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IMMUNOLOGY IN TUBERCULOSIS

Robert Koch made two signal contributions in the field of tuberculosis, viz. demonstration of the tubercle bacillus and proving it to be the causative organism and the Koch's phenomenon, the basic immune reaction. Very little work was done in respect of Immunology till very recently, though, if immunological studies had been pursued as assiduously as the more spectacular microbiological ones, many of the unsolved problems of pathogenesis and evolution of tuberculosis would probably have been solved by now.

Despite all microbiological advancements, however, the diagnosis of an active case of tuberculosis is still far from satisfactory. If the number of bacilli in the sputum of a pulmonary tuberculous patient is less than 50,000 per ml, the laboratory may report the sputum as negative. Culture has its own deficiencies. Diagnosis of non-pulmonary tuberculous manifestations where neither X-ray examination is helpful nor biopsy and/or microbiological proof always possible, diagnosis is still more difficult and is mostly presumptive.

Serology was introduced in the diagnosis of tuberculosis for the first time in 1901 by Bordet and Gengou. Their complement fixation test was soon followed by agglutination, precipitation and diffusion tests which were all antibodies-based. Since these tests failed if antibodies were scanty, more sensitive techniques like radio-immuno-assay, enzyme related immuno-assay and fluorescence techniques were evolved. The sensitivity improved but not the specificity. It is well-known that antibodies persist after infection even if it is not followed by disease, after BCG vaccination and even after the disease is fully healed. In other words, a positive antibodies-based serological test fails to distinguish between infection and disease and between active and inactive lesion. Another difficulty in serological tests based on antibodies is that the cut-off value of the antibody titre is neither reliable nor consistent and may vary considerably with technique, subject, etc. In fact, the immunologists today agree that if immunology is to help in the diagnosis of tuberculosis, the test will have to be based on detection of antigen rather than antibodies.

The difficulty in having a specific and a sensitive test, however, still persists. Some fraction of the antigen is specific and some fraction is shared by other species of mycobacteria. Attempts to identify and isolate the species-specific fraction of the antigen determinant (epitope) have failed so far. Monoclonal antibodies have raised the hopes very high once again. But their contribution has still to be established and may not serve the desired purpose

unless the species-specific antigen becomes available. The papers published in this issue bring out the role of the available immunological tests in the present day diagnosis of tuberculosis.

Studies carried out by the Radiation Medicine Centre, Bombay have made it possible, by using sonicates, to detect even minute quantities of antigen or bacillary count as low as 1000 per ml as against 50,000 per ml by smear examination. Another significant achievement concerns the diagnosis of meningitis. It has been shown that antibodies are not ordinarily present in CSF, pleural and ascitic fluids in healthy individuals or those with non-tuberculous disease of the respective organs. Thus, detection of antibodies even in small quantities in these body fluids tends to establish the diagnosis of tuberculosis, though it fails to distinguish between disease caused by *M. Tuberculosis* and that caused by other atypical mycobacteria. If these findings are replicated elsewhere and in large numbers, it will provide the requisite break through.

An ideal serological test should be capable of differentiating between disease and infection, between infection with virulent *M. Tuberculosis* and BCG or Atypical Mycobacteria. It should be capable of distinguishing between active and arrested lesions, and between tuberculous and non-tuberculous disease not only of the lungs but of all organs in the body. It should facilitate monitoring the response to chemotherapy. And above all, it should be simple and easy, not requiring highly sophisticated equipment or expertise, so that it could be deployed even at the extreme periphery in developing countries where majority of the cases are found but the health facilities are rudimentary. Needless to say, no test available today fulfils these requirements. With world-wide interest now being evinced in this much neglected field and the large numbers of research studies being carried out particularly in molecular biology, monoclonal antibodies, detection of mono-specific antigens etc., it is hoped that many unsolved problems in tuberculosis will be solved and an adequate test will finally be evolved which will give a new direction to case-finding and ultimate control of tuberculosis.

IMMUNOLOGIC TESTS FOR THE DIAGNOSIS OF TUBERCULOSIS

SOTIROS D. CHAPARAS*

Summary: The extensive antigenic sharing between species of mycobacteria often complicates the diagnostic value of serologic tests and tests for cellular hypersensitivity in tuberculosis. Purification of antigens or the preparation of hybridoma monoclonal antibodies offer only a partial solution in that the immunologic response to *Mycobacterium tuberculosis* is variable so that a battery of "pure" antigens or monoclonal antibodies, highly specific for the tubercle bacillus, may be required for satisfactory sensitivity. The multideterminant nature of antigens further complicates a full solution to specificity. Although a single antigen molecule may possess epitopes specific for *M. tuberculosis* it may also bear nonspecific ones. The tests available today are sensitive and useful as aids in the diagnosis of tuberculosis. In many situations a high titer or a strong tuberculin reaction may provide strong indications of a tuberculous infection. However, a diagnosis must consider other criteria as well, such as, clinical symptoms, radiological findings and, especially, isolation and identification of the etiologic agent.

Introduction

Several advances such as the production of hybridomal monoclonal antibodies (MABs) and the cloning of specific T-lymphocytes have opened new vistas of research and applications in tuberculosis immunology. However, we are still far from solutions to very important problems such as the isolation of totally monospecific diagnostic skin test antigens for tuberculosis, determining the significance of antibody in the serum of tuberculosis patients, and understanding the various factors which destroy the bacillus and result in successful resistance to the disease.

