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TRANSPLACENTAL TRANSFER AND DEVELOPMENT OF IMMUNITY TO PPD IN YOUNG INFANTS

by

NAJMA JAMIL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled TRANSPLACENTAL TRANSFER AND DEVELOPMENT OF HUMORAL IMMUNITY TO PPD IN THE INFANT submitted by DR NAJMA JAMIL in partial fulfillment of the requirements for the degree of MASTERS OF SCIENCE in PEDIATRICS.

Supervisor

Date: May 27, 1987

DEDICATED TO MY PARENTS AND

MY FAMILY

Abstract

Immunization against tuberculosis with the bacillus Calmette-Guerin (BCG) in infants is of controversial value. Protective efficacy for newborns has never been accurately defined. Since protection may be influenced by passive immunity transfer from mother to fetus during pregnancy, or by acquisition of active antimycobacterial immunity before vaccination it is essential to investigate these influences. The objective of this thesis was to assess the extent of humoral antimycobacterial immunity in newborns and their mothers and in infants under 2 years of age.

The organism causing tuberculosis possesses antigenic properties very similar to many other pathogenic bacteria as well as non-pathogenic environmental mycobacteria. Cross-reacting immunity towards these various microorganisms have the potential to influence the reaction to the vaccine organism. The test antigen utilized in these studies was purified protein derivative (PPD), an extract of a cell wall component of mycobacterium tuberculosis.

The following findings were made by measurement and titration of anti-PPD antibodies using the enzyme linked immunosorbent assay technique (ELISA) for blood samples from mother-infant pairs and children up to 2 years of age as well as in some patients infected with mycobacterium tuberculosis and others with atypical mycobacteria.

1. The newborn human baby receives anti-PPD antibodies of

the IgG isotype from the mother. The level is comparable to that of the mothers.

2. This passively transferred maternal antibody is detectable up to 4 months of age by the technique applied in this thesis.

3. [redacted] months of age, the infant actively develops anti-PPD antibodies [redacted] these are cross-reactive antibodies due to other mycobacteria, has not been established.

Taking this evidence together with other findings of anti-PPD cell-mediated immunity in infants, it is concluded that the outcome of BCG vaccination may be influenced by pre-existing specific immunity in infants. This immunity is passive during the first few weeks of life but becomes an active one during the first year in many infants.

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It was with the kindness of Dr. Kaare Haslov at Copenhagen Serum Institute, that the "environmental mycobacterial" antigens were made available to me.

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OBJECTIVES: Maternal antibodies after being passively transferred to the fetus in-utero may have important effects on immune reactivity of the newborn. These effects may be expected to exist only for the lifespan of the immunoglobulin, and therefore be short lived. The test antigen used in this work is the purified protein derivative (PPD) of Mycobacterium tuberculosis (the organism that causes tuberculosis). To assess the duration of time for which the newborn will have these maternal antibodies in the circulation and the age when the infant begins to produce certain specific antibodies of its own was one of the objectives in this study. Theoretically, this passive transfer of maternal antibodies to the fetus could interfere with effectiveness of the protection offered by (bacillus Calmette-Guerin) BCG- vaccination if given at birth. Similarly, exposure to environmental "atypical"mycobacteria could lead to production of antibodies cross-reactive with the Myc.TB antigens. This could either adversely or favourably affect the development of active immunity to BCG, interfering with the expected protection offered by the vaccination. The type and degree of humoral and cell-mediated immunity to PPD, as it develops in the infant, have been investigated in this work. The presence of cross-reactive antibodies in patients infected with different mycobacterial strains was also analyzed.

INTRODUCTION: The present study was initiated following the observation that 48% of infants born to mothers who were responsive to PPD by blast transformation (BT), showed positive BT reaction to this antigen though there was no obvious exposure to mycobacteria during pregnancy (Pabst *et al*, 1987). The clinical significance of tuberculosis led to the present study which was designed to investigate humoral immune responses to PPD in maternal and infant populations.

Tuberculosis and its causative organism: In spite of the fact that the incidence of tuberculosis has shown a marked decline in developed countries, it is still a major problem in the developing and underdeveloped countries of the world (Grange *et al*, 1979) where an estimated five million people per year die from tuberculosis. The annual rate of tuberculous infection is determined by tuberculin skin testing in a representative sample of unvaccinated children and has been found to range from 2-10% in under-developed countries. Even in Canada, the province of Saskatchewan has an incidence of 1-2% (Personal communication, Dr. V.Hoeppner) in the native Indian population. It is extremely important to search for a reliable screening test for the diagnosis of this infection. The methods used so far are chest X-ray, sputum test, skin test and culture. The only test that proves to be 100% reliable is the culture of the infective agent from sputum, while no other test can be relied on individually. But by the time the sputum becomes positive, the infection is already widespread and undoubtedly the patient has infected many contacts.

Once the diagnosis has been made, there may be a lack of compliance to the treatment regimen by most patients, and instead of a full 18 months course of chemotherapy many patients stop their medication after a few months as soon as they feel better. This has led to reactivation in an estimated 17% of cases, in whom investigations and treatment have to be reinitiated (Personal communication Dr. V. Hoeppner, University of Saskatoon, 1987).

This necessitates the search for a reliable screening test for the diagnosis of TB before the sputum is positive. An improvement in the vaccination programme is also essential in order to achieve maximum benefit and protection for the community and the world population at large.

The causative organism of this disease is Mycobacterium tuberculosis (Myc.TB), which is a gram-negative acid-fast bacillus. On first exposure to the micro-organism humans develop a primary lesion which in 95% of cases occurs in the lungs. After the bacilli enter the body via the respiratory route, they undergo a latent period during which they localize in the lung parenchyma and pass into regional lymph nodes through the draining lymphatics. The defences of the body are activated, attracting polymorphonuclear leucocytes and macrophages which contain the infected area, preventing spread to the rest of the lung tissue. This area is then invaded by cells of the immune system. Such an isolated lesion surrounded by different cells, along with its draining lymphatics and regional lymph nodes is called a primary

complex. This primary complex usually passes unnoticed and at the end of about ten weeks the patient develops delayed hypersensitivity (DTH) to the antigens of this bacillus which can be detected by Mantoux testing. Later the lesion usually undergoes caseous necrosis and calcifies without any further symptoms. Even if the lesion is completely healed, the bacilli can persist and remain dormant in reticuloendothelial sites of the body for many years. In ill health and low resistance they are capable of reactivation to produce active disease. This is called secondary tuberculosis.In some children however the primary lesion may never heal and develop progressively. This is commonly seen in infants born to tuberculous mothers, unless they are separated from the mother after delivery. The child then develops various signs and symptoms of the disease, from pulmonary, meningitic or other disseminated forms of tuberculosis. In the older individuals reactivated tuberculosis is usually pulmonary, but may also be present in other organs. When the disease clinically presents some time after the primary infection has occurred, there are equal chances of it being either due to reactivation of dormant endogenous bacilli, or due to fresh exogenous infection. The two processes cannot usually be discriminated .

Methods used for testing of infection

Radiographic investigation: The primary lesion may present as a small lung opacity but it must be differentiated from the opacities due to malignancy or other causes and thus it can only be a first step in the series of investigation.

Sputum testing and culture of micro-organism: As mentioned earlier this is the only reliable single test but if positive, it indicates that the infection has already progressed and disseminated.

Mantoux test or tuberculin skin-test: Mantoux testing detects the delayed hypersensitivity reaction against the mycobacterium bacillus antigens. It is a reliable method of tuberculin testing and used in general surveys of the population for assessment of immunity to tuberculosis. A measured amount of tuberculin solution of known concentration is injected intra-cutaneously on the volar surface of forearm. Unless a definite wheal follows the injection the test is not satisfactory and a false negative reaction may result. Such a situation may occur if the injection is given subcutaneously or due to leakage at the injection site. For routine testing, a dose of either 10 TU (Tuberculin units) of Old Tuberculin (OT) or 5TU of PPD (may even give only 1TU if the person is known to have had tuberculous infection in order to avoid severe reactions) is injected intracutaneously and the result is read after 48-72 hours by measuring the area of induration at its greatest diameter. An induration of less than 5 mm. constitutes a negative reaction by WHO criteria (These criteria refer only to the clinical diagnosis of "negative" or "positive" cases while immunologically even a smaller reaction is positive and indicates sensitivity). For diagnosis of infection if the test area measures 10 mm. the test is positive. Between 5-9 mm. the reaction is doubtful and the test is repeated with the same dose of antigen. When even the second

injection gives a negative result or yields the same degree of reaction, then PPD testing with a higher dose of 10 TU is arranged. In rare circumstances where the atypical mycobacterial antigens are available, they are injected simultaneously. If the latter is positive as well as the response to a higher dose of PPD, then it suggests that the response to PPD is a heterologous reaction. When positive the test indicates past infection with Myc.TB, and if the patient presents with any obvious symptoms, a further evaluation is done because tuberculosis can occur in any organ of the body and so can present itself with widely varying symptoms. In endemic areas, for individuals who have a negative Mantoux test but live in households with TB patients BCG vaccination is recomended to induce active immunity to TB. Unfortunately the Mantoux test is useful only if the result is positive, as negative tuberculin test results have been seen to occur in individuals with mild to moderate as well as severe forms of TB infection, and many such patients could therefore be missed if the skin test alone is carried out. This negative response in patients could result from factors to be discussed later.

The skin test response to mycobacterial antigens shows an inverse relationship between the immediate and 48 h. reaction (Kardjito *et al*, 1981, and Pepys *et al*, 1955). Possibly the early reaction by causing vasodilation leads to a more rapid loss of antigen from the injection site.

Immediate reaction: This occurs within a few minutes of skin testing. It is not clear whether this reaction is of IgE antibody

origin or is due to short- term sensitizing IgG antibody (Pepys *et al*, 1979).

At 6-8 hours: This appears to be an antigen-antibody complex mediated response, because Kardjito *et al* (1980) found that reactions at 6-8 h correlate to some extent with serum levels of antimycobacterial antibody in the IgG and IgM class.

At 48-hours: Most of the immunologically reactive patients suffering from TB give a maximum skin reaction after 48 h, and this correlates with cell-mediated immunity to antigens of Myc. TB. This is a classical DTH response, and most of the immunologically reactive TB patients give this typical 48 h. skin test response (Lenzini *et al*, 1977).

Discrimination between TB patients and healthy controls: Most of the less immunologically- reactive TB patients can give a maximum skin test reading at 24 h, but problems arise when a false positive response occurs at same time in healthy individuals. This is considered to be a non-specific immune reaction and it commences at 3-6 h, is more pronounced between 12-24 h. and then disappears at 48-72 h. In such cases the early response at 3-6 h. is the reaction that differentiates, because it only occurs in TB patients, and not in healthy controls. Therefore it discriminates the patients from skin test positive healthy control individuals, when both are read at 48 h. and found to be positive (Kardjito *et al*, 1982, and Lenzini *et al*, 1977).

In 1979, Rook and Stanford observed that delayed hypersensitivity may not be a correlate of a protective immune response in the

patient. Instead a marked degree of the tuberculin sensitivity appears to be detrimental to the patient. This was also observed by Youman (1979) who then postulated that hypersensitivity to antigens of Myc. tuberculosis does not confer protection from disease and rather low grade sensitivity favours immunity more than high grade sensitivity.

Relevance of Mantoux testing: The DTH response examined by Mantoux testing is a T-cell dependent immune phenomenon manifested by an inflammatory reaction at the site of antigen deposition, reaching its peak intensity 24 to 48 hours after initiation. It is indicative of previous antigenic contact and immunologic response to Myc.TB. There is no significant change in vascular permeability of the area. Thus induration and not erythema is characteristic of the lesion. It is distinguishable from immediate hypersensitivity or Arthus reaction which is an antibody-dependent phenomenon that peaks in a few minutes or hours.

DTH response being an in vivo process has a variety of in vitro tests correlating with it, such as T-cell proliferation assays, and production and assay of lymphokines such as lymphocyte migration inhibition factor (LIF). An in vivo correlate of DTH is the contact sensitivity (CS). This is also a T-cell phenomenon induced by injuring the skin with highly chemically reactive compounds such as trinitrochlorobenzene (TNCB).

DTH and CS are transferable only by T-cells and the lesions are characterized by gross induration and mononuclear cell infiltrates.

Basophils in these reactions are usually absent.

Specificity of DTH responses: As studied in murine models the DTH reaction seems to be mediated by specific cells. The T-cells involved in a DTH reaction (TDH) bear the surface antigen Lyt 1+, and are Vicia villosa lectin non-adherent (Loveren et al,1984). They have been shown to mediate specific responses. The DTH reaction can occur in response to protein antigens, a variety of bacteria and their products and to some viruses like influenza and reovirus. Although T-cytotoxic cells are thought to be effective in vivo against viral infections by recognition of virus infected host cells, it has also been determined that TDH are involved in limiting viral spread. Using recombinant reovirus different only in the haemagglutination gene, a detailed examination of DTH has shown that most TDH are specific for this particular gene product (Bianchi *et al*, 1981). Thus immunity to a single virion component may be sufficient for complete protection.

The Lyt 1+ TDH like cells have also been implicated in skin graft rejection and tumours. Tamura *et al* (1983) have indicated that in skin grafts transfer of such cells into T-cell depleted recipients resulted in specific skin graft rejection of MHC incompatable grafts. Also immunizing A/J mice with one type of tumour cells followed by resection of the growing tumour, led to a tumour-specific classical DTH reaction, so that animals primed with irradiated tumour I cells will develop marked DTH response when challenged with tumour I but will manifest little reaction when challenged with tumour II.

The specificity of the DTH reactions to this extent would indicate that the in vitro correlates of this test may be equally specific and therefore could be reliable immunological assays.

Antibodies and DTH reactivity:

According to the hypotheses put forward by Peter Bretscher in 1981, that the induction of any type of an immune reponse depends on many variables. Different conditions favour the induction of different classes of immunity, and induction of each class is exclusive, for example the induction of a strong humoral response is not associated with induction of DTH and vice versa. The conditions known to favour DTH are just those known to produce an unresponsive state at the humoral level and vice versa. Depending on the type of antigen and the frequency of antigenic determinants, the cellular co-operation required for an immune response is made available. Thus the greater the number of reactive sites available on the foreign antigen, the greater is the inter-cellular co-operation possible. He postulates that the effector inductive complex is formed between the receptor, antigen and helper factor released from or on the surface of the helper T cells. It has been observed that IgG antibody response requires maximum amount of help for induction or in other words the maximum number of inductive complexes, while DTH can be induced with the least amount. IgM antibody response has an intermediate requirement for them. Thus if an antigen has a series of repeating determinants or is available in large quantities it would induce a humoral response, and if the determinants are few or there is less antigen

available then DTH would result. Also each class of immune reaction is induced by activation of the respective precursor cells which depend on the availability of antigen-specific T-helper cells. This is known to be true for all types of immune reactions whether cellular or humoral. When the T-helper cells for one class induce that specific reactivity, there is a simultaneous induction of regulatory cells for the other class of reactivity. For instance DTH response favours development of repressor T cells inhibitory for humoral response, and when a humoral response occurs it induces suppressor T cells (inhibitory for cell-mediated responses). The repressor cells are inhihibitory T cells bearing surface markers of $Thy1^{+}Ly1^{-}Ly2^{+}Ia^{+}$ which are envisaged to act on the induction of helper T cells for a humoral response and therefore prevent induction of a humoral response. This ensures that when the immune system is chronically stimulated by an antigen with a few foreign antigenic sites, only a cellular response is mounted. As mentioned earlier the amount of help required for DTH is less than that for a humoral response. Therefore the repressor cells should not inhibit the induction of helper T cells until there is sufficient help for CMI response. The suppressor cells for the CMI bear the surface antigens of $Thy1^{+}Ly1^{+}Ly2^{-}Ia^{-}$ (Ramshaw *et al*, 1976,1977).

It has been observed and postulated that a foreign cell with few antigenic sites is not susceptible to IgG dependent complement mediated cytolysis. The immune system responds to such a foreign cell by inducing cytotoxic T cells, it is also advantageous for the

body to produce exclusively an effective response against it. Even more important is the fact that an ineffective humoral response in this situation can block the cytotoxic process. Active inhibition of the humoral response with the help of repressor T cells appears to ensure this. This theory should predict that the anti-PPD antibody response be associated with production of regulator cells that should inhibit or modulate the DTH response against PPD. But the observations have been different.

Kapoor & colleagues in 1985, with respect to PPD-induced delayed hypersensitivity and antibodies have observed a direct relationship between the two types of responses. Since IgA provides surface immunity to mucosal linings it prevents satellite infections in other parts of lungs, and thus facilitates elimination or phagocytosis of the infecting mycobacteria. Also, observations by other workers indicate a positive relationship between Mantoux reactivity and antibody responses. This may be explained as (1) serum IgG and secretory IgA by virtue of forming immune complexes may enhance the DTH and (2) the fact that the mycobacterium by virtue of certain peculiar antigenic properties can modify or deregulate a normal immune response as has been experienced in the use of complete Freund's adjuvant. Mycobacterial antigens produce skin sensitivity due to sensitized T cells and simultaneously have a mitogenic effect on B cells.

