mechanism fails.
2. Post primary or adult tuberculosis (often erroneously called 're-infection tuberculosis') which is the result of progress of infection years later in spite of specific immunity.

In 1993 WHO declared tuberculosis as a global emergency. Teeming millions are groaning today under the relentless ravage of tuberculosis. In developing countries like India especially North Eastern part of India, enjoying no exemption. The Pulmonary tuberculosis (PTB) in Gurkhas has been reported to be higher as early as in 1964 (Large). The etiology of the disease is very well known. It is due to the tubercle bacillus *Mycobacterium tuberculosis* the one of more than 30 well- characterised and many unclassified member of the genus *Mycobacterium.* The clinical presentation of the disease depends on the host immune response to the offending agents. But what is not clearly understood

that infection with *M. tuberculosis* does not necessarily develop overt tuberculosis. It can infect a host and then be immediately eliminated (no infection), become dormant indefinitely inside the host (latent infection) or cause disease soon (primary tuberculosis) or many years (reactivation tuberculosis) after the infection. Only a small portion of individual infected with *M. tuberculosis* develop disease and most of those infected remain disease free. Following *M. tuberculosis* infection of the immunologically normal host, there is a 5% risk of developing tuberculosis during the first year and a 5% additional risk spread over the lifetime of the host (Centre for Disease Control, 1992).

After a continuous decline for over 70 years, in the mid 80's tuberculosis emerged in epidemic form hastened by the onset of AIDS. WHO predicts 90 million new case of tuberculosis will occur between 1990-2000. 30 million people are expected to die of tuberculosis by the end of this decade. Aging, diseases such as diabetes mellitus and HIV infection, and our socio-economic condition are some of the factors which are responsible for weakening the immune system of the infected individuals and tip the balance in favour of microbe. Unravelling this is of great importance both for understanding the immune mechanisms in PTB and developing future immunological strategies to control it, especially on the face of emergence of drug-resistant forms of the bacillus.

The unique feature characterising human tuberculosis is its pathogenesis. The pathogenesis of tuberculosis involves cell-mediated immune responses against *M. tuberculosis*. The cell involved in tuberculosis immunology and pathogenesis include mainly T cells (α/β, γ/δ, CD4, CD8), macrophages and secondarily B cells. T cells play a crucial role because they constitute the element which trigger the subsequent immune events. Tuberculin skin testing is the simple method to see whether an individual is infected and sensitized with *M. tuberculosis*, is clearly a proliferation of T cells subsets. In patients with PTB, PPD skin test are negative at the time of diagnosis in 20-25% of cases. Even when the reactions are technically positive, there often barely exceed the cut point.

Hypergammaglobulinemia is a well described concomitant of tuberculosis.

So, we can't ignore the role of B cells also. Tuberculosis pleurisy also presents an interesting phenomenon from an immunological point of view.

The role of genetic factors as determinants of the risk of tuberculosis has been established in humans by virtue of twin studies and in murine models by cloning of the gene for BCG resistance. The Human Leucocyte Antigen (HLA) is a group of genes where lurk some crippling diseases like ankylosing spondylitis, SLE, rheumatoids arthritis, tuberculosis and may be many other diseases which are still in dark.

HLA, the small part of chromosome number 6, comes in two stretches, called Class I and Class II. The class one antigen consists of 3 loci HLA-A, B and C. Antigen defined by these loci are expressed mostly on the cell membranes of leucocyte. The Class II loci specifies antigen on B cells.

T cell response to proteins is directed against different epitopes of an antigenic protein. The T cell will target which epitope of the antigen depend upon the gene product of the patient through Ir genes (Beneceraff *et al*, 1972). So an exact definition of the influence of HLA- linked genes is delineation of T cell mechanisms is of critical importance. In the context of how to protect people against infection with *M. tuberculosis* and /or against developing tuberculosis, Bacillus Calmette Guerin (BCG) remains the controversial of all the currently used vaccines. If we can characterise that particularly which protein (antigen) could draw out a strong cell mediated immune response (CMI) that may be a better candidate for vaccination. While characterising a protein for T cell activity, it is important to consider the context of HLA phenotype of the subject concerned.

Reference study reveals that there is no one or two gene products which are universally responsible for the disease and moreover, not a single datum is available on the HLA antigen frequencies in the population of Eastern and North Eastern India.

Justifications for taking this study :

1. According to the report of WHO tuberculosis is an epidemic in the Eastern -Hill Region of India specially Darjeeling district.

2. Incidence of the disease is fairly high (in a survey conducted by the author)
3. The population studied consisted of many endogamous, inbreed caste groups with desperate genetic make up, both in anthropological, anthropometric and HLA polymorphism.

Thus it is appropriate to study a population which is an admixture of different major groups and wherein most of the individuals have been exposed to tubercle bacilli. Furthermore, the knowledge of HLA association promise new ways of treatment when the era of molecular medicine arrives.

In view of the above in the present investigation different parameters have been studied.

1. To evaluate the association of HLA Class I and Class II antigens in the patient with pulmonary tuberculosis and their various clinical groups and pleural effusive patients;
2. To understand the role of T cells/macrophages in the pathogenesis of pulmonary tuberculosis;
3. To investigate the incidence of the types and sub-types of lymphocytes of pleural effusion;
4. To evaluate the degree of antibody mediated immune response by quantitative estimation of immunoglobulins like IgM and IgG; and
5. To evaluate the degree of cell mediated immune response *in vitro* by using PHA.

REVIEW OF LITERATURE

2.1 Epidemiology :

Human existence since the mutation of *M. tuberculosis* is too short for completion of even one full epidemic cycle. So instead of a single world wide epidemic, there have been many concurrent epidemics of tuberculosis (TB) of varying duration. An inhibited TB epidemic generally reaches its peak in a new host population within 50-75 years after onset and then shows a steady but much slower decline as the more resistant survivors reproduce. The rate of natural decline in incidence is generally 1 to 2 percent per year.

TB first become an epidemic disease in the crowded condition of the industrial revolution. As shown in figure (Fig. 1) Grigg constructed a theoretical plot of TB. Mortality and morbidity peaks. After about 300 years each epidemic has run its course although the incidence never falls to zero (ER Grigg, 1958).

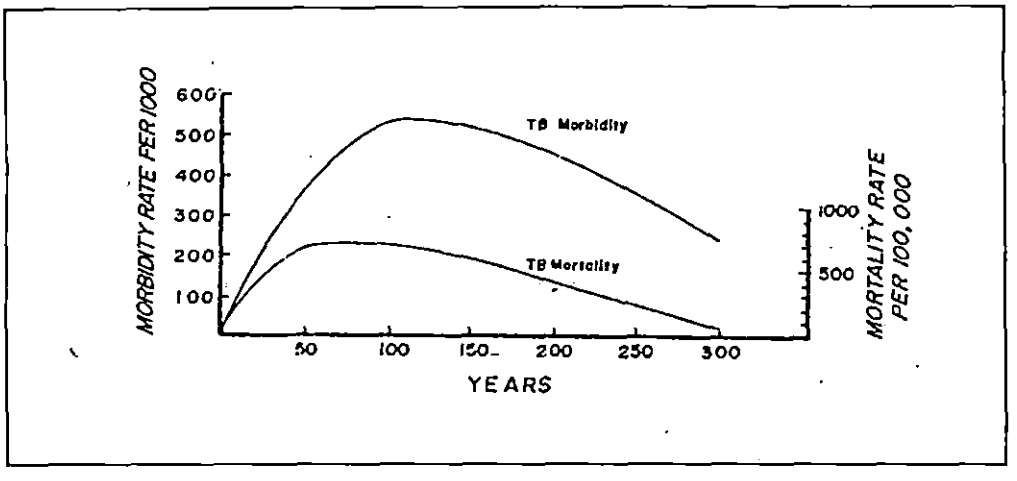


Fig. 1. A theoretical plot of tuberculosis morbidity and mortality after the disease is introduced into a population having no previous experience with it.

Events such as the discovery of tubercle bacillus as the etiologic agent, the use of tuberculin skin testing, the development of the chest radiograph, the sanatorium concept movement, the wide spread use of Bacille Calmette Guerin (BCG) vaccination, the two world wars and the advent of effective chemotherapy

have minimal impact on the shape of the curve. A guide to determine the stage of the epidemic the disease take a heavy toll among children and young adults. As the epidemic matures, the at-risk groups shift gradually to older persons. In most Western countries before the HIV epidemic, TB had become mainly a disease of older people.

The total number of case recorded in South East Asian Region (SEAR) in 1993 was 1,623,980 with a prevalence rate of 89.8 per 100,000 for the region as a whole. In 1992, the total number of cases recorded was 2,116,006. Over the years, there has been a resurgence of TB is posing a mounting threat in the region. The table (1) presents the tuberculosis scenario in the region.

Table 1. Tuberculosis in SEAR, 1993

Country	No. of cases recorded	Prevalence rate (per 100,000)
Bangladesh	54001	44.2
Bhutan	4259	266.9
DPR Korea	-----	-----
India	1412970	156.7
Indonesia	62966	32.4
Maldives	175	74.8
Myanmar	20207	47.2
Nepal	13161	62.4
Srilanka	6573	36.7
Thailand	49668	87.3

Source : **WHO/SEARO, 1993**

The acquired immunodeficiency syndrome (AIDS) epidemic has been added a new dimension to the problem of TB. Using TB notification data and HIV seroprevalence information with estimates of future trends, the WHO has projected the morbidity and mortality of TB world wide and the relationship to HIV infection. An overall increase in total TB cases from 7.54 million in 1990 to 8.77 million, 10.22 million, and 11.87 million in 1995, 2000 and 2005 respectively, is predicted. This presents an increase of 57.6% over 15 years (Fig 2). WHO also estimated that total TB deaths will increase by 38.7% from 1990 to 2000 but those TB deaths related to HIV will rise by 33.1%. The percentage of HIV attributable TB deaths was estimated to increase from 4.6% in 1990 to 14.2% in 2000 (WHO, Tuberculosis Programme, 1992).

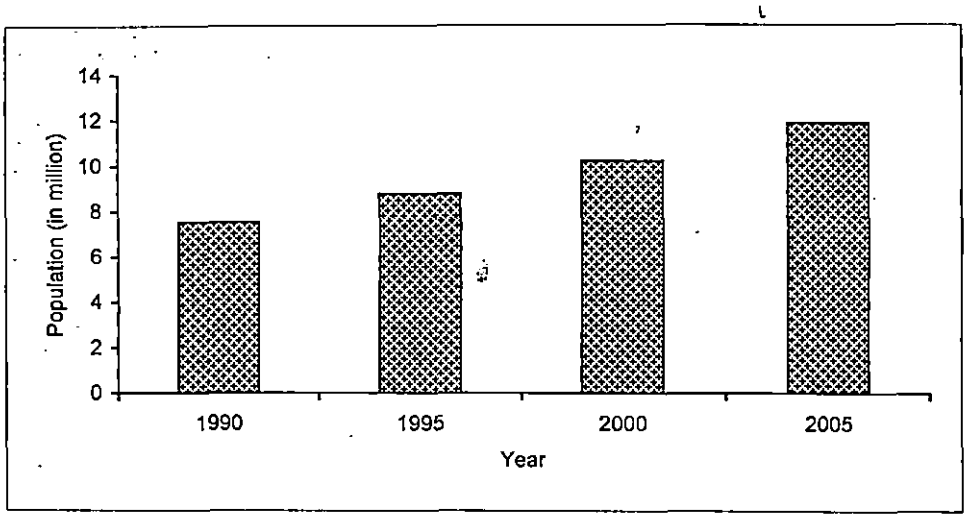


Fig. 2 WHO projected percentage increases in T.B. cases compared with 1990.

2.2 Historical Background

2.2.1 Pulmonary Tuberculosis

Tuberculosis is an ancient disease traced in neolithic and paleolithic era (Bartels,1907). The disease which termed 'Leoping' in China is similar to TB. Rikveda described the disease as '*Yokshma*'. Sushtuta in *Sushruta Samhita* found difficulty in curing the disease.

In ancient Rome Celsus made a hall mark in diagnosis and treatment of the disease. It is a matter of interest that even in recent times another Roman doctor Carlo-Formalina came forward with the idea of collapse therapy in the form of 'Artificial Pneumo-thorax'.

In 7-8th century AD an Arab physician described the disease in great detail. The sanatorium conception appeared in the 19th century. Gideon Harvely and Richard Morton are the two names specialised in pthisiology at that time. During 19th century Rene Theophile Hyacinthe Laennec (1781-1826) truely laid the foundation of our present day knowledge.

In 1882 another major breakthrough occurred when Robert Koch reported the isolation and culture of tubercle bacillus and successful reproduction of the disease in animals by these isolates.

In 1947 streptomycin, an effective antitubercular drug established. In 1953 Isoniazid established which is a higher order drug than former and TB became medically curable in most cases.

2.2.2 Pleural Effusion

Pleural effusion is a clinical diagnosis which does not denote any hints as to its etiology and prognosis. The disease entity of pleural effusion is known from ancient times as has been aptly mentioned in several historical medical literatures in India and abroad. In our country though Ayurveda did not

directly mentioned about this disease yet while described the effectiveness of a drug named 'Kalyansundara Rasha'. It was said to be effective in 'URASTOA' (Urak means chest and Toa - water). Famous 'Kabirajans' DN Sengupta and UN Sengupta later on compiled in one of the 'Sangrahans'.

The disease was mentioned in ancient Egyptian text book of medicine 'Ebers Papyrus' (1553-1650 BC). Hippocrates (460-355 BC) also knew this condition and called them 'peripneumonia'. This term is in vogue for over 2000 years.

Thomas Sydenham (1624-1689) regarded 'peripneumonia' the same as pleurisy except that it effected lungs more extensively.

The different notes on precaution was first discovered by Leopold Aunbruggar (1722-1809) in 1761 but its clinical importance was unaccepted for nearly 50 years.

Rene Theophill Hyacinthe Laennec (1781-1826) first by his epoch making discovery of stethoscope gave our modern conception of pneumonia, pleurisy, PTB and other different lung infection.

It was Joseph Skoda in 1839 who added another useful diagnostic point in his work on percussion and auscultation, in the form of 'Skodial Resonance' was described in relation to lobar pneumonia and over air containing upper lobe above a lower consolidated one or above the fluid level of effusion.

Bowditch, WHI (1808-1892) is the first man to tap pleural effusion by means of a pump (invented by Eyman) and trochar and cannula in 1852 (Castiglioni).

2.3 Influencing factors

2.3.1 Age and Sex

Infants and young children of both sexes are prone to develop tuberculosis. Though in industrialised countries tuberculosis is mainly an older man disease (Horne, 1996), but in developing countries the vast majority of cases occur between the ages of 15-59 years, the most economically productive individuals in society.

2.3.2 Socio-economic condition

The association between unprivileged section of the society and tuberculosis is well known. (Spence *et al*, 1993). Poor housing conditions and overcrowding have been reported to faster the development of tuberculosis.

Nutritional Status

Poor nutritional status is believed to be liable for tuberculosis (WHO, Tuberculosis Programme, 1992). But exactly how and which dietary elements (proteins, vitamins, micronutrients) favours the development of the disease is not very clear. Recently, experimental studies have led credit to an important contributory role of protein undernutrition (Mc Murray and Bartow, 1992).

Living Accommodation

Air borne transmission of *M. tuberculosis* from infectious patients to susceptible hosts is obviously enhanced by unhygienic and crowded living conditions (Barry, *et al*, 1986)

Occupation

Workers with silicosis (Snider, 1978) and medical professionals and laboratory of microbiology personnel (Sugiat, *et al*, 1990) have a increased rate of tuberculosis.

2.3.3 Cigarette Smoking

Studies have shown that cigarette smokers are more prone to tuberculosis than nonsmokers (Horne, 1996).

2.3.4 Other Diseases

Patients taking immunosuppressive drugs reported to have an increased likelihood of developing clinical tuberculosis. Patients suffering from diabetes mellitus, hypothyroidism and also the patients underwent gastrectomy are at increased risk of tuberculosis, although the mechanism underlying their susceptibility are unknown (Murray *et al*, 1990). It has been suggested that emotional factors may contribute to the development of clinical tuberculosis apart from AIDS.

2.4 Immune Responsiveness

2.4.1 Pulmonary Tuberculosis

PTB is first an infectious disease, however, PTB is also an immunological disease which makes its pathogenesis so complicated. Without this immune response by the host, human PTB might have been a more easily controllable disease. So it is about fourty years that the scientists are trying to find out the path of regulation of human immune response in tuberculosis.

Antibody Level

The levels of IgG, IgA and IgE in PTB patients are increased. The patients bearing B18 and B14 antigens tend to show significantly low levels of IgG which suggest a possible genetic influence on the expression of immunoglobulin levels (Paphia *et al*, 1985). The levels of IgG antibodies to PPD are also indicative of favourable and unfavourable processes of pulmonary tuberculosis. In addition to IgG and IgE, IgM is also increased in patients as well as household contacts (Ellner *et al*, 1996). Very recently it has also been suggested that elevated level of anti-IgG antibodies in PTB patient show a relation of it with infection/exposure to *M. tuberculosis* (Mehra *et al*, 1996).

T cells

T cell mediated DTH response is responsible for the pathogenesis of PTB. In case of the PPD stimulated activation of T cells, not only the specifically PPD sensitized T cells but also a large number of non-specific T cells are recruited and involved in the proliferation (Tsuyuguchi *et al*, 1982). Recently it is reported that combination of interleukin 2 (IL-20), tumor necrosis factor alpha (TNFα) and IL-6 activated naive (CD 45RA+) and memory (CD 45RO+) resting CD4+ T cells to proliferate (Unutmaz *et al*, 1994). It is confirmed by getting CD4 and CD8 cells from TB patients (Ellner *et al*, 1996). Later it is revealed that PPD induced proliferating lymphocytes belonged to CD4+ and not CD8+ lymphocytes (Shiratsuchi *et al*, 1984) The existence of suppressor macrophages in peripheral blood mononuclear cells from tuberculous patients with low tuberculin response was also found (Ellner, 1978).

2.4.2 Pleural Effusion

The traditional explanation for the development of tuberculous effusion is that a small sub pleural focus of *M. tuberculosis* ruptures into the pleural space, setting up an interaction between the live bacilli and sensitized CD4+ T lymphocytes. The clinical syndrome is a reflection of an *in situ* delayed type reaction in which plasma protein exude into the pleural space and CD4+ helper T cells accumulate, proliferate and produce and release inflammatory mediators (Ellner *et al*, 1988).