The diagnosis of tuberculosis requires a consideration of a number of observations including isolation and identification of *Mycobacterium tuberculosis*, signs and symptoms revealed by the patient, history of exposure to tuberculosis subjects, epidemiologic and social situation analysis, as well as the serologic and cellular immunologic status of the patient. Where the tuberculosis rate is high, diagnosis of tuberculosis is often presumed simply by the repeated demonstration of acid fast bacilli in the sputum and by radiologic indications. Serologic tests, skin tests, and *in vitro* tests for cellular hypersensitivity are helpful in diagnosis and often of prognostic value. In regions where the tuberculosis rate is relatively low, skin tests are useful in determining infection with the tubercle bacillus. Appropriate measures may then be taken to prevent the development of disease.

The demonstration of antibodies in serum of subjects may be confusing or helpful in diagnosis as will be discussed. Of greater value is the presence of mycobacterial antigen or

antigen-antibody complexes in blood or tissues of a patient.

Underlying infections with nontuberculosis mycobacteria complicate the diagnostic value of tests for cellular hypersensitivity and serology of tuberculosis. The value and disadvantages of antigens and antibodies with increased specificity for the diagnosis of tuberculosis will be examined.

In this review it is not possible to give credit to all investigators who, over the decades, have explored and developed various techniques to detect antibody in patients with tuberculosis and to determine its significance. Nor is it possible to credit many investigators who have laboured towards the isolation of monospecific antigens to be used as serologic or as skin tests in the diagnosis of tuberculosis. Rather, I shall focus principally on selected, relatively recent reports which exemplify application of the most useful serologic procedures and tests for cellular hypersensitivity used as aids in the diagnosis of tuberculosis.

Antigen preparations

Reliable test results for skin testing, for *in vitro* tests for cellular hypersensitivity or for serology require standardization of reagents and procedures. The standardization of complex mixtures of antigens such as tuberculin is especially difficult and in some cases impossible to achieve. Old tuberculin and purified protein derivative (PPD) are prepared from cultures which have been autolyzed and heated and consist of probably a hundred or more antigens in different stages of denaturation. Each preparation, even if it is made from the same

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strain, in the same medium and by identical procedures, will vary in the nature and quantity of the various antigens. When we consider that persons become sensitive to or form antibodies to different antigens to various degrees, it is a wonder that skin tests and serologic tests, with all their shortcomings, perform as well as they do.

Greater reproducibility may be obtained with the sonication of young, actively growing cultures of *M. tuberculosis* (53). Sonicates of various species of Mycobacterium are sometimes referred to as new tuberculin (84, 86, 87). Of course, it is mandatory to grow the organisms in a completely synthetic medium under defined conditions. With the same strain, comparable sonicates can be made which display similar electrophoretic and immuno-electrophoretic patterns (53). However, depending on their use and conditions of storage, such sonicates may pose special problems. Freeze-drying often results in insoluble denatured material. After reconstitution the presence of numerous enzymes may degrade some of the antigens over a period of time. Additionally, certain antigens are relatively labile and lose their characteristics with relative ease. This may render the "newer" tuberculin less stable. Old tuberculin and PPD which have been sterilized by heat and already digested by several months exposure to autolytic enzymes and conditions tend to be more stable due to the fact that the more labile and enzyme-susceptible antigens have already been largely inactivated. However, much of the specific character of the antigens has also been lost.

Standardization problems of tuberculin, PPD, have been reduced somewhat by the preparation of very large batches of material which can be freeze-dried and stored under conditions favourable to the stability of the reagent. In this way the International Standard PPD-S, which was prepared by Seibert (83) in 1941 has been relied upon, for over forty years. Standardization of other products to this standard, however, relates only limited information about the product's overall performance in human populations. Different products standardized to the same standard are not equivalent to each other. Nor does it mean that the standard and a standardized product will always give the same results in skin tests. As long as the tuberculin being used is not from the same lot, there will be differences.

Sensitivity and antibodies to tuberculin may be induced by infection (but not necessarily disease) with endemic species of mycobacteria other than *M. tuberculosis* (21,23,35,40,87). In some regions of the world they pose an important problem in the diagnosis of tuber-

culosis infection. To overcome this problem a number of investigators have attempted to prepare antigens of fractions which are monospecific or possess considerably increased specificity for skin testing (16,17,18,21,30,31). Results have been greatly disappointing. The status of fractionation efforts has been reviewed (15,18,19,31,43,). Efforts at purification by fractionation with procedures of high resolution and with the use of affinity chromatography with apparently specific antibodies have been attempted (28,29). Although specificity for serological tests has not been increased, specificity for skin tests has been convincingly increased over that for the unfractionated parent tuberculin. In serologic reactions, antibodies recognize epitopes and form lattice networks enlarging to precipitates, activate complement, cause agglutination and other manifestations of antigen-antibody interactions. In contrast, for a positive skin test to occur a macrophage must present to a sensitive lymphocyte an antigen recognized in conjunction with a major histocompatibility complex (MHC) gene product. The lymphocyte possesses receptors not only for an epitope of the antigens (which may be different from epitopes that respond in serologic reactions) but also receptors for polymorphic determinants (Ia) encoded by the MHC. The ensuing activation of the lymphocyte results in the release of mediators from the T-cell, some of which cause the inflammatory response recognized as a positive skin test. Triggering for a skin test reaction is, thus, far more complex than that for a serological reaction.

Determinants which are specific for *M. tuberculosis* can be presumed by results of skin tests (23,35,36) and in serologic tests (28,29) but isolation of totally monospecific reagents has not yet been accomplished. Such reagents would find wide applicability and value in skin testing and serology. Dual or multiple skin testing may be helpful in distinguishing whether a sensitivity is due to infection with *M. tuberculosis* or some other mycobacterial species (35,36,66). A single skin test with a PPD preparation for tuberculin or for another species e.g. *M. avium* may not offer a solution. However, if both are properly standardized and applied simultaneously and the reaction to the *M. avium* PPD is greater than the tuberculin it may be possible to rule out infection with *M. tuberculosis*. One cannot however, say that *M. avium* is the infecting species, because of the rampant cross reactivity among mycobacteria. Short of isolation and identification of the infecting species one cannot be certain of the etiologic agent. The lack of specificity of antigens isolated with affinity chromatography using monoclonal antibodies may be due to a multideterminant nature of antigens.