Factors that modify skin testing:

1. Serum factors: In 1985, Kapoor *et al* observed that the DTH may be directly related to some immune as well as non-immune factors

in the serum. For instance they found a direct relationship between anti-PPD IgG_2 antibodies and the DTH reaction induced by PPD. These antibodies are found in the majority of patients with active or previous TB infection, and a similar relationship between anti-PPD IgA and PPD induced DH suggests that as IgA antibody provides mucosal surface immunity, it can in combination with mycobacterial antigens form immune complexes leading to production of skin reactive factors thereby modulating the delayed cutaneous response. The direct relationship between anti-PPD IgG and DH may be explained by the fact that IgG antibody activates complement and thus augments monocyte-mediated uptake of mycobacteria (Kumar, 1979) leading to their elimination from circulation. This results in a lesser amount of available antigen, so that a good DTH reaction can now occur (Bretscher, 1981). It may be concluded from this study that the two antibody mediated processes can lead to the prevention of spread of the disease and simultaneous elimination of mycobacteria from the blood stream. On investigating the effects of malnutrition on DTH, Kapoor *et al* (1985) detected a direct relationship between the haemoglobin levels and the anti-PPD DTH response. This indicates that a malnourished patient with a low haemoglobin, often has a decreased DTH response.

In 1981, Kardjito *et al* also observed that the DTH response is directly related to serum levels of albumin. In malnourished individuals, a decrease of this protein is associated with a lesser DTH response. By virtue of lesser tissue damage due to

inflammation this may be advantageous to the already compromised host.

Gupta *et al* (1983) made similar observations and found a significant correlation between the level of anti-PPD IgG antibody and PPD-induced DTH (r=0.468) in patients and (r=0.208) in controls.

2. Mycobacterial infections and their mutual cross-reactivity :

The so-called environmental "atypical" mycobacteria are known to possess determinants that may be antigenically related to the epitopes of Myc.TB (Anderson *et al*,1986) leading to cross-reactivity among the antibodies and receptors recognizing them. This may result in some of the phenomena observed in this study. The atypical mycobacterial infections may therefore have an influence on tuberculin sensitivity of the population. Previous observations have shown that different populations showed considerable variations in response to BCG vaccination.

Hart in 1932 had shown that a dilution of 1/1000 of OT was usually sufficient to elicit a positive response in TB patients. When the subjects were skin-tested with tuberculin in Europe, they either developed a pronounced local induration and erythema or no reaction at all. A few individuals in the latter group showed very slight or no reaction, when tested again with a higher dose of tuberculin. Clearly these small reactions were 'non specific'. Palmer and Edwards (1968) on testing navy recruits obtained evidence that the positive response following dual skin testing could be associated with immunity against TB- infection. The

incidence of TB disease was seen to be 50% less in men with such low grade sensitivity than in men with no such immunity when the recruits were followed. This indicates the presence of a low but effective immunity against Myc.TB disease in these individuals. This low grade sensitivity detected by dual skin-testing with tuberculin was thought to identify different sensitizing agents (Hart & Sutherland,1977).

Furculow *et al* (1941) observed that healthy individuals who did not respond to a dose of tuberculin which could normally elicit a reaction in TB patients, were injected subsequently with a higher dose of tuberculin, did give a positive reaction. This response to only a high dose of tuberculin was also 'non specific' and could reflect priming of the immune reactive cells by the lower dose given first, or it could be attributed to previous immunization by atypical mycobacterial infection causing an initial weak sensitization enhanced by cross-reacting with the skin test antigen given later. The two possibilities cannot be distinguished because it is unethical to give a high dose in the first instance.

Antigenic structure of Mycobacterium tuberculosis:

The structure and antigenic features of the mycobacteria are reviewed briefly.

The structure of mycobacteria is highly complex, containing many different proteins, sugars and lipids which are closely associated in macromolecular complexes. Thus it would be expected that the number of antigenic determinants or epitopes occurring on each bacillus is enormous. The numbers demonstrated by various tests

depends upon the preparation used and the specificity of the test. Therefore no single technique can reveal the total set of antigens in a bacterial cell. To date immunoelectrophoresis of cytoplasm (Petroff *et al*, 1917) in concentrated culture filtrates of Myc.TB and immunodiffusion (Stanford *et al*, 1974) of bacterial ultrasonicates, have been utilized to study the antigenic properties of Myc.TB. Crossed immunoelectrophoresis permits detection of up to one hundred distinct antigens of Mycobacterium TB, which is probably a significant underestimate of the total number. Some molecules such as polysaccharides may consist of repeating units with similar antigenic properties, but other molecules such as proteins may bear more than one distinct epitope on the same molecule (Daniel and Janicki 1978). Variability due to evolution may lead to these protein molecules possessing both species-specific and species-shared epitopes.

Janicki *et al* (1971) have demonstrated 11 major antigens by electrophoresis. The antigens 1,2 and 3 have been identified as being polysaccharides and are common to all mycobacteria,while the antigen 5 has been identified as a glycoprotein with antigenic specificities apparently restricted to Myc.TB. Information concerning the physicochemical nature of the other ten antigens is being acquired.

Another important and likely source for the variability and antigenic diversity is the culture method used. Boyden and Sorkin (1956) suggested that the bacilli could produce special adaptive enzymes when growing in vivo and these might generate formation

of antibodies, which by neutralizing the enzymes could play a protective role in favour of the host. Such antibodies however cannot be elicited by use of antigens from bacilli grown in culture. It may lead to postulating that the bacilli contain a much greater number of antigenic determinants for immune cell reactivity in vivo than in vitro.

Immune cellular interactions:

Like with any foreign antigen, the immune reaction of the body to any mycobacterial infection may be regulated differently in each individual, depending on the genetic susceptibility and existing immunity to the specific and related antigens. This could be explained by the observation of immune reactions in different individuals exposed to the same antigen. The major cell types involved in an immune reaction are the antigen presenting cells (APC), T-cells and B-cells.

The APC include different cell types with related functional activities present at various sites in the body. In an immune response these cells are capable of "taking up" the antigen intracellularly and breaking down or processing it and later re-expressing some parts of it on the cell surface to be recognized by other effector cells. The recognition of antigen differs for different cell types. B-cells recognize native antigen, while helper T-cells can only recognize antigen in the context of self histocompatibility antigens. The antigen presenting cells are the macrophages, dendritic cells, Kupffer cells, Langerhan's cells and cells of reticulo-endothelial system. It has also been shown that

the B-cells are capable of antigen processing and presentation to other cells of the immune system.

The T-cells may either induce or suppress both humoral and cell-mediated immune responses. These opposing regulatory activities are mediated by T-helper (Th) or T-suppressor (Ts) cells respectively. When a foreign antigen is recognized by a B-cell bearing specific receptors with affinity for this particular antigen, that B-cell will proliferate, provided T helper signals are also available. Clonal growth gives rise to daughter B-cells each with the parent specificity. Some of these progeny cells mature into antibody-secreting plasma cells and others become memory cells. As a result large amounts of antibodies specifically reactive to this antigen are released into the circulation. The cellular as well as the humoral responses are specific both in the induction and effector phases.

Non-immune defence mechanisms:

The macrophages are involved not only in immune responses but also in other defence mechanisms due to cytoplasmic phagosomes and lysosomes containing a variety of bacteriolytic agents. These phagocytic and bacteriolytic activities operate when any infective agent enters the body.

If an infection progresses despite an immune response, the survival of the infecting organism could be explained by the absence or inhibition of an effective non-immune reactivity. For instance as proposed by Adams in 1982, certain bacteria can avoid

the body's defence mechanisms of phagocytosis by either of three courses: firstly they may escape the phagosomes and lie free in the cell cytoplasm, secondly they could prevent fusion of lysosomes and phagosomes, and thirdly, they may resist the bactericidal effects of the lysosomes.

While studying macrophage activation in Myc.tuberculosis infection, Lowrie *et al* observed in 1979 that the mycobacteria were readily ingested by the macrophages, but they markedly impaired the fusion of bacterium-containing phagosomes to the lysosomes. Dead bacteria did not cause this impairment, and live BCG were less able to impair fusion than the more virulent strains of Myc.TB. On the other hand antibody-coated bacteria were unable to inhibit the process of fusion and therefore the organism could readily be eliminated by specific antibody. Although the in vivo significance of this phenomenon is not known it suggests that anti-mycobacterial antibodies produced in the host, could play a protective role in the host-pathogen interaction by enhancement of a non-immune reaction.

The monocytes and macrophages can also regulate the activation of reactive T-cells through the level of IL-1 production. It has been shown by Fujiwara *et al* in 1986, that in the presence of lipopolysaccharides (LPS) or PPD, the monocytes from patients with tuberculosis produced significantly higher activities of IL-1 than did those from healthy individuals, when both showed positive skin reaction with tuberculin. In contrast PPD-induced blast transformation response was lower in patients than healthy

controls, and IL-1 production by monocytes showed a positive correlation with monocyte suppressor activity for PPD-induced blastogenesis of peripheral blood mononuclear cells from healthy individuals. Also, exogenous IL-1 was capable of suppressing PPD-induced blastogenesis, suggesting that monocytes from TB patients are activated to produce increased IL-1 which in turn may be a mediator of suppressor cell function. The suppressor cells could be a different subset of monocytes from those involved in IL-1 production.

In 1979, Katz *et al* attempted to study the effects of TB and subsequent chemotherapy on B cell function and on the subpopulations of T cells that have immunoregulatory activities. They observed a marked decrease and in fact absent plaque forming cells (PFC) responses in patients with active TB as compared to healthy adults, this improved significantly ($p<0.05$) after chemotherapy instituted for 4-6 weeks, but it was still much lower than PFC responses in normal donors ($p<0.001$). They also observed that both treated and untreated TB patients have circulating adherent cells that are capable of suppressing autologous PFC responses, and that removal of these cells partially corrected the suppression in untreated cases and completely reversed the suppression in treated patient cells. Acute TB patients had relatively increased percentages of TgR cells (with Fc receptors for IgG) ($p<0.001$) and a concomitant decrease in TμR cells (with Fc receptors for IgM) ($p<0.05$). After 4-6 weeks of chemotherapy a rise in TμR and a fall in TgR cells

occured leading to correction of the proportion to levels comparable to normal donors ($p<0.02$).

Antigen-antibody reactions for testing of Myc. TB. antigens

(a) Complement fixation tests: Many investigators have used this test for the diagnosis of TB. The principle of the method is that complement binds to appropriate antibodies causing lysis of erythrocytes which have adsorbed mycobacterial antigens on their surface. Thjotta and Gunderson in 1929, obtained positive results in 94.6% of pulmonary TB patients, but only in 44% of extrapulmonary TB. The results in these early studies varied enormously and the general opinion was that the test is positive in the majority of active TB patients. But it is also positive for inactive TB, in healthy controls as well as in other diseases. This makes the test not very discriminatory and therefore of less value to distinguish between healthy individuals, inactive TB patients, and active TB patients.

(b) Haemagglutination test: This is also referred to as the Middlebrook & Dubas (1950) test. In this procedure red blood cells (RBCs) sensitized with water soluble mycbacterial antigens are added to serial dilutions of test serum, which has been heated to inactivate complement. Agglutination at a serum dilution of 1:8 was found for many cases of TB but not in sera from healthy controls. Later, in order to attain more specific results modifications of this test system have been used. The tests were extensively evaluated by various investigators and showed

enormous variation in the sensitivity and specificity using tuberculin antigen and Rhesus negative Group-O human, or sheep erythrocytes. As for sensitivity, the percentage of sera giving positive reactions at a titre of 1/8 or greater varied from 0-50% in non-tuberculous sera. The range of discriminatory power was very wide, varying from 92% of TB patients and none of controls, to the other extreme of 50% of controls and 60% of patients giving positive results. Sheep erythrocytes on the whole gave better results, but the test has not gained popularity because of its complexity and has been replaced by technically simpler tests for anti-tuberculin antibody assays.

(c) Radioimmunoassay (RIA): This highly sensitive technique is based on binding of a radiolabelled antibody to antigen or vice versa. In the Farr technique, radiolabelled antigen combines with antibody in the fluid phase and this complex is then precipitated out by a protein-precipitating agent such as ammonium sulfate. The radioactivity of the co-precipitated antigen is measured. If antigen itself is a protein then anti-immunoglobulin serum is used for precipitating out the labelled antigen-antibody complex. But this technique proved not to be very sensitive and was unable to discriminate culture proven TB patients from healthy controls.

In contrast to this, in the solid-phase RIA, unlabelled antigen is used for binding to a suitable surface. Antibody binding to this antigen is then detected by addition of a radiolabelled antiglobulin. This gave much better sensitvity and specificity to the assay system.

(d) Enzyme linked immunosorbent assay (ELISA) is a useful tool to quantitate antibody levels in various immunoglobulin classes to a range of soluble and insoluble mycobacterial antigens. The principle involves binding of antigen to a solid phase such as polystyrene plates, and testing the antibody-containing serum, by incubating it in the antigen coated plate under optimum conditions for the reaction. The amount of antibody binding is then assessed by incubating the complex in the presence of an anti-immunoglobulin linked to an enzyme. The enzyme is then allowed to combine with its specific substrate, yielding a colored reaction in contrast to a colorless background in control wells. The ELISA has gained popularity because of its reproducibility, minimum operator's subjectivity in evaluation of results and safety as compared to RIA because there are no radioisotopes used.

In order to select the antigen for TB testing, it is useful to refer to the information given by Winters and Cox (1981) who found comparable readings with different antigens in RIA using plastic coated beads as carriers. The antigens used were

(1) BCG whole cell —
(2) Myc. TB whole cell
(3) PPD
(4) Myc. TB cell wall and
(5) BCG cell wall.

The results were positive respectively in 59, 56, 52, 46 and 37% of patients with bacteriologically proven TB infection. Slightly higher levels of antibodies were found in tuberculin skin-test

positive than in tuberculin negative controls. It is clear that PPD can safely be used as the best choice because of its competitive results, and also because it is available for research purposes as synthetic preperation with standard reactivity. These conditions would be impossible to attain with the whole cell preperations.

LITERATURE REVIEW:

Serum antibodies : Serum antibodies to tubercular antigens in man have been observed by various investigators, since the beginning of this century. The methods have varied and therefore, the levels of sensivity and specificity between the different test systems have also varied.

In 1903, Bordet and Gengou demonstrated a complement fixation test in animals using whole bacillus as an antigen. This test system involved complement lysis of erythrocytes which had mycobacterial antigens adsorbed onto their surface, provided there were appropriate antibodies present. Later in 1918, Brown and Petroff utilized this test in patients with pulmonary tuberculosis and found the test to be negative in extrapulmonary TB. Pulmonary TB patients appear to produce much more antibody to mycobacterial antigens than patients with extrapulmonary TB. Therefore the test was not considered to be sufficiently sensitive for the diagnosis of TB disease.

Later, Middlebrook and Dubos (1950) used a hemagglutination assay for detecting anti-TB antibodies. In this procedure, sheep erythrocytes were sensitized with water soluble mycobacterial antigens. Serum (heated to inactivate complement) from TB patients and not healthy controls, at dilutions of 1:8 or more was seen to agglutinate the sensitized sheep erythrocytes. Middlebrook used human group O, Rhesus negative erythrocytes, to avoid agglutination by heterophilic antibodies. Using this assay, he

discovered that polysaccharides which were common to all mycobacterial species as well as some other bacteria (Janicki *et al*, 1978) were the antigens binding to erythrocytes, rather than protein moeities of the bacillus which were more species-specific (Daniel *et al*, 1981). This led to further modifications by Boyden who treated erythrocytes with tannic acid and found that mycobacterial protein antigens were now adsorbed by these cells. Sera that agglutinated PPD-coated tanned RBCs at a titre of 1:160 or greater were considered positive. In this study the test appeared to be more discriminatory and specific than the test with untreated RBCs. But the system was too complex for assaying the anti-tuberculin antibody, therefore it did not gain much popularity.

Another type of assay utilized for this purpose is the radioimmunoassay (RIA). Different investigators have used modifications of the solid phase for RIA, for example polystyrene microtitre plates or plastic coated metal beads for coating of antigen. Nassau & Parson (1975) used this technique to quantitate antibodies binding to polystyrene plate wells coated with concentrated culture filtrates of Myc. TB. After addition of I^{125}-labelled antiglobulin the wells were cut out from the plates and counted in a scintillation counter. The results were expressed as percentage of the activity of a 'standard' highly positive serum. Values greater than 30% of the control were obtained with sera from 63% of 140 patients with TB and in one of 179 control individuals, while values greater than 20% occurred in 88%

patients and 12% control subjects. These results indicated that solid-phase RIA was superior to the precipitation RIA.

Subsequently Mauch *et al* (1980) quantitated antibody binding levels in sera from 90 healthy individuals and 40 patients with pulmonary tuberculosis. The 95.3 and 99.7% confidence limits for healthy individuals were established and it was found that 64 and 52% of sera from patients with TB had binding levels of antibodies above these two confidence limits, respectively. Although RIA is a powerful tool in the study of antibodies for TB the cost of equipment and reagents may make it unsuitable for routine use in the study of TB outside major research centres. It is likely therefore to be replaced by enzyme immunoassay in many instances.

The technique that has gained popularity recently is the Enzyme-linked immunosorbent-assay (ELISA). It utilizes a stable alkaline phosphatase linked anti-human globulin conjugate, and the assay is reproducible. It is therefore a very useful tool to quantitate antibody levels in various immunoglobulin classes reactive to a range of soluble and insoluble antigens. It also has the advantage of providing results in a short time.

The sensitivity and specificity of the ELISA system has been addressed by different investigators and their results are quite comparable.

In animal models where responder and non-responder strains have been studied to a great extent, the immune response to an antigen may vary considerabely from host to host, owing to genetically

controlled variations in antigen processing by APCs (Weiner *et al*, 1974) or in the regulatory cell functions. Moreover, the humoral response may be affected by antigenic competition (Taussig, 1973). Prior exposure to certain shared mycobacterial antigens which may have similar epitopes to Myc.TB, accelerates subsequent responses to these antigens and may suppress formation of antibodies to the newly introduced specific antigens. For instance, a molecule may possess two determinants, x and y, but for spatial reasons only x may be able to bind to the B cell. In such a case, an excess of B cells reactive to antigen x would inhibit by competition the attachment of cells reacting to the y determinant. This is termed intramolecular antigenic competition. Competition also occurs when the epitopes are located on different molecules, in which case Taussig has postulated that the limiting relative amount of help from co-operating T-cells is responsible. Irrespective of the mechanism, the occurrance of antigenic competition may considerably reduce the humoral immune response to specific mycobacterial antigens in tuberculosis.