A remarkable increase in percentage of autologous erythrocyte resetting T cells was also demonstrated in pleural fluid after stimulation *in vitro* with PPD (Tsuyuguchi *et al*, 1982). Several studies demonstrated that the T lymphocytes found in the pleural fluid of patients with tuberculous effusion are specifically related to PPD of *M. tuberculosis* and produce interferon when cultured with PPD (Fujiwara *et al*, 1982). This is in contrast to peripheral blood lymphocyte from the same patient that hyporesponsive when cultured in the presence of PPD. It is suggested that in pleurisy patients PPD reactive T cells in peripheral blood did not decrease in activity but were depressed by suppressor cells (Fujiwara *et al*, 1982). Indeed the occasional observation of cutaneous anergy to intradermal infection of PPD in patient with tuberculous pleural effusion has been explained by the preferential sequestration of antigen-specific T cells in the pleural space (or by the presence of suppressor cells in the blood) (Ellner *et al*, 1988). Enumeration of lymphocyte subsets in tuberculous pleural effusion has revealed a range of CD4+ T cells with ratios as high as 65% and CD8+ T cells with a ratio upto 19% (Shimokata *et al*, 1986).

IFNγ production was attributed to CD4+ T cells in one study (Shimokata *et al*, 1986). Subsequent studies confirmed high levels of IFNγ in tuberculous effusion, but did not find a correlation with the distribution of lymphocyte subsets (Ribera, E. *et. al*, 1988) and did not find statistically significant higher CD4/ CD8 ratio in tuberculous effusion (Gugman *et al*, 1989).

More recently, memory T cells (CD4+, CDw29+T) from tuberculous

effusion were shown to produce IFNγ specifically in response to PPD of *M. tuberculosis* (Shimokata *et al*, 1986) but not when stimulated by an irrelevant antigen. Additionally, IFNγ levels are markedly elevated in the pleural fluid, suggesting local production of the lymphokine (Ribera *et al*, 1990).

PPD stimulated release of IL-1 is higher in pleural TB (Kurasawa, T. *et al*, 1990).

Elevated NK cell activity is found in tuberculous effusion with increased number of IL-2 receptor being expressed on CD16+ cells in the effusion. Thus it was believed that IL-2Rs played an important role in activation of NK cells (Ota *et al*, 1990). High NK cell activity was also confirmed in tuberculous pleural effusion by Okubo *et al*, in 1988.

Cytotoxic T cells are thought to be important in the eradication of *M tuberculosis* infected macrophages and generation of PPD specific cytotoxic T cells at the site of pathology (Lorgot *et al*, 1992).

That tuberculous pleurisis represents a resistant response manifested by mild disease is supported by the demonstration of expansion of the γ-δ-+T cell population which produces IFNγ, granulocyte-macrophage colony-stimulating factor, IL-3 and TNFα, cytokines that stimulate macrophages and may enhance mycobacterial elimination (Barnes, 1992).

Transforming growth factor β (TGF-β) plays an immunosupressive role in inflammatory response and may be responsible for regression of granulomatous inflammation in tuberculous pleurisy (Maeda *et al*, 1993).

Determination of anti mycobacterial antibody in tuberculous pleural effusion has been shown to have potential diagnostic utility. However, passive diffusion of antibody into pleural space, and occasional phase positive results due to malignant limit the utility of such tests (Levy *et al*, 1990).

2.5 HLA System

The Major Histocompatibility Complex (MHC) is a large cluster of closely linked genes on a chromosome, responsible for encoding characteristic cell surface glycoproteins and provides a strong barrier to allotransplantation. The cell surface glycoproteins defined by the MHC are referred to as major histocompatibility antigens; they differ between member of a species and are thus called alloantigens.

Around 1937, the MHC coded antigenic system was first described in mouse (now known as H2 complex) by Gorer in England, after its existence has been predicted by Haldane. The term 'Major Histocompatibility Complex' was first introduced by Snell and his collaborators in 1948 to distinguish between gene(s) responsible for acute (quick) rejection of allogenic tissues and tumor grafts, from those which are associated with chronic (slower) rejection of allogenic tissue grafts and usually do not cause rejection of tumor grafts.

The history of HLA system in man is equivalent to the H2 complex in mouse began with the description of an antigen MAC (totally HLA A2 + A28) by Dauset in 1958. HLA alleles were initially recognised by the human alloantibodies that appear following multiple blood transfusion in which the recipient lacks the antigen present on the donor's leukocytes; in pregnencies where the mother differs in HLA with the father; multiparous woman having the same husband; volunteers immunised with a skin graft and a booster injection of buffy coat cells from donors differing from the recipient only by one antigen. The definition of the antigens determined by the different loci of the HLA complex was initially made by the leukoagglutination test with these antisera; currently they are defined by a microlymphocytotoxicity test where lysis of specific antigen bearing leukocytes in a small number in presence of the antiserum and complement is examined.

The pioneering work of an international group of investigators like Dausset, Bodmer, Batchelor, Ceppellini, Payne, Terasaki, Amos, Kissmeyer-Nielsen, Van Rood, Walford formulated the basic genetic model of the HLA system. One of

the turning point in the history of HLA system was in the beginning of 1964 when productive international collaboration in the form of International Histocompatibility Workshops (IHWS) was started. Presently 12 such Workshops have already been completed.

The HLA defining gene complex present on the short arm of chromosome number 6 in the 6p 21.3 band (Lamm *et al*, 1985). It is encoded by genes that span a region about 4000 kb or 4 x 106 nucleotides (Hardy, 1986). A large number of genes with variable expressions and functions arranged in the form of three regions : Class I (36 genes), Class II (27 genes) and Class III (39 genes) (Campbell and Trousdale, 1993) (Fig. 3).

2.5.1 HLA Class I Loci :

This loci are analogous to the K and D loci of the mouse H2 and specify antigens that are present on almost all cells and are responsible for eliciting strong allograft reactions. Although 20 - 30 genes have been defined in the class I region, HLA-A, -B and -C are the only products which are expressed, rest all being pseudogenes. Other human Class I genes with less defined gene products have been identified like HLA-E, -F,-G, -H, and -J (Fig. 4). Table 2 gives a recent listing of the recognized class I alleles as defined by serology. The Class I antigens consist of peptide chain of 44,000 dalton and is non-covalently linked to a 11,500 dalton β2 microglobulin molecule encoded by a gene in chromosome 15 (Ploegh *et al*, 1981). Class I heavy chain is folded into 3 globular domains, each of which contains stretches of about 90 amino acids α1, α2 and α3. The amino acid sequence differences causing rich polymorphism of HLA Class I molecules are found in 7 variable regions in α1 and α2 domains corresponding 9-12, 40-45, 62-83, 94-97, 105-116, 137-163 and 174-194 (Lopez de Casro *et al*, 1985). The two α helix and 8 antiparallel β pleated strands α1 and α2 domain interact to form antigen binding groove (Bjorkman *et al*, 1987) The α3 domain non covalently associated with β2 miroglobulin and constitute 8 antiparallel β strands which supports the α1 and α2 domains as a platform. Alleles at A locus are usually indicated by numbers like HLA-A1, -A2, -A3. The distribution of HLA antigen is distinctive for certain racial groups and can serve as anthropologic markers in the study of migration pattern and disease. It can be mentioned here that since chromosomes are paired, each individuals have six serologically defined

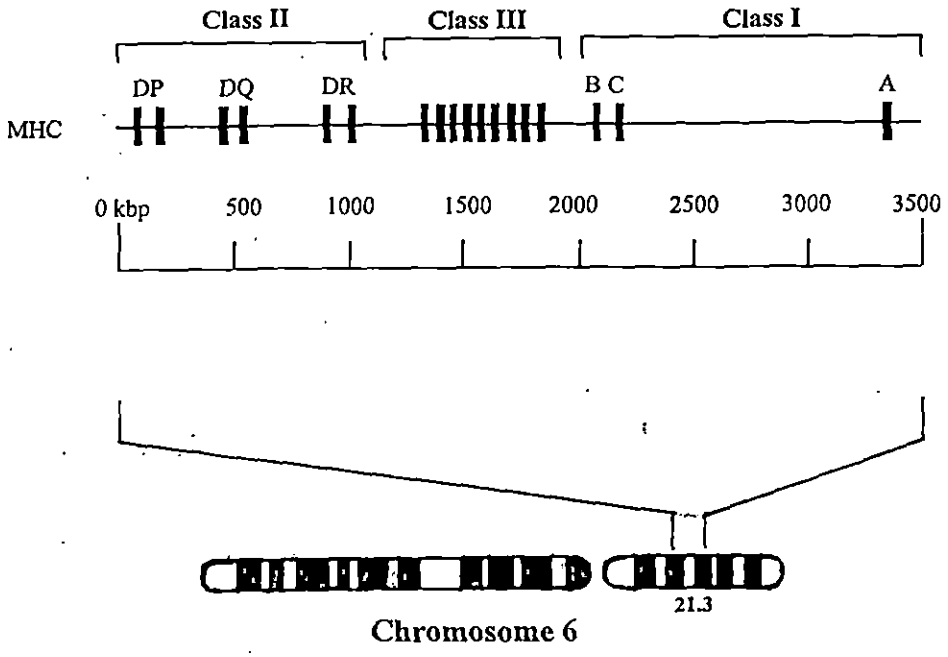


Fig. 3. Schematic presentation of human chromosome 6 (bottom) with Giemsa light and dark band shown in white and black, respectively. The chromosome segment in the 6p 21.3 band corresponding to the MHC is shown enlarged in the middle. The extent of class I, class II and class III region is marked, the genes are represented by bars. The HLA antigens (class I and Class II encoded from MHC genes are shown in top.

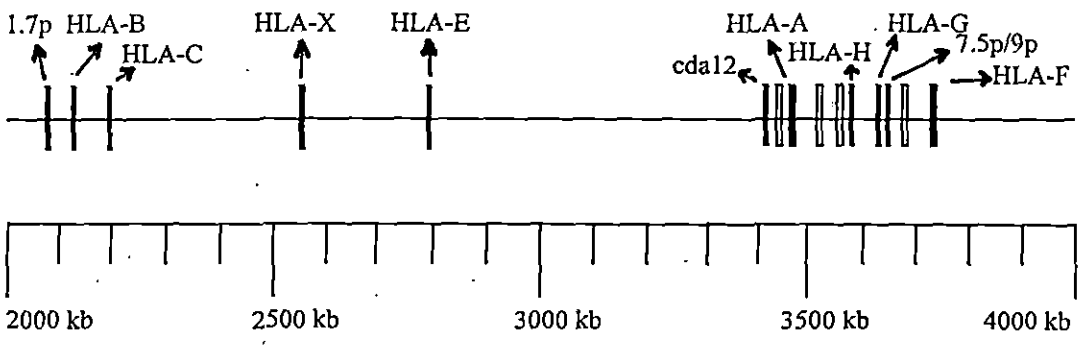


Fig. 4. Molecular map of the MHC class I region. Apart from three classical class I genes HLA-A, HLA-B, HLA-C, several new loci designated as HLA-E, -F, -G are also shown. HLA-X have been newly described. The hollow bars represent pseudogenes.

Table 2. Serological specificities of HLA-A, -B and -C alleles.

HLA-A		HLA-B		HLA-B		HLA-C	
A1		B5		B21		CW1	
A2			B51		B49	CW2	
A3			B52		BW50	CW3	
A9		B7		BW22			CW9
	A23	B8			BW54		CW10
	A24	B12			BW55	CW4	
A10			B44		BW56	CW5	
	A25		B45	B27		CW6	
	A26	B13		B35		CW7	
	AW34	B14		B37		CW8	
	AW66		BW64	B40		CW11	
A11			BW65		BW60		
AW19		B15			BW61		
	A29		BW62	BW41			
	A30		BW63	BW42			
	A31		BW75	BW46			
	A32		BW76	BW47			
	AW33		BW77	BW48			
	AW74			BW53			
A28		B16		BW59			
				BW67			
	AW68		B38	BW70			
	AW69		B39		BW71		
		B17			BW72		
AW36			BW57	BW73			
AW43			BW58				
		B18		BW4			
				BW6			

Specificities listed on the left hand side of each column represent the broad specificities. The narrower specificities or 'splits' are shown on the right hand side of the column eg A9 (broad specificity) has two 'splits'; A23 and A24.

Usually there is a tight linkage of the multiple alleles that are inherited together from one generation to the next. However, sometime in about 0.8% of children undergo recombination which corresponds to about 1/200 gamets, allele at HLA -A and -B undergo recombination which is even much rarer between HLA -A, -B and -C loci.

2.5.2 HLA -D (Class II) Loci :

The HLA-D region is centromeric to Class I loci. There are at least 6 sub regions DR, DQ, DP, DO, DN and DM of the class II molecule. Each molecule is made up of a 34,000 dalton α chain and a 29,000 dalton β chain. There are different types of genes for a particular chain. The molecular map of HLA class II region is shown in Fig 5. List of HLA -D region specificities have been shown in Table 3.

DR sub-region contains 9 DRB genes (DRB 1-9) and only one invariant A gene. DRB2, DRB6, DRB7, DRB8 and DRB9 are pseudogenes without a first exon (Rollini *et al*, 1987). DRB1 is the most polymorphic that contains the most classical DR alleles. DRB3 and DRB4 are products of separate loci that code for DR52 and DR53 specificities respectively (Gorski *et al*, 1987). DRB5 encodes HLA-DR2 associated with allelic subtypes. DRB2 is in association with DR52 group haplotypes. DRB6 is found with DR1 and DR10 haplotypes. It is also found on all DR5 group haplotypes. DRB7 and DRB8 are found in association with DR53-group haplotypes. DRB9 probably on all haplotypes.

DQ subregion contains five genes, DQA1, DQA2, DQB1, DQB2 and DQB3 of which DQA2, DQB2 and DQB3 are not known to be expressed because no protein or mRNA product has been defined in them. In contrast both DQA1 and DQB1 are functional and polymorphic.

Three new subregion DO, DM and DN have been named recently lying between the DQ and DP loci. The extent of polymorphism in these two loci is not too well known yet.

Two A and two B sets of genes for DP subregion exists and extensive polymorphism confined only to DPB1 and DPA1 displays limited polymorphism.

Table 3. Serological specificities of HLA-DR, -DQ and -DP alleles.

Serological specificities	T cell defined specifities
DR1	Dw 1, Dw 20
DR2	Dw 2, Dw 12
DR17 (3)	Dw 3
DR18 (3)	Dw 'RSH'
DR4	Dw 4
DR11 (5)	Dw 5
DR12 (5)	Dw 'DB6'
DR13 (6)	Dw 18, Dw 19, Dw 'HAG'
DR14 (6)	Dw 9, Dw 16
DR7	Dw 17, Dw 'DB1'
DR8	Dw 8.1, Dw 8.2, Dw 8.3
DR9	Dw 23
DR10	----
DR51	Dw 21, Dw 22, Dw 2, Dw 12
DR52	Dw 24, Dw 25, Dw 26
DR53	Dw 4, Dw 10, Dw 13, Dw 14, Dw 15, Dw 17, Dw23
DQ5 (1)	Dw 1, Dw 21, Dw 9
DQ6 (1)	Dw 12, Dw 8, Dw 2, Dw 18, Dw 'FS', Dw 19
DQ2	Dw 3, Dw 7
DQ7 (3)	Dw 4, Dw 5, Dw 8, Dw 13
DQ8 (3)	Dw 4, w 10, w 13, w14
DQ9 (3)	Dw 23, w 11
DQ4	Dw 15, Dw 8, Dw 'RSH'
DPw1	
DPw2	
DPw3	
DPw4	
DPw5	
DPw6	

All three sets of Class II molecules are expressed on B lymphocytes and activated T lymphocytes, where 60-90% monocytes do not express DQ antigens but all of them are DP and DP positive.

2.5.3 HLA Class III Loci

At least 39 genes are encoded within a 100 kb segment between the HLA-B and HLA-DR region. This includes complement genes C4, C2 and Bf (Fig. 6). The order of complement genes in HLAIII region is uncertain. No crossovers have been found between the C2, Bf and C4 loci. The two genes for the tumor necrosis factor (TNF α, β) lie between HLA-B and the complement genes. Apart from these there are also G7a (valyl t RNA synthatase) and Cγp 21B (steroid 21-hydroxylase) genes which are not associated with the TNF genes, BAT2 (G2) and BAT3 (G3) have been describes.

Klein (1987) has argued that the central region of the MHC has no structural or functional correlation with the Class I or Class II regions and hence it should not be considered as a part of the MHC. However, interestingly certain haplotypes containing fixed alleles of Class I and Class II region carry specific central region alleles i.e. extended haplotypes or supratypes.

2.5.4. HLA Polymorphism

An important feature of the HLA system that sets it apart from other genetic complex systems is the extraordinary high polymorphism that exists in it. It provides an exception inter-individual variable HLA antigen profile. The high degree of polymorphism in HLA appears to have resulted from recombination and exchange of genetic material between alleles of the same locus and also from point mutations and other genetic events. Theoretically several million genotypic combinations (approximately 150 billion or even more) are possible in the HLA system. According to Klein (1987) such a polymorphism is not only advantageous for an individual, but even more for the survival of the species surrounded by many different and often changing pathogens. Thus the polymorphism is probably essential for efficient functioning of the system.

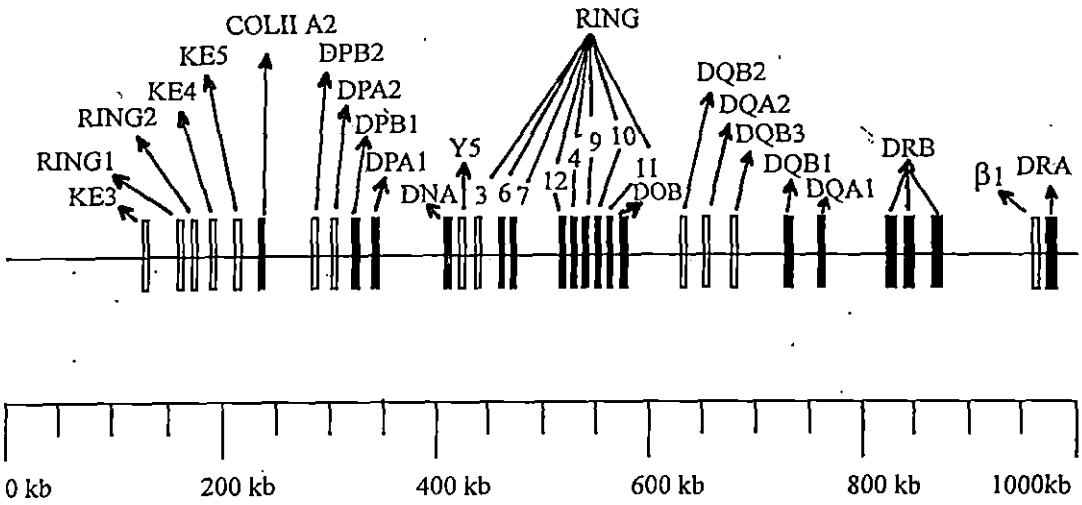


Fig. 5. Molecular map of the MHC class II region. The number of DRB genes vary within the haplotypes. The hollow bars represent pseudogenes.