Thus, although a molecule may possess a predominance of a single epitope structure it may also possess minor epitopes which are cross reactive, albeit at a low level, with other species (see figure 1). The monoclonal antibody may also react with other antigens which possess minor epitopes capable of reacting with it. In skin testing, the presence of even a minor contaminating antigen may cause reactivity with sensitive lymphocytes and the release of interleukin 2 and inflammatory mediators which amplify the reaction. Serologic tests on the other

hand are generally constructed so that cross reactions are reduced close to background levels. That is, preliminary titrations with antigen and antiserum permit the selection of dilutions of those reagents which favour manifestations of only the specific antigen-antibody interaction.

Monoclonal Antibodies

The immune response of persons infected with *Mycobacterium tuberculosis* is generally polyclonal wherein numerous clones of

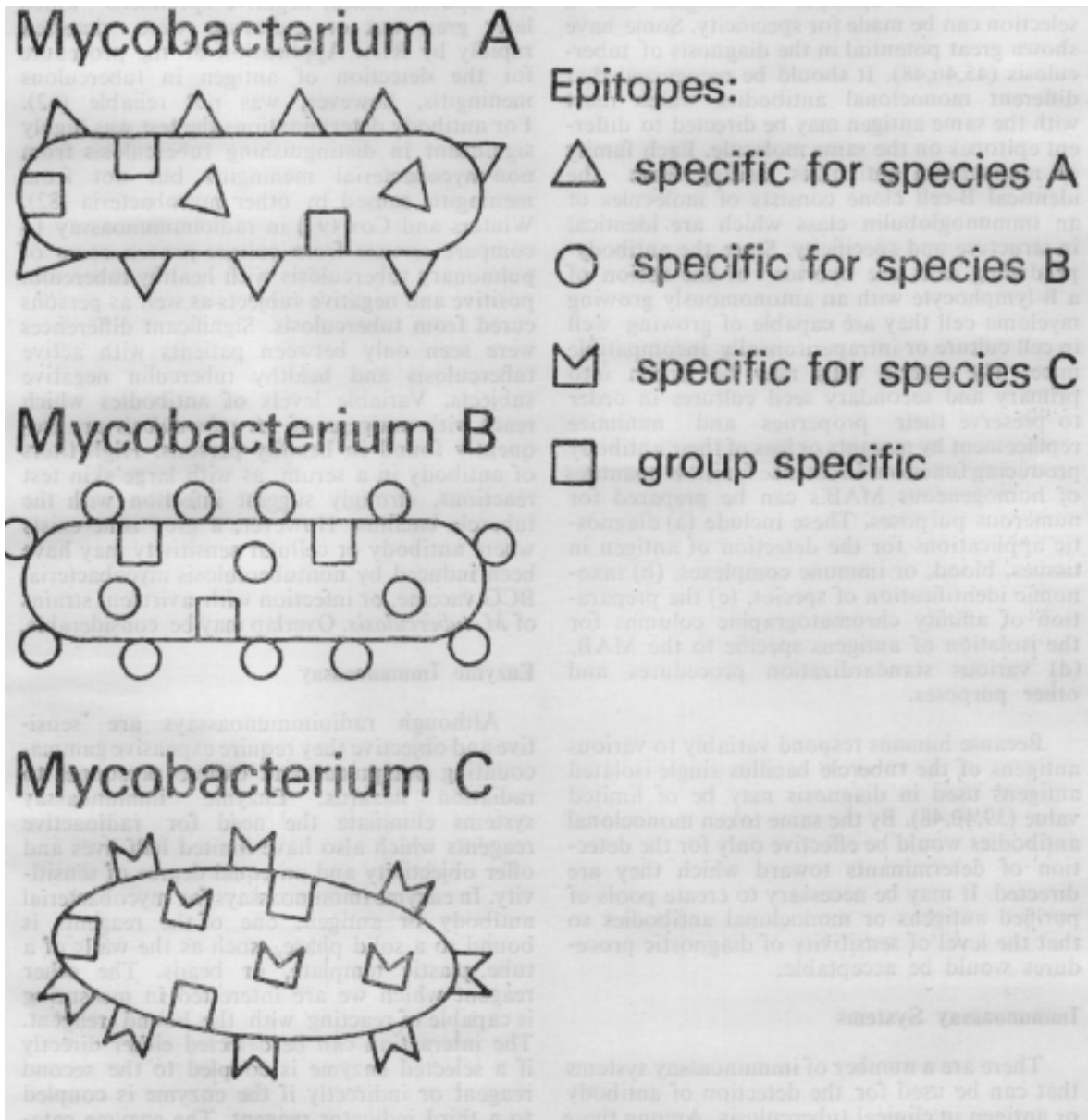


Fig. 1

Cross reactive determinants on mycobacteria. Depicted are molecules from three different species of mycobacteria showing specific and non-specific determinants.