Thus for discrimination of TB patients from non-TB controls, an antigen unique to Myc. TB would be more useful as compared to its other antigens. It is yet to be seen if it can be of practicable value. Zeiss *et al* (1982) have used a modified RIA in parallel with the ELISA for assaying IgG antibodies reactive to PPD in sera of patients with active TB. Both assays demonstrated a marked increase in IgG antibody activity in TB patients as compared to

skin-test positive and negative healthy controls, with a highly significant correlation between the two methods (Spearman's rank test; $r=0.976$; $p<0.001$) on the basis of the results obtained. Because of the independent observations made both by Favez *et al* (1966) and Freedman *et al* (1966), that an IgG antibody response was more specific in correlating with the disease state than the total antibody response, these investigators assayed only for IgG and then suggested that the RIA and ELISA methods were both equally useful in discriminating TB patients from non-TB controls when the control group were also skin test positive to PPD.

Kalish, Radin and colleagues (1983) used ELISA to detect and evaluate IgG, IgM, IgA and IgE antibodies against PPD (produced by Parke Davis Co.) with respect to diagnosis of active TB. They found that serum IgG levels and secretory IgA levels both showed a significant increase in patients with active TB, while there seems no correlation between levels of IgM and IgE with the presence or severity of the disease. The IgG level was also found to decline with treatment and the duration of therapy had a direct effect on the serum IgG antibody level. There was no significant difference in healthy skin test positive or negative individuals, whereas diseased individuals showed IgG titres as high as 1/1000. Radin, Zeiss and Phair (1983) found that active TB patients showed a significantly higher titre ($p<0.00001$) of IgG antibody activity against PPD (Parke Davis) than healthy skin-test positive individuals. Serum IgM and IgA antibodies were much less discriminatory .

In 1980, Tandon *et al* attempted to test the usefulness of ELISA in immuno-diagnosis of tuberculosis. They assayed for anti-PPD IgG antibody, and found that approximately 80% of blood samples from sputum positive TB patients gave positive results on assaying for the antibodies reactive to purified tuberculin. The test detected disease also in 56% cases in which no bacterial evidence of disease was present yet. The investigators therefore believe that if preliminary clinical and radiological examination suggest a tuberculous infection then ELISA can safely be recommended as a test for additional diagnostic information.

Kalish and colleagues (1983) made similar findings by ELISA experiments. They observed that active TB patients with pulmonary infection had significantly higher mean levels of IgG antibodies reactive against PPD than the normal controls or patients suffering from atypical mycobacterial disease ($p=0.005$), sarcoidosis ($p=0.0001$), histoplasmosis ($p=0.004$), blastomycosis ($p=0.008$) or cryptococcosis ($p=0.017$), patients who had recieved BCG vaccination ($p=0.003$) and PPD skin test-positive and skin-test negative control subjects ($p=0.001$).

Grange *et al* (1980) used ELISA to study the levels of antibody binding to BCG belonging to the IgG, IgM and IgA classes in patients suffering from granulomatous diseases of TB , sarcoidosis and Crohn's disease. They studied the three diseases simultaneously because of the suspicion that the etiology for the other two diseases is also mycobacterial in origin. These antigens they suspect may be in the cell-wall-free or filtrable forms which may

exist in circulation after the phagocytic processes have degraded the non-pathogenic mycobacteria. Patients with TB as compared to Crohn's disease and sarcoidosis showed significantly elevated levels ($p>0.001$) of IgG antibodies binding to antigen prepared by ultrasonicate of BCG (Glaxo). Many patients with sarcoidosis and Crohn's disease by contrast, had significantly higher levels of IgM class (27.5%) and IgA class (45%) antibodies.

Relevance of Mantoux test in tuberculosis:

Aziz and Haq (1985) in Pakistan, investigated 181 clinically diagnosed TB patients who were either positive or negative for culture of gram-positive acid-fast bacilli in their sputum. When Mantoux testing was done 93% of the sputum positive TB patients were positive as compared to only 12% of the sputum negative patients. This led the authors to suggest that review of the diagnosis of pulmonary tuberculosis in Mantoux negative cases was necessary. This observation underlines the importance of doing both Mantoux testing and culture from each patient.

Spirer *et al* (1977) have tested in vitro T-cell function in young children, by utilizing the leucocyte migration inhibition test (LMIT) mediated by cells sensitive to tuberculin. This factor which is capable of inhibiting migration of normal leucocytes in culture can be produced by the antigen sensitized lymphocytes. LMIT in conjuntion with the Mantoux test were used as parameters of cell mediated immune response (CMIR). They demonstrated that the absence of skin reaction does not mean the absence of LMIT reaction. The Mantoux test was positive in only 32.4% cases as

compared to LMIT which was positive in 50% cases. Maximum waning of CMIR occurred in the first three years of life and malnutrition accelerated this waning. The investigators did not report on the state of humoral immunity at the time of waning CMIR. Youman (1979) had experimentally demonstrated that immunity against TB is not related to delayed hypersensitivity to mycobacterial antigens. It is important though to keep in mind, that the negative Mantoux results obtained in TB patients may be due to generalized anergy in some of the patients.

Seth *et al* (1985) considered the symptomatic Mantoux positivity (SMP) and primary pulmonary complex (PPC) as milder forms of disease in children and studied the immunoglobulin levels in these patients.

Lenzi *et al* had shown that the immune spectrum of TB in children was different from that in adults. In children the fresh infection is accompanied by circulating antigen alone, and antigen antibody complexes are formed with treatment of infection, whereas antibody alone is present in circulation when the infection is controlled. The level of IgG was significantly increased ($p<0.05$) whereas IgM level was significantly decreased ($p<0.05$) in children with SMP and PPC as compared to healthy controls. No significant difference was seen between the two disease groups for IgG and IgM. The level of IgA was comparable in the SMP, PPC and control groups.

In India, Gupta *et al* (1983) and many other investigators, have observed a positive relationship between the Mantoux test

reaction and anti- PPD antibody levels in the blood. They found that Mantoux positive individuals had high IgG antibody titres, whereas Mantoux negative individuals had low antibody titres when tested by ELISA.

Grindulis *et al* (1984) investigated children at 22 months age who had been vaccinated with BCG at birth, and who either developed a scar at the injection site or not. The absent scar could be a result of unsatisfactory vaccination, or it indicated failure of sufficient reactivity by the child to produce a scar. They were all tested for cell-mediated immunity against tuberculosis by means of Mantoux test reaction and by in vitro blast-transformation with tuberculin (OT) as well as phytohaemagglutinin (PHA) and pokeweed mitogen (PWM). The latter two were used as non-specific mitogens. They showed that amongst those chidren who were given BCG at birth and had a scar about 50% showed a negative skin reaction to low dose of10 TU PPD (that is 0.1ml 1/1000) . When these negatively reacting children were reinjected with a larger dose of 100 TU PPD (0.1ml 1/100) subsequently, about 20% of them still showed no response even to the higher dose of antigen. When peripheral blood lymphocytes (PBL) of these skin test negative children were cultured with PPD, they showed a decreased lymphocte blast transformation, that is less than 50 % of the value of control (PBL from healthy adults cultured under similar conditions) against tuberculin, but not so with other mitogens.

Impaired reaction to tuberculin in a previously vaccinated individual may be caused by a defect of initial recognition of the

antigen, or failure of sensitized lymphocytes to react because of malnutrition or a serious infection. Defect in immune recognition could also be due to immaturity of the preterm infant's immune system, though in this study all such infants were excluded. Also there were no clinically ill or overtly malnourished children in the study, but children who showed no response to primary BCG vaccination were shorter, lighter and thinner than the other group which had shown a good response to BCG. This still is not enough reason to explain the decreased DTH or blast transformation response in so many children. Antibodies were not studied in these children which might have given an indication of Ts cell activity. Some babies have been shown to have impaired CMI at birth which may persist up to 12 months age (Ferguson *et al*, 1978 & Chandra *et al* ,1981). The children who had no scar were smaller for gestational age babies compared to those who reacted with a scar. But this again does not explain the reduced CMI in babies who reacted with a scar.

There is one major problem with the study, which is the use of lymphocytes from an adult as control. The adults are expected to differ greatly because of exposure and to a much wider variety of organisms that can modify the immune reactivity. The maturity of the immune system due to age is an important factor and the controls should therefore be age-matched.

Regulators of the immune response:

T-cells: In 1976, Moretta *et al* worked at identifying different subsets of T cells in humans that may have regulatory effects on

the antibody response. First they identified two distinct subpopulations which showed similar responses to Con A but different patterns of reactivity to PHA. One carrying surface receptors for the Fc portion of IgG (Tg) showed decreased response to PHA and included cells with suppressor capabilities for the PWM-induced production of intracytoplasmic immunoglobulin in B-cells, and others carrying surface receptors for the Fc portion of IgM antibodies (Tμ) which showed good response to high doses of PHA but minimal response with lower doses. The lower dose was the same as considered optimal for unfractionated T cell responses. They noted that these cells were present in approximately 20 % and 75 % concentrations respectively in the total T-cell population. The total T-cell population included some that did not carry receptors for either antibody, possibly due to the purification processes. However, it was attempted to exclude this possibility by the fact that Tg depleted and Tg^+ cells when mixed in the same proportion as in the original suspension gave a response to the mitogen phytohemagglutinin (PHA) identical to the unfractionated T-cell response. The investigators explained this discrepancy by suggesting that the T-cell subpopulations may exert some mutual regulatory control when responding simultaneously to a mitogen.

Katz *et al* (1979) observed that TB patients had a decrease in the absolute T- cell numbers, and showed a relative increase in Tg cells ($p<0.001$) with a concomitant decrease in Tμ cells ($p<0.05$). This may explain the reduced cellular response in active TB

allowing the infecting organism to go unchecked thus resulting in significantly increased antibody response. The T-cell numbers in this study were proportionally reduced in untreated patients ($p<0.001$). All these alterations returned to normal after anti-tuberculous therapy for 6-8 weeks.

Later when monoclonal antibodies (MoAb) to T-cell receptors were developed, in particular the OKT and Leu series, more precise definition of various T-cell subsets could be made, thus facilitating the study of interaction processes in immune reactions.(Reinherz and Schlossman 1980a, Ledbetter *et al* 1981). It was therfore realized that Tg and Tμ receptors alone were not very good markers for defining functional properties of cells. Instead these Fc receptors in conjunction with the MoAb appear to play an important role in T-cell function definition. (Reinherz and Schlossman 1981, Moretta *et al* 1981, Heijnen *et al* 1982 and Ballieux *et al* 1983).

OKT3 is a pan-T-cell marker and is present on all subtypes. OKT8 is expressed on both the suppressor and cytotoxic T-cells and OKT4 is expressed on the helper T-cells. Although expressed on the variously defined subsets of cells, these markers do not correlate solely with the function of T8/Leu2 or T4/Leu3 cells because the cytotoxic T-cells can possess both phenotypes. The alloreactive cytotoxic $T8^+$ cells have been seen to react with Class I MHC and $T4^+$ cells react with Class II MHC antigens. The suppressor cells in the $T8^+$ group are either Ts-effector ($T8^+T3^+DR^+$) or Ts-activator ($T8^+T3^+DR^-$) cells. DR is an antigen expressed on most of the

activated cell subsets.

A small subset of peripheral T-cells that do not have receptors for either IgM or IgG and are $T\mu^-g^-$ can be induced to express Fc receptor for IgM (FcμR) after activation by 5h. of culture with the antigen. (Heijnen *et al* 1980). These activated helper T-cells which were primed $T\mu^+$ cells were also capable of exerting suppressor-inducer activity. Therefore it was realized that the same subset of $T\mu^+$ cells simultaneously possessed both helper and suppressor inducer activities. They were characterized further by OKT4 and Leu3 antigens. (Heijnen *et al* 1982a). The subpopulation of OKT4+Leu3+ cells therefore included T-cells subsets with different functional activities, that is T-helper cells which required the presence of APC for activation and recognized antigen in the context of Class-II histocompatibility antigens (Rees *et al* 1981, Uytde *et al* 1982) while the T-suppressor-inducer cells that could recognize the antigen in its native form could therefore be generated in the absence of adherent monocyte APC. (Heijnen *et al* 1981, Rees *et al* 1981, Uytde *et al* 1982).

Unprimed T cells when given an inducer signal by these suppressor-inducer T4+Leu3+ cells can develop into suppressor effector cells. They are therefore said to be precursor-suppressor cells passing into the effector state. Both are phenotypically T8+. The Ts effector cells express FcgR at the surface. Suppressor activity has also been attributed to mononuclear cells which are $T8^+3^+OKM\text{-}1^+$ phenotype. The mononuclear leucocytes,

granulocytes and the natural killer or NK-cells are all recognized by OKM-I marker (Kleinhenz *et al*, 1985 and Katz *et al*, 1979) but it remains to be established if there is a subset of suppressor effector T-cells which also are OKM-I positive.(Heijnen *et al*, 1982). The Tc or cytotoxic cells were found to vary in their phenotype, that is they could either be $T8^{+}T3^{+}$ or $T4^{+}8^{-}3^{+}$.

Monocytes: In TB infection apart from T-cells other cells shown to regulate antibody responses are the monocytes. Katz *et al* (1979) observed that patients with active or treated TB have a subpopulation of circulating monocytes that are capable of suppressing autologous PFC responses by PWM-stimulated lymphocytes against SRBC. This suppression was reduced by removal of an adherent cell population from the suspension of mononuclear cells, and it led to a significant increase in the antibody response in both treated as well as untreated TB patients. Neither group of patients had increased proportions of monocytes as compared with the normal subjects ($p<0.2$), and so this finding cannot be explained on a purely quantitative basis. Kleinhenz *et al* (1985) defined these cells as an adherent cell population reactive to the monoclonal antibody OKMI, which were non-erythrocyte-rosetting, and radio-sensitive mononuclear cells. These monocytes due to their exaggerated IL-1 production (Fujiwara *et al*, 1985) were also capable of causing suppression of PPD-induced blastogenic responses which were lower in TB patients than in healthy subjects

($p < 0.005$). Thus IL-1 may be a mediator of suppressor cell

function.

Effect of materno-fetal relationship on PPD reactivity:

Mohr (1973) studied the skin-test response in children (age range from 12 months to14 years) and observed that 5 out of 9 children born to tuberculin skin-test positive mothers and who were breast fed were reactive to intradermal tuberculin. None of the 78 children born to and breast fed by skin test negative mothers reacted to tuberculin. Also 2 children born to tuberculin positive mothers but not breast fed showed negative reaction to tuberculin. The investigator therefore proposed that this aquisition of cellular hypersensitivity was associated with ingestion of colostrum. There was no evidence of placental transmission of TB sensitivity occurring in case of mothers who were tuberculin positive and who did not breast-feed their children.

Schlesinger and Covelli in 1977 in a similar study on lymphocytes observed that 8 out of 13 children born to tuberculin positive mothers had tuberculin reactive peripheral blood T-cells after 4 weeks of breast feeding as compared to 1 out of 13 bottle fed infants of positive mothers and none of 9 breast fed infants of tuberculin skin-test negative mothers. There was no evidence of transplacental transfer of tuberculin responsiveness in mothers who were positive in response but did not breast feed.

Caspary and Field in 1971 while investigating for tumors, identified in young infants of 7 months and 4 years ages, the occurrance of sensitized peripheral blood lymphocytes which on incubation with PPD produced a factor called the macrophage

slowing factor (MSF). This could be identical to the macrophage inhibiting factor (MIF). This strong sensitivity to PPD was a very unexpected finding, therefore they studied lymphocyte sensitization in the newborn with reference to the corresponding state in their mothers, and found that CMI to the same antigen was present in each infant to which the respective mother was sensitized. It led to various speculations concerning the mechanism whereby immunologic information was passed from mother to newborn.

One possibility is passage of the maternal lymphocytes via the placental route (Schroeder *et al*, 1974), as assesed by the chromosomal study. It is postulated that if lymphocytes pass into the fetus they may lead to chimerism causing either graft vs host disease or immuno- deficiency in the baby. This cannot however be considered a common or normal occurance as in the present study the PPD-positive children were normal and none suffered from immunodeficiency. Secondly, some circulating maternal antibody passed on to fetus may render the baby's cells reactive. This is under study and cannot be excluded completely. Thirdly, subcellular components like maternal lymphocyte DNA may be utilized in the make up of fetal lymphocytes. This has not been studied in humans yet. Antigen might pass from mother to fetus. Gill, Kunz and Bernard (1971) stressed that antigen metabolism played a crucial role in the induction and regulation of the immune response. When radio-labelled aggregated antigen was injected into rats under conditions which would not favour degradation of

antigen, later testing of the offspring indicated passage of the antigen into the newborn where it localized in bone marrow and in a few cases in thymus and spleen also. The newborns showed circulating antibodies reactive to the antigen and an enhancement in specific antibody response to the antigen on secondary challenge, as compared to control newborns.

Abrahams (1970) in Brisbane, has shown that after BCG vaccination in infancy, these children at a later age, react more strongly to PPD-B (from Myc.TB) rather than to PPD-S (from Myc. Avium). The investigators propose that exposure to environmental atypical mycobacteria may provide a continuous antigenic stimulus, resulting in the regulation of immunity which could reverse the tendency of these children making them more reactive towards PPD from Myc.TB (PPD-S), although they were vaccinated with BCG during infancy which contains PPD-B. For this reason we see the immunity from TB after BCG vaccination.