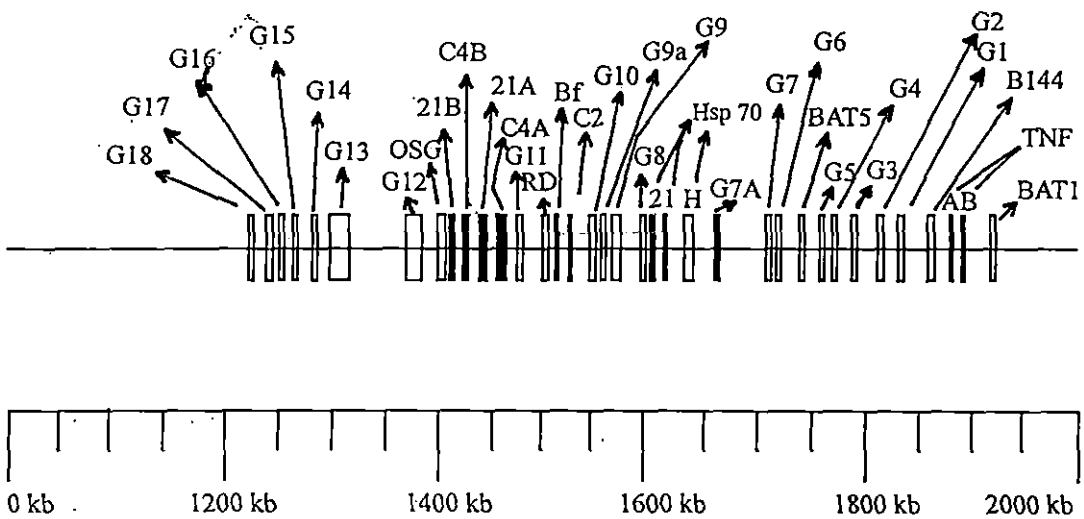


Fig. 6. Molecular map of the MHC class III region. Pseudogenes are indicated by open boxes, expressed genes by black boxes. Number of C4 and CYP21 genes vary between haplotypes.

2.5.5 HLA and Linkage Disequilibrium

Certain combinations of alleles in different HLA loci occur in much higher frequencies than expected. For example, the haplotypes of HLA-A1 and HLA-B8 are expected to have frequency equal to the product of their gene frequencies i.e. 0.15 x 0.1 = 0.015. However, in reality, the haplotype A1, B8 is observed to be 0.079, which is significantly more than the expected frequency. Certain other haplotypes like this are A2, B12; A3,B; A29, B12, A11, Bw35; Dr3, B8 etc. This unusual and preferential association between alleles of different loci is called linkage disequilibrium and is expressed in terms of delta.

The reasons for linkage disequilibrium are not yet known. This may be due to the evolutionary selection for some species or due to the reproductive isolation of population with different proportions in their gene pool. As the recombination frequency is very low (0.8%) once a linkage disequilibrium is established it will take a large number of generations to approach equilibrium. This phenomenon suggest that these combination of genes represent preserved ancient or ancestral haplotypes.

2.5.6 Biological Significance of MHC

The first indication that MHC could play a key role in the regulation of immune response came from the studies of Beneceraff and co-workers (1967) in guineapigs using haptane derivatives of Poly L-lysine. Mc. Devitt *et al*, (1972) mapped the actual position of Ir gene within the murine MHC in a series of experiments using H2 recombinants. The term immune response (Ir) gene was introduced by Beneceraff and Mc Devitt (1972). Later, Rosenthal and Shevac (1973) using a group of a-glutamic acid plus lysine (GL) and L-glutamic acid plus L-tyrosine (GT) demonstrated that the immune response in the guineapig is under Ir gene control. Recently studies concerning immune responsiveness to streptococcal cell wall antigen demonstrated that Ir genes were actually linked to HLA (Sasazuki *et al*, 1980).

Doherty and Zinkernagel (1974) further implied that cytotoxic killing of virus infected isogenic cells is possible when cytotoxic T cells (CTCS) and the target cell expressed the same H2 haplotype. These studies revealed that cytotoxic

T cells could recognise a virus infected target cell only in the context of class I molecules, a phenomenon called 'MHC restriction'. The initiation of cellular and humoral responses requires successful interaction between T lymphocytes and antigen presenting cells (Feldman *et al*, 1979). While class I MHC molecules are important for the presentation for foreign peptide to CTL and suppressor T cells, helper T cells recognised the antigen only in association with class II molecules (Meurer *et al*, 1984). The fact that class II histocompatibility molecules themselves act as immune response factors by binding and presenting the processed antigens to T cells led to the demonstration of association of disease with HLA linked genes.

2.5.7 HLA and Disease Association

This concept was originally suggested by Amiel in 1967 (Amiel, 1967) for Hodgkin's disease. He demonstrated a HLA association between an antigen 4C (now B5 + B35 + B18 + B15) and Hodgkin's disease. It opened a new vista in HLA studies. The search for such association increased in an exponential number. Table 4 gives some of the most significant association between HLA antigens and diseases.

2.6 Genetics of PTB

There are several lines of evidence indicating the existence of host genetic factor(s) regulating disease susceptibility or resistance to the disease. Different races have different susceptibility/resistance to the disease.

PTB has been reported to occur more commonly in twins even when living apart (Kallmann *et al*, 1943). In house hold contacts tuberculosis is more likely to occur in siblings than in husband to wife and vice versa latter is in much physical contact (Emerson, 1960).

Trobridge in 1956 showed that negative persons are more susceptible to tuberculosis than Rh+, though others have failed to confirm the observation (Lewis and Woods, 1961; Kothari, 1959; Shenoy and Daftary, 1962).

Table 4. Some significant HLA and disease association

Disease	Race	Studies N	Patients No	%+	Control (%+)	RR
Rheumatoid Arthritis						
B27	C	17	861	16	9	2.0
DR4	C	17	1127	68	25	3.8
BW54	O	3	221	24	11	2.5
DR4	O	5	348	66	39	2.8
DR4	N	3	109	40	10	5.4
Juvenile Rheumatoid Arthritis						
B27	C	15	1146	25	9	3.9
DR5	C	5	422	34	15	3.33
Ankolysing Spondilitis						
B27	C	40	2130	89	9	69.1
B27	O	7	211	85	15	207.9
B27	N	2	33	58	4	54.4
Acute Anterior Uvetis						
B27	C	10	520	47	10	8.2
Reiter's Disease						
B27	C	25	906	80	9	37.1
Juvenile Diabitis Mellitus						
B8	C	39	4322	40	21	2.5
B15	C	36	4052	22	14	2.1
DR3	C	13	1174	46	22	3.3
DR4	C	12	1051	51	25	3.6
BW54	O	8	453	39	11	5.6
DR3	O	4	139	38	14	4.8
DR4	O	4	139	49	25	2.6
B8	N	6	337	19	11	2.4
B15	N	5	299	6	5	2.22
DR3	N	3	135	57	28	3.2
DR4	N	3	135	46	11	6.7
Graves Disease						
B8	C	18	1445	43	23	2.5
DR3	C	4	333	56	25	3.7
B35	O	3	162	42	14	4.4
Celiac Disease						
B8	C	16	696	68	22	7.6
DR3	C	5	194	79	22	11.6
DR7	C	4	137	60	15	7.7
Nacrolepsy						
DR2	C	2	45	100	22	129.8
DR2	O	1	92	100	34	358.1

Disease	Race	Studies N	Patients No	%+	Control (%+)	RR
Psoriasis Vulgaris						
B13	C	36	2579	19	5	4.1
B17	C	35	2515	19	7	5.3
B37	C	15	804	7	2	3.9
CW6	C	7	353	56	15	7.5
DR7	C	5	296	48	23	3.2
B13	O	5	336	24	8	3.3
B17	O	4	224	12	9	1.9
B37	O	3	206	20	2	8.4
CW6	O	4	262	27	4	8.5
DR7	O	2	148	10	1	7.6
Pemphigus Valguris						
A26	CJ	5	117	60	20	4.8
B38	CJ	5	117	59	21	4.6
DR4	CJ	3	62	91	32	14:6
Darmatitis Herpetiformis						
B8	C	14	498	75	22	9.8
DR3	C	4	126	82	20	17.3
Behcet's Disease						
B5	C	6	150	31	12	3.8
B5	O	6	199	68	33	4.5
Idiopathic Hemochromatosis						
A3	C	11	493	72	28	6.7
B7	C	11	493	48	26	2.9
B14	C	10	481	19	6	2.7
Sjogren's Syndrome						
B8	C	6	184	50	24	3.3
DW3	C	4	105	64	24	5.7
Systemic Lupus Erythematosus						
B8	C	17	855	40	20	2.7
DR3	C	9	316	42	21	2.6
Goodpasture's Syndrome						
DR2	C	2	25	88	27	13.8
Multiple Sclerosis						
B7	C	38	4964	37	24	1.8
DR2	C	13	1051	51	27	2.7
Myasthenia Gravis						
B8	C	12	747	44	19	3.3

N = Number of Studies
No. = Number of Patients

C = Caucasian
O = Oriental
N = Negro
J = Jewish

%+ = Percent Positive
RR = Relative Risk

Chinese with blood group 'O' have been found to be more resistant to PTB than those with other blood group. 'O' and 'AB' has also been reported in Danish patients (Viskum, 1975).

There are many reports in PTB where immune response of the host to the bacillus is responsible for clinical expression of the disease.

Antigens of MHC complex, HLA in man, play a critical role in mounting immune response against infectious organisms (Beneceraff, 1981).

2.7 HLA and PTB

It is now widely accepted that the HLA genes and its products (the HLA antigens) controls various vitally important functions of the immune system and plays a significant role in the genetic susceptibility to disease. HLA typing has had a significant clinical impact on the diagnosis and it was noted that several diseases were associated with HLA. Several studies have been under taken in different ethnic population to determine whether there is an association between susceptibility/resistance to tuberculosis and HLA antigens (Table 5).

One of the first reports of an association between the HLA antigens and tuberculosis was that by Selby *et al* in 1978 who showed that tuberculosis patients in Canada had a increased frequency of HLA-B8 antigen. Before that in 1973 I. Rosenthal *et. al*, in European Caucasian population and Takata *et al* in 1978 in Japanese population conducted association and family studies but find no significant association. Al-Arif *et al* in 1979 revealed a positive association between tuberculosis and HLA- B15 in N. American black population whereas Cox *et al* in 1982 reported a lack of association between the phenotype frequencies of Class I antigens (HLA -A, -B, -C) and tuberculous disease in a population of Mexican-Americans. Nonetheless, stronger and more consistent associations were found with Class II antigens particularly with HLA-DR2 and later in 1988 he reported that HLA-DR3 was significantly decreased in patients

to HLA DR2 or for recognition by T cell receptor. He also suggested that Class I molecule (HLA -A10, -B8) may present antigen to $CD8^+$ cytotoxic or suppressor T cells and this might contribute to the risk of developing tuberculosis following infection: the Class II molecule (DR2) may present antigen to $CD4^+$ helper or delayed hypersensitivity T cells with cytolytic functions and this might be instrumental in the developing of smear positive tuberculosis. In support of this Bothamley *et al* in 1989 showed that in Indonesian population there is a significant association between smear positive PTB and HLA-DR2 and -DQ1 while DQw3 was more strongly associated with health. Furthermore, the presence of DR2 was also associated with a high antibody titre to selected antigens of *M. tuberculosis*. In 1988 Chandanayingyong *et al* studied the HLA antigenic profile of the tuberculosis patients and he found an increase of HLA Bw46 and DR4 and a decrease of HLA B12. In 1989 Nikolalian *et al* suggested that the presence of antigen DR2 was a certain risk factor as to development of tuberculosis and its presence in tuberculous patients could indicate unfavourable outcome of the disease. In 1980s different studies on the various major groups and caste of Tamilnadu have shown appreciable differences in their HLA allelic and haplotype frequencies. Khomenko *et al* in 1990 also worked on six ethnic groups of the USSR and confirmed the idea that patients with tuberculosis has a increased frequency of DR2 and reduced frequency of DR3 in all groups and variably increased A1 in Armenian, B5 in Moldavian and Russian, B7 in Russian, B12 in Armenian and Yzbekh, B35 in Armenian and Kazakh, B14 in Kazakh, Cw4 in Armenian and decreased A2 and A3 in Kazakh and A3 in Turkman, A10 and Cw9 in Moldavian, B79 in Turkman, Cw1 in Kazakh. Recently in 1996 Pospelov *et al* also confirmed the association of DR2 and DR53 with PTB in Terminian population.

Table 5. Association between HLA Class I antigens and Pulmonary Tuberculosis

Year	Population	HLA	P values <	Reference
1973	Europian Caucasoids	NA		Rosenthal *et al*, 1973
1976	Korean	↑B12		Lee and Ko, 1976
1978	Japanese	NA		Takata *et. al*, 1978
	Canada	↑B8	0.01	Selby *et. al*, 1978
1979	North American Black	↑B15	0.0006	Al Arif *et. al*, 1978
1982	Mexican American	NA		Cox *et al*, 1982
1983	North Chinese	↓A19	0.0021	
		↑B15	0:0001	Jiang *et. al*, 1983
		↑B27	0.01	
		↑B35		
1985	Egyptians	↑A2		Hafaz *et. al*, 1985
		↑B5		
	North American	↓B5	0.046	
	Blacks	↑DR5	0.028	Hwang *et.al*, 1985
		↓DR6	0.0095	
	North Indians	↓DR2	NS	Singh *et. al*, 1983a
		↓DR6	0.05	
1986	North Chinese	↑A29	0.01	
		↑B47	0.01	
		↑DR2		
		↓DR5		
	North Indians	↑B12		
		↑B44		
		↑DR2		Mehra *et. al*, 1986b
	South Chinese	↑B44	0.01	
		↑A11	0.025	
		↑B15	0.025	
		↓Cw3	0.05	
		↑DR2		
	South Indians	↑B49	0.01	
		↑DR2		
1987	South Indian	NA		Paphia *et. al*, 1987
1988	Mexican American	↓DR3	0.01	Cox *et. al*, 1988
	Hongkong Chinese	↑DR8	0.028	Hawkins *et. al*, 1988

Year	Population	HLA	P values <	Reference
	Thai	↑Bw46		
		↓B12		Chandanayingyong *et al*, 1988
		↑DR4		
1989	Indonesians	↑DR2	0.01	Bothamley *et al*, 1989
1990	Armenian	↑A1	0.001	
		↑B12	0.01	
		↑B35	0.01	
		↑Cw4	0.001	
		↑DR2	0.01	
		↓DR3	0.001	
	Kazakh	↓A2	0.05	
		↓A3	0.001	
		↑B14	0.01	
		↑B35	0.05	
		↓Cw1	0.05	
		↑DR2	0.001	
	Moldavian	↓A10	0.05	
		↑B5	0.01	Khomenko *et al*,1990
		↓Cw9	0.01	
	Russians	↑B5	0.05	
		↑B7	0.01	
		↑DR2	0.001	
		↓DR3	0.05	
	Turkman	↑A3	0.05	
		↓B79	0.05	
		↑DR2	0.01	
		↓DR3	0.05	
	Yzbekh	↑B12	0.05	
		↑DR2	0.05	
1991	South Indian	↓A24	0.05	
		↓B52	0.05	
		↓B57	0.01	
		↓B61	0.001	
		↑A10	0.0001	Brahmajothi *et al*,1991
		↓A19	0.025	
		↑B8	0.001	
		↑B14	0.05	
		↑B17	0.025	
		↑DR2	0.001	
1996	Turinian	↑DR2	0.01	Pospelov *et al*, 1996
		↑DR53	0.001	

↑increase; ↓decrease and NA = No association

MATERIALS AND METHODS

Materials

3.1 Geographical Uniqueness

India is a vast country with wide density of population. North Bengal, a part of one of the North Eastern States of India consisting of quite a few different ethnic groups.

Geographically it can be divided into three distance zones running North to South. These are mainly as follows: a. The Hills, b. The Terai and c. The plains. The racial groups belonging to different ethnic population are composed of different elements of great diversity of different creeds, customs and even different colours.

The three principal groups of population belonging to Mongoloid origin of North Bengal and adjoining areas are 1. Hill tribes, 2. Kirati complex and 3. The Rajbansis.

Ecologically, the Hill tribes and the Kirati complex are the native of Darjeeling district and Dooars of Jalpaiguri district, dwelling mainly at altitude of 1,500 ft. to 8,500 ft and above, while Rajbansis are the permanent residents of plains of North Bengal.

Racially the different groups of population are the descendants of Mongolian stock (Dalton, 1872; Moris, 1933).

1. Hill tribes : The Himalayan people scattered among the many high river valleys. They are mainly represented by Sherpa, Bhutia and Lepcha. All these Himalayan people have their origin from the Mongolian stock. According to their linguistic position the Hill tribes belong to the Tibeto-Himalayan groups of Tibeto-Chinese family, but now they speak mostly in Nepali (Das *et al*, 1966).

a. Sherpa : They are the most famous among the Hill tribes. Despite their close affinity with the Tibetans, they feel as much Nepali as many other people. Sherpa society is divided into a number of exogamous clans like

Pankarma, Gole, Mopa etc. So a person must marry outside his or her clan, exogamous with the clan but endogamous within cast. Sometimes fraternal polyandry is also found among the Sherpas. Cross cousin marriage is not allowed.

b. Bhutia : The term Bhutia is applied to all the Tibetan races being derived from 'Bhot' the Indian name for Tibet. They don't practice the polyandry and cross cousin marriage. However, they are endogamous within their caste.

c. Lepcha : They call themselves as "Rong". Their physical characteristics stamp them as being members of the Mongolian race, while certain peculiarities of language and religions render it probable that the tribe is a very ancient colony from Southern Tibet (Dalton, 1872). Socially they are divided into different clans, a few are Sadamu, Sompome, Simick, Namchu, Rongong, Fudong, Forning, Karthack etc. and the marriage system is exogamous within clans and endogamous within caste.