B-lymphocytes produce antibodies to various antigens. Antibodies to some of the antigens may be pronounced and precipitate with the respective antigens while some may be barely perceptible or require very sensitive techniques for detection. Standardization with polyclonal antisera demands the production of a single large pool that will last for many years (52). Each immunization scheme, indeed each bleeding, results in antisera that may differ in the number of antibodies and in their specificities and affinities for the antigens (14). Monoclonal antibodies (MAB) on the other hand react with epitopes on antigens and a selection can be made for specificity. Some have shown great potential in the diagnosis of tuberculosis (45,46,48). It should be recognized that different monoclonal antibodies which react with the same antigen may be directed to different epitopes on the same molecule. Each family of monoclonal antibodies, arising from the identical B-cell clone consists of molecules of an immunoglobulin class which are identical in structure and specificity. Since the antibody-producing cells are hybrids of the fusion of a B-lymphocyte with an autonomously growing myeloma cell they are capable of growing well in cell culture or intraperitoneally incompatible mice. The hybrid cells may be frozen into primary and secondary seed cultures in order to preserve their properties and minimize replacement by mutants or loss of their antibody producing function. In essence limitless quantities of homogeneous MAB's can be prepared for numerous purposes. These include (a) diagnostic applications for the detection of antigen in tissues, blood, or immune complexes, (b) taxonomic identification of species, (c) the preparation of affinity chromatographic columns for the isolation of antigens specific to the MAB, (d) various standardization procedures and other purposes.

Because humans respond variably to various antigens of the tubercle bacillus single isolated antigens used in diagnosis may be of limited value (39,40,48). By the same token monoclonal antibodies would be effective only for the detection of determinants toward which they are directed. It may be necessary to create pools of purified antigens or monoclonal antibodies so that the level of sensitivity of diagnostic procedures would be acceptable.

Immunoassay Systems

There are a number of immunoassay systems that can be used for the detection of antibody or antigen in clinical tuberculosis. Among these are included objective and sensitive procedures such as radio immunoassay and enzyme immunoassays (3,55,63,82,97); and less objective and/or sensitive tests such as hemagglutination,

(10,49,50,51,70,71,81) various immunoprecipitation procedures (4,20,21,24,42,43), and fluorescent antibody assays (2,6,7,8,9).

Radioimmunoassays

Radioimmunoassays (RIA) are among the most sensitive procedures for the detection of antibody or antigens. Recent examples of application of the technique in tuberculosis include those by Kadival et al. (1982, 1983) who have been able to detect *M. tuberculosis* antigen as low as 1mg/ml or 1x 10³ organisms/ml. Sputum smear-negative specimens which later grew out on culturing were detected rapidly by RIA. Application of the procedure for the detection of antigen in tuberculous meningitis, however, was not reliable (82). For antibody determinations the test was highly significant in distinguishing tuberculosis from non-mycobacterial meningitis but not from meningitis caused by other mycobacteria (82). Winters and Cox (97) in radioimmunoassay to compare serums from culture-proven cases of pulmonary tuberculosis with healthy tuberculin positive and negative subjects as well as persons cured from tuberculosis. Significant differences were seen only between patients with active tuberculosis and healthy tuberculin negative subjects. Variable levels of antibodies which react with antigens of *M. tuberculosis* are frequently found in healthy persons. High titers of antibody in a serum, as with large skin test reactions, strongly suggest infection with the tubercle bacillus. However, a grey zone exists where antibody or cellular sensitivity may have been induced by nontuberculosis mycobacteria, BCG vaccine, or infection with avirulent strains of *M. tuberculosis*. Overlap may be considerable.

Enzyme Immunoassay

Although radioimmunoassays are sensitive and objective they require expensive gamma-counting equipment and expose personnel to radiation hazards. Enzyme immunoassay systems eliminate the need for radioactive reagents which also have limited half-lives and offer objectivity and an equal degree of sensitivity. In enzyme immunoassays for mycobacterial antibody or antigen, one of the reagents is bound to a solid phase, such as the walls of a tube, plastic template, or beads. The other reagent which we are interested in measuring is capable of reacting with the bound reagent. The interaction can be detected either directly if a selected enzyme is coupled to the second reagent or indirectly if the enzyme is coupled to a third indicator reagent. The enzyme catalyzes a substrate and converts it to a coloured product which can be measured spectrophotometrically. The intensity of the colour correlates with the amount of antigen or antibody for

which we are assaying. There are numerous variations in this procedure which can be modified for use in direct or indirect measurements or in inhibition assays. The reader is referred to a comprehensive review of enzyme immunoassay (ELA) procedures and principles by Yolken (98). The following exemplify recent clinical application of EIA in tuberculosis. Sera from patients with culturally proven tuberculosis and from subjects who were healthy and tuberculin positive or negative have been examined by enzyme-linked immunosorbent assays (ELISA) to detect antibody levels for tuberculin PPD in IgG, IgA, secretory IgA, IgM and IgE in studies by Radin et al. (56,75, 99). Clearest differences between the healthy and ill subjects were seen in measurement of IgG antibody.

Significant but lower differences were also seen with IgA. There were no significant differences between the three groups for the IgM and IgE classes of antibody. The distinction found for sera from tuberculous patients and healthy subjects is not a usual finding. In order to discriminate for IgG a dilution of 1: 100 of serum was used. For IgA a dilution of 1:10 gave the best discrimination. One problem with this type of discrimination is that cross reactivity exists. A cutpoint dilution is usually used. When small numbers of patients are examined an apparent distinction can be made from sera of healthy persons. With larger numbers of subjects greater overlap occurs and a sharp discrimination can not be seen. With serum specimens from new patients in which a differential diagnosis needs to be made rapidly and prior to results of cultures, diagnostic information from ELISA may be useful but on tenuous grounds especially if the results are in a borderline area.