Effect of pregnancy on immune reactivity:

Covelli and Wilson (1978) have observed a progressive depression in pregnant patients in the cell mediated immune response to specific mitogen like PPD, while sparing the non-specific mitogens like PHA which stimulate the lymphocytes polyclonally. This depression in antigen-specific responses attains significance at 36 weeks of gestation and continues through delivery ($p<0.001$) but the reactivity returns as soon as 24 h. postpartum. It is likely that a soluble serum factor is produced during the later part of pregnancy which alters the reactivity of sensitized lymphocytes.

This explanation is supported by an observation made by Maraz & Petri (1974), that supernatants from the lymphocytes of gravid women and subjects with choriocarcinoma were capable of depressing nonspecific mitogen stimulation of unwashed lymphocytes. Jones & colleagues (1973), were also able to demonstrate a factor in the maternal plasma from gravid women in the third trimester that was capable of inhibiting blastogenesis.

Anderson *et al* (1962) observed that washed maternal PBL normally show allogeneic responses against paternal antigens. A serum factor appears during pregnancy which blocks this response to paternal antigens. This may be one important reason for the survival of the fetus to term despite being immunogenic to the maternal immune system. Rocklin *et al* (1976) demonstrated in some patients with spontaneous repeated abortions, this blocking factor is sometimes absent which may be the reason that fetal cells carrying paternal histocompatibility antigens are a target for maternal lymphocyte reactions. It is therefore conceivable that emergence of sensitized lymphocytes is accompanied by the production of a serum regulator, which acts as a braking mechanism to prevent lymphocytes from abnormally high responses under physiological conditions.

Lang & Noren reported in 1968 that antigenic stimulation by viral infection during intra-uterine life not only results in specific IgM production by the fetus in utero, but may also result in elevated levels of IgM antibodies at birth in neonates. Fetal IgM levels

increase rapidly after the 20th week, and reach values comparable to those in maternal serum by the 40th week in intra-uterine infections. Fetal IgG production against viral antigens is also stimulated, but this is masked by the high maternal IgG levels transmitted through the placenta.

Smith, Field and Caspary in 1972 studied the lymphocyte reactivity to PPD antigen in pregnancy and observed that reactivity diminishes as pregnancy advances and is restored to normal during the first 10 days after delivery. There is an associated hormonally controlled corresponding rise in level of lymphocyte depressing factor in serum which falls after delivery.

Effect of BCG vaccination:

The immune response of the individual to BCG vaccination depends on various factors :-

1. Route of vaccination- intradermal injection is noted to be the most reliable route.
2. Dose of vaccine.
3. Type of vaccine - freeze-dried BCG is found to result in least variability due to environmental effects on the vaccine.
4. Immune responses - Influences on the cell-mediated immune response (CMI) and humoral response (antibody formation) in the host and their possible relationship to atypical mycobacterial infections.

This last factor has been studied rather extensively by various groups of investigators. It has been observed by Fusillo *et al* (1958) that the BCG vaccination didnot induce any detectable increase in anti-BCG antibody response until 6-18 weeks later.

Immune responses: The delayed hypersensitivity reaction is a T-cell dependent phenomenon manifested by an inflammatory reaction at the site of antigen deposition usually the skin, reaching its peak intensity 24-48 hrs. after initiation. When an infection occurs the body develops hypersensitivity to the infective organism due to generation of memory cells which are known to persist in the circulation for many years, a well known fact in tuberculosis . In such individuals an antigenic challenge via the intracutaneous route, can cause these presensitized cells to

build up an acute and heightened response called the classical DTH response. This can also be elicited by previously induced active immunization with BCG vaccination, as well as due to acquired clinical or subclinical tuberculous infection. If the test is positive it indicates a previous exposure to Myc. TB. and the host has either immunity or disease which can be investigated further clinically. If on the other hand, the skin test is negative it does not rule out disease, though it certainly indicates lack of detectable immunity on part of the host.

Other cell-mediated immune responses: Kathipuri *et al* (1982) examined differences in response to giving BCG vaccination to children either at birth or at three months of age. The objective of this study was to compare the immunity induced [illegible] newborn with that in older infants. They found that infants [illegible] capable of exhibiting sufficient anti-tuberculin CMI responses when tested with Mantoux testing and lymphocyte migration inhibition test (LMIT) whether they were given BCG at birth or at 3 months age. The Mantoux positivity was comparable in the group of infants vaccinated at birth and that vaccinated at three months of age. Also the LMIT results were comparable in the two groups. The two cell-mediated responses could be elicited in 72.5% of infants vaccinated at birth and 78.2% of children vaccinated at the age of three months. The investigators therefore suggested that the BCG vaccination be given at birth especially in areas where the disease is prevalent, because of the greater chances of aquiring disease relatively soon after primary infection during infancy.

Relevance of cross-reactivity to protection by BCG:

Orme and Collins (1985), examined the possibility of cross-protection to infections by other mycobacterial species, offered by BCG in mice. They observed that Myc. kansasii and Myc. avium infections were controlled when the mice were exposed to these organisms after BCG immunization, but no protection was observed against Myc. simiae or Myc. intracellulare. There is a possibility that responses to some antigenic determinants on BCG cross-react with determinants of the two former species, and not with the antigens of the two latter species, and thus the immune response generated due to BCG is effective in one case but ineffective in protection against the other .

This may be applicable to humans based on an observation by Romanns (1983) in Sweden of an increase in the incidence of atypical mycobacterial infections after discontinuation of the regional BCG vaccination policy. It is possible that the strains susceptible to anti-BCG cell-mediated immune response, which were kept under control by vaccination were unchecked by not giving BCG vaccination caused the newly seen clinical infection. Explanations were sought during a large project in India (Madras Trial) for testing the efficacy of BCG-vaccination, which resulted in a controversial report that BCG is ineffective in preventing disease. After the trial, Paramasivan and colleagues (1985) identified some non-tuberculous mycobacteria from 8.6% of the 16,907 sputum specimens examined. The species most commonly detected were Myc. avium-intracellulare, Myc. terrae and Myc.

scrofulaceum. In order to explain this lack of protection against TB in adults in this trial, the interpretation given by Palmer and Long (1966) was that it may be due to the influence of the 'atypical' environmental mycobacteria, which was also supported by experimental studies in guinea pigs and mice. In guinea pig studies with Myc. avium the immunity produced was as good as with BCG. For the Madras trial therefore it was proposed that the non-pathogenic mycobacteria may immunize the population against challenge with the Myc.TB to an extent similar to BCG, so subsequent BCG vaccination provides little if any additional protection.

Stanford, Shield and Rook (1981) on the other hand proposed a slightly different hypothesis. According to them certain species of mycobacteria might so prime the immune response that subsequent vaccination with BCG may be affected adversely. They proposed that there exist two types of CMI mechanisms in the response to mycobacteria, both of which produce tuberculin positive tests. However, one mechanism is more protective than the other against mycobacterial infections. These are referred to as "Listeria-type" (described originally by Mackaness) and "Koch-type" (described by Koch) responses. When the environmental mycobacteria prime for a Listeria-type response, the pattern of reaction depends on the activation of antigen-presenting-and-processing-macrophages by specifically primed T-cells. These activated macrophages are phagocytic and bactericidal so they can provide protection to the host. In such cases subsequent BCG will be recognized by T-cells

and will markedly boost that response enhancing the capacity of T-cells to recognize further mycobacterial species by lowering the threshold of immunological recognition. BCG would therefore afford good protection by reinforcing the already induced phenomenon. In the case where environmental mycobacteria prime for Koch's type of response which results mainly in destruction of cells containing live mycobacteria or mycobacterial products on their surface, a tissue damaging hypersensitvity reaction is induced. BCG can have a boosting effect on this reaction leading to a temporary decrease in the level of immunity that already exists. Edwards *et al* (1982) confirmed the above hypothesis in ginuea pig model in which Myc. avium-intracellulare induced a protective effect similar to that of BCG but there was no evidence of an immuno-suppressive effect by subsequent BCG vaccination in this species.

Smith, Reeser and Musa (1985) working on guinea pigs also found that infection with Myc. simiae prior to BCG vaccination resulted in reduced skin reaction to PPD-S. However this was not reflected in a suppression of the protective effect induced by BCG vaccination. There was also no evidence of immunosupression by avium-intracellulare infection.

Madras BCG trial: This was a controlled community trial of BCG in south India by collaboration between WHO and the Indian Council of Medical Research. It started in 1968 and was completed in 1971. The entire population including all ages, reactors as well as non-reactors, were eligible, and the study included 260,000 of the

360,000 population. Vaccine was used from two different strains of BCG and each was given in two different doses.

Clearly neither of the vaccines in full or reduced dose gave any protection against pulmonary TB. Actually in persons with initial tuberculin reaction of 0-7mm there were more cases among vaccinated than among those given placebo. However this tendency reversed and after 10 years the number of cases developing pulmonary TB was similar in all groups of vaccinated individuals; 93 in those given strong vaccine, 99 in those given weak vaccine and 93 in those receiving placebo.

This report led to various explanations offered by those in favour of BCG. Some obvious ones are listed below:-

Discrimination of infected from non-infected: On the basis that only few persons had no sensitivity to tuberculin, persons with a skin reaction of 0-7 mm to the initial tuberculin test with PPD (3TU) were considered non-infected. These could have been misclassified and could in fact have included infected cases showing up later as actively infected patients.

Strain of infecting organism: The so-called "south Indian variant of Myc.tuberculosis" which caused infection in this region is different from the Myc.Tb and notably of low virulence in the guinea pig. The BCG may not be protective against this variant of Myc.TB, or the low grade sensitivity produced by environmental mycobacteria in that region may have protected as well as the BCG vaccination used.

Exogenous reinfection: This is an unsolved problem, and may have

played an important role in failure of the South Indian trial. In an English BCG trial the risk of reinfection was greatly reduced and it affected the results enormously. The incidence in tuberculin positivity, that is already infected cases, was 149 per 100,000 and after BCG dropped to 14.1 per 100,000 (Quoted in *British J. Chest Dis.* by Thoracic Assoc. 1980).

Manchester, Germany, Malaysia, Singapore and Korea all showed positive protection by BCG ranging from 60% to 90% (Quoted in *"Research on BCG vaccination"*. H.G. ten Dam. WHO)

As the children rarely produce bacterial positive sputum and tend to develop serious forms of disease such as miliary TB and TB meningitis which are often detected too late for treatment, it is essential to give them protection by whatever methods are available until further clarifications are available.

MATERIAL AND METHODS: The main objective was to study the development of immunity to PPD in infants, born to the mothers who possessed good cellular and humoral imunity to the antigen. Later, older children up to 24- months of age were included in the study to observe changes occuring with development. The plasma samples from some of these children were also tested for the presence of antibodies to "atypical" environmental mycobacteria, which are antigenically related to tubercle bacillus but do not produce similar disease. Finally, it was attempted to study the antibodies to various mycobacterial antigens in patients who were clinically proven to have infection either due to Myc.TB or by the "atypical" mycobacteria. This evaluation was undertaken with the future aim of seeing if either the existing antibodies in the newborn, or the cross-reactive antibodies produced later in infancy against another strain could affect the active immunity to be induced by BCG vaccination for protection against TB.

Throughout this study the enzyme-linked immunosorbent assays (ELISA) method was used for measuring antibodies. The assay system was sensitive and could detect antibodies at very high dilutions of plasma or serum samples. Thus it was relatively easy to overcome the usual problem faced with laboratory investigations in very young infants, as very small amounts of blood were sufficient for the test.

Specific anti-PPD antibody was detected by the indirect method. First, the multiwell polystyrene plates were coated overnight with the antigen, and after thorough washing the next morning the

plasma sample was added to allow the antigen-antibody reaction. If the sample contained antibody specific for PPD, that would bind to the coated antigen and form a complex on the solid phase. The bound antibody was detected with an enzyme-linked anti-human antibody. This second antibody was affinity purified goat anti-human antibody linked to the enzyme alkaline phosphatase. Finally enzyme substrate was added and the alkaline phosphatase gave a colour reaction by degrading the substrate. The optical density was measured on a Titertek multiscan plate reader and the relative concentration of the antibody in each sample was calculated from the OD reading of each well. For purposes of standardizing the assay, to avoid the effect of day to day variations, a positive serum pool was initially made, for use as a positive control in each assay. One row in each plate contained only the dilution buffer without plasma sample and it served as the background control. If high reactivity was seen in the background, it indicated some fault in the system that needed correction and then that particular assay was considered unreliable.

Positive pool: After obtaining an informed consent of each patient, blood was obtained from some TB patients receiving treatment at the Aberhart Hospital, and also from a few healthy adults who were known to give a high response to PPD when screened by blast transformation of PBL with PPD. These samples were screened in several ELISA tests for antibody titre, and the three with highest antibody content were pooled in equal amounts

to obtain a positive control pool. The assay was then standardized by repeating10 times and running the pooled plasma with different samples in serial dilutions. The standard deviation for the positive pool was calculated. Later it was used as positive control for calculating ODI in all the single dilution assays. OD readings for the positive pool for use as controls :

Anti-PPD IgG Mean ± S.D. = 1.52 ± 0.23

Anti-PPD IgM Mean ± S.D = 0.74 ± 0.17

The optical density index (ODI) was calculated for each sample in an assay as follows :

$$ODI = \frac{\text{Average OD of sample}}{\text{Average OD of positive pool.}}$$

Blood samples: For the purpose of analysis plasma samples were obtained from various sources:-

Group1: Normal mother and cord paired blood samples were obtained from mothers delivering at the University of Alberta Hospital and the Charles Camsell Hospital. The mothers of latter hospital were all Canadian native Indians.

Group 2: A large group of samples came from children receiving treatment at the UAH for diseases other than TB. The ages of these children ranged from one to twenty-four months.

Group 3: Nine patients with "atypical" mycobacterial infections, who were diagnosed on the basis of culture and were being treated at the Aberhart hospital.

Group 4: Six patients with TB admitted at the Aberhart hospital, or visiting the outpatient TB clinic. These last two groups included

patients ranging in age from 6 to 40 years.

The children were categorized into four groups, on the basis of age. Thus the studied groups contained thirty-nine infants of 1-4 months, twenty-four of 5-8 months, fifty-five of 9-12 months and fourteen of 13-24 months age (total of 103).

Standardization: It was felt useful to obtain the positive control in pure form if possible, to exclude control variability and therefore be certain to have a standard pool. Therefore IgG was isolated from the whole plasma pool to get rid of other factors that may interfere with the assay. Affinity purified IgG was obtained by Fast Protein Liquid Chromatography (FPLC) from the positive pool, then aliquoted and stored at -20 degrees for use as a positive control. Further standardization of the assay involved testing for the following variables which could affect the ELISA readings :-

a) Plasma lipids : It was suspected that the lipids by virtue of non-specific binding to the plate or antigen may interfere with the assay. This supicion was alleviated because there was no change in the OD readings when samples were tested from the same individual in either fasting state or after a fatty meal.

b) Antigen coating: It is important that the antigen be coated on to the plate as evenly as possible leaving a minimum number of unbound sites on the plastic. In case of some antigens, surface vesicles may form, which is prevented by the use of NP40 detergent. This results in an improved antigen coating and therefore different OD readings before and after the NP40

treatment would be observed. Coating with or without adding NP40 to PPD made no difference in the OD readings in the experiments described here.

c) General assay conditions: Type of plate, the kind of coating buffer for plates, the concentration of antigen and test samples were decided by using each in serial dilutions. Optimum conditions were noted and therefore selected for use throughout.

d) Inhibition assay : This test was done to ensure the specificity of antibodies that were being detected by the assay. If the sample is mixed with excess antigen before assaying, the antibodies in the sample readily form complexes and subsequent testing of the sample for antibody reactivity results in a much lower OD reading. This is because less antibody is available in free form to react with the same antigen coated on to the plate. This is known as the inhibition or competition assay, and indicates the specificity of the antibody that is detected by the test.

e) Antigen : The antigen used for the present study was PPD from Parke Davis Co, Detroit, Michigan Cat # 00719526-10 (Appendix I). The antigen was dissolved in sterile distilled water and aliquoted in sealed glass tubes and frozen for use.

Antigens of atypical mycobacterial species of Myc. avium-intracellulare, kansasii and scrofulaceum were very kindly provided by Dr. Kaare Haslov at the Tuberculin Department of Statens Serum Institute, Copenhagen.

f) Plasma samples: The dilution of 1/100 of plasma samples was used for the single dilution assays. Later serial dilutions of test

samples were used in titration aasays. Only trends of increase or decrease in antibody activity were detectable with single dilution assays, which were later differentiated into high and low titre groups after the serial dilution titrations.

g) Multiwell Plates: Polystyrene microtitre Immulon:2 Dynatech plates were used, originally, lot # 3000 for IgM, lot # 3002 for IgG and later lot # 52002 for both IgG and IgM detection. Because it was noticed that a change in the lot number of plates resulted in non-specific results (which could be due to differences in the treatment of the plastic used in manufacture) plates from the same three lots were used throughout.

h) Enzyme-linked antibody: Alkaline phosphatase-labelled goat anti-human antibody $F(ab)_2$ fragments directed to IgG and IgM (gamma-chain and mu-chain specific) were obtained from Sigma. A dilution of1:200 was found to be optimum after testing different dilutions. Tests for IgM are more sensitive than for IgG. Initially while using the antibody in 1:500 to 1: 1000 range, anti-human IgG was unable to detect human IgG while anti-IgM could detect its counterpart.