2. Kirati Complex : Kirati people mainly residing in hills and few in plains, more commonly known as Rai, Limbu, Magar and Gurung form together one of the largest single ethnic groups. As many as they are further subdivided into a number of smaller clans or a tribal units.

a. Rai : The term Rai means 'headman' but over the years become the popular genetic term of reference for an entire ethnic subgroup (Bista, 1980). The social structure of the Rai tribe includes a number of clans like Chamling, Thulong, Yakha, Hamkhim etc. The clans in turn may be further subdivided into kindred. Ideally all marriages are monogamous.

b. Limbu : Limbus are the descendants of great Mongolian family and the earliest reference was made in Visnupuran (Dalton, 1872). They are further divided into different clans like Yangdanbay, Lahuti, Thabay etc. Marriage system is strictly exogamous within clan, monogamous and patrilocal.

c. Tamang : They maintain a belief that they originally came from Tibet. Tamang means Horse traders. Now a days they are well settled in Darjeeling and

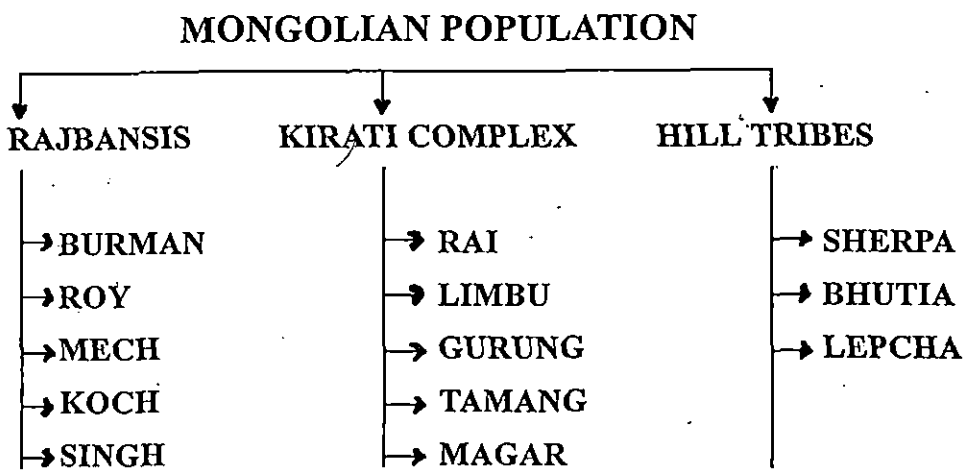
adjoining areas. The entire community of Tamang is vertically divided into several clans like Bomjan, Yonzone, Walba, Moktan, Ghising etc. Marriage is exogamous, each clan member can intermarry with any other clans except his own and mothers clan. Polyandry is absolutely forbidden.

d. Gurung : A Gurung community is broadly divided into two main groups known as 'Char-jaata' literally mean four clans and 'Shorrah- jaata' of sixteen clans. Again each are subdivided into several clans like Ghaley, Ghonde, Lamichane etc. The two groups do not intermarry under normal circumstances, they are strictly endogamous units divided strictly into exogamous clans. They are monogamous.

e. Magar : They believed their original home was Bara-Mangarant which includes a major part of hill districts of Nepal. But this is a question of dispute (Bista, 1980). Now they are well settled in Darjeeling district and adjoining areas. Magar tribes consists of number of clans like Alley, Rana, Thapa etc. Magar as an ethnic group are endogamous and exogamous within a clan. A few Magar women do marry outside the group but men unlike Chettri or Brahmins almost always marry within the group (Bista, 1980).

3. Rajbansis : They constitute relatively large and dominant group of the people living in the plains of North Bengal, namely, Jalpaiguri, Cooch Bihar and West Dinajpur. They have been offered to as 'Koch' or 'Koche' as this is said to be their historical and original home (Rishley, 1891 and Sanyal, 1973). According to Rishley (1891) and Dalton (1872), the Rajbansis have originated from Dravidian stock with suspected admixture of Mongolian blood. They are divided into different clans like Roy, Koch, Singh, Mech, Rai-Burman, Barman etc. Marriage as a rule monogamous, exogamy and polyandry by either sex is not tolerated. We tried to include all the groups in an equal proportion. The investigated groups are represented in the following flow chart.

Flow Chart showing Mongolian groups and subgroups of North Bengal and adjoining areas



3.2 Study Subjects

3.2.1. Patients with pulmonary tuberculosis

A total of 168 unrelated sporadic patients with active pulmonary tuberculosis attending the out patients chest ward of the North Bengal Medical College, Darjeeling for a period of over four years and admitted in the S.B. Dey Sanatorium, Kurseong, Darjeeling were considered for the study. Detailed record as well as the history of the treatment of each patient was completed through data information sheet and followed up carefully for more than a year. Subjects belonging to the same socio-economic status and ethnic background were treated as controls. The average age of the patients were 35 ± .08 years. The ratio of male and female was 10:3.

According to the radiographic extent, the disease was classified as unilateral limited (UL), bilateral limited (BL), unilateral extensive (UE) and bilateral

extensive (BE) lung lesions. The disease was considered "limited" if it involved less than one third of the lung fields in a hemithorax and "extensive" if the lesion were more profuse.

Patients were also classified according to their response to chemotherapy. Those who responded to the first line anti-tuberculosis chemotherapeutic agents (i.e. combination of isoniazid, rifampicin, pyrazinamid and streptomycin or ethambutol) were classified as drug responders. Response to treatment was defined as clinical and radiologic improvement with disappearance of AFB in the sputum or smear examination within three months regimen and who consistently has AFB positive sputum smears for at least three months were considered to having drug failure. All the patients are hospitalized to ensure drug compliance.

Out of the120 patients enrolled for the study 79 were AFB^+ and 41 were AFB^-.

3.2.2 Patients with Pleural Effusion

A group of 48 patients were taken who had moderate to massive pleurisy with effusion. The details of the clinical groups of patients included are depicted in table.

3.2.3 Healthy controls

A total of 202 unrelated healthy individuals formed the control group from the same endemic area. The patients and controls belonged to the same region. Immediate blood relations of both patients and controls were not taken for the study. The controls were excluded for any family history of tuberculosis or related diseases.

3.3 Diagnosis

All patients (pulmonary tuberculosis and extra pulmonary tuberculosis) were diagnosed according to the standard clinical parameters which include sputum/smear culture examination, X-ray chest investigation and clinical features.

Bacteriology

Sputum smear microscopy was readily done to all patients who have pulmonary tuberculosis. Spontaneously produced sputum was the specimen of choice. Promptly 0.1 to 0.2 unconcentrated sputum was aspirated and slowly spreaded 2-3 drops of the liquid uniformly on a slide to make a thin smear. The smear was fixed at 80°C for 15 minutes (modified from Allen, J., 1981, *J. Clin Pathol.* **34:** 719). The Tuberacle bacilli were stained with carbolfuchsin stain and examined under oil-immersion (100x) for presence of acid-fast bacilli. The technique is known as Ziehl-Neelsen acid fast stain. In order to reduce the false positive diagnosis, three smears were observed. A positive report was given only if two or three typical bacilli had been seen. Those with two or more positive smear results are classified as 'Smear Positive'. Those with positive smear out of three, should have an X-ray evidence of pulmonary TB. Three negative results and x-ray evidences were classified as 'Smear Negative' (V.H. Balasangameshwara, XXII National Congress, 1998). Demonstration of acid fast bacilli microscopically provides only presumptive evidence. So all sputum negative cases were re-examined by the sputum culture. Cultures were very sensitive for detection of tubercle bacilli and may be positive with as few as 10-100 bacilli/ml of sputum.

The patients with pleural effusion were included by smear test of pleural fluid for AFB and pleural biopsy.

Radiology

Chest radiograph was done both for diagnosis and evaluation of the disease. Multinodular infiltration in the apical posterior segments of the upper

lobes and superior segment of the lower lobes is the most typical lesion of pulmonary tuberculosis. The activity of tuberculosis was judged from serial films. The radiographs were read on the basis of the classification of the National Tuberculosis Association of USA (NTA, 1962).

Clinical Pathology

Other routine clinical laboratory tests were also done like Hb, WBC count, ESR and blood sugar level though they contribute little to the diagnosis of tuberculosis. In all cases of pleural effusion, tuberculin test was done with 1TU of PPD - RT 23.

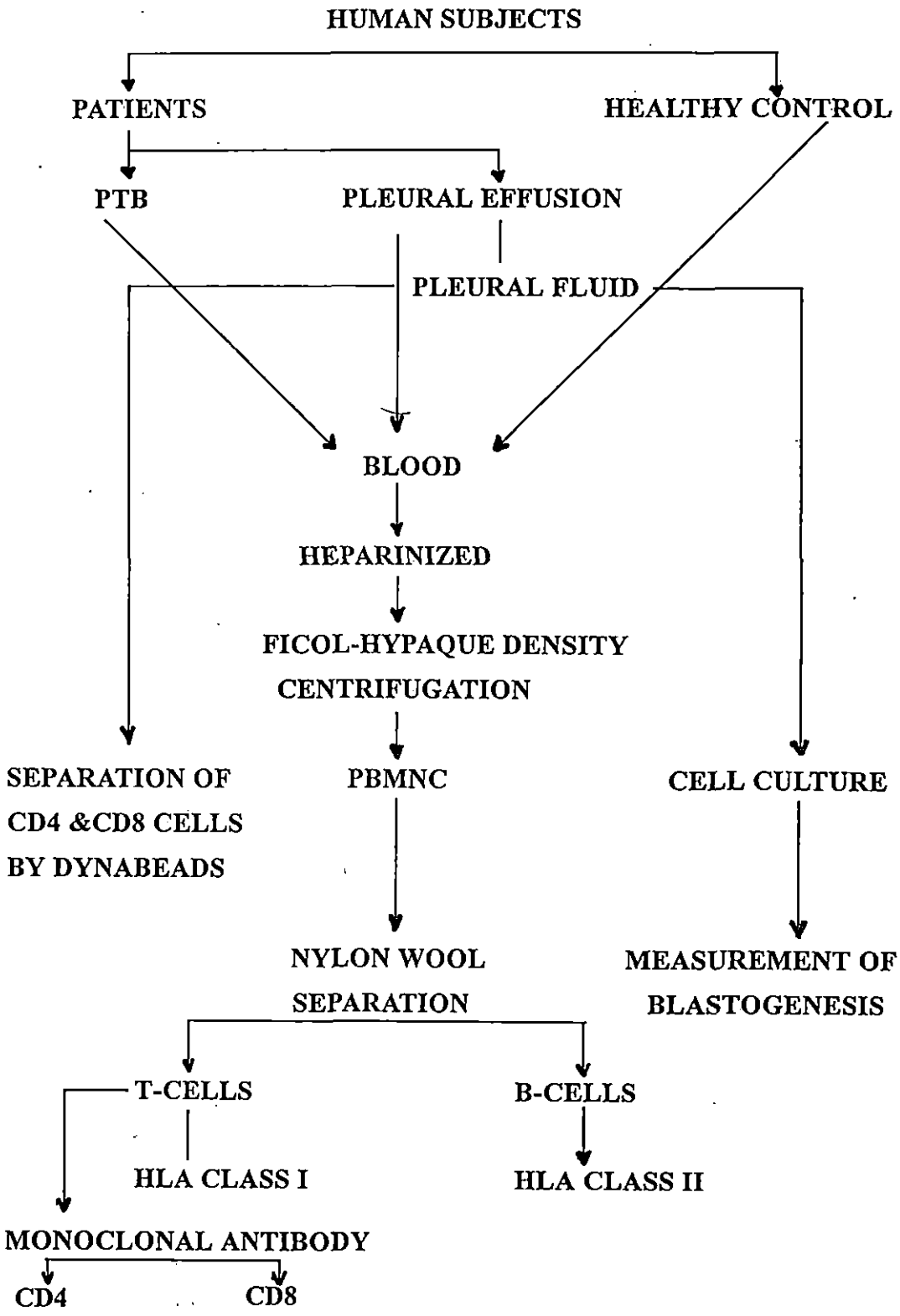
Physical Examination

Chronic cough, fever, scant and nonpurulent sputum, Hemoptysis are few typical symptoms. But, generally these are appreciated only in the presence of extensive diseases.

Methods

The study was performed on an ethnic population of North Eastern India particularly of hill region. One hundred and sixty eight patients (168) with 48 having pleurisy and 200 unrelated healthy individuals were included in the present study. All patients were diagnosed by the standard criteria. All the subjects were HLA typed by standard microlymphocytotoxicity method to determine a possible association of HLA allele(s) with PTB in North Eastern people. The lymphocytes were cultured for 72 hours to investigate the role of T-cell in pathogenesis and the degree of cell mediated immune response. CD4+ and CD8+ T cells were separated to investigate the incidence of the subsets of lymphocytes in case of pleural effusion. Further, to understand the degree of antibody mediated immune response, the levels of IgG and IgM were quantitated of the patients. Following flow chart is the diagrammatic representation of the methodology employed in the present study.

DIAGRAMMATIC REPRESENTATION OF THE EXPERIMENTAL DESIGN OF THIS STUDY



3.4 Reagents

1. HLA antisera

A set of 52 well defined HLA antisera (2 sets were used ; one from c6 Diagnostics and another distributed for 12th International Histocompatibility workshop and Conference) were used for HLA specifications. The HLA Class I serological trays identified 18 locus of A alleles, 34 locus of B alleles and 8 locus of C alleles. The HLA Class II antisera defining specificities from DR1 to DR18 including DR52 and DQ1 antigens are also specified.

2. Complement

Pooled rabbit serum was used as the source of complement. Peripheral blood was collected from a batch of 20 young healthy rabbits by intracardiac puncture and kept in clean test tubes in slanting position for 30 min. at room temperature after which it was kept at 4°C for 2-3 hours for clot retraction. Then it was centrifuged at 2000 rpm at 4°C for 15 min. and clear serum was collected by pasteur pipette. Sera from all rabbits were mixed together and sterilized by membrane filtration (Millipore. USA, 22 μm) in sterile environment (Laminar Flowhood, Thermodyne, India). The sterile pooled sera was then aliquoted in microtubes (Tersons) and stored at -20°C freezer until use.

3. Phosphate Buffered Saline (PBS, pH 7.2)

Solution A : 0.2M $NaH_2PO_4 . 2H_2O$ (MW 156.01)

31.302 g/ l H_2O

Solution B : 0.2M $Na_2HPO_4 . 2H_2O$ (MW 177.99)

35.598 g / l H_2O

Solution C : 0.163 M NaCl (MW 58.44) 8.5 g /l H_2O

140 ml solution A, 360 ml solution B was mixed adjusted the pH 7.2 using either solution A or B. With this mixture 500 ml of solution C was added to make PBS.

4. Heparin : (Span Diagnostics, India)

5. Lympho prep : (C6 Diagnostics, Wisconsin, USA)

6. Phytohaemaglutinin

PHA-P (Sigma) was reconstituted with 5 ml sterile triple distilled water.

7. Cell Culture Media

Minimum Essential Medium (Hi Media, Bombay) and Medium TC199 (Welcome, UK) were used. 10 gm of the dry media was dissolved in 950 ml of sterile triple distilled water and 50 ml of 4.4% sodium bicarbonate solution was added to make the total volume 1000 ml. The media was of a light red to pinkish colour having an approximate pH 7.0-7.2. The media was supplemented with antibiotic penicillin-streptomycin 50 (IU/ml) and nystatin (50 IU/ml) procured from Sigma as an antifungal agent. The media were filtered through a millipore filter and cooled in tightly stoppered conical flasks and stored at 4°C.

3.5 Collection of Blood

20 ml of blood samples were collected from each patient aseptically into sterile siliconized glass tube containing 20 ml preservative free heparin (10 IU/ ml) and mixed by gentle shaking.

3.6 Isolation of Lymphocytes (Boyum A, 1968)

a) 20 ml freshly drawn heparinized venous blood was diluted approximately with equal volumes of PBS (pH 7.2)

b) The diluted blood was gently layered over lymphoprep (sp. gravity of 1.077) with a pasteur pipette in a ratio of 2:1 thus for each sample 3-4 tubes were used.

c) All tubes were centrifuged at 400 g (2000 rpm) for 20 min at room temperature (22-24°C).

d) The interphase with foggy layers of the mononuclear cells were aspirated with clean centrifuge tubes.

e) The cells were washed twice with PBS (pH 7.2) for 10 min and supernatent was discarded.

f) Finally cells were resuspended in RPMI-1640 supplemented with 10% heat inactivated goat serum (Chaudhuri and Chakravorty, 1983)

g) Their viability was checked by the trypan blue dye exclusion method and counted in a haemocytometer and adjusted a concentration of 2 x 10^6 cells/ ml in the same medium.

Lymphocytes were separated in the same way from the freshly drawn pleural fluid of tuberculosis pleural effusive patients and counted.

Nylon wool column separation (Danilovs, *et al*; 1980)

As HLA DR/DQ antigens are expressed on the surface of only a few cell types like lymphocytes, monocytes, macrophages and activated T cells, the B cells were used for HLA Class II antigen detection. These cells were purified from peripheral blood lymphocytes (PBLs) suspension using nylon wool column. The principle involved in this procedure is that the T lymphocytes do not adhere to the nylon wool while B lymphocytes and granulocytes adhere. T cells get eluted out by washing the nylon wool columns with prewarmed RPMI at 37°C and B lymphocytes remain adherent to the nylon wool and can be harvested by gentle squeezing of the column. The nylon wool columns are designed to accommodate upto 50 x 10^6 purified PBLs. The columns were prepared from plastic straws of approximately 3-4 mm diametre as mentioned below.

a) One end of the straw was heat sealed at an angle of 45°.

b) Approximately 0.1 gm of scrubbed nylon wool (Robbins Scientific, USA) was tested and soaked in RPMI 1640 medium for a few min and gently packed into the straw evenly to a height of approximately 4-5 cm for 10-50 x 10^6 cells.

c) The bottom sealed tip of the straw (2 mm) was cut off so that column was pre heated to 37°C in an incubator.

d) The column was then washed with 5 ml of RPMI-1640 with heat inactivated goat serum already warmed to 37°C.