Daniel and his group investigated the potential utility of purified fraction 6 (89) and fraction 5 (28,29,30) in differentiating antibodies in sera from tuberculous subjects from healthy tuberculin positive and negative persons. Antigen 5 appeared in precipitation studies (29,89) to be unique for *M. tuberculosis* while antigen 6 was not. With antigen 6 geometric mean titers (gmt) for patients who were undergoing therapy was 1:179. For those who had already achieved inactive disease status the gmt was 1:9. Healthy persons who were either skin test positive or negative had a titer of 1:6. When the antigen coating the wells of the microtiter plates was antigen 5 sensitivity was between 84% and 94% with serum of patients with active pulmonary tuberculosis at a cutoff dilution of 1:40. Specificity at this dilution was reported to be 86.5%. However, a one tube dilution difference (1:20) decreased specificity considerably. For example, for healthy

tuberculin negative subjects using a cutoff dilution of 1:40, a 4.2% of the sera reacted, but for the 1:20 dilution 33.3 % of the sera reacted. One cannot place too much reliance on a single two-fold dilution. The results also emphasize that the specificity reported for antigen 5 by less sensitive precipitation procedures breaks down in the ultrasensitive ELISA procedure. This may be due to the presence of other mycobacterial antigens or to the presence on antigen 5 of minor determinants which are not specific for *M. tuberculosis*.

Reggiardo et al. (78,79) used three purified glycolipids (phosphatidyl-inositol-dimannoside, phosphatidyl-inositol pentamannoside, and an unidentified glycolipid A) for coating tubes in ELISA tests for antibodies in sera of patients. A high degree of sensitivity was seen in that 44/46 tuberculous sera was detected when all three glycolipids were used for assays. Use of only one of the glycolipids resulted in fewer positive sera. This investigation speaks for the heterogeneity in the antibody response of patients to different antigens. This will again be referred to in the discussion on precipitation procedures.

Meningitis in children is the most common cause of death in tuberculosis. Diagnosis in the absence of a positive culture is based on clinical and nonspecific diagnostic finding. Of greatest importance is rapid diagnosis. Since many of the populations of the world have already been sensitized with inapparent infections with various mycobacteria, serum samples of healthy subjects frequently contain antibodies to antigens of *M. tuberculosis* which may complicate diagnosis. Hernandez and co-workers (44) found that with cerebrospinal fluids (CSF) an impressive distinction could be made between tuberculous meningitis and nontuberculous meningitis in ELISA assays. The test was 100% sensitive and specific. It appeared that the antibodies in CSF may have been synthesized within the central nervous system. One advantage of Hernandez's procedure was that antigen was covalently bound to plastic discs which could be washed more quickly and effectively than when the antigen is passively coated to walls of a container. Covalent binding of the antigen further assured that bound antigens would not be displaced as they might be in procedures which rely on passive adsorption of antigen. Sada (81) successfully demonstrated the presence of antigen in tuberculous meningitis with ELISA.

Immunoprecipitation

Various procedures of immunoprecipitation have been developed including those

occurring in a gel matrix. In gels, immunodiffusion with antigen and antibody may occur in one or two dimensions (74). These methods are among the least sensitive tests for antibody but permit direct comparison of precipitation bands to establish identity or non-identity. To detect antigen in clinical specimens requires large amounts for precipitation unless one of the reactants is coupled with gamma emitting isotopes which can be detected with the appropriate photographic emulsions. Various types of immunoelectrophoretic (IE) analyses can be performed for the determination of antibody in serum. These have been detailed elsewhere (4). One of the problems in tuberculosis immunology has been standardization of reagents. One effort at standardization under a U.S.-Japan Tuberculosis Programme (52) applied conventional immunoelectrophoresis with a standard goat anti-tuberculosis serum (referred to as 001 and later replaced by 002) and an antigen preparation. Eleven precipitation bands were detected by this system and although this was far less than the actual number of precipitating reactants present it offered investigators an opportunity to relate their findings with those of others. Immunoelectrophoretic systems with a much greater resolving potential than conventional IE include rocket IE (94) and 2 dimensional (2D-IE) also referred to as crossed IE (95). In rocket IE if wells are placed close to each other or overlapping, fusion of bands produced with antigens from adjacent wells and the antiserum occur (14,20,91). This allows a direct comparison of antigen preparations and a better understanding of species relationships. The percent of shared antigens has been assessed for several species of mycobacteria (14,10). Closs et al, (25), and Harboe (42,43) have employed 2D-IE for examining antigens of *M. bovis*. They have detected as many as 89 antigens. By imposing an intermediate layer of an antigen preparation or antiserum it was possible to alter the patterns of precipitin arcs formed and to identify the presence of an antigen or antibody placed in the intermediate gel. Closs (25) remarked that an excessive number of precipitin bands makes it difficult to relate antibodies in a patient's serum with the reference. For this reason the antiserum of the reference is diluted so that 30 or fewer bands, which appear to be key components, persist and are identifiable.

Kaplan and Chase (57,58) investigated with immunodiffusion and crossed immunoelectrophoresis (CIE) the presence of antibody in tuberculous patients. Only 46% of forty patients with newly acquired pulmonary tuberculosis showed antibodies prior to treatment but this number rose to 60 % upon treatment. With relapsed tuberculosis 66% of patients were positive and this value rose to 75% upon treat-

ment. Tuberculin positive or negative healthy subjects showed no precipitating anti-tuberculosis antibody. The sparse antibody levels in untreated tuberculosis subject was attributed to suppressor cells. The test appeared to be very specific in differentiating between tuberculosis and nonmycobacterial diseases such as bacterial pneumonias, bacterial sepsis and various forms of cancer. These investigators also determined by CIE that nine major antibodies to antigens of *M. tuberculosis* sonicate were produced in the serum of tuberculosis patients. The response in individual patients to the antigens varied greatly possibly due to genetic determinants. One common antigen to which all patients responded was the nonspecific arabinogalactan which is linked to mycolic acid and peptidoglycan in the bacterial cell wall. Kaplan and Chase were able to relate the nine antigens as to their frequency in tuberculosis cross reactivity with *M. leprae* and other mycobacteria and most importantly to standards and descriptions of antigens reported by other investigators.