Concentrations of anti-IgG between 1:100 to 1:500 were tested and 1:200 was selected as one that detected antibodies causing reaction and colour development to proceed at a reasonable rate. The reaction was stopped between 20-40 minutes.

i) Substrate: p-nitrophenylphosphate substrate from Sigma was used at 1mg. per ml.

j) Buffers :

(a) ***Dilution buffer*** : PBS-Tween for diluting plasma and enzyme.

1x PBS + 0.5% Tween 20 + 0.2 g % sodium azide.

(b) ***Coating buffer*** : Carbonate coating buffer for coating the polystyrene plates.

2.1465 g. Na_2CO_3. 10 H2O (0.795 g. Na_2CO_3)

1.465 g. $NaHCO_3$

0.1 g. NaN_3. (0.5 ml of a 20 % solution.)

(c) ***Diethanolamine buffer*** : (10 %) for colour development.

97 ml. diethanolamine.

800 ml. distilled H_2O.

0.2 g. NaN_3

100 mg. $MgCl_2 6H_2O$

Adjust to pH 9.8 with 1 M. HCl, and adjust volume to one litre.

Store at 4^0 C in dark.

Warm volume sufficient for assay before use.

(d) ***Reaction stopping solution*** : 3M NaOH solution to stop enzyme-substrate reaction.

(e) ***Washing buffer*** : 0.1 % Tween 20 in distilled water for washing plates in the automatic washer.

ELISA PROCEDURE:

Flat bottom microtitre plates were coated with PPD solution in the coating buffer (1 µg/well in 200 ul) overnight at 4^oC to allow binding of the antigen to the surface of the well. The following

morning, the plates were washed 5x with washing buffer in the au[redacted] plate washer and the wells flooded with 1% BSA for 5 min[redacted] order to block all unbound sites on the polystyrene surface. The BSA was shaken out and plates were washed 5x again. The plasma or serum was centrifuged to get rid of all precipitates. Then the samples were diluted 1:100 for single dilution assays. For titration, the samples were used in 2-fold serial dilutions ranging from 1:80 to 1:5120. The first row of each plate contained only the dilution buffer to serve as the background control, which would detect the amount of non-specific binding of enzyme-linked antibody to the coated antigen or to the plate itself. One row contained appropriately diluted positive control antibody, which provided a source for standardizing the readings in each assay every day and for each plate. To the remaining ten rows were added the samples at 200 µl/ well in duplicates. The average background reading was deducted from the averages of positive control as well as the sample readings before any further calculations. This was also the method for screening of different lots of plates before the final selection was made. The plate was incubated at 37°C for 1h. at the end of which, the plates were washed with the washing buffer in the automatic washer, and then 200 µl. of the 1:200 diluted enzyme was added to each well, followed by an incubation for another 1 hr. at 37°C.

The plates were washed again as before and 100 µl/well of the substrate added which was 1mg/ml of p- nitrophenylphosphate in the diethanolamine buffer. The plates were left in light which

allowed the alkaline phosphatase reaction to proceed. The reaction was stopped after 20-40 minutes by addition of 25 µl of 3M NaOH to each well. The plate was then read on Titertek Multiscan (Fisher) plate reader at 405 nm, reference wavelength 690 nm and the optical density measured. The background control reading was averaged and substracted from the average OD reading for each sample.

Procedure for Inhibition assay : Plates were coated with PPD as usual. Before addition of the plasma sample it was first mixed with excess of the PPD antigen in a test tube, making a final mixture of 1/10 dilution of sample and 5 µg/ml of PPD. This was incubated for 1 hour at 37°C. At the end of one hour this mixture was plated and the assay proceeded in the usual manner. A standard control assay was done simultaneously without addition of PPD to the sample.

Single dilution assays : Analyses of the OD readings of all samples when testing only the single dilution of 1:100 gave no indication of the portion on the binding curve the reading would fall. Therefore it was not a good indicator of the relative concentration of the antibody.

Titration : In order to overcome the difficulty in single reading assays, all the samples were retested in serial dilutions. From the binding curve thus obtained, the titre was noted which was the sample dilution selected as the reliable lower limit of positivity of the antibody reactivity.

Procedure of dilution : The sample dilutions were started by

making a first dilution of1:4 (a) by mixing of 30 µl of sample to 90 µl of the dilution buffer. This was a reasonable amount of plasma to start with, because it could be measured with more accuracy than any smaller quantity. This was diluted a further 5-fold to obtain a 1:20 dilution (b) by mixing 30 µl of 'a' + 120 µl dilution buffer. This finally was tranferred to the test plate in which it was diluted again 4-fold thus obtaining a starting dilution of 1:80 of the original plasma sample by adding (50 µl of 'b' + 150 µl dilution buffer in each well). From this starting dilution 2-fold dilutions were made on the plate into each consecutive vertical pair of wells. Some samples which contained a high antibody content detected with one testing were further diluted and retested at higher dilutions. Titres were calculated by plotting the OD as function of dilution on a semilog graph paper. On approximating the different OD readings, different dilutions gave a curved line. From this the titre was read off which was the dilution of plasma giving an OD reading of 0.1 and titres of all samples in each group, were plotted, with the mean titre thus calculated for each clinical group.

RESULTS:

The work for this thesis initiated with the antibody testing of mother and cord blood samples, later young infants up to two years of age,and finally some patients with culture proven mycobacterial infections. Age matched controls were used for the last group of individuals. The results are given in the same order.

Section A:

Standardization of assay.

For each set of experiments with different antigens, it was attempted to standardize the assay before running test samples for actual data. Reproducibility of the assay was a major factor rigorously examined, and the conditions which could affect that were noted. The main difficulty in reproducing assay results were storage of samples and the antigen used. Any change in the lot numbers of the plates or reagents made a great difference too. It was found necessary to centrifuge samples for removal of precipitates, and then to store them frozen in small aliquots to avoid frequent thawing and refreezing of the same specimen. The PPD was best stored in sealed glass tubes after reconstitution, because storage in plastic tubes could lead to binding of the antigen to the plastic surface. The next important condition was to ensure that the antibodies being detected in this assay were adequately specific for the PPD antigen. This was assessed with the help of a competition assay for antibody binding to the antigen, also known as the inhibition assay.

I only started testing samples when the assay was reproducible

with minimum deviation from the standard.

INHIBITION ASSAY

Basis of the assay: The test sample is allowed to react with excess of antigen by mixing and incubating them together for one hour at 37°C. If the determinants on this antigen can compete with the epitopes recognized by the PPD-specific antibodies, it results in the majority of free antibodies in the sample getting bound, leading to antigen-antibody complex formation. The sample on subsequent assaying for PPD-reactive antibody determination, shows a lower content of free antibody which is available to react with PPD bound to the solid phase and therefore the titre is much lower as compared to that in a control assay in which the sample was not subjected to pre-incubation with PPD.

When inhibition was performed with some random samples and the positive control, the inhibition assay indicated that in fact the antibodies being detected were specific for PPD. These samples were tested at a single dilution of 1:100 and the inhibition ranged from 30 - 75%. The results of 17 samples assayed on two occasions including the positive control each time are shown in the following Table A1 and then plotted in Fig A1, to show the relative difference in readings before and after treatment with excess antigen, indicating inhibition of binding.

TABLE A1 **Results of Inhibition Assay**

DONOR	IgG/OD	IgG/OD (excess PPD)	%inhibition
1. +ive.pool	1213	200.	83.51
2. +ive pool	1098	370	66.30
3.	522	384	26.40
4.	714	410	42.57
5.	950	450	52.63
6.	1013	380	62.49
7.	1333	776	41.78
8.	777	309	60.23
9.	465	168	63.87
10.	340	159	53.24
11.	390	175	55.13
12.	325	227	30.15
13.	367	148	59.67
14.	439	142	67.65
15.	414	138	66.66
16.	353	135	61.76
17.	520	197	62.12

OD = optical density at 690 nm.

% inhibition = inhibition of antibody binding due to incubation with excess PPD antigen.

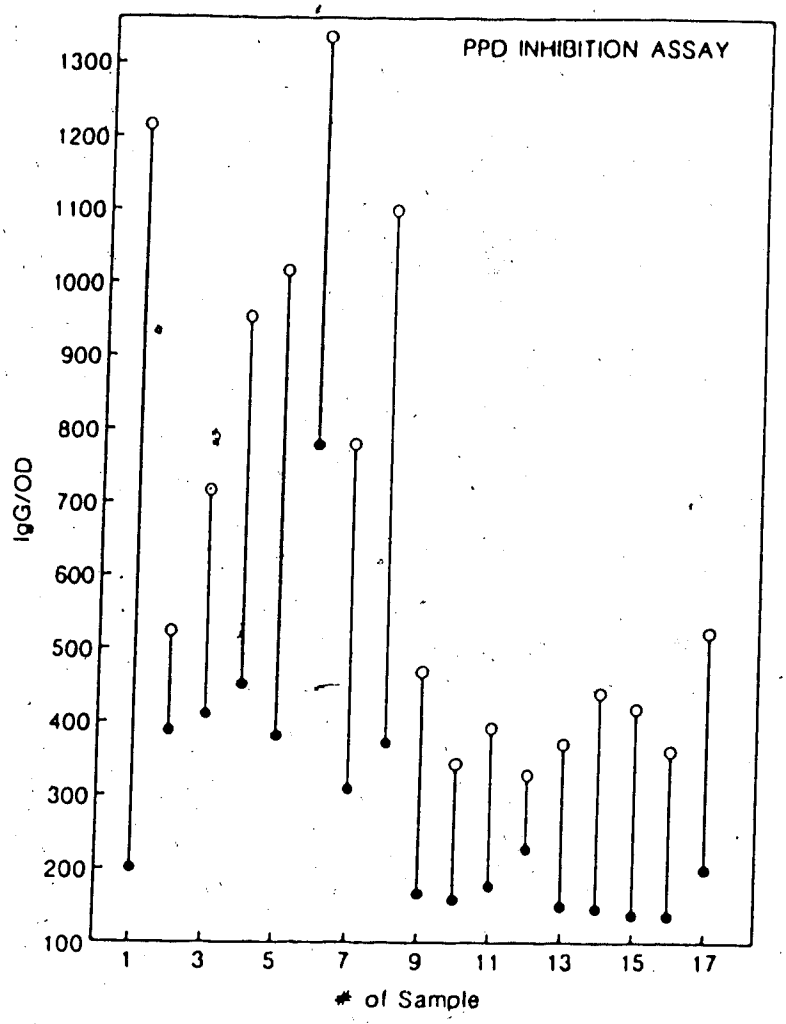

FIGURE A1 PPD-INHIBITION ASSAY.

Each bar represents the difference in the optical density (OD) readings in the same sample before (o) and after (•) treatment for an inhibition assay. Abcsissa indicates the ODI readings and the ordinate shows the number of the samples tested.

Section B :

Anti-PPD antibody in mother-cord pairs. Relationship between Stimulation index and and Optical Density Index.

The results summarized in Figure B1 show the data obtained for anti-PPD IgG and IgM antibody which were measured in mother-cord pair blood samples. These samples were tested at only a single dilution of 1/100 and the ODI was calculated and analyzed for each individual sample.

$$ODI = \frac{\text{Average OD of sample}}{\text{Average OD of positive control.}}$$

On comparing anti-PPD IgG and IgM antibodies in the mother-newborn pairs there was a very low level of IgM antibody in the cord blood, irrespective of its level in the mothers while the IgG antibody level was comparable within each pair. Consequently the level of IgG anti-PPD antibody in cord blood followed closely that of its level in venous blood of the mother.

Later the same paired samples were titrated in serial dilutions ranging from 1/80 to 1/5120. Results are depicted in Figure B2, and these data confirm the observations made with single dilution assays in Figure B1. The titre of anti-PPD IgG antibody in each cord sample is comparable to the respective maternal level.

Figure B3 (reproduced with permission from Dr. H. Pabst data) depicts the results obtained from the proliferation assays performed on mother-cord paired blood samples. The stimulation index (SI) was calculated on the basis of the uptake of tritiated

thymidine by PBL during the blast tranformation induced by culture

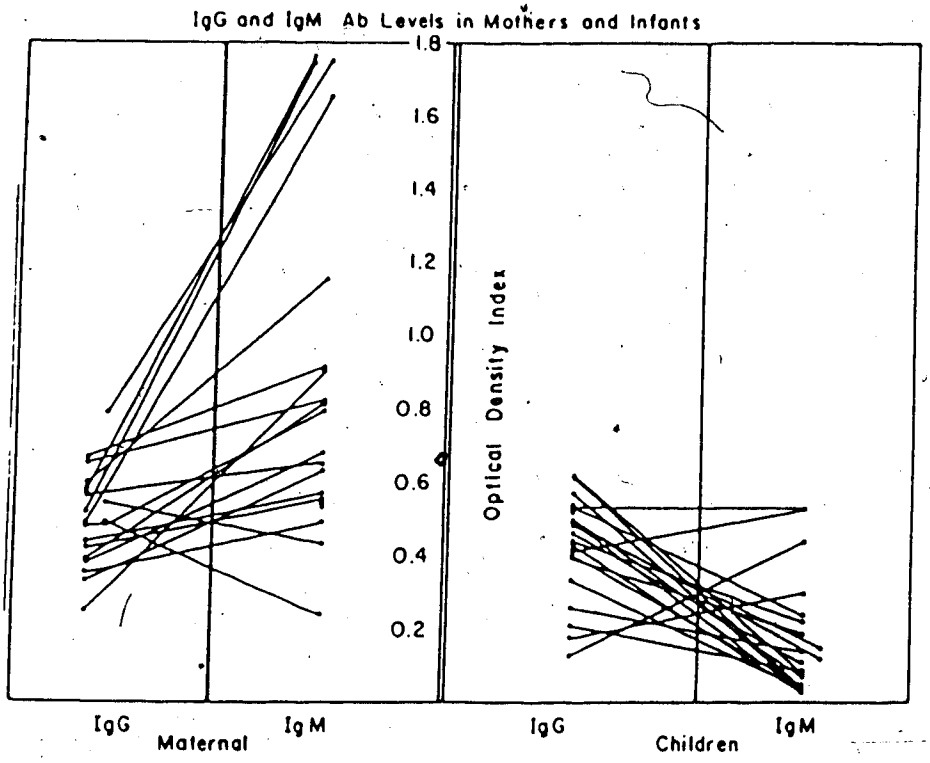

FIGURE B1 ANTI-PPD ANTIBODIES IN MOTHER-INFANT PAIRS.

Anti-PPD IgG and IgM antibody were tested in maternal and cord (including babies up to 3 days age) paired blood samples. Samples were tested in 1:100 dilution and the optical density index is indicated on the central line. Each bar connects the ODI of IgG and IgM in a single sample. Results show that the IgG level is comparable in mother-infant pairs, but the IgM antibody level is very low in the cord blood sample irrespective of its level in maternal blood.
Conclusion: Maternal IgG antibody can cross the placenta but IgM does not.

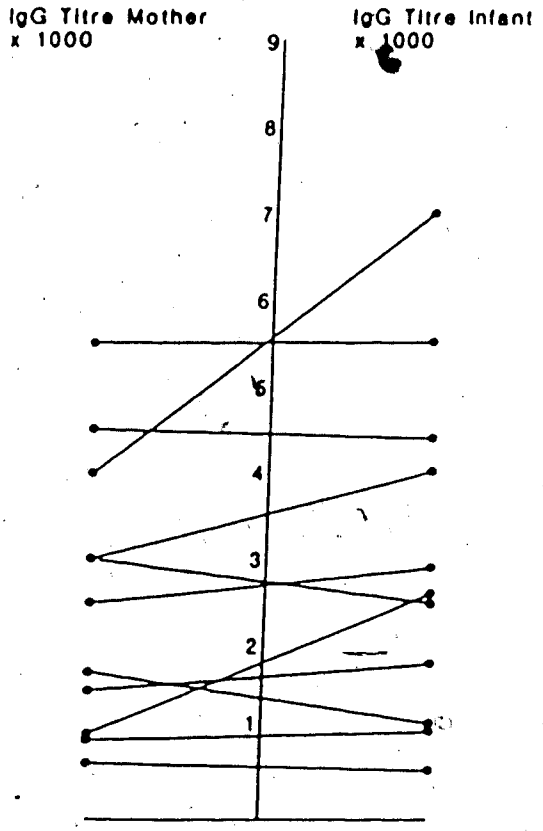

FIGURE B2. ANTI-PPD IgG ANTIBODY TITRES IN MOTHER-INFANT PAIRS.

11 Mother and infant paired blood samples were titrated for anti-PPD IgG antibodies. The titres in each pair had comparable values. Some cord blood samples even showed a higher titre indicating some concentrating mechanism during cross-placental transfer.

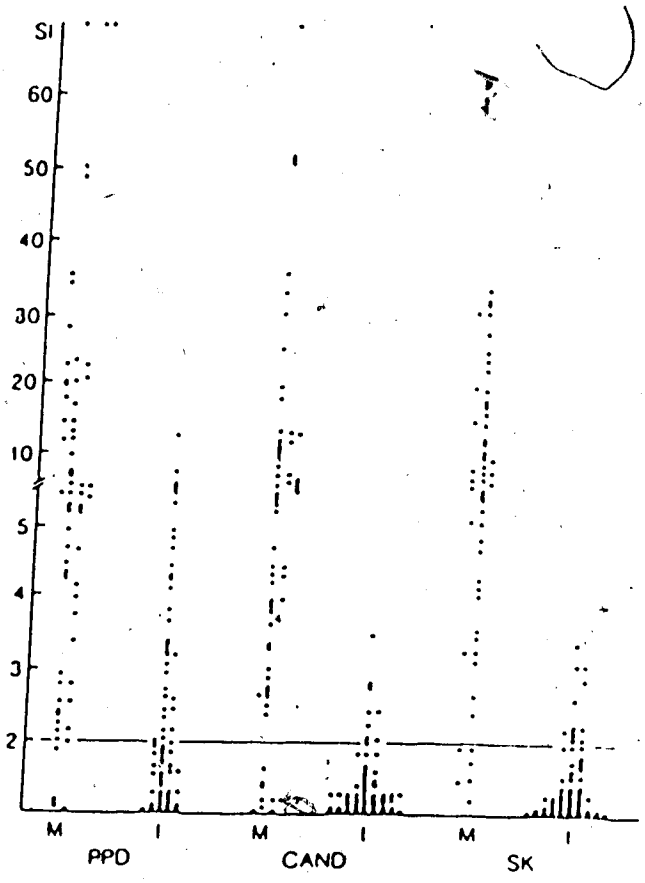

FIGURE B3. LYMPHOCYTE PROLIFERATION IN RESPONSE TO ANTIGENS.