Separation of T and B cells:

a) The lymphocyte pellete was resuspended in 0.5 ml of RPMI-1640 medium and added gently to the top of column pre-heated at 37°C. Cells were allowed to move all the way into the nylon wool.

b) 0.2 ml of RPMI-1640 added to the top of the column to prevent the nylon wool from drying. It was then incubated at 37°C for 30 min by placing horizontally in an incubator. This allows B cells to adhere to the nylon wool.

c) The column was placed vertically in a test tube so as to collect the non adherent T cells by allowing 10 ml of medium pre-heated at 38°C to pass through the column. T cells were then centrifuged, resuspended and were used for HLA Class I typing.

d) The enriched B cells were collected in another tube by adding chilled (4°C) medium to the column and gently squeezing the straw. This was repeated at least 5-8 ml of medium was used. B cells were pelleted by centrifugation, resuspended, counted and adjusted to a concentration of 2 x 10^6 cells. These cells were used for HLA Class II typing. T and B cells were also separated from the lymphocytes of pleural fluid in the same way.

3.7 HLA Class I and Class II serological typing

HLA ClassI antigen typing

This was done by using cell fraction as obtained above. 60 well HLA Terasaki trays (NUNC, Denmark) were used for typing.

a) The typing trays with HLA antisera were thawed immediately before use.

b) 1 µl of lymphocyte suspension (2 x 10^6 cells/ml) containing approximately 2000 cells were dispensed carefully into each well by a repeating dispenser (Hammilton Co., USA).

c) The trays were incubated at room temperature (22-24°C) for 30 min.

d. 5 µl pooled, titrated and non-toxic rabbit complement was added to each well and the trays were incubated at room temperature for 60 minutes.

e) 4 µl of 5% water soluble Eosin (Qualigen Fine Chemicals, India) was added to each well to stain the dead cells and after 5 min 5 µl of 40% neutralised

formalin (pH 7.0) was added, to fix the cells.

f) The trays were kept in a refrigerator and read in the next morning.

HLA Class II antigen Typing

B cells were adjusted at a concentration of 2.5 x 10^6 cells /ml. The procedure used was same as for Class I typing except that the incubation period was doubled at both steps i.e. 1 hour for cells and serum followed by 2 hours after adding the complement.

Microscopic evaluation of the test

The trays were read using an inverted phase contrast microscope (Leitz, Labovert FS, W. Germany). Score was done by the system adapted for the percentage of dead cells. The stain eosin will be taken up only by the dead cells and therefore will look like red under the microscope. Typing trays were scored by the system adapted in the 8th International Histocompatibility Workshop. In case the negative control has 20% or more dead cells, the test was repeated.

%dead cells	Score	Interpretation
0-15	1	Negative
16-25	2	Doubtful negative
26-50	4	Weak positive
51-80	6	Positive
81-100	8	Strong positive
0	9	Not reliable

3.8 Separation of CD4+ and CD8+ T cells

a) Lymphocytes separated from peripheral blood and from pleural fluid were washed twice at 4°C in RPMI-1640 supplemented with 5% heat inactivated goat serum.

b) cells were resuspended in RPMI-1640 and adjusted the number at 2 x 10^6 cells/ml.

c) Cell suspension was divided into two parts.

d) In case of separating CD4+ T cells 0.5 ml cell suspension was mixed with 0.02 ml Dynabead M-450 CD4+ [Dynal UK Ltd., UK; number of Dynabeads 3 x 10^6] in the tube and was placed in a shaker at 4°C which provides both gentle tilting and rotation and incubated for one hour.

e) On another tube 0.5 ml cell suspension was mixed with 0.02 ml Dynabeads TM M-450 CD8 to separate CD8+ cells and incubated for 10-15 min in the same way.

f) Rosseted cells (both CD4+ and CD8+) were isolated by placing the tubes in the Dynal MPC-e-1 for 2-3 min. g) Cells were resuspended in the medium and counted in a heamocytometer under the microscope.

3.9 *In vitro* Cell culture

Preparation of human AB serum

Blood was collected in a sterile tube and kept at room temperature for about 2-3 hours until all RBCs and fibrinogens clot and settled down. The tubes were then entrifuged for more time to get clean serum on the top of the clot The serum was then pipetted into a sterile centrifuge tube and again centrifuged at 3000 rpm for 20 min to avoid cell contamination. The serum was pipetted into a screw bottles and was put in water bath at 55°C for 45 min. The heat inactivated serum was stored at -20°C.

Autologous serum was prepared in the same way for cell culture.

Preparation of Cell culture

a) Lymphocytes from peripheral blood and pleural fluid were adjusted at 2 x 10^6 cells/ml and resuspended in media.

b) In an universal culture tube 4.5 ml of prepared media was pipetted and was supplemented with 0.5 ml of autologous serum/ AB serum (10%) as the case may be.

c) To each culture tube 0.1 ml of reconstituted PHA and 0.3 ml of the cell suspension was added.

d) Culture tubes were incubated in a vertical position at 37°C in humified atmosphere of 7.5% CO_2 in air for different hours like 24, 48 and 72.

Cell Viability Test

The percentage of viable cells was counted by heamocytometer in percentage of trypan blue (Sigma Chemical Company, USA. Viability of the cells after in vitro culture was determined at different hours like 24, 48 and 72 and the percentage of viable cells at different hours was calculated by considering the number of viable cells at the beginning as 100 percent.

Measure of Blastogenesis

After binding with the mitogen or antigen, metabolic activities of the small lymphocytes get augmented and they gradually transform into bigger cells known as 'blast' cells. The blastogenesis is considered as one of the indicator of activation of lymphocytes and the percentage of the blast cells in a lymphocyte population stimulated with mitogen or antigen can be a measure of the degree of activation of lymphocytes. Blasts were counted by haemocytometer in presence of trypan blue under microscope fitted with an oculometer. Cells with diameter greater than approximately 7 mm were scored as medium sized and cells with diameter greater than 10-11 mm were scored as large. The proportion of transformed or 'blast' cells is determined from the sum of viable medium plus (Chaudhuri and Chakravarty, 1983). Blast cells were counted at the end of 24 and 48 hr of culture.

3.10 Measure of Immunoglobulins

Collection of Serum

A little portion of blood was collected without heparin and allowed to stand at room temperature for an hour. After clotting of the blood, the serum was collected by centrifugation at 2000 rpm for 10 min, aliquoted and preserved at -20^{0}C in freezer. 2% sodium azide was added as preservative in each case.

Estimation

The immunodiffusion was followed for quantitation of IgG and IgM of normal and patient groups. HC-partigen IgG and IgM Immunodiffusion plates (Bhering, USA) were used for the estimation of IgG and IgM respectively.

a) The plate was opened and leave for about 5 min at room temperature to allow any condensation water that may have accumulated in the wells to evaporate.

b) Well 1 number was filled with control serum and well 2-12 were filled with undilute 5 µl patient's sera.

c) The plate was closed tightly and left at room temperature for 50 hours. In case of IgM it was 80 hours

d) At the end of diffusion the diameters of the precipitin rings were measured accurately (to 0.1 mm) using a calibrated scale.

e) The immunoglobulin concentrations related to the measured diameters were read directly from the table of reference values. The results were reliable only when the value was found for the control serum applied to well 1 was within the confidence range taken from the table of values, enclosed each of the control serum statistical analysis.

3.11 Statistical Analysis

Phenotype frequencies

Phenotype frequencies of the 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:

$$\text{Percent Phenotype frequency (PF)} = \frac{\text{No. of times a phenotype is present}}{\text{Total No. of subject studied}} \times 100$$

Significance

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

	+	-
Patient	a	b
Control	c	d

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

$$n1 + n2 + n3 + n4 = N$$

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

The level of significance is reported in terms of probability (P) value. The P value was considered significant when the value was < 0.5%. Relative Risk was ascertained by a modified method of Woolf (Haldane, 1956) using the following formula:

$$\text{Relative Risk (RR)} = \frac{\text{No of positive patients x no of negative controls}}{\text{No of negative patients x no of positive controls}}$$

The significance of difference between the levels of IgG and IgM antibodies in controls and patient groups were calculated by using Student's 't' test. P values lass than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Page No.

RESULTS AND DISCUSSION

4.1 Pulmonary Tuberculosis : The radiographic and bacteriological extent of the disease and response to chemotherapy in patients of North Eastern India

The present investigation deals with the radiographic extent and sputum status of pulmonary tuberculosis patients in this demographic region. The dynamics of chemotherapeutic response varied widely from rapid recovery (resolution of inflammatory infiltrative changes, disappearance of tubercle bacilli and cavities during the first six months of treatment) to slow recovery (slow regression of destructive lung changes).

It has been observed that high number of patients (n = 89) were having lesions of both the lungs and 41 of these were having severe extensive disease. The disease was considered 'limited' if it involved less than one third of the lung before and 'extensive' if the lesions were more profuse. 25.8% of the total patients were unilateral limited, whereas, there was no unilateral extensive patients.

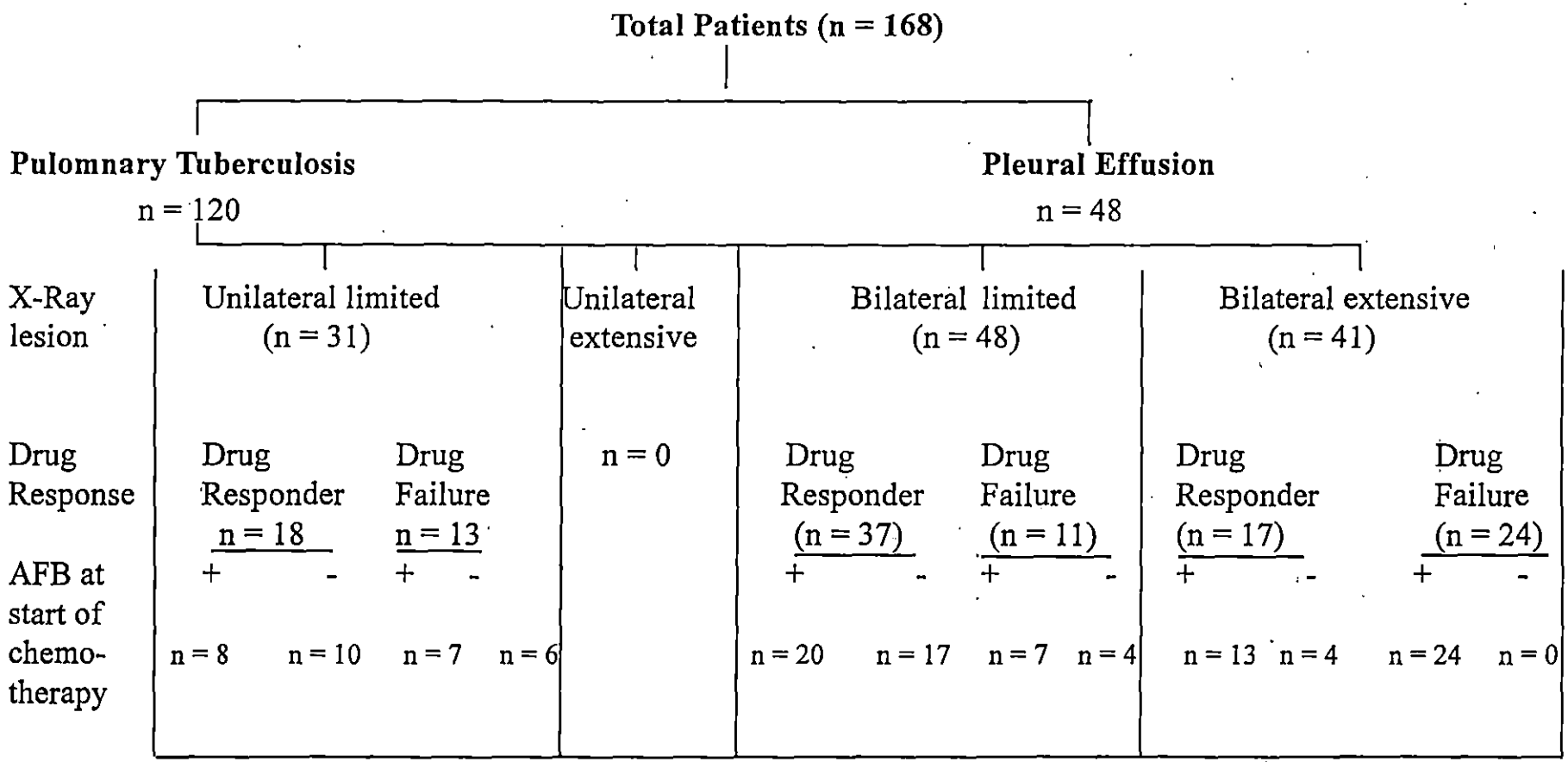
41.9% (13) of unilateral limited and 20.8% of bilateral limited were drug failure and 58.5% of bilateral extensive diseased groups were multidrug resistant.

34.16% of the patients were sputum negative when the chemotherapy was initiated (Table 6).

Discussion

In this study there were altogether 168 patients of pluro- pulmonary tuberculosis among which 120 patients were found to be suffering from pulmonary parenchymal disease without pleural effusion. Among this group there were only 11 patients classified as suffering from unilateral limited disease whereas 89 patients had bilateral disease. Bilateral extensive involvement was noted in 41 patients. This shows that in these patients bilateral limited and extensive involvement were more common.

Table 6. Distribution of study subjects and clinical groups



When we consider the sputum AFB status of the same patients as many as 79 patients out of this 120 were found to be sputum positive at the initiation of chemotherapy.

This extension and bacteriological positivity may have several etiological basis:

1. Our patient population were all high landers and according to the concept of herd immunity this population has got rather recent exposure to mycobacteria. The evolutional list of tuberculosis as established by epidemiological methods shows a characteristic trend in a fixed group of population. In the initial stage the disease produce by mycobacteria is acute and devastating with high mortality. With passing of centuries the disease takes a more chronic form with less mortality and more morbidity. This second type of response is now seen in average Indian population while the high landers are still in the high group.

2. As this region is tuberculosis prone, most of the inhabitants are more or less exposed to *Mycobacterium tuberculosis*. Hence, they possibly developed a low zone tolerance to that antigen. This low zone tolerance may effect their clinical manifestation and only a high amount of antigen shows the sign of the disease.

3. Tuberculosis is a contagious disease spread by droplet infection. The droplets carrying bacilli after being expectorated from a patient may remain suspended in air for a long duration. The bacilli are very susceptible to sunlight, drying and desiccation. Closed indoor inhabitation, lack of ventilation and lack of adequate exposure to sunlight are the three criteria easily met in the dwelling of this patient population under study. The low socio-economic condition with associated under nutrition and thus undermining natural immunity is another contributory factor.

4. That the disease classification seems more as bilateral than unilateral denotes that we are getting the patients in advance stage of the disease as also substantiated by sputum bacteriological status. An interesting study revealed by Toman showed that extensive disease is not necessarily preceded by limited stage disease in most of the cases. Here in this group the first chest X-ray showed

bilateral disease in 89 out of 120 cases probably reflecting their susceptibility and lack of immunity.

5. The number of drug failures was 40%. In a study done on the same group (De Sarker, S., unpublished data) reported high percentage of multi drug resistant tuberculosis with high infectivity and geographical clustering. The data shows that the infectivity and again close contact indoor dwelling with lack of ventilation leading to the clustering.

4.2 Association of HLA

Study on the immunogenetic aspect of disease is most useful in identifying not only the mode of inheritance of a particular disease process but also in understanding the immunopathogenic mechanism underlying it. Incidentally, most diseases that show strong HLA association have unknown etiology and known mode of inheritance, for example various autoimmune and rheumatological diseases. In this respect, immunogenetic studies of mycobacterial infectious diseases are important since the causative organism is already known and the relevant peptides involved are being to be characterised. Association of HLA class-I and class-II antigens with the disease has been studied by serological method.

4.2.1 Association with HLA class I antigens

The phenotype frequencies of HLA class I antigens in patients with pulmonary tuberculosis were compared with that of healthy controls (Table 7). Significantly increased frequencies of HLA -A9 (33.3% vs-8.33, $p< 0.01$, RR = 5.63); HLA -B7 (33.3% vs 8.33, $p< 0.01$, RR = 5.63) and decreased frequency of HLA-A2 were observed (3.3% vs 25.0%, $p< 0.01$, RR = 0.10) in the patient group as compared to controls.

4.2.2 Association with class II antigens

Among the class-II antigens, only HLA -DR2 showed significantly deviated frequencies among patients as compared to controls. It occurred with a frequency of 36.6% in PTB patients as compared to 4.16% in healthy controls, $X^2 = 19.9$, RR = 13.02 (Table 8).

Table 7. Phenotypic frequency of HLA class I antigens in pulmonary tuberculosis and control.

HLA antigen	Phenotypic Frequency (%)		X^2	Relative risk
	PTB n = 120	Control n = 282		
A1	10.00	25.00	0.2	0.33
A2	3.30	25.00	5.48*	0.10
A3	13.30	4.10	1.90	3.79
A9	33.30	8.33	9.92*	5.63
A10	6.60	4.16	2.32	1.60
A11	16.60	8.33	0.90	2.20
A19	10.00	4.16	0.60	2.50
A28	3.30	8.33	0.80	0.30
A29	6.60	25.00	3.66	0.21
B5	20.00	20.83	0.30	0.94
B7	33.30	8.33	9.92*	5.63
B8	13.30	6.00	1.90	2.39
B12	3.30	4.16	0.40	0.77
B15	10.00	16.60	1.20	0.55
B16	16.60	6.00	2.50	3.11
B18	6.60	4.16	0.70	1.60
B22	3.30	4.16	0.90	0.77
B35	3.30	4.16	0.90	0.77
B37	3.30	8.33	0.60	0.30
B40	3.30	4.16	0.90	0.77

n = number of subject studies
X^2 = Chi square
* = $p < 0.01$

Table 8. Phenotypic frequency of HLA class II antigens in pulmonary tuberculosis and control.

HLA antigen	Phenotypic Frequency (%)		X^2	Relative risk
	PTB n = 120	Control n = 282		
DR1	13.30	8.33	1.80	1.73
DR2	36.60	4.16	19.90*	13.02
DR3	23.30	20.83	0.50	1.15
DR4	13.30	8.33	1.80	1.73
DR5	10.00	4.16	0.60	2.50
DR6	10.00	4.16	0.60	2.50
DR7	13.33	8.30	1.80	1.73
DR8	6.60	4.16	0.30	1.60
DR9	6.60	11.95	1.22	0.57
DQ1	13.30	33.33	3.58	0.31
DQ2	13.30	33.33	3.58	0.31
DQ3	53.30	37.50	1.70	1.90

n = number of subject studies
X^2 = Chi square
* = $p < 0.01$

4.2.3 HLA association with the radiographic extent of the disease

No significant correlation was found between HLA class-I antigens and the extent of chest X-ray lung lesions of the PTB patients. The DR2 distribution was correlated with different radiographic lung lesions (UL, UE, BL, BE). The data is summarized in Figure 7. Only 6 (19.3%) of UL and 14(30%) of BL were DR2 positive. On the contrary 21 of the 41 BE (51%) had DR2 positive.