A standard terminology for the various immunodiffusion and immunoelectrophoretic procedures needs to be developed as well as standard preparations. Proposed guidelines for this have been published (22) which include descriptions and abbreviations of the tests and proposals for reporting description and designation results and their interpretation. Application of such guidelines should eliminate some of the confusion that exists in the literature.

Agglutination tests

A number of agglutination tests for antibody to *M. tuberculosis* have been used over the years. Middlebrook and Dubos in 1948 (68) found that carbohydrates from the tubercle bacillus passively became adsorbed to untreated sheep red blood cells and that antibody from some tuberculous patients caused hemagglutination. The large red cell provided an amplification of the carbohydrate-antibody interaction on the red cell surface which resulted in hemagglutination. Later Boyden (10) found that tuberculoproteins could effectively adsorb onto sheep erythrocytes if the cells were first treated with high dilutions of tannic acid. Because agglutination of particles increased the sensitivity of an antigen-antibody reaction, particles other than sheep erythrocytes were used in efforts to avoid problems attendant with the use of an organic biological substrate. Thus, latex (26), bentonite (33,34) kaolin (92) and other particles were employed. Although these procedures increased the ability to detect antibody, the significance of the antibody still was not clear since sera of healthy tuberculin positive and negative persons also reacted.

More recently hemagglutination tests have been re-examined for potential application in tuberculosis (49,50,51,77). Jagannath and Sengupta (49,50,51) reported that aldehyde treated red cells were stable for months and passively adsorbed tubercular antigens. The tests were more sensitive than CIE tests with intermediate gel and could be performed with a drop of blood obtained from a finger prick. The test was not, however, specific enough for serodiagnosis. The results were comparable with those obtained by ELISA. Glycolipids of *M. tuberculosis* adsorbed onto the erythrocytes (78,79) were found to be highly sensitive 82.5 % in detecting antibodies in serum of 211 tuberculosis patients and in 21 % of close contacts. However, as with ELISA studies, a battery of different glycolipids was required to reach this level of sensitivity. This again emphasized the variability in the antibody response of persons to different antigens.

The hemagglutination assay was made even more sensitive by Jagannath et al. (49,50,51) by the use of *Staphylococcus aureus* bearing protein A (SAPA) which binds to immunoglobulin. SAP A was added to the system just prior to the addition of sensitized erythrocytes. Sensitivity was increased 2-8 times that over the passive hemagglutination system, depending on the species source. The procedure, however, offers sensitivity but does not appear to have any increased specificity.

Fluorescence procedures

Nassau and Merrick (70,71) presented very encouraging data with the use of a fluorescent antibody test using sera from 248 bacteriologically confirmed cases of tuberculosis. Sera were collected 4-8 weeks after culture identification. The test consisted of smears of *M. tuberculosis* fixed to glass slides and reacted with test serum. Fluorescence staining was accomplished indirectly with the use of goat anti-human IgG to which was coupled fluorescein. Ninety percent of patient sera from pulmonary tuberculosis were positive at a dilution of 1/50 and 97 percent were positive at a dilution of 1/20. Comparable sensitivity was seen with sera from extrapulmonary tuberculosis. Of random healthy subjects, 97.5 percent were negative at a 1/20 dilution. Thus, the test displayed considerable specificity, especially at the 1/50 dilution.

Because the procedure of Nassau and Merrick (70,71) expressed reactivity only to surface antigens of the tubercle bacillus, several investigators examined the usefulness of soluble antigens in the diagnosis of tuberculosis (2,3,9, 60,93). The test was referred to as the soluble antigen fluorescent antibody (SAFA) test.

Kiran et al. (60) examined sera of children from age 1 month to 12 years who had lymphadenitis due to different causes. It was difficult to diagnose a tuberculosis etiology since the tubercle bacillus could not be demonstrated in most lymph nodes. The histological picture was also nonspecific in many cases. The test used by Kiran and co-workers (60) was a convenient simplified procedure which employed cellulose acetate discs that had been impregnated with saline extract of *M. tuberculosis*. Fluorescence of the discs after reaction with antiserum and fluorescein labeled conjugate indicated degree of reactivity. The SAFA test with mycobacterial saline extracts was useful in providing serological clues but interpretations should be made with caution because several patients who were identified as being tuberculous were negative in SAFA tests. The tests were strongly positive only in about 55% of 31 cases of proven tuberculous lymphadenitis and for 5 of 106 cases which could not be identified as tuberculous. None of 10 tuberculin positive healthy adults were strong reactors.

Bhardwaj and Shriniwas (8,9) examined tuberculin preparations prepared by different methodologies and widely used in serologic techniques for suitability in the SAFA test. Panels of sera from healthy control subjects and from patients with confirmed tuberculosis were used for comparison of the tuberculins. Great variations were noted with the various preparations which re-emphasized the variability in antigen composition and of the human response to various antigens. Additionally nonspecific fluorescence of the preparations drastically curtailed their use in the SAFA test. A saline extract of mycobacteria was most suitable for this test. One would have to question the ability of ordinary saline to extract antigens from thick lipoidal cells. Response to many of the antigens is probably not detected.