Stimulation indices (SI) for mothers (M) and infants (I) of PBL or cord lymphocytes, using PPD, Candida or Streptokinase as antigens.

$$SI = \frac{H^3T \text{ uptake by lymphocytes with antigen (cpm)}}{H^3 \text{ T uptake by lymphocytes without antigen (cpm)}}$$

with PPD. Two other commonly encountered antigens, Candida-albicans and Streptokinase-Streptodornase, were used as controls. It was observed that 53% of the newborns born to PPD positive mothers were also PPD-positive and showed an SI of higher than 2.0.

In Figure B4 the cellular immunity (CMI) and humoral response is compared in each mother-cord pair to see if any relationship exists between the two types of immunity.

On plotting the data, a cut-off at 0.4 for the ODI seems to divide the maternal data into two categories. The majority of samples with an ODI of less than 0.4 have a high SI reading, which is greater than 2.0 (SI>2.0 is PPD-positive) and the majority of samples with ODI greater than 0.4 show a low SI reading of less than 2.0. This is seen only in the maternal samples and does not hold true in cord samples.

From these results there is no evidence of a primary in-utero mycobacterial sensitization, which should have induced measurable anti-PPD IgM antibodies in the newborn. It is concluded that the maternal anti-PPD IgG antibody is transferred to the newborn by the placental route.

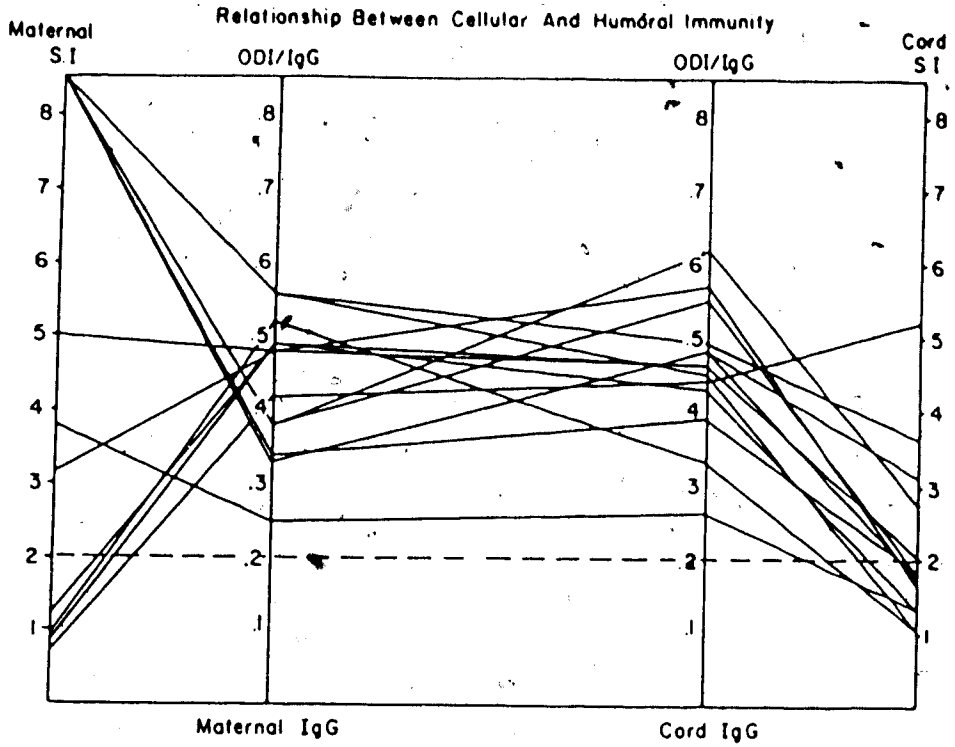

FIGURE B4 RELATIONSHIP BETWEEN THE CELLULAR AND HUMORAL IMMUNITY.

Relationship between the cellular and humoral immunity to PPD in mother and infant were evaluated. Results show that there exists an inverse relationship between the two types of immunity in the mother, but in cord blood the values of SI (due to CMI) and ODI (due to Ab) bear no relationship to each other. 5 out of 8 mothers with high SI have cord samples exhibiting a high SI (>2.0), and 6 out of 7 mothers with an ODI>0.4 have cord samples with a high ODI.

Section C :

Anti-PPD antibody levels in developing infants.

Plasma samples from babies aged between 1 to 24 months of age were tested for anti-PPD antibody. Age and sex distribution is as shown in Table C1. At the time these children were tested there was no evidence of mycobacterial infection in any child.

The levels of anti-PPD IgG and IgM antibody were initially tested in these children. Each sample was tested only at a single dilution of 1/100 and the optical density index (ODI) was measured. The OD readings for anti-PPD IgG and IgM in each child thus obtained were compiled and then statified into separate groups according to age. The results were stratified into four groups (A) 1-4 months, (B) 5-8 months, (C) 9-12months and (D) 13-24 months of age, and the mean value of ODI ± S.E. of the mean were individually calculated for each group as shown in Table C2.

ODI/IgG antibody. The mean ODI for anti-PPD IgG showed an increasing tendency of ODI levels with age. A lower mean ODI level for anti-PPD IgG was seen in group A=0.25 ± 0.02, with a slight increase in the older age groups B=0.31 ± 0.04; C= 0.31 ± 0.03 and D= 0.32 ± 0.04 respectively. There is no statistically significant difference between the different age groups.

ODI/IgM antibody. Table C3 gives results of the level of IgM antibody in these children. Again a lower mean ODI for IgM was seen in group A, with an increasing ODI with age. The mean ODI ± S.E. values were 0.24 ± 0.03; 0.31 ± 0.03; 0.42 ± 0.03 and 0.47 ±

0.07 in groups A, B, C and D respectively. There was no evidence of

TABLE C1. Age and sex distribution of 113 children in the antibody study.

Age Groups	Males	Females
1-4 months	23	16
5-8 months	12	5
9-12 months	20	14
13-24 months	15	8

Blood samples were obtained from children being treated at the University Hospital for unrelated illnesses. No evidence of mycobacterial infection was present in any child.

TABLE C2. ODI of anti-PPD IgG antibody in children at different ages.

Age(months)	Sample size	Mean ODI ± S.E.
1-4	39	0.25 ± 0.02
5-8	24	0.31 ± 0.04
9-12	55	0.31 ± 0.03
13-24	14	0.32 ± 0.04

Anti-PPD IgG antibody was measured in single dilution (1:100) of the blood samples from children and the ODI was calculated. The results were stratified into four groups according to age, and are expressed as mean ODI ± S.E. of the mean in each age group.

TABLE C3. ODI for anti-PPD IgM antibody in children at different ages.

Age	Sample size	Mean ODI ± S.E.
1-4 m.	34	0.24 ± 0.03
5-8 m.	22	0.31 ± 0.03
9-12 m.	46	0.42 ± 0.03
13-24 m.	13	0.47 ± 0.07

Anti-PPD IgM antibody was measured in single dilution (1:100) of the blood samples from children, and the ODI was calculated. The results were stratified into four groups according to age, and are expressed as mean ODI ± S.E. of the mean in each age group.

the transfer of maternal IgM to the fetus it was considered reasonable to follow only IgG antibodies in all future analyses.

Titre/IgG antibody. This apparently slight trend of increasing level of antibody with age, as seen in single dilution assays, showed more pronounced differences and clear discrimination amongst the different age groups, when the plasma samples were titrated. In Figure C1 are the results of blood samples from the children when they were titrated for anti-PPD IgG by testing in serial dilutions. The OD readings were noted at each dilution and then plotted as function of dilution in a semi-log fashion which were fitted to form a curve of antibody binding. The application of such a curve is shown in Figure C2. From this curve the dilution of the sample that gave an OD reading of 0.1 was read off as the titre. It was an arbitrary choice made which represented the reliable lower limit of positivity at which antibody activity was detectable. From this the mean titre was calculated separately for each age group. Table C4 gives the statistics on these data.

The mean anti-PPD IgG titre of group A was found to be significantly lower ($p < 0.001$) than in groups B, C and D. The reciprocal mean values for titres were 382 ±34 ; 920 ± 186 ; 2412 ± 55 and 1279 ± 310 for the groups A, B, C and D respectively.

This observation led to the conclusion that the children belonging to group A have the lowest titre of anti-PPD IgG antibody. The level was even lower than that in the cord blood, showing a decline in titre in this group probably due to catabolism. However,

there was a definite increase in titre from 4 months of age on.

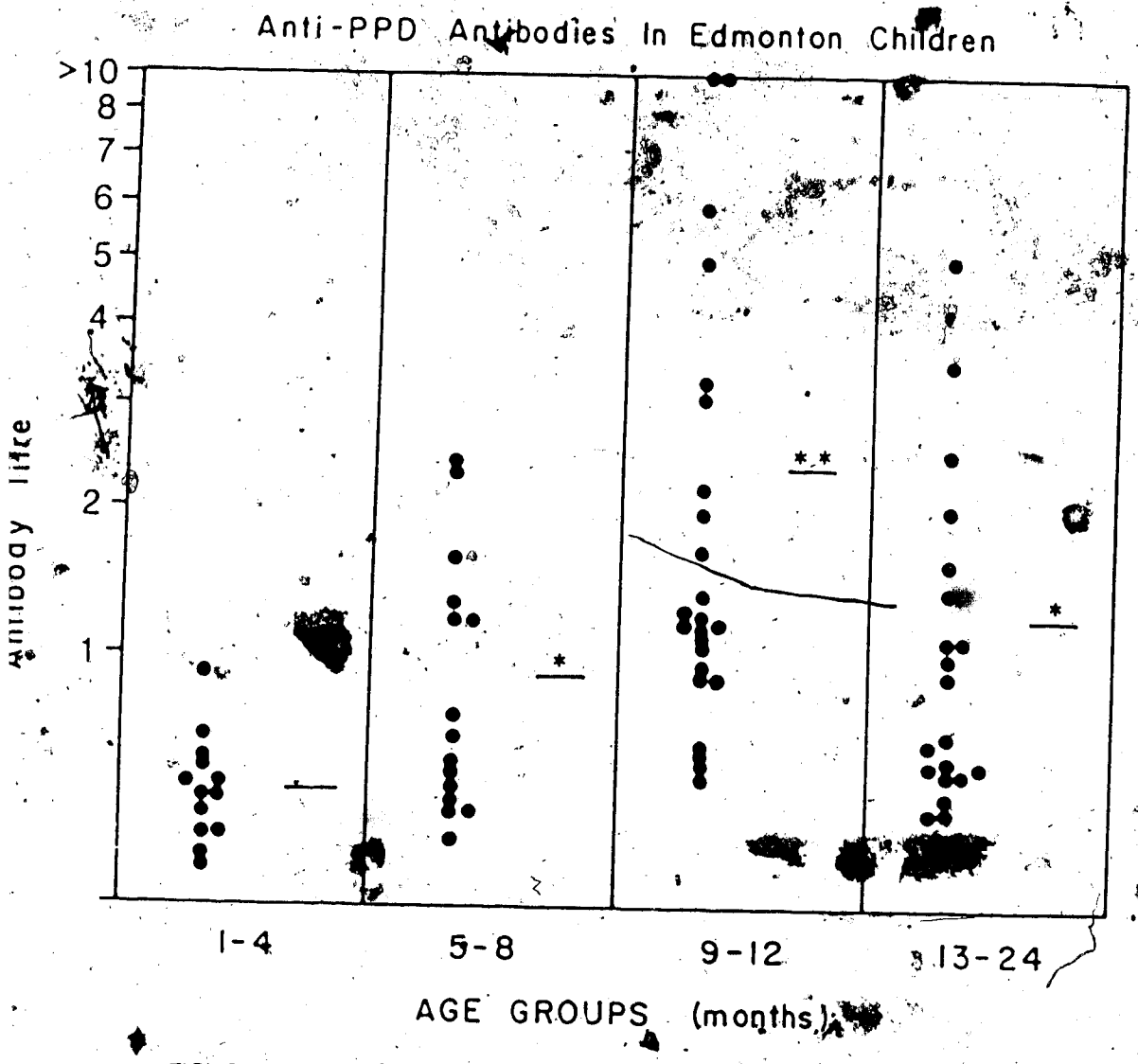

FIGURE C1 ANTI-PPD IgG ANTIBODY TITRE IN YOUNG CHILDREN

n=34 n=15 n=24 n=20

Titres of anti-PPD antibody in Edmonton children. Blood samples of children ranging from1-24 months of age were titrated for anti-PPD IgG antibody. The results are expressed as log of the reciprocal value of the titre obtained. Each dot represents one sample and the data are stratified according to age groups in months. Mean titre for each age group is indicated by a horizontal bar. Significant increases in mean titres with increasing age were found compared to the 1-4 months age group.

* = $p < 0.05$

** = $p < 0.001$

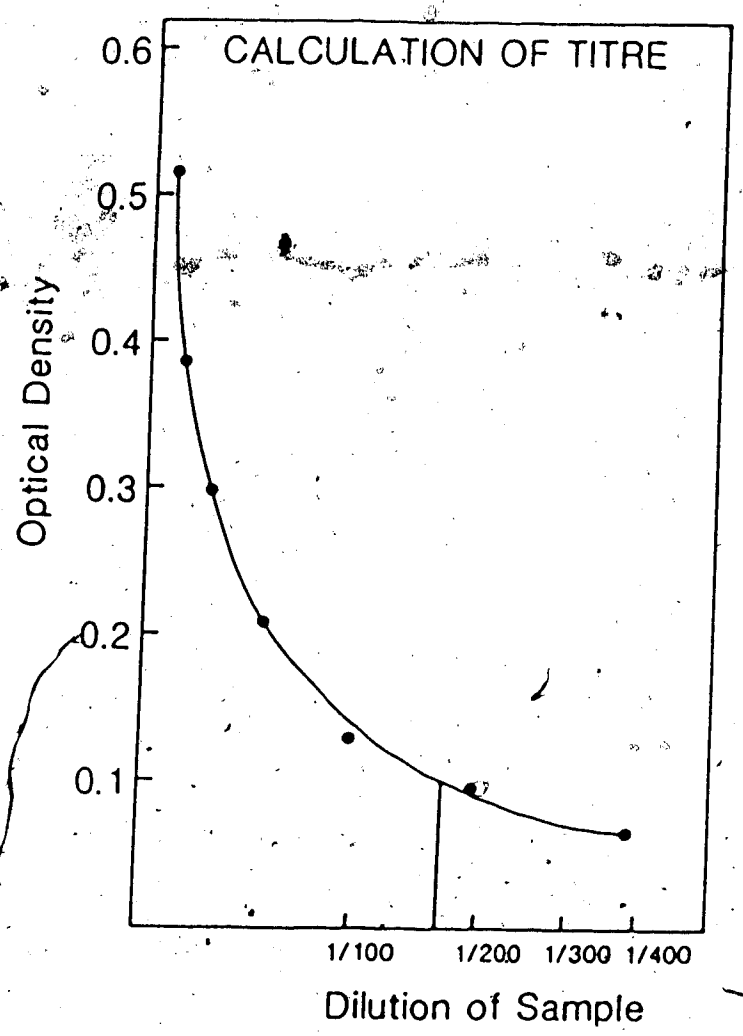

FIGURE C2 CALCULATION OF THE TITRE.

Serial blood sample dilutions are tested for anti-PPD IgG antibody and the optical density (OD) noted for each dilution. The OD values are plotted as function of dilution on semi-log graph to obtain the binding curve. Dilution at which the OD reading is 0.1 is taken as the titre.

TABLE C4. Anti-PPD IgG antibody titre in children at different ages.

Age Groups	Sample size	Mean titre ± S.E.
1-4 m.	34	382 ± 34
5-8 m.	15	920 ± 186
9-12 m.	24	2412 ± 55
13-24 m.	20	1279 ± 310

Blood samples from children were tested for anti-PPD IgG antibody in serial dilutions that ranged from 1:80 to 1:5120, and the OD was noted at each dilution. Titre was read off the binding curve at OD of 0.1. Results are expressed as mean titre ± S.E. of the mean in individual age groups.

Clearly, antigenic confrontation of the infant must have initiated this rise (see Discussion).

Section D:

Changes in PPD-immunity with age and disease.

In this section it was attempted to examine how efficiently and how soon the infant's immune system would develop anti-PPD antibodies, when the maternal supply of antibodies is lost. In this respect it was necessary to take into account the effect of exposure to the so-called "atypical" or environmental mycobacteria. These are commonly encountered but do not cause disease similar to or to the same extent as the Myc. TB. It is significant that antibodies produced against different mycobacterial strains in rabbits exhibit cross-reactivity on immuno-electrophoresis. It is reasonable to assume that this cross-reactivity also holds true in humans. This could have important effects on the developing anti-tuberculous immunity in the early years of life. Therefore I studied antibodies to the environmental mycobacteria simultaneously with the anti-PPD antibodies. The species of the atypical mycobacteria tested were intracellulare, kansasii and sorofulaceum.