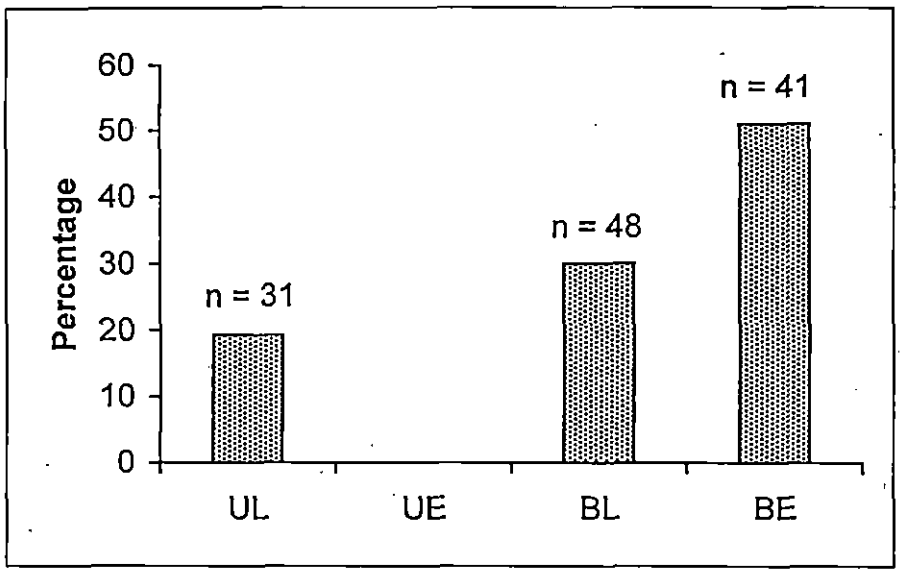


Fig. 7. Distribution of DR2 in the four clinical sub-groups, based on lung lesion.

4.2.4 HLA association with dynamics of chemotherapy

To determine whether chemotherapeutic response is related to host genetic make up, the distribution of HLA antigens in the drug responders and drug failure patients were analyzed.

No deviation was found in the distribution of HLA class I antigens between controls and drug responders and multidrug resistant patients.

HLA -DR2 was found to be most significantly higher in drug failure group of patients with a frequency of 62.5% as compared to 37.5% in drug responder group ($X^2 = 13.57$) giving a relative risk of 0.36. No significant difference was found in HLA class II specificities between the two groups [a. rapid recovery where cavities and all disappeared in the first six months of treatment, b. slow regression of destructive lung changes] of drug responders (Table 9 and Fig 8).

Table 9 Association of HLA -DR2 with drug responsive PTB and drug failure PTB.

	Total no.	No. of antigen bearing	Phenotype frequency (%)
Total patients	120	44	36.6
Drug responder	72	27	37.5
Drug failure	48	30	62.5

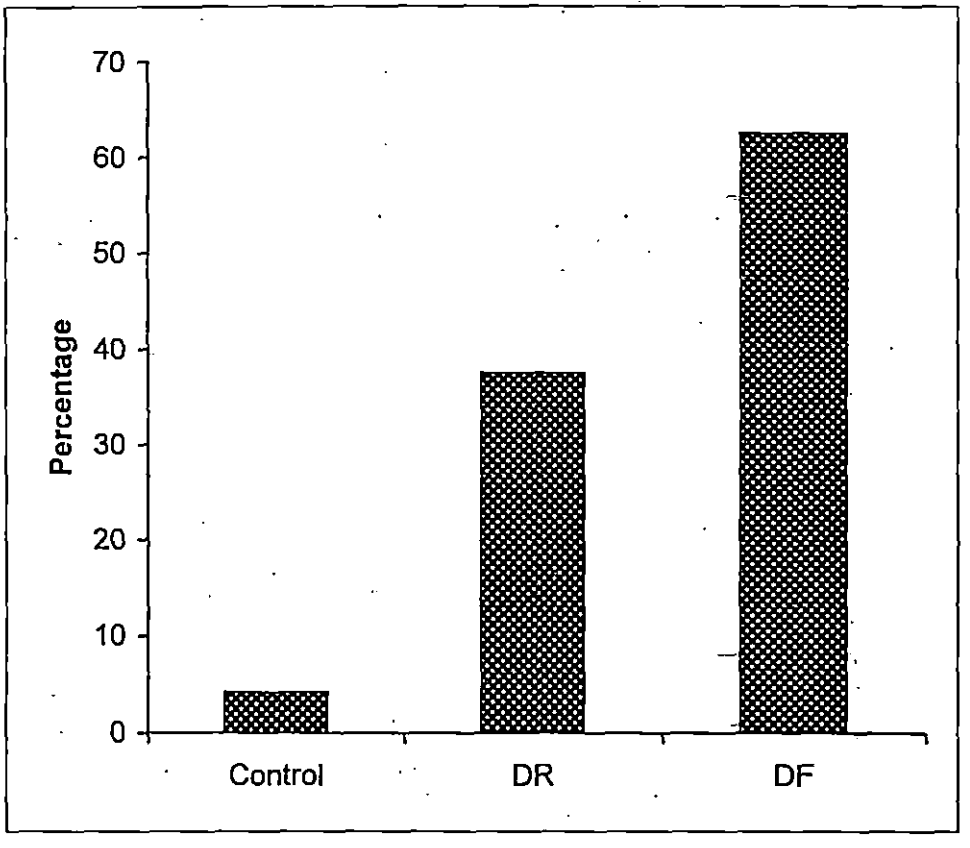


Fig. 8 Distribution of HLA DR2 in drug responder and drug failure group of PTB patients as compared to controls. DR and DF represents drug responder and drug failure respectively.

4.2.5 Discussion

The available literature shows a divergent result in HLA association with the disease (Table 4) which might be attributed to several factors including the ethnic variability of the HLA association and endemicity of the environment. The decreased frequency of HLA -A2 observed in the present study is in accordance with the finding reported in Kazakh patients (Khomenko *et al*, 1990). It is probable that HLA -A2 give some sort of resistance to the host against the disease. Molecular sub typing of this antigen may further lead to the identification of T cell receptor binding portion makes possible its involvement in the mediation of suppressor T cell activity. The higher incidence of A9 is a unique finding in this population. However, in Russian population an increase in B7 was noted (Khomenko *et al*, 1990) which is similar to the findings of the present investigation. Although various investigators have tried to correlate HLA phenotypes with clinical course and severity of the disease that is different stages of radiographic lung lesions, sputum smear positivity (Al- Arif, 1979; Brahmajothi *et al*, 1991) and breakdown to active tuberculosis of inactive pulmonary lesions (Hawkings *et al*, 1988), no report including the present study revealed consistent association of HLA class I antigens with the severity of disease in PTB. This class I molecules (HLA -A9, -B7) may present antigen to CD+ T cells and this might contribute to the risk of developing tuberculosis.

A positive association of HLA -DR2 with PTB patients in the population under study confirms by the findings of others (Singh *et al*, 1983a, 1983b; Mehra *et. al*, 1986; Bothamley *et. al*, 1989; Khomenko *et al*, 1990; Bramajothi *et al*, 1991; Pospelov *et al*, 1996) in other Asian population. However, in North American blacks (Hwang, *et al*, 1985), Mexican American (Cox *et al*, 1988) and Hong Kong Chinese (Hawkings *et al*, 1988) does not favour DR2 association with PTB.

When the patients were divided into drug failure and drug responder, a high frequency of DR2 was found among the drug failure forms of PTB patients. Generally, drug resistance occurs when a single drug is given and when the viable bacterial population in the lesions is large. Drug resistant mycobacteria are most

likely evolve through a chromosomal mutation mechanism to escape or prevent drug induced metabolic damage, probably by a mechanism of decreased permeability of the cell membrane against the drug, or decreased affinity of the drug binding sites, or due to the loss of a drug activating enzyme (Mitchison, *et al*, 1984). Immunomodulators particularly recombinant INF-γ have been shown in animal models to increase the efficacy of the early stages of chemotherapy of tuberculosis (Khor *et al*, 1986). The unresponsiveness or anergy - failure to react to purified protein derivative, PPD (Chan, 1991) has been described. DR2 may play a critical role in inducing anergy/an unresponsiveness to *M. tuberculosis* which leads to the development of infection in the drug resistant form of PTB. However, nature of the immunological imbalance in this drug failure cases needs intensive investigation.

In South Indian patients with PTB DR2 was found to be strongly associated with far advanced cases than those with minimal and moderate radiographic lung lesions (Bramojothi *et al*, 1991). In the present study extensive disease has been found to occur more significantly in whom DR2 was overwhelmingly present. Anergy induction role of DR2 may lead to a spurt in the bacillary population.

4.3 Antibody levels in pulmonary tuberculosis

Although PTB has been characterised by cellular immune reactions (Kaufmann, 1993), the importance of humoral arm in the pathogenesis has been recognised by the presence of *Mycobacterium tuberculosis* specific antibodies both in patients (Grange *et al*, 1980) as well as in healthy contacts (Pitchappan *et al*, 1991). An immune spectrum has been demonstrated in tuberculosis where in the cell mediated and humoral immunity are mutually exclusive, at opposing ends of the spectrum (Daniel *et al*, 1981).

The present investigation have been undertaken to determine antibody levels in patients with PTB in relation to various clinical manifestations. The status of HLA antigens in patients was also correlated with the level of these antibodies in order to understand the HLA- linked genetic involvement of the host for generation of these antibodies.

4.3.1 IgG and IgM antibodies in PTB patients

The level of IgG and IgM were compared with that of healthy controls and the data is presented in Figure 9. The base levels of IgG and IgM in healthy

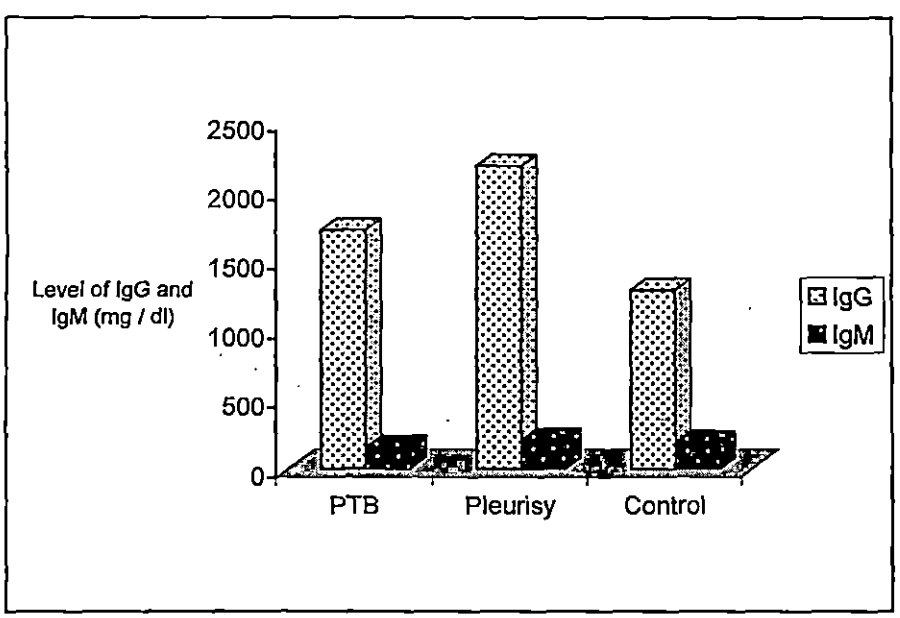


Fig. 9 The level of IgG and IgM antibody in PTB, Pleural effusion and healthy controls.

controls were 1288.6 mg/dl and 190.52 mg/dl respectively. PTB patients showed increased level of IgG (1723 mg/dl, t = 0.1%). Interestingly the level of IgM was lower among patients (154.5 mg/dl, t = 0.1%).

4.3.2 IgG and IgM antibodies in Pleural effusive patients

When the data was compared with pleural effusive patients it was found that the levels of IgG (2188 mg/dl, t = 0.1%) and IgM (210 mg/dl, t = 0.1%) were increased in relation to healthy endemic control (Fig.9).

4.3.3 IgG and IgM antibodies and sputum examination

To evaluate a possible association of IgG and IgM antibodies with disease activity, the PTB patients were further grouped on the basis of sputum culture examination. The levels of IgG were significantly increased in both AFB positive (2651 mg/dl, t = 0.1%) as well as negative patients (2653 mg/dl, t = 0.1%) compared to healthy controls (Fig. 10). The levels of IgM did not differ significantly in the two groups of patients from that of healthy controls.

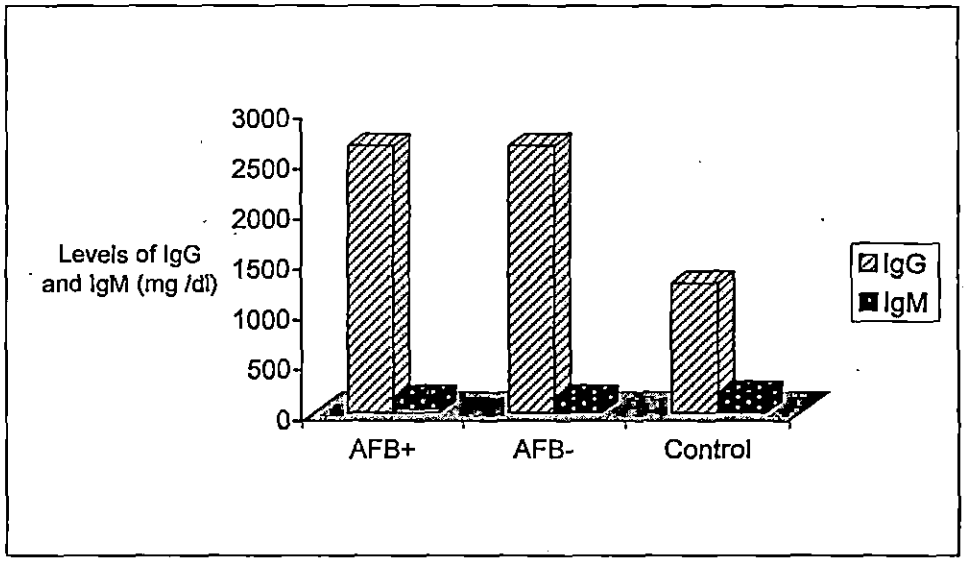


Fig 10 The level of IgG and IgM antibody in patients with pulmonary tuberculosis and controls in relation to sputum examinations.

4.3.4 IgG and IgM antibodies and clinical manifestation

The levels of antibody correlated with the extent of pulmonary lesions based on chest roentogram examination of the patients (Table10). The level of IgG was increased with increasing extent of the disease as judged clinically and radiologically. The increase was significant in all three groups (UL, BL and BE) as compared to healthy controls. Patients with bilateral extensive disease had higher levels than those with other groups but the IgM level did not differ significantly among the three groups.

Table 10 Antibody levels in PTB patients with various chest X-ray lesions

Chest X-ray lesions	IgG (mg/dl)	IgM (mg/dl)
Healthy controls	1288.6	190.52
Unilateral limited	1614	161.73
Bilateral limited	1803.1	126.58
Bilateral extensive	2653	144.5

4.3.5 IgG and IgM antibodies and antituberculosis chemotherapy

Concentration of IgG was significantly increased in both the drug responder (1658.8 mg/dl, t = 0.1%) as well as drug failure (1928.8 mg/dl, t = 0.1%) when compared to healthy controls. The increased was more pronounced in the drug failure cases. The significant difference was not observed in the levels of IgM between drug failure and drug responders (Fig.11).

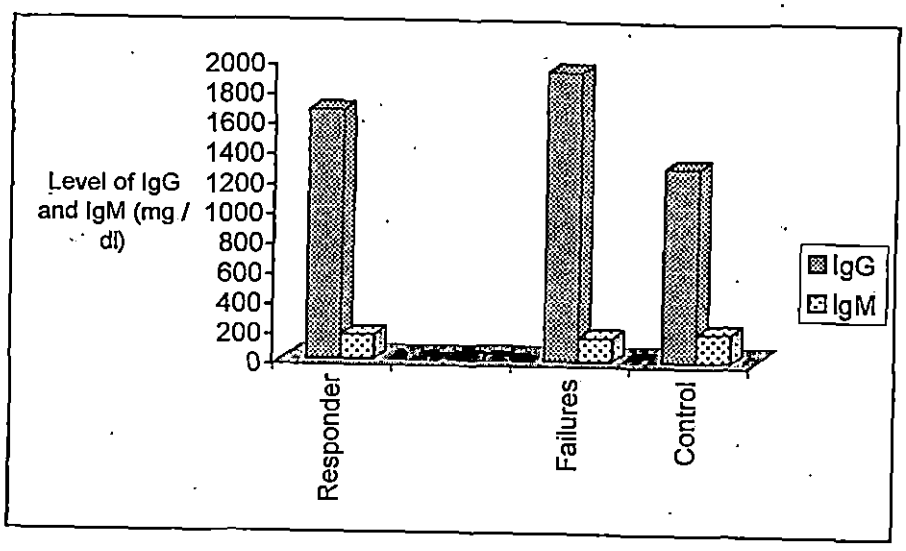


Fig. 11 IgG and IgM antibodies in PTB patients and controls in correlation with antituberculosis chemotherapeutic response.

The impact of chemotherapy on the levels of serum antibody was studied in 10 patients who had all three types of X-ray lesions found in our study. The serum samples were drawn before the start of chemotherapy as well as at 3, 5 and 12 months following chemotherapy (Figs.12 and 13). Among the 6 patients who showed clinical improvement following institution of antituberculosis chemotherapy, the serum IgG levels were significantly decreased from 2703 mg/ dl to 1497 mg/dl ($t = 0.1\%$) after 3 months of therapy. The dynamics of decrease was variable in individual patients. In 2 of them levels of IgG return to normal within three months of treatment both of which were in limited status of X-ray lung lesion. The remaining 4 patients, the levels of IgG dropped to 1831.12 mg/ dl. In remaining 4 patients another serum sample was obtained after 5 months of the start of chemotherapy. Although these patients did not take any specific anti-tuberculosis therapy in the next two months, the levels dropped further and returned to normal level in 3 cases. The remaining patient had a bilateral extensive lung lesion. On the other hand, the 4 drug resistant patients did not reveal any significant alteration in their antibody level after three months of chemotherapy as compared to their levels at the start of treatment. In 1 of the 4 patients studied in this group, the IgG levels were measured again after 12 months of the start of chemotherapy. Again no significant deviation was observed.