Chemical markers and nonspecific tests

Several laboratories (3,63,64,96) have employed a radioactive bromide partition test to assist in the diagnosis of tuberculous meningitis. The test is based on the determination of an increased permeability in the blood-brain barrier. This is done by an estimation of the partitioning of ^{82}Br , in the form of ammonium or sodium bromide, between blood and cerebrospinal fluid (CSF). In healthy subjects serum $^{82}\text{RR}/\text{CSF}^{82}$ ratio averages about 3.1. The value tends to drop in cases of tuberculous partition test with CSF adenosine deaminase activity with CSF obtained from 58 children with suspected tuberculous meningitis. Thirty-three patients were eventually diagnosed as having tuberculosis. Four of these patients had

false negative results with the bromide test and five by the SCF adenosine deaminase test. Of the 25 patients for whom a diagnosis of tuberculosis was excluded one was false positive by the bromide test and four by the CSF adenosine deaminase test. Both the tests provide a useful adjunct in the differential diagnosis of tuberculous meningitis. Even though the test appeared to be specific within the confines of the experiment it is methodologically a non-specific test. Tuberculostearic acid is a useful specific marker for tuberculous meningitis (65). Tuberculostearic acid extracted from spinal fluid could be detected to the picogram (10^{-12} gin) level by gas chromatography/mass spectrometry. Thus the test is ultrasensitive and results are obtainable in 1-2 days but limited to facilities which possess this type of esoteric equipment. Since the number of species possessing tuber-culostearic acid are limited to mycobacteria and perhaps a few nonpathogenic genera, its demonstration should weigh significantly on the criteria used for diagnosis.

Brooke et al. (11) reported on the increase of a basic indolic compound in CSF of patients with tuberculous meningitis. The presence of the compound was valuable in differentiating between tuberculous, cryptococcal and aseptic meningitides. However, its origin is unknown and it may be a nonspecific substance.

Detection of Antigens or Immune complexes

The detection of mycobacterial antigen in tissues or fluids of a patient is very significant of an infectious process involving a member of the genus *Mycobacterium*. It may be possible to identify the infecting species with the appropriate specific reagents. The detection of immune complexes of mycobacterial antigen with antibody is also of significance. Soluble complexes usually reflect an advanced disease stage and provide a potential for immune damage via complement activation.

Sensitive procedures such as radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) are recommended since they can detect nanogram levels of antigen. Unfortunately, the relationship between sensitivity and specificity is such that as the sensitivity increases specificity decreases. The problem is minor when trying to distinguish the infections due to mycobacteria from those of different genera. With all species of mycobacteria there is appreciable cross reactivity so that with the usual reagents a definitive answer as to the infecting species cannot be made. Straus and Wu (88) performed radio-immunoassays with labeled tuberculo-protein and determined the equivalent amount of substances in sputum cultures of patients infected

with five species of mycobacteria. Crossreactivity was significant. Immunoreactive substances were not found for cultures of diverse bacteria and fungi. Kadival and coworkers (55,82) showed that RIA could detect 1×10^5 tubercle bacilli or the equivalent of 1 mg/ml of sonicated antigen. They were able to detect antigen in clinical samples such as sputum which were negative by smear but turned out to be culture positive. However, for cerebrospinal fluids (CSF) of patients with tuberculous meningitis the procedure was not useful for the detection of antigen (82) RIA tests for antibody in CSF were sensitive and satisfactorily specific which makes RIA a promising approach to the diagnosis of tuberculous meningitis. SADA et al. (81) found the ELISA system to be effective in the detection of mycobacterial antigen in CSF of patients with tuberculous meningitis. Rapidity with which ELISA can be performed offers an advantage over culture techniques. Additionally whereas culture tests for tuberculous meningitis are reportedly positive about 55-75% of the time, ELISA was found to be highly sensitive (81.3%) and specific (95%).

Specificity can be increased considerably with the use of purified antigens which bear a large complement of specific determinants and the use of monoclonal antibody reagents. It should be remembered that antigens may not be totally free of contaminating antigens from other fractions and that protein antigens are by nature multideterminant. Antigenic molecules then may bear in addition to the major specific determinant, cross-reactive determinants. Usually in serologic tests with major monoclonal antibodies such cross-reactivity is low and close to background levels. Occasional patients' sera, however, may contain significantly increased antibody levels to minor cross-reactive determinants. Conversely, monoclonal antibodies may react predominantly with epitopes on major molecules of homologous antigens. The possibility exists that other antigens may bear the same epitope to a very minor degree. Isolation of antigens with affinity chromatographic procedures, using monoclonal antibody, in addition to the homologous antigen may also react with and immobilize other antigens. Such a preparation may have useful increased specificity but it will not be fully monospecific. Circulating immune complexes can be expected when the antigen concentration relative to the antibody concentration is in excess. Small circulating immune complexes should be rapidly cleared by phagocytic cells. Although the significance of immune complexes in the tuberculosis is unknown, they have the potential of being deposited on endothelial cells of small blood vessels in various organs and induce complement activation and cytotoxins and other mediators which eventually lead to tissue damage.

To detect antigen which is complexed with circulating antibody may require dissociation of the complex. Since antigen-antibody reactions activate the classical pathway of complement activation a number of techniques have been developed for the detection of immune complexes with the use of complement components. Carr et al. (12) detected complexes in the sera of 68 % of patients with active tuberculosis and in 58 % of sera of patients with *M. intracellulare* infections. They employed the first component of complement Clq, and measured its binding activity to immune complexes (Clq-BA). Successful cure reduced the reactor rate to 15 %. May et al. (13,67) employed conglutin in affinity chromatography to isolate circulating immune complexes in tuberculosis. The affinity for certain complement components for conglutinin permits their separation from most of the other serum components. The complexes were found by ultracentrifugation to have sedimentation constants between IIS to 13S. They consisted of human serum albumin, IgG, IgA, and the Clq, C3, and C4 components of complement. By acid dissociation of the complexes antigen could be detected. A more sensitive procedure was the detection of inhibition of precipitation of free antigen with antibody by the complexes. Limitations of the system include the disadvantage that large more recently formed complexes preferentially bind to the conglutinin affinity column; thus the detected complexes may not be representative of the entire population. Of greatest value is the fact that bound mycobacterial antigen can be dissociated and detected which is of greater diagnostic significance than the demonstration of antibody.