In Table D1 are shown the mean IgG antibody levels in the plasma samples which were tested against PPD as well as the three antigens of atypical mycobacteria. Besides the normal young children, in this section were also included healthy adults in order to see the effect of age, and some patients with culture proven mycobacterial infections to study the effect of their disease on antibody development.

TABLE D1. ODI for IgG antibodies reactive against mycobacterial antigens in 44 individuals from different clinical groups.

Clinical state	Sample size	Mycobacterial Antigens Used (Mean ODI ±S.E.) PPD	kansasii	scrof	intracell
Atypical Myc. patients.	9	0.70 ± 0.17	0.51 ± 0.11	0.53 ± 0.08	0.58 ± 0.10
Tuberculosis patients	6	0.33 ± 0.10	0.84 ± 0.21	0.63 ± 0.06	0.69 ± 0.12
Children 1-4 m.	10	0.16 ± 0.03	0.33 ± 0.02	0.35 ± 0.03	0.38 ± 0.02
Children 5-8 m.	8	0.30 ± 0.09	0.42 ± 0.05	0.67 ± 0.10	0.59 ± 0.10
Children 9-12 m.	13	0.58 ± 0.13	1.22 ± 0.23	1.08 ± 0.17	1.16 ± 0.15

IgG antibody reactive against various mycobacterial antigens was tested by ELISA at a single dilution of 1:100 in each sample, and optical density index (ODI) was calculated.

The results are expressed as mean ODI ± the standard error of the mean (S.E.) in the different clinical groups for each mycobacterial antigen.

Therefore the individuals tested belonged to the following groups:-

(a) Known TB patients who were being treated at the Aberhart Hospital by Dr. Fanning.

(b) Patients with culture-proven atypical mycobacterial infections, also through Dr.Fanning.

(c) Normal children between 1-24 months of age were stratified into groups 1-4, 5-8 and 9-24 months of age. After the age of 9 months up to two years age, the children had almost identical values of antibody levels. The aim was to see if cross-reactivity existed between antibodies reactive to the different mycobacterial species. Each sample was simultaneously tested at 1/100 dilution for IgG antibodies against PPD and the three "atypical" antigens . The ODI for each sample for each of the four antigens was calculated and tested statistically. Analysis of variance (ANOVA) was done between all the groups against each antigen.

ODI/IgG antibody. In Table DI the left hand column lists the clinical state of the individual groups. The antigens are listed horizontally on top. Mean ODI values and the standard error of the means for antibodies reactive to the four antigens are listed. The results of this set of assays, therefore represent the five groups of individuals,.

(1) The group with lowest content is the group A of children1-4 months age. The analysis of variance (ANOVA) done between the other four groups and this group indicates that the mean level of

antibody against each of the four antigens is significantly ($p<.05$) higher in all other groups as compared to the group A.

(2) Patients with atypical mycobacterial infections had significantly greater ($p<0.05$) levels of anti-PPD antibodies as compared to the group A and TB patients. It has been shown that high levels of anti-PPD antibodies that develop in active TB patients decline with chemotherapy . This may be the reason why we find lower anti-PPD antibody levels in TB patients who were receiving treatment for their illness.

3) Levels of anti-kansasii, anti-scrofulaceum and anti-intracellulare antibodies are significantly higher ($p<0.05$) in TB patients than in the group A. This finding probably reflects cross-reactivity of the atypical mycobacterial antigens used with the antibodies formed in the patients during their M. tuberculosis infection.

4) There is a significant ($p<0.05$) increase in the level of IgG antibodies against atypical antigens after the age of 4 months indicating development of immunity to the environmental mycobacteria on exposure.

Titre/IgG antibody. In Figures D1 to D3 are results of antibody titration when serial dilutions were tested for IgG antibody reactive to each of the antigens in these samples. It was also realized at this stage that the patients with mycobacterial infections belonged to varying ages ranging from childhood to late adulthood. Therefore besides1-4 month old infants, patients-age matched healthy adults were also included in the testing, which

led to further interesting observations. The titre of IgG antibody reactive to PPD, Myc.kansasii, Myc.scrofulaceum and Myc.intracellulare showed a significant ($p<0.001$) increase in the adults as compared to the young infants (Fig D1).

Comparison of normal adults with TB patients showed that the anti-PPD IgG titre is 4-fold lower in TB patients, anti-kansasii IgG is similar in the two, whereas anti-scrofulaceum and anti-intracellulare are slightly lower in TB patients than normal adults.

Comparison of atypical mycobacterial patients with normal adults showed that the anti-PPD titre is one half of the titre in normals, anti-kansasii is similar in the two groups, while anti-scrofulaceum is slightly greater and anti-intracellulare IgG titre is slightly less in the patients than in normal controls.

Table D2 summarizes the results of titration assays of blood samples from individuals of the four clinical groups. It lists the statistical mean values for the titres and their standard errors for the antibodies reactive to all the four mycobacterial antigens.

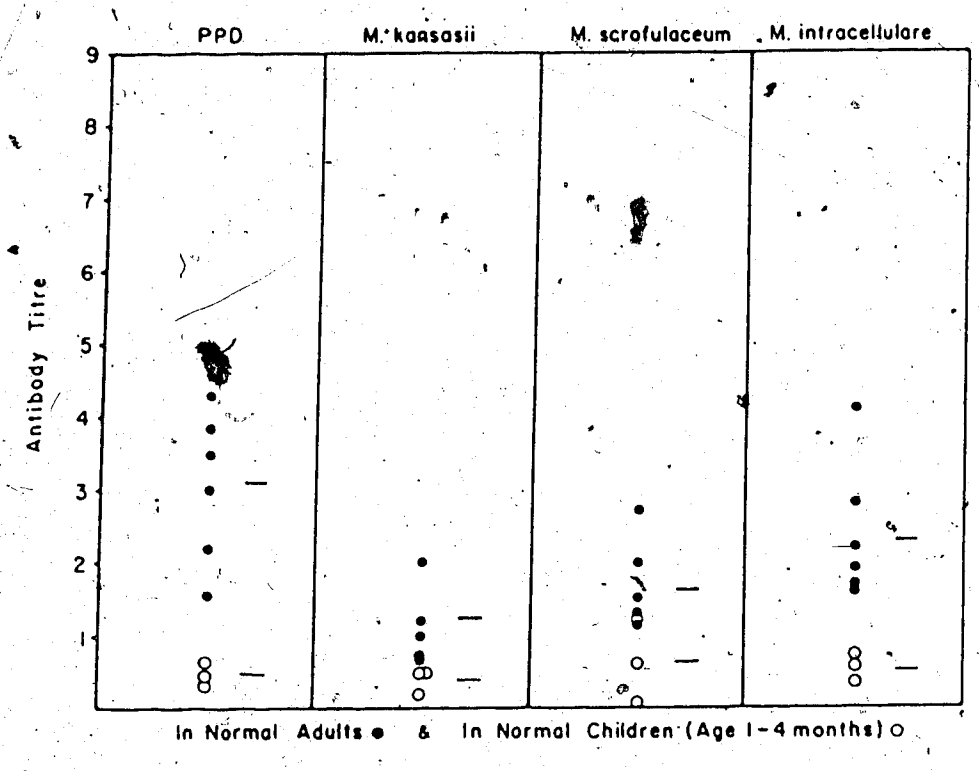

FIGURE D1 IgG TITRES FOR MYCOBACTERIAL ANTIGENS IN YOUNG CHILDREN AND HEALTHY ADULTS.

Results of IgG antibody titration of blood samples from 6 healthy adults and 3 young children between 1-4 months of age. For each sample the titre of every antigen is represented by a dot. The abscissa represents the titres of IgG antibody against mycobacterial antigens.

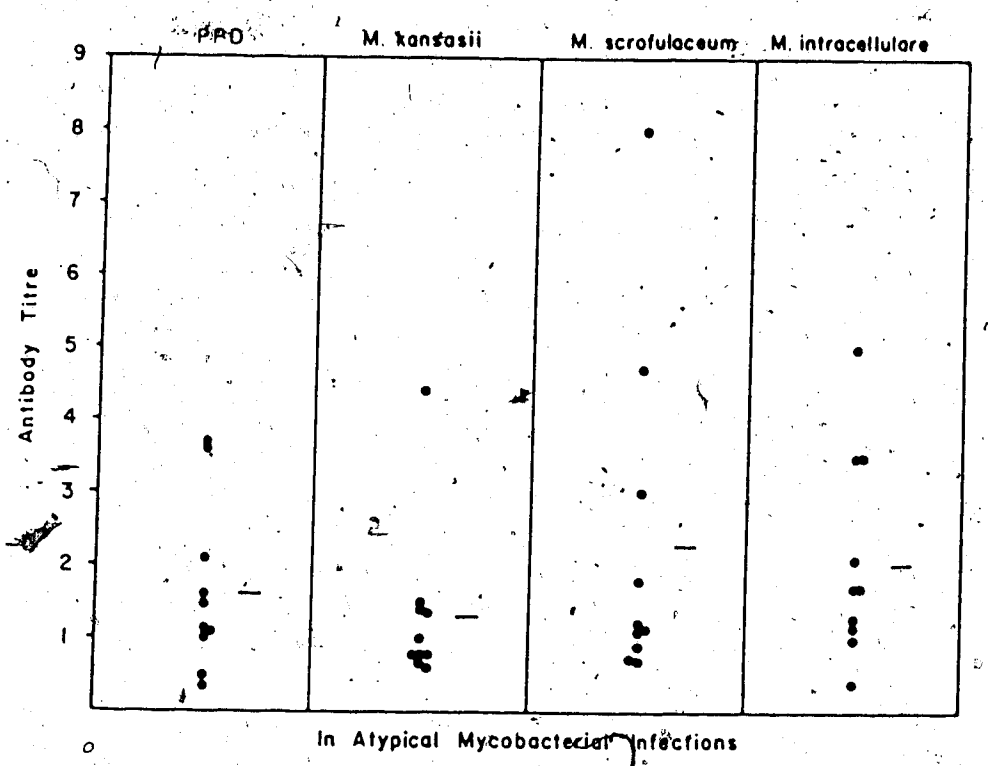

FIGURE D2 IgG TITRES FOR MYCOBACTERIAL ANTIGENS IN ATYPICAL MYCOBACTERIAL INFECTIONS.

n=10

Results show the IgG antibody titration of blood samples from patients suffering from atypical mycobacterial infections. For each sample the titre of every antigen is represented by a dot. The abscissa represents the titres of IgG antibody against mycobacterial antigens.

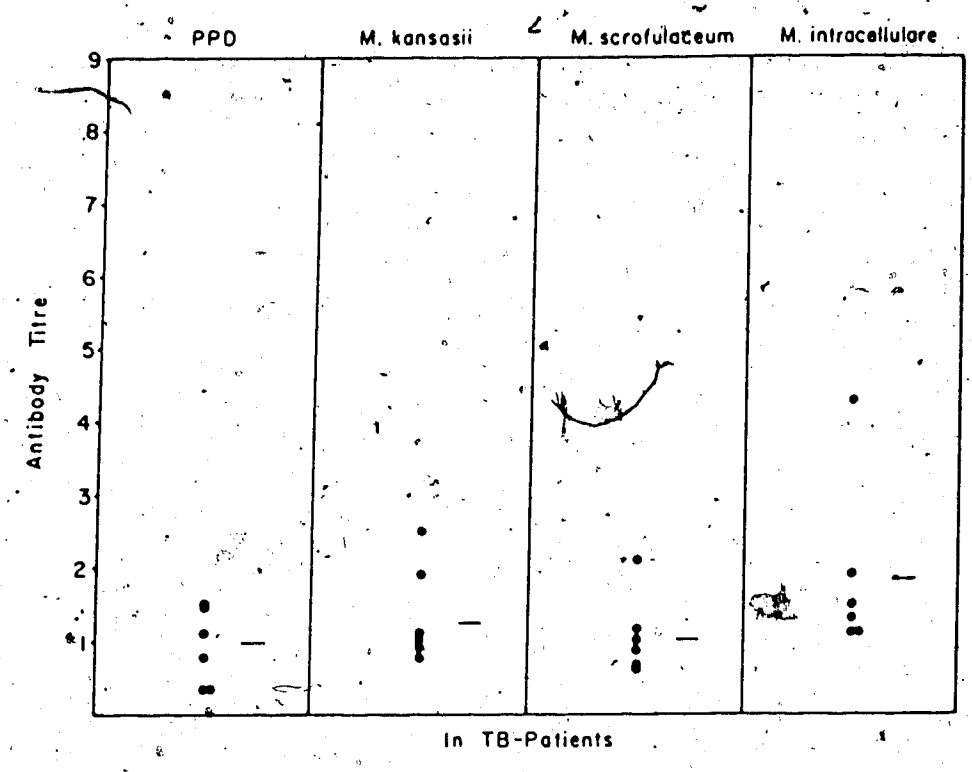

FIGURE D3 IgG TITRES FOR MYCOBACTERIAL ANTIGENS IN TUBERCULOSIS.
n=6

Results show the IgG antibody titration of blood samples from tuberculosis patients. For each sample the titre of every antigen is represented by a dot. The abscissa represents the titres of IgG antibody against mycobacterial antigens.

TABLE D2. Anti-mycobacterial IgG titres of 25 individuals from different clinical groups.

Clinical state	Sample size	Mycobacterial antigens used (Mean±S.E.) PPD	kansasii	scrof	intracell
Atyp. Myc. patients.	10	1655 ± 368	1330 ± 355	2330 ± 744	2135 ± 450
TB patients.	6	916 ± 210	1233 ± 193	1058 ± 224	1866 ± 501
Normal Adults.	6	3066 ± 423	1241 ± 238	1608 ± 259	2383 ± 385
Children 1-4 m.	3	450 ± 86	400 ± 100	606 ± 340	520 ± 113

IgG antibody reactive against various mycobacterial antigens was tested in serial dilutions ranging from 1:80 - 1:5120 in each sample. Titre was read off as the dilution giving OD of 0.1.

The results are expressed as mean IgG titre ± standard error of the mean (S.E.) in the different clinical groups for each mycobacterial antigen.

DISCUSSION

An intriguing and thought provoking observation was made in our laboratory (Pabst et al) while testing PPD-reactivity in mothers and infants which led to the present study. This observation was made in case of mothers who were PPD-positive as assessed by testing their peripheral blood lymphocytes (PBL) in a six-day-culture in the presence or absence of PPD for blast transformation (BT), and were found to be "positive" with stimulation index (SI) of greater than 2.0. (SI of 2.0 taken as the cut-off point between "negative" and "positive" responders.)

As shown in Figure B3 48% of babies born to such mothers, also gave a positive response (i.e. SI>2) to PPD-induced blast transformation. This response was on subsequent testing, however found to decrease with time, and it was gradually lost so that the infants became PPD-negative by the age of 4 months. This phenomenon was not observed in the babies born to PPD-negative mothers.

The proliferation assay is dependent on the presence of immune reactive cells, and therefore blast transformation of the PBL is considered to be an in vitro analogue of the DTH response seen in vivo. It indicates the degree of cellular sensitization of the newborn to the antigen. Another reaction mediated by sensitized cells is their capacity to produce a lymphokine known as the lymphocyte-inhibition-factor (LIF) in response to the sensitizing antigen. The production and assay for LIF was also done on lymphocytes from the cord blood samples after determining the

response by blast transformation. There was a positive relationship between the results of both assays in 7 cases.

On observing the transfer of cell mediated immunity in the newborn, it was necessary to study also the humoral immunity to PPD in these mother-infant pairs. As the immune regulation involves both types of immunity it is reasonable that they be studied simultaneously.

The objective of this thesis work was to evaluate and study the presence of specific anti-PPD antibody immune reactivity, and the method used was the ELISA technique. Blood samples were first tested in the newborns and their mothers, later followed by older infants between the ages of 1-24 months. Finally, the effects due to age in healthy adults and effects of infection due to environmental atypical mycobacteria was studied in patients.

In the newborn, the level of anti-PPD IgG antibody was found to follow closely that in the mother, while negligible IgM could be detected in the cord blood. This finding supported the fact that the transplacental transfer of IgG antibody molecules can occur, but not that of IgM antibodies. It also indicates that there had been no intra-uterine exposure in these infants, which would have led to sensitization and the development of active specific immune reactivity against TB antigens.

For comparison of the CMI and humoral immunity against PPD, besides assaying for anti-PPD antibodies, blast transformation of PBL of the same mother- newborn pairs was being done in Pabst's laboratory, and the stimulation indices (SI) were calculated. These

SI results were plotted together with the ODI readings to see if any relationship existed between cellular and humoral immunity to PPD in each mother-infant pair (Fig B4). It showed that 5 out of the 8 mothers with high SI had infants with high SI as well. Also mothers with high ODI had infants with similarly high levels. In mothers taking 0.4 of IgG/ODI as the arbitrary cut-off showed that the mothers having an ODI above this value had a low SI (less than 2.0) while those having an ODI below 0.4 had a high SI (greater than 2.0). It could be interpreted therefore that cell-mediated and humoral immunity to PPD are inversely related in the mothers.

When beyond the newborn period infants were tested, significant changes inanti-PPD antibody titre occurred at different ages (Fig C3). This was seen on comparing the results of children samples which were then stratified into different age groups. There were four groups made of 1-4, 5-8, 9-12 and 13-24 months amongst healthy children. Table C1 shows the age and sex distribution of these children.