Out of the 6 drug responder patients, 5 showed normal IgM level after three months of treatment. In the remaining 1, though initially there was a slight increase but no further change in IgM level was observed in subsequent months. In another 4 patients categorised later as 'drug failure cases', the level of IgM were unchanged even after one year of treatment from the levels at the start of chemotherapy.

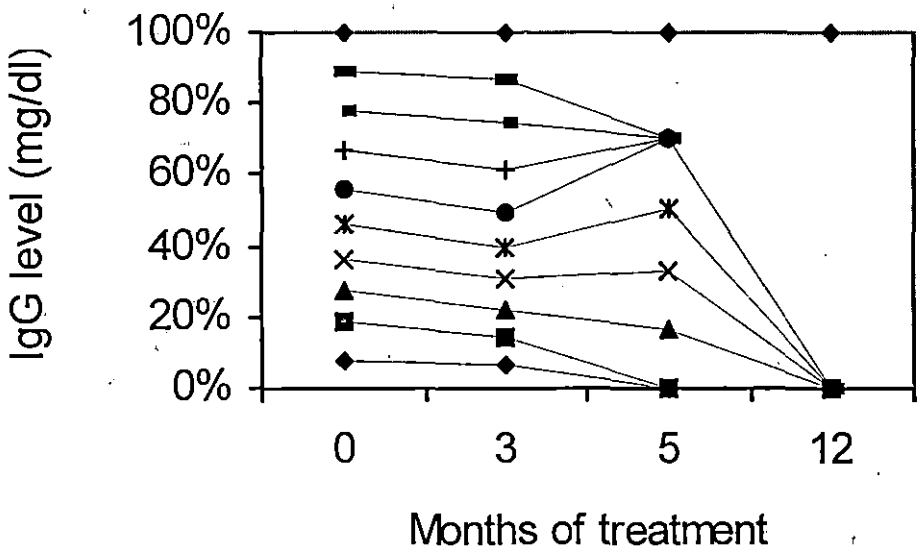


Fig. 12. Levels of IgG antibodies in PTB patients before and after anti-tuberculosis treatment

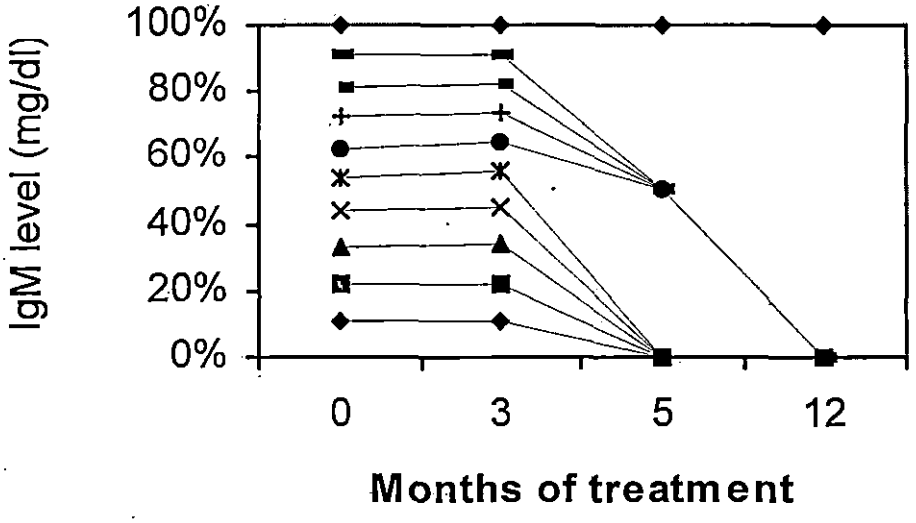


Fig. 13. *Levels of IgM antibodies in PTB patients before and after anti-tuberculosis* treatment

4.3.6 HLA and its role in the levels of IgG and IgM antibodies

Among the HLA class-I and class-II antigens studied, only HLA-DR2 was found to be positively correlated with the levels of IgG antibodies. The data were shown in Table 11. In HLA -DR2 positive patients, a statistically significant increase of IgG levels were observed (2946.6 mg/dl) as compared to DR2 negative patients (1678 mg/dl, t = 0.1%).

Table 11. Association of HLA.-DR2 and IgG and IgM antibodies in patients with pulmonary tuberculosis.

	HLA	Healthy Subjects	PTB patients
IgG (mg/dl)	DR2+	1282	2946.5
	DR2-	1288	1678
IgM (mg/dl)	DR2+	195.5	154.15
	DR2-	194.0	163.8

4.3.7 Discussion

In the present study it has been observed that both the IgG and IgM immunoglobulin levels in case of pleural effusion were higher than the PTB patients. It is well known that in case of the chronic illness both primary and secondary immune responses may be triggered and therefore the levels of both IgM and IgG may be increased. Interestingly we did not get any statistically significant difference of IgG and IgM level between the PTB patients of AFB^+ and AFB^-, indicating that exposure to pathogen rather than the bacterial load may induce the humoral immunity. Therefore, from that point of view possibly

the question does not arise whether the pleural effusive patients were AFB^+ or AFB^-. But indeed it was essential to know what was the ratio of T and B cells in peripheral blood circulation among the pleural effusive and PTB patients. We have observed that in our case the percentage of T cells were migrated to the pleura and therefore the ratio between T and B cells were diminished in the peripheral blood circulation. This has been discussed in detail in Section 4.4 (Cellular Immunity). Therefore it seems that due to encapsulation of the bacteria in the pleura which is having less blood supply may act like a store house of antigens and giving the continuous supply of bacterial antigens slowly by diffusion to the immune system of the patients.

It has also been documented from the present study that the level of IgG was kept on increasing with the increment of the degree of infection which supports the existence of an immune spectrum in PTB, wherein the cell mediated and humoral immunity are mutually exclusive, at opposing ends of the spectrum (Daniel *et al*, 1981). Further support to the hypothesis of immune spectrum comes from the observation of the present study on the increased levels of IgG antibodies in the drug responsive vs drug failure group of patients. The latter are mostly severe cases with bilateral extensive lung lesion and high mycobacterial antibody titer. Antibody levels were declined as DTH responses increase during the course of treatment but it was found that the levels remained unaltered even after three months of antituberculosis chemotherapy, indirectly indicating that the production of the immunoglobulins in tuberculosis is independent of treatment.

Though in case of *M. tuberculosis* infection antibodies have no known protective value and may even antagonise protective immunity (Averbach,1980), the data suggests that these antibodies are related to infection. Similar increased activity of IgG documented by others (Khomenko, *et al*, 1990; Ellner,1996) suggesting that a spurt in the activity of this antibody is a consequence of disease. Non-deviating values of IgM in different groups of patients suggesting an absence of IgM antibody mediated B cell immune deviation in pulmonary tuberculosis.

Following initial studies demonstrating existence of immune response (Ir)

and /or immune suppression (Is) genes in the major histocompatibility complex (Mc Devitt and Chinitz, 1969; Mc Devitt and Benacerraf, 1969), several HLA alleles have been found to be positively associated with antibody production in malaria (Osoba *et al,* 1979), rhesus immunization (Durandy *et al,* 1986), insulin antibody production (Reeves *et al,* 1984) and influenza H3 hemagglutinin response (Durandy *et al,* 1986). It was found that the patients those were having DR2 positive showed higher level of IgG among the PTB patients. The same was also noted by Bothamley *et al* in 1989. As such it is really very difficult to comment clearly what is the relationship between the DR2 antigen and the higher level of IgG but it suggests a possible role of HLA-DR2 or a linked gene in the production of antibodies. However, a detailed study using an *in vitro* system is necessary to make the relationship clear. It is also necessary to characterise the various sub-population of immunoglobulins before examining their likely role as immunoregulatory factors in pulmonary tuberculosis.

4.4 Cellular Immunity

Pulmonary tuberculosis is a disease regulated entirely by the cell-mediated, delayed type immune response of the host against *Mycobacterium tuberculosis*. Inspite of the attention for several years the individual role and mode of action in disease resistance or response is still unknown. T cells with their different subpopulations participate in both cellular and humoral immune response to the disease with a helper or suppressor role. So it is important to study the different cell types, compare them with various markers of disease like roentgenographic lung lesions, AFB status, antituberculous chemotherapy and finding the factors which influence their response to *M.tuberculosis*.

For a quantitative assessment for the extent of sensitization with *M.tuberculosis* an *in vitro* cell culture was performed where peripheral blood mononuclear cells (PBMC) from PTB infected patients and PBMC and pleural fluid from pleural effusive patients were cultured with PHA.

T cells

4.4.1 T cells in PTB, Pleural Effusive patients and Controls

The percentage of T cells were 53% in pleural fluid (PFL) from the tuberculous pleurisy, 51% in peripheral blood from the same patients and 64% in controls. Percentage of the T cells in the PBMC of pulmonary tuberculosis patients were 59.8%, which was higher than the tuberculous pleurisy patients but still lower than the healthy controls (Table 12).

Table 12 Percentage of lymphocytes and blasts in case of pulmonary tuberculosis, pleural effusive patients and controls.

Immunologic Parameters	PTB (%)	Pleural Effusive PBMC (%)	Pleural Effusive PFL(%)	Control (%)
Total Lymphocyte	2.0	2.1	1.3	3.2
T-cell	59.8	51.0	53.0	64.0
B-cell	34.0	39.0	44.0	16.0
T-blast	29.0	29.4	23.0	3.0
B-blast	19.0	19.3	25.0	

4.4.2T cells and X-ray lung lesion

Taking the radiographic extent of the disease as a criteria it has been observed that lower percentage of T cells in bilaterally extensive group (the differentiation has already been defined in previous chapter) of patients (56%). Most nearer to normal were unilateral limited (60%) and bilateral limited groups (59.5%) (Fig.14).

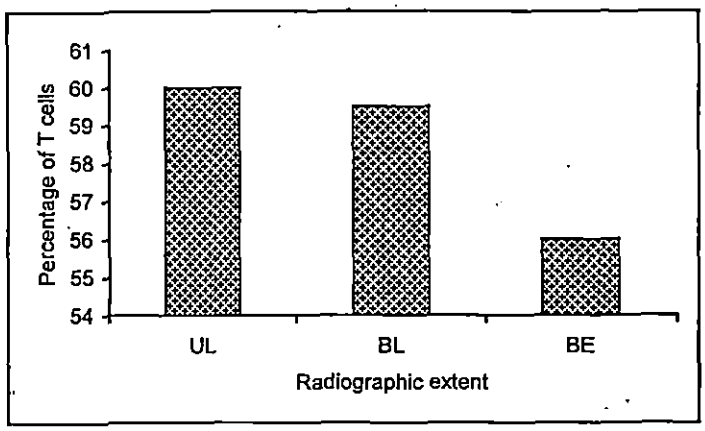


Fig. 14.T cell and the radiographic extent of the disease. UL, BL and BE stands for unilateral limited, bilateral limited and bilateral extensive respectively.

4.4.3 T cell and sputum status

T-cells were found to be lower in AFB positive (57%, AFB+ vs Control; t = 0.1%) and AFB negative (61%, AFB- vs Control; t = 5%) as compared to controls (64%). Again, when two patient groups were compared with each other no statisticallysignificant differences were observed.

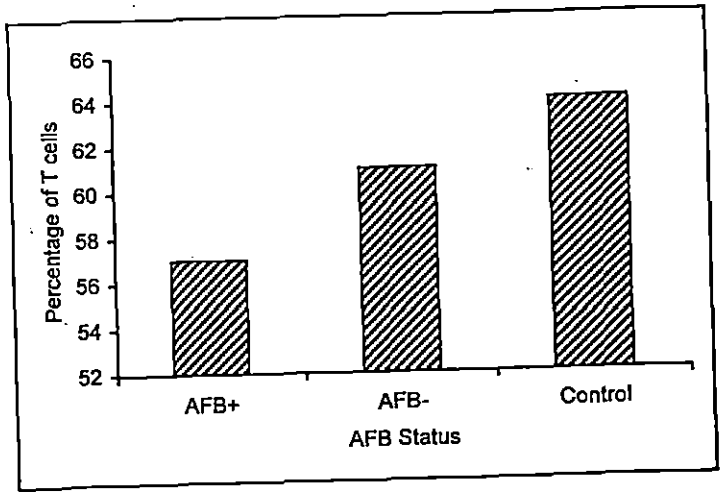


Fig. 15. T cell and the AFBstatus.

4.4.4 T cell and anti-tuberculosis chemotherapy

The T cells were also analyzed based on the response to antituberculosis chemotherapy. As compared to values in healthy controls (64%) the T cells were found to be lower in drug failure group of patients (57%, drug failure vs control; t = 0.1%). In drug responsive groups the number of T cells were 59% (drug responsive vs control, t = 1%) (Fig. 16).

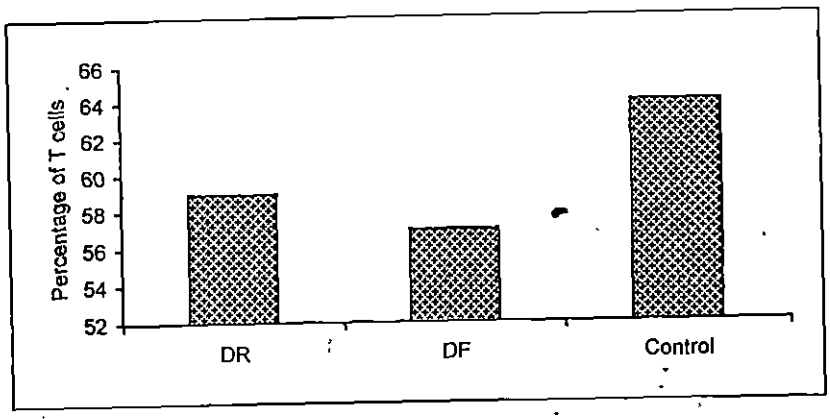


Fig. 16. T cells and anti-tuberculosis chemotherapy. DR and DF stands for drug responder and drug failure respectively.

4.4.5 T cell subsets

Using monoclonal antibodies T lymphocyte subsets were examined in patients with PTB and tuberculous pleurisy before and after *in vitro* culture with PHA (Table 13). Lymphocytes obtained from pleural fluid from patients with tuberculous pleurisy showed 44.1% CD4+ and 18.03% CD8+. The ratio was 2.2:1 whereas from PBMC of the same patients it was 44.8% of CD4+ and 16.1% CD8+. The ratio was 2.7:1. In healthy controls the percentage of CD4+ and CD8+ were 45.05 and 16.05 respectively. The ratio was 2.8:1.

Table 13. Percentage and ratio of CD4 and CD8 positive T cells

Subjects	CD4+ (%)	CD8+ (%)	CD4/CD8
PTB	33.00	16.50	2:1
Pleural Effusive			
PBMC	44.80	16.1	2.7:1
PFL	41.10	18.03	2.2:1
Healthy Control	45.05	16.05	2.8:1

B cells

4.4.6 B cells in PTB, Pleural Effusive patients and Controls

The percentage of B cells was higher in case of all the patient groups than control (16%). Highest percentage was observed in the pleural fluid (44%, vs control, t=0.1%). In the PBMC of pleural effusive and PTB patients the B cells were 39% (vs control, t=0.1%) and 34% (vs control, t = 0.1%) respectively (Table 12)

4.4.7 B cells and X-ray lung lesion

B cell percentage was increased significantly in all three groups (UL =33%, BL=35%, BE=36%) as compared to healthy controls (16%). No statistically significant deviation was observed among these patient groups (Fig.17).

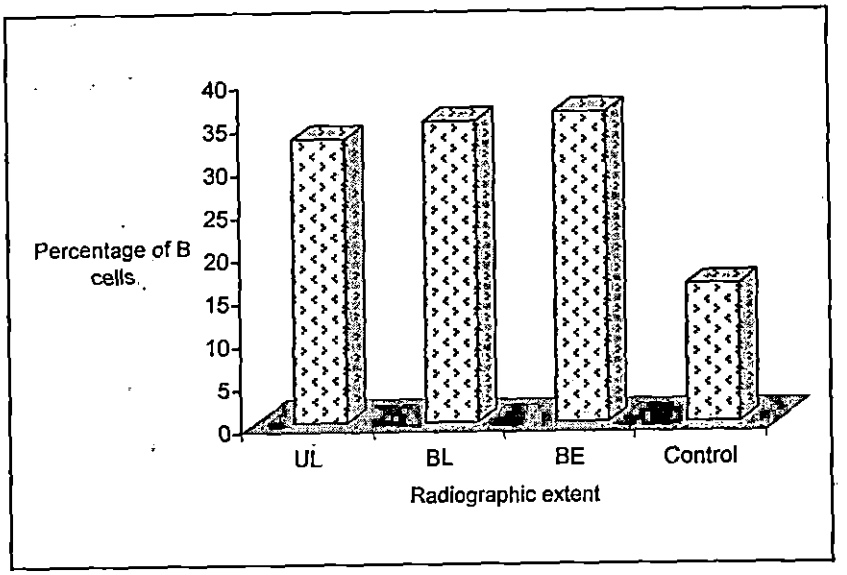


Fig. 17. B cell and the radiographic extent of the disease. UL, BL and BE stands for unilateral limited, bilateral limited and bilateral extensive respectively.

4.4.8 B cell and sputum status

B-cell was found to be increased in both AFB positive (31%, t = 0.1%) and negative (37%, t = 0.1%) as compared to controls. No statistically significant differences were observed when the two patient groups were compared to each other (Fig.18).