Skin tests and *in vitro* tests for cellular hypesensitivity

Skin tests. Some important aspects of skin testing have been discussed in an earlier section. Interpretations of a tuberculin skin test must consider 2 major factors: first, whether the subject had been exposed to any persons with tuberculosis; second, the epidemiologic situation including the case rate and the degree to which nontuberculous mycobacteria are found in an environment. When non-tuberculous mycobacteria induce sensitivity, determination of whether the subject has had contact with tuberculosis patients is often very helpful in determining whether a tuberculin skin reaction is due to *M. tuberculosis*. In table 1 are listed reaction sizes and probabilities of infection with the tubercle bacillus. Segregation of subjects into contacts and noncontacts permits a more valid interpretation of skin test reactions (17). In regions where tuberculosis case ratio is high a large proportion of persons are tuberculin positive. Other criteria then become very important in a differential diagnosis such as the

TABLE 1

Probability of Tuberculosis Infection in U. S. Naval Recruits (1961-1968)

Reaction Size mm	Percent	
	Contacts	Non-contacts
0	0.0	0.0
2—3	12.7	0.6
4—5	9.0	0.4
6—7	38.4	2.5
8—9	57.3	5.2
10—11	82.5	16.1
12-13	90.7	28.4
14—15	96.0	49.7
16—17	97.8	64.8
18—19	98.9	78.9
20—21	98.9	78.6
22 +	100.0	100.0

From: Rust P. and Thomas J. 1975, Am. J. Epklem. 1—1: 311.

repeated demonstration of acid fast bacilli in the sputum, radiologic findings and, most importantly, isolation and identification of *M. tuberculosis*. Additionally, a high prevalence of *M. tuberculosis* often masks infections acquired earlier by usually nonpathogenic species of mycobacteria. In regions of the world in which the tuberculosis case rate is low and the infection rate with nontuberculous mycobacteria is also low, conversion from a tuberculin negative state to a tuberculin positive state becomes very significant. If left untreated the risk of developing tuberculosis is about 5 per cent per year (95). Where resources are available screening for converters and preventive treatment is often a cost effective measure. A detailed review of skin testing, interpretation and significance has recently been published (17). The reader is also referred to an insightful review on the tuberculin skin test by Snider (85).

A positive tuberculin skin test with a standardized low dose of PPD (2-5 TU) informs

the practitioner that the subject has been exposed to some mycobacterial species. Because nontuberculous mycobacteria have low or no pathogenicity, only a limited degree of proliferation generally occurs in the host and development of sensitivity to homologous antigens is not very great.

Cross reactivity to the tuberculin PPD is of a low order (<10 mm) and is usually reflected by a small skin reaction. Reactions to the homologous species antigens of nontuberculous mycobacteria are usually larger than those to the cross reacting tuberculin. *M. tuberculosis* is the most virulent of the mycobacteria and will usually multiply in the host to a greater extent than nontuberculous mycobacteria even when disease does not develop. As a consequence many lymphocytes become stimulated and a high level of sensitivity to tuberculin is produced. A very large reaction (>16mm) is usually indicative of extensive replication by the tubercle bacillus and is a matter of serious concern. Appropriate other parameters should be examined to decide whether chemoprophylaxis or chemotherapy should be instituted. Small reactions may result early in a tuberculosis infection as sensitivity is beginning to develop or in chronic or progressive tuberculosis when energy is developed. Less virulent strains or strains which are usually restricted in growth may induce only a mild sensitivity to tuberculin. Figure 2 presents patterns of distribution of reaction sizes in four population groups.

Panel B shows a typical distribution pattern for patients with tuberculosis; most subjects develop large reactions. Panel A being an area low in nontuberculous mycobacterial sensitivity shows only a few persons producing small reactions with the curve dipping close to baseline between 5 and 10 mm. In panel D, the dip is filled in by reactors sensitized because of infection with nontuberculous mycobacteria which produce many reactions between 5 and 9 mm in diameter. Panel C may represent an epidemiologic picture applicable to some regions of India where the case rate is relatively high and nontuberculous mycobacteria are significant. The sensitivity due to *M. tuberculosis* may be pure or superimposed on top of that induced by another mycobacterial infection.

The presence of a positive skin reaction to tuberculin indicates that the principle defense systems known to be required for immunity to tuberculosis is present. Virulent organisms possess a heavy lipoidal coat and other structures which serve as barriers to adverse substances. Mycobacteria engulfed in phagosomes may produce sulfatides which hinder the fusion

of lysosomes to phagosomes (37,15). Therefore, many bacilli continue to grow even when macrophages become highly activated. Continued growth may lead to stimulation of suppressor macrophages and lymphocytes with the onset of energy and a poor prognosis. This subject has been reviewed elsewhere (15,16) and will not be reviewed further here.

A low level of sensitivity may be boosted by skin testing to a size that may be interpreted as positive and indicative of a current tuberculous infection. For the United States, Comstock and Woolpert (27) recommend that two serial tuberculin tests at least one week apart be performed. Persons who develop reactions of less than 10 mm should receive the second injection which may or may not reflect a boosted reaction. The second reaction constitutes a baseline for interpretations of future reactions. In the USA a person is considered to be a converter if in subsequent tests the baseline reaction is exceeded by at least 6 mm and a reaction is at least 10mm. For India, the increase above the baseline reaction size may need to be considerably greater to be considered a true conversion.

Margileth (66) found that