When anti-PPD IgG antibody titrations were done in the children samples, it showed that the youngest age group had a significantly lower titre ($p<0.001$) than all the older infant groups. Therefore children up to the age of 4 months have a titre less than 1:400, but there is a significant increase ($p<0.001$) after the fourth month and the mean titre reaches almost adult values (1:10,000) around one year of age.

It may be hypothesized that the majority of the anti-PPD IgG antibody detected during the early age of 1-4 months is maternal

in origin that has passively been transferred by the placental route. This antibody transfer reaches generally the same concentration in the fetus at term as in the mother, but it is catabolized with time.

Griffith *et al* in 1982 studied 100 mother cord pairsed samples and measured the levels of antibodies against different viruses (herpes simplex, measles, respiratory syncitial and rubella). The investigators found that higher level of antibody could be detected in the cord than in maternal sera. It was therefore concluded that IgG antibody against most if not all viruses were concentrated on the fetal side of circulation.

As the child grows and the immune system matures and develops, the exposure to various environmental antigens would stimulate de novo antibody production. In a study done at the University of Alberta in 1986 (Gill *et al*). it was demonstrable that infections with atypical mycobacteria may cause childhood lymphadenitis throughout the year in harsh inland Northern climate of Alberta about 480 km of Edmonton.

Interactions between the passively transferred humoral and cellular anti-PPD immunity to the newborn at birth and the newly developing active immunity later after the fourth month is of special interest.

Transfer of maternal IgG antibodies via the placental route to the newborn is a well known fact, but most interesting fact is the timing of the presence of this antibody in the newborn. The maternally transferred antibodies wane during the child's

development and are continuously replaced by newly arising antibodies due presumably to the child's own immune response. This occurs almost during the same time when the infant loses the cellular immunity which was also transferred at birth. Although the present data do not suggest any correlation between the extent of cell-mediated or humoral immune response in children, it is likely that the transferred maternal antibody may have an important role to play in the development of immunity during early infancy. It may provide protection or immunity to TB similar to BCG vaccination, or it may interfere with the "take" of BCG.

On the other hand, the decreased amount of blast transformation of PBL on stimulation with PPD in some infants and its presence in others, may reflect the differences in the maternal immune reactivity during pregnancy, which she is able to transfer to her baby.

This may be explained by one of the following hypotheses:-

(1) The mycobacterial antigens that have already been processed by mother's cells and degraded into transferrable particles could cross the placental barrier and sensitize fetal T-cells. These cells can then be induced to proliferate in vitro in response to PPD. Because the reactivity remains for only a few months, because the degraded antigen may be lost with time. The proliferation of PBL observed in the cord blood therefore is not an active immune response. It diminishes progressively over the first few months of life, with decreasing quantities of antigen for activating the cells. As a result the child becomes PPD negative by the age of 4 months.

(2) Some newborns may have PPD specific T-cells in the repertoire bearing an idiotype to which the mother may possess anti-idiotypic (anti-id ab) antibodies which get transferred via the placenta. This anti-id IgG on crossing the placenta could bind to the idiotype on the T-cell surface and sensitize them, so that the in vitro challenge with PPD gives sufficient signal to induce proliferation. It needs further study, but it is the observation of simultaneous disappearance of maternally transferred IgG antibodies to PPD and loss of specific cellular reactivity to PPD that favours this hypothesis.

It may be argued with regard to anti-idiotypic antibodies, that in organ transplantation especially in renal transplant patients the anti-idiotype antibody is inhibitory for cellular reactivity, and is deliberately induced in the host by giving repeated transfusions of blood from the organ donor over a preoperative period. Its usefulness has been established and has proved to be beneficial for survival of the graft due to specific regulatory mechanisms which suppress the donor cells reactivity. It is postulated that this can turn off the host T-cells specific for the donor's organ. It should be noted that this procedure involves whole blood transfer including all cellular elements from the donor, and the materno-fetal situation is not comparable. The latter transfer involves only IgG antibody and some possible lymphokines, or plasma soluble transfer factors.

Bernard *et al* in Columbia University investigated the disease called Myasthenia gravis in which muscles are afflicted with

extreme weakness and fatigue due to auto-antibody in these patients against the acetyl choline receptor (membrane molecule through which muscle recieves signals from nerves). The investigators raised antibodies to a substance that binds to the anti-receptor antibody, and then made anti-id antibody to its idiotype. The anti-id was capable of binding the receptor and by either blocking or destroying the receptor, led to symptoms of the disease.

In experimental models it has been demonstrated that anti-id antibody is capable of causing suppression or stimulation of T-cell responses under different circumstances.

(3) It may be proposed that some cytophilic anti-PPD antibodies or specific T-cell inducing molecules may enter the newborn from the mother and attach to PPD-specific T-cells, and could act as signal mediators when the antigen is also present. This hypothesis should predict that the reactivity of T-cells should be present as long as the sensitizing anti-idiotypic antibodies/factor remain in circulation. This is supported by the results of the newborns studied in this work, showing that the reactivity persists for a 3-4 month period which is the average lifespan of much of the transferred maternal antibodies. If on the other hand it is a soluble factor, metabolic degradation with time could similarly lead to loss of signal for cell activation.

In older children, it is possible that the antibodies being detected as reactive to PPD could have been produced in response to some different mycobacterial species or other environmental

micro-organism to which the children are exposed. These may exhibit cross-reactivity with the antigenic determinants of PPD. This was analyzed by following the antibody development in response to atypical mycobacterial infections. It included some patients, who either had infections due to Myc.TB or the so-called "atypical" mycobacteria. Patients suffering from tuberculosis exhibited a significantly ($p<0.05$) higher level of antibodies against atypical mycobacterial antigens than the young healthy infants.

Patients with atypical mycobacterial infections had significantly higher levels ($p<0.05$) of antibodies reactive with PPD as compared to young infants.

Since there is a great deal of homology between the antigenic determinants of various mycobacterial species (Anderson *et al*, 1986), cross reactivity could be expected amongst the antibodies reactive to the different strains. This is indicated by some of the data in this study.

The decreased levels of antibodies against the corresponding mycobacterial antigens could be explained by the anti-TB chemotherapy that was being instituted to all these TB patients under study (Kalish *et al* 1983).

Titration of the antibodies reactive to each of the mycobacterial antigens showed that age played an important role in the development of humoral immunity, and the titres significantly increased with age. The normal adults when compared with the groups of patients with mycobacterial illness revealed no

significant differences, possibly because the number of samples tested is very small. Therefore statistically significant cross-reactivity amongst these antibodies needs yet to be established.

CONCLUSIONS: For studying the antibodies to various mycobacterial species, I selected the ELISA system as it is currently considered to be a good and reliable antibody assay (Malvano *et al*, 1982, Zeiss *et al*, 1982 and Kalish *et al*, 1983). It was essential to stantardize it for each antigen when used. Therefore to test specificity of the assay and detection and exclusion of artefacts, inhibition assays were performed which provided strong support that the antibodies being detected were reactive to PPD. From the results thus obtained, the following conclusions have been made.

When tested the mother-cord blood samples for anti-PPD antibody, it showed that the majority of maternal IgG antibody crossed the placenta and that is why the levels are comparable in cord blood and maternal peripheral blood. IgM antibody does not cross the placental barrier and therefore any IgM detectable in the cord blood would be of infant origin. The samples used in the present study came from infants without evidence of intra-uterine mycobacterial infection (an extremely rare event in any case) which would have caused a specific anti-mycobacterial antibody response by the baby. There seems to be an inverse relationship between cellular and humoral immunity against PPD in mothers of the newborns included in this work. In the newborn the passively transferred IgG persists in circulation up to the age of about 4 months, which is the expected lifespan of most of the antibody transferred. After this age the newly arising high titre is

presumably an indication of the child's own immune reactions to environmental exposure. The data in this study confirms that increasing age plays an important role in the development of a rising titre of antibodies reactive to various mycobacterial antigens. The high titre in normal healthy adults could be due to polyclonal activation by subclinical infections with environmental mycobacteria or cross-reactivity due to other micro-organisms. The passively tranferred maternal antibody and cellular immunity in the newborn seem to play an important role in the development of immune reactivity in the infant. In early infancy, this transferred immunity may affect the response to active immunity induced by the BCG vaccination given for prevention of tuberculosis. During later childhood, the environmental exposure to mycobacteria may affect immunity to BCG by causing cross-reactive immune responses. The data in this work provides a foundation for further study of anti-tuberculous immunity in greater detail. This would help to assess the optimum age for vaccinating against tuberculosis which is still one of the major health problems in the world today.

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APPENDIX I

BLAST-TRANFORMATION ASSAY

Heparinized venous blood was collected after consent from mothers admitted in labour rooms at Charles Camsell Hospital and University of Alberta Hospital. Samples were collected from the cord as well as mother soon after delivery and if possible on the third day after delivery from the baby again.

Lymphoid cells were seperated from each sample by Ficoll-Hypaque gradient and after three washes with MEM were suspended in RPMI 1640 medium + 20% AB serum at a concentration of $2x10^6$/ml. These cells were then cultured in microtitre-well polystyrene plates in the presence or absence of PPD at 37°C in 5% CO_2 and air for six days. The PPD- induced blastogenic tranformation is then measured by the incorporation of tritiated thymidine added during the last 6-8 hours of culture. The stimulation index

(SI) is = $\frac{\text{cpm of culture with PPD}}{\text{cpm of culture without PPD}}$

APPENDIX:II

Variability due to PPD from different sources.

* Previous to the experiments of the present data, sera from 58 mother-infant pairs and 70 children upto 2 years of age were assayed using PPD from Connaught Laboratories, Toronto. The procedure for the assay was exactly similar, but all the samples showed considerably high levels of IgG antibodies reactive to PPD. After excluding several variables, PPD antigen from a different source was substituted (Parke Davis, Detroit). Also on searching the literature, it was observed that most of the investigators had used PPD from Parke Davis Co. for measuring antibodies in TB patients. It was observed by testing a random batch of samples simultaneously with the Connaught PPD and the Parke Davis product, that the latter appeared to give us more specific results and less evidence of cross-reaction. Better discrimination of the levels of anti-PPD antibody between the positive and negative blood samples was obtained and therefore experiments in the present study was done using the Parke Davis PPD antigen.

APPENDIX: III

THE ELISA AS A SYSTEM:

Kockwa *et al* (1967) and Oss *et al* have made the observation that proteins apparently attach to plastic surfaces by hydrophobic interactions. The proteins adsorbed may form a monolayer film (Canteriro *et al*, 1980) and Kockwa *et al*, , 1967). At small concentrations of pure proteins, the amount bound to the plastic is proportional to the concentration of protein in solution, and at saturation, a constant amount of protein is attached, regardless of concentration. The binding capacity of the proteins to plastic has an inverse relationship to the molecular weight (Canteriro *et al* , 1980). If antigen mixtures are used, as for example in an extract of a micro-organism, the components are likely to compete with each other for the limited sites on plastic surfaces.

ELISA tests may be competitive, indirect or double antibody sandwich assays.

(1) In the competitive method the antibody is bound to the solid phase. The test solution containing the antigen is allowed to react and then enzyme labelled antigen is added. Substrate is then added, and the reference wells containing only enzyme labelled antigen

show a colour change. The amount of antigen in the test sample would be equivalent to the amount of inhibition of colour development.

(2) In the indirect method the antigen is bound to the solid phase. Test serum containing specific antibodies is allowed to react with and bind to the antigen. Then an enzyme-labelled, anti-human antibody is added and allowed to react with the sample antibody complexed to the bound antigen. This is then detected by adding substrate its reaction with the enzyme leads to colour development. The amount of colour development is thus equivalent to the amount of antibody in the serum being tested.

(3)In the double antibody sandwich method the principle is the same,except that a known specific antibody is bound to the solid phase. The test solution containing the antigen is added, then enzyme labelled specific antibody is added,which binds to the antigen too. This enzyme on addition of the substrate, degrades the latter and gives therefore an indirect estimate of the amount of antigen present in the test sample.

Enzyme immunoassays depend on the assumption that either an antigen or antibody can be linked to an enzyme whilst retaining both enzymic and immunologic activities and this has been shown to occur satisfactorily by Avrameas *et al,* (1969), Nakane *et al,* (1974) and Sternberger *et al,* while working in the related fields. In a study done by the Dynatech laboratories, it was realized that the Elisa also has pitfalls besides possessing a considerable potential. These aspects may be related to:-

(a) Solid phase: Polyacrylamide, cellulose, cross-linked dextrans, polystyrene or polypropylene and various plastics are used for making tubes, plates and beads. The polystyrene plates of a special formula are best suited for coating with proteins and lipoprotein antigens.The sensitization is carried out by passive adsorption in alkaline solution and precise optimal conditions for concentration of reactant, time, temperature, and pH are best determined by trial and error for each system. Once established, the conditions have to be followed very carefully, because a slight departure from the optimum can lead to big variations in the result.

(b) Dilution of sample: in a buffer like PBS, and a wetting agent like Tween 20 can help prevention of non-specific adsorption to the solid phase.The sample can be tested in serial dilutions or at a single dilution simultaneously with a control reference preparation, and the results then analyzed accordingly.

(c) Conjugate: The enzyme used to conjugate for antigen/antibody labelling should be stable, highly reactive, cheap and safe, easy to prepare and assay. The two enzymes that best fulfill the above criteria are horseradish peroxidase and alkaline phosphatase. These are linked by glutaraldehyde cross linking to the antigen/ antibody. Sheep or rabbit anti-human antibodies are used for the enzyme conjugation. It is critical for these antibodies to be in pure form and they are therefore prepared by affinity chromatography, and they have been observed to give lower background levels in indirect ELISA and the conjugates can be used at higher dilutions. They are also very stable when preserved at

4°C, and alkaline phosphatase conjugates in particular have been observed to retain full activity for at least one and a half years.

(d) Substrate: Enzyme substrate should be cheap safe and easy to use. Mostly chromogenic substrates are used which are initially colourless but yield a coloured product after enzymic degradation. If the substrate reaction is too slow , then more concentrated conjugate is to be used, and if it is too fast then the assay is repeated using more diluted con.jugate. This would lead to assessment of a level which appropriately should give colour in about 20 to 40 mins.

Alkaline phosphatase is used with p- nitrophenylphosphate as substrate.The reactivity can be stopped with a highly alkaline solution like NaOH, and the yellow product remains stable for some time.

(e) End result: It may be subjectively assessed by eye or it can be measured in a spectrophotometer. The visual testing would give a positive as coloured and the uncoloured wells as negative. It may be used to determine the end point of serial dilution titrations. Otherwise the result may be recorded as:-

1) Positive or negative for samples, above or below a threshold level, taken as the cut-off point, which is determined after running several negative samples.,

2) Absorbance value- as a direct measure of the samples' antibody reactivity.

3) As end point titre- several dilutions are made and titre is the test dilution that gives a reading higher than the known negative

control.

4) As a ratio-in relation to the negative control (established as in the first case). Then the absorbence of the sample above a certain defined limit is considered positive.

5) As a unit ,-A standard curve is constructed (by running samples with known contents simultaneously with the test samples). The absorbence of unknown sample is calculated from the standard curve .

The reaction is stopped when colour/absorbence of the positive control reaches a predetermined value.

Analysis of the results. Malvano *et al* (1982) have discussed the modes of analysis of the results obtained by ELISA, as used in the classical infective diseases. According to them, the single absorbence readings are valid to indicate the trend, but may not prove to be adequate indicators of the parallelism of response-antibody activity. They also indicate systematic and random variability.

The other way is to titrate in serial dilutions, and then read the assay. Titre for each sample is calculated as the dilution at which the reading is just above that for the negative control. This method gives parallelism between the antibody activity and titre, but it also has lack of practibility due to excessive manipulation and reagent costs. Excessive manipulation may make it more prone to high level of systematic and statistical errors, and therefore less reliable.

Malvano *et al* (1982) observed that if in a group of dilution curves,

sequential vertical or horizontal intercepts were drawn and ranked (isoabsorbance or isoconcentration lines), the choice of any cut-off value before the absorbance extinction value which is the absorbance of the blank or diluent, did not affect the sequence of absorbance single readings, or the sequence of measurable titres. The titres also unequivocally correlate with the titre at any given dilution, through a sigmoidal regression, defined by a saturation value for absorbance reading at high positive level, as well a plateau of the blank or diluent absorbance.

It is seen that the optical response, and therefore shape of the binding curve, is dependent on a multitude of factors. These are related either to the method used, or within method variables such as the reagents and assay conditions, which are subject to variability. Therefore, untransformed data cannot be very precise, in representing absorbance readings or titrations. Also the signal intensity is dependent on the experimental conditions, and there is a non-linear correlation of signal with antibody activity.This leads to less and less discrimination on increasing antibody activity, which again implies, that the use of untransformed data may be unreliable.In titration, there is limited discrimination at either end of the scale, because precision of the assay relates to the sample dilution scale chosen, after defining the sequential dilutions of the control, for definition of a cut-off value. Due to this, the continuity of the assay response is lost, and samples are classified into groups. Moreover, the dilution at which extinction of the antibody response occurs, depends on the sensitivity of the

assay, which may vary under different circumstances. The only advantage to this type of analysis, is the parallelism obtained, between the antibody reactivity and titre.

A good alternative, is then, the use of a reference scale. This is especially useful if an internationally accepted reference serum is available. In the absence of such a serum, a provisional arbitrary scale for antibody activity greatly improves the test reliability.

In my test system single absorbance readings were taken and analysed at first. The results from that part of the data showed only the trends of anti-PPD antibody activity in the newborns, older infants at different ages, healthy adults and finally the patients with mycobacterial infections.

Later the titration for anti-PPD antibodies of samples in serial dilution experiments have allowed a greater discrimination in the different groups and therefore are statistically more significant than the single absorbance values.