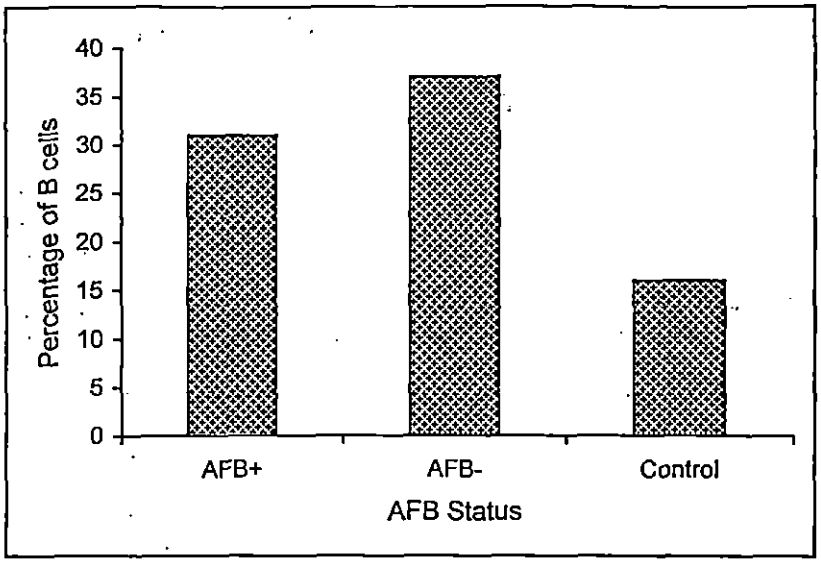


Fig. 18. B cell and the AFB status.

4.4.9 B cell and anti-tuberculosis chemotherapy

The percentage of B cells in PTB patients was also anlysed based on the response to antituberculosis chemotherapy. As compared to values in healthy controls (16%) B cells were found to be significantly increased in both drug responsive (30%, t = 0.1%) as well as drug failure group of patients (38%, t = 0.1%). No significant deviation was found between the groups (Fig.19)

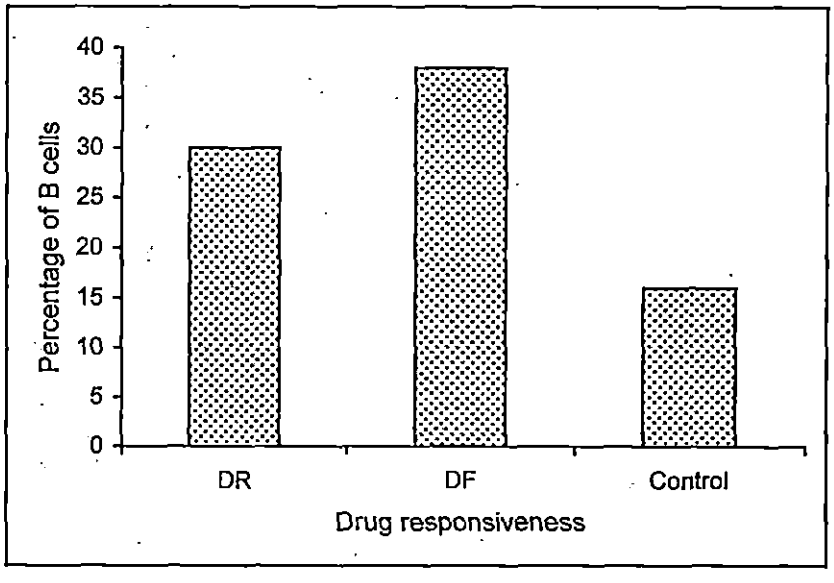


Fig. 19. B cells and anti-tuberculosis chemotherapy. DR and DF stands for drug responder and drug failure respectively.

4.4.10 Blasts

Both T and B blasts were higher in patients when stimulated with PHA (Table 12).

4.4.11 Discussion

The present study reveals that there was an overall diminution in the percentage of T cells in PTB patients than the healthy controls. The result support the view of Tsuyuguchi in case of tuberculosis (Tsuyuguchi, 1996). The reactive T cells were concentrated in PFL compared to peripheral blood in pleurisy patients. The density of T cells in tuberculous PFL could represent selective accumulation or *in situ* expansion of this cell population, thereby causing peripheral depletion. In contrast PBMC of patients with PTB were more responsive than the PBMC of tuberculous pleurisy patients and shows a higher percentage of T cells hence, they have a more chance to be PPD positive.

But when we categorised the PTB patients under standard radiographic criteria, the patients with bilateral extensive disease had lower number of T cells. Advanced TB patients showed low level of IL-2 (Ellner *et al*, 1986). This report corroborates the finding of the present study. This may be a cause for poor differentiation of T cells. Though the PPD positiveness is comparatively higher in pulmonary cases than pleural effusion but there was a report of the trend toward smaller skin test reaction in the group with advanced PTB patients in Karachi, Pakistan (Hussain *et al*,). The lower number of T cells in bilaterally extensive PTB patients in this study may account for this.

The absence of a difference in T cell percentage between AFB positive and AFB negative patients indicates that exposure to pathogen rather than the bacterial load is responsible for the alteration of the number of T cells.

Lymphocytes obtained from PTB patients showed lowest CD4/CD8 ratio compared to either PFL and PBMC of TB pleurisy or PBMC from healthy controls. Mantoux test is always a first attempt to find out whether the patient is infected with *M. tuberculosis* antigen or not. Tuberculin skin test is a T cell mediated phenomenon and it shows the positive reactions when the number of CD4 cells doubles the number of CD8 cells and infiltrate at the site. Patients with tuberculous

pleurisy usually show an impaired tuberculin skin test in most of the cases. The CD4/CD8 ratio was lower in pleural fluid (2.2:1) than the healthy control (2.8:1). This finding recently confirmed by flow cytometric analysis as well (Kleinhenz *et al*, 1987). Result obtained in the present study suggest that there may be different reasons other than CD4/CD8 ratio for the impairment of tuberculin skin test in pleural effusive patients as the ratio was not significantly changed in the cases of PPD negative.

PFL from pleurisy patients were highly responsive to antigens show more activated T lymphocytes. But the investigation with T cell subsets revealed that CD8 cells were more in number than CD4 cells which may have a major role in protective immunity because these CD4 T cells when activated express Ia antigens on their cell surfaces. The existence of suppressor macrophage in low tuberculin response was shown by Ellner *et al*, 1978. The suppressor T cells in addition to suppressor macrophages are responsible for impaired immune response in tuberculosis. In all the three samples (PBMC of PTB and pleural effusive and PFL of pleural effusive) lower number of CD4 cells were obtained which was most in PBMC of PTB patients in comparison to healthy controls. This CD4 deficiency provide nonsurveillance of latent foci of infection (Ellner, 1996) causing the reactivation of tuberculosis which is most common in the area under study, because being a prone area more or less all the people possibly were already infected with the pathogen. CD4 T-cells are known to produce IL-2, GMCSF, IFNγ and TNFα which are the key cytokines for mediating protection. These cytokines activates macrophages to kill intracellular pathogens. As CD4+γ/δ T cells were higher in healthy contacts (Tsuyuguchi, 1996), it seems that these cells might be playing a role in preventing the host from developing PTB following frequent mycobacterial infection. The decrease in CD4+ cell might drive the individual into the disease. So, the present study suggests that activation of CD4+ cells may be a novel approach for the protection of the disease.

The present investigation revealed a high immunoglobulin level which accounts for the higher percentage of B cell. It is possible that certain lymphokines like BCGF, BCDF, TGFB overexpressed during microbial infection and promote differentiation of B cell. The increased activity of (ab')2 γ in patients with PTB (Rajlingam, *et. al*,1996)

which were involved in the immunoregulatory processes and as B cell mitogens (Parker, 1980) also suggests a substantial degree of B cell activation in patients. It has been shown that the ideotypic antibodies react with surface immunoglobulin receptor on B lymphocyte (Birdsall *et al*, 1984) and the elevated activity of heterologous anti-immunoglobulins (LCA) (Rajlingam, 1997) denotes a substantial degree of B cell activation in patients.

The linear increase of B cell percentage from UL group to BE group according to the radiographic extent of the disease, showed a parallel correlation with disease advancement. Though the relevance is not clear, but the stimulus for B cell activation is abundant in patients with bilateral extensive disease.

The absence of significant difference in B cell percentage between AFB positive and AFB negative indicates that the exact mechanism for B cell proliferation lies somewhere else rather than the bacterial load.

Though the B cell percentage is more profound in drug failure patients there is no significant decrease following successful treatment with anti-tuberculosis chemotherapy.

SUMMARY

Tuberculosis is a chronic mycobacterial disease characterised by cellular immune reactions (Kaufmann, 1995). The present study deals with pulmonary tuberculosis (PTB) and also extra pulmonary tuberculosis like pleural effusion. This has been a major health problem for many developing and industrialised countries (Sudre *et al*, 1992). One third of the world's infected population (500 million) lives in India alone (Dollin *et al*, 1994). PTB is a prevalent disease in our study area i.e. North Eastern Hill Region. In addition to the environmental, nutritional and epidemiological factors, several lines of evidence point towards an important role of genetic factors in influencing the susceptibility/resistance to tuberculosis.

In this study, HLA class I and class II polymorphism has been studied in 120 pulmonary tuberculosis, 48 pulmonary effusive patients and 282 health controls from North Bengal region using two stage microlymphocytotoxicity technique. In addition to HLA, the antibody (IgG and IgM) levels, sub population of T and B cells were also studied to find out the pathological, clinical and immunological relevance of these serological markers to the clinical disease expression and to host MHC genes.

The present investigation revealed that a high number of patients (n = 89) were having lesions of both the lungs and 46.06% (n = 41) of these were having severe disease. 31 patients were having unilateral limited disease. No examples of unilateral extensive disease was found. Among bilateral severe diseased group 58.5% were drug failure. 34.16% of the total patients were sputum negative when the chemotherapy was initiated. This proves their position among high landers and in the low zone of tolerance.

HLA-A9 and B7 were more frequently present in PTB patients than in controls (33.3% vs 8.33%, $p < 0.01$, RR = 5.63). HLA-A2 was decreased in the patient group as compared to controls (3.3% vs 25.0%, $p < 0.01$, RR = 0.10).

HLA -DR2 was more frequently present in PTB patients than in control (36.6% vs 4.16%, $p < 0.05$, RR = 13.02). No significant correlation was observed in HLA allelic frequencies in various patient groups based on radiological lesions on the lung, though a slight higher percentage of DR2 was found in bilaterally extensive group. DR2 association was strongest among the drug failure group of patients while no such association was found in drug responder group.

Immunoglobulins IgG and IgM are known to have important clinical and biological implications. As compared to control values (IgG = 1288.6 mg/dl, IgM = 190.52 mg/dl), the level of IgG was significantly higher in PTB patients (1723 mg/dl, significant at 0.1% level), possibly suggesting that the occurrence of this antibody is related to infection or exposure to *M. tuberculosis*. The lower level of IgM in the patients (154.5 mg/dl, significant 0.1% level) may prove a significant marker. In pleural effusive patients both the levels of IgG (2188mg/dl, significant at 0.1% level) and IgM (210 mg/dl, significant at 0.1% level) were increased. IgG antibodies were significantly increased in both sputum positive (2651 mg/dl, significant at 0.1% level) and sputum negative (2653 mg/dl, significant at 0.1% level) patients, though no significant deviation was observed between these two groups. The level of IgG was positively correlated with bilaterally extensive disease. The drug failure patients had higher activity of IgG than drug responders. A statistically significant increase of IgG level (2946.6 mg/dl) was observed in HLA-DR2 positive patients as compared to the DR2 negative groups (1678 mg/dl, significant at 0.1% level). No significant deviation was observed in the levels of IgM between any groups of the patients.

T cells, their sub-population, B cells and blasts those are responsible for the immune reaction of the disease were quantitatively assessed. The total lymphocytopenia was observed in the diseased patients of both pulmonary tuberculosis (2.0%) and pleural effusive (1.3%) as compared to controls (3.2%). Lower number of T cells was noted in peripheral blood mononucleocytes (PBMC) of PTB patients than in control. In pleural effusive patients the number was still lower in pleural fluid (PFL = 53%) and PBMC (51%). T cells revealed a significant decrease in bilaterally extensive disease. There was no correlation

between T cell activity and sputum positivity. Further T cells were found to be lower (57%) in drug failure patients. The percentage of CD4 cells was least in PBMC of PTB (33%) and lower in PBMC (44%) and PFL (41.1%) of pleural effusive patients as compared to healthy control (45.05%). There was no significant deviation in CD8 cells as compared to control except in pleural fluid where slightly higher percentage was observed. The ratio of CD4/CD8 was almost same in these cases. Further T cells were found to be lower (57%) in drug failure patients. The percentage of CD4 cells were least in PBMC of PTB (33%) and lower in PBMC (44%) and PFL (41.1%) of pleural effusive patients as compared to healthy control (45.05%). There was no significant deviation in CD8 cells as compared to control except a little higher in pleural fluid. The ratio of CD4/CD8 was almost same. The percentage of B cells was higher in all the patients group than normal and was highest in pleural fluid though no significant deviation was observed among the patient group.

Immunogenetic studies of the mycobacterial infectious diseases are important since the causative organism is already known. The importance of the study of association between PTB and HLA was the variability of the alleles associated with the ethnic population. HLA association with tuberculosis in geographically varied population may provide crucial information on the involvement of immunopathogenetic mechanisms.

In the present study a strong association of HLA DR2 with PTB patients was observed. In this regard it is need to be mentioned that this association was very strange among the drug failure patients. In addition with Chinese (Mehra *et al*, 1986), Indonesians (Bothamley *et al*, 1989), Kazakh, Russians (Khomenko *et al*, 1990) and in North (Singh *et al*, 1983) and South Indians (Brahmajothi *et al*, 1991) the present study also confirms the DR2 association with PTB patients in our population as well. So one can postulate that specific epitopes lies on the DR2 molecule may preferentially bind pathogenic mycobacterial peptides leading to the stimulation of CD4 T cell clones resulting the detrimental immune response for causing the disease clinically. Further molecular subtyping is needed to be done to find out the particular sequence of DR2 molecule where the epitope

exactly lies. This may provide further information for the identification of critical amino acid residues involved in peptide binding. Deleting such disease inducing epitopes and combining multiple immunity inducing peptides that can bind to all or most of the DR molecules will be the most useful in developing an effective/ protective antimycobacterial vaccine at the population level. In addition to HLA molecules the search for other disease susceptibility genes that are very closely linked to the HLA complex may yield useful information.

Quantitation of antibody levels can prove to be useful indicators of disease activity in term of active/detrimental cell mediated immunity against the invading pathogen. Specific epitopes derived from *M. tuberculosis* antigens/antibodies should be used to study the exact specificities of these antibodies. This will provide an important insight into the host anti-idiotypic network during anti-mycobacterial immunity.

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ANNEXURE

UNIVERSITY OF NORTH BENGAL

Cellular Immunology Lab.
Department of Medicine

HLA No. :
Sample No :

INFORMATION SHEET

Pulmonary Tuberculosis B.A. Gr.

PATIENT IDENTIFICATION

Name.....
S/O, W/O, D/O.....
Age/Sex.....Caste.....
Address:.....
.....
.....

HOSPITAL RECORD

Hospital.....
Regn. No.....
Date.....

SOCIO-ECONOMIC STATUS

Occupation :
Monthly income : Rs. <500> 1000> 1500> 2000> 2500> 3000> 3500> 4000> more
Living accommodation : Rented/Govt.Qurt./Own separate/Flat/Hutmet
(No. of rooms:) Crowded area/Not much.

Sanitation

Bath and toilet : In the house/community
Washing : Laundry/Local arrangement
Hygienic condition around house : Good/Fair/Poor
Nutrition : Poor/Moderate/Good/V.good
Smoker/Non-smoker :

CLINICAL DETAILS

Past illness : Enteric/Malaria/Bronchitis/G.I.Condition/Piles/other

Present illness:

Duration	:	Night sweating	:
Caugh	:	Haemoptysis	:
Expectoration	:	Loss of appetite	:
Fever	:	Weight loss	:
Chest pain	:	Anorexia	:
Diarrhoea	:	Dysphagia	:
Abd. pain/Gola-formation	:	Others	:

GENERAL PHYSICAL EXAMINATIONS

Glands-neck	:	Resp. rate	:
Axilla (Inguinal)	:	Pulse	:
Oral mucosa	:	BP	:
Skin lesion	:	Weight	:

Others

Chest :

CVS :

Abd :

LABORATORY INVESTIGATIONS AND REPORT

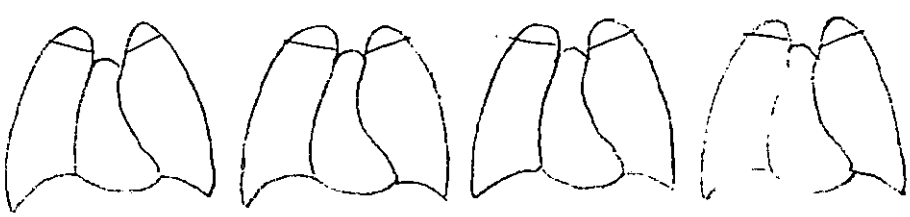
Hb	:	Urine.R/E	:
TLC	:	M/E	:
DLC	:	Lymphocyte count	:
Pan T cell count	:	T4 cell count	:
T8 cell count	:	Mantoux test	:

Sputum for AFB

Smear : Culture : Atypical :

Date						
Result						
	Sm	Cul	Sm	Cul	Sm	Cul

X-ray Chest



Other investigations:

Clinical diagnosis :

Nature and duration of treatment :

Dynamics of treatment :

Quick recovery :

* Resolution of inflammatory infiltrative changes
* Disappearance of tubercle bacilli
* Healing of cavitier during first 6 monthe of TX

Slow recovery :

* Slow regression of destructive lung changes
* Healing or no healing of lung cavities

Repeated tests:

Sputum :

X-ray :

Remarks:

Date:

LIST OF PUBLICATIONS

1. Sreejata De Sarker, Alok G. Ghosal and T.K. Choudhuri. Study on the role of HLA antigens and the host immune responsiveness against the pulmonary tuberculosis. *Flora and Fauna*, 1996; **2(2)**: 171-174.
2. S. De Sarker, TK Chaudhuri and KB Datta. 2000 Certain facts and findings about Immunogenetics of human Pulmonary Tuberculosis. Paper Communicated to *Perspectives in Cytology and Genetics* and **accepted** for publication.
3. Sreejata De Sarker, Alok G. Ghosal and T.K. Chaudhuri. Immunogenetic factors associated with Pulmonary Tuberculosis. Paper presented in *12th International Histocompatibility Conference*, held on June 9-12, 1996 Paris, France.
4. Sreejata De Sarker, Alok G. Ghosal and T.K. Chaudhuri. Compartmentalization of lymphocytes in Pleural Effusion and association of HLA antigens with human Pulmonary Tuberculosis. Paper presented in the *All India Immunology Congress* held onVisakhapattanam, Visakhapattanam, India.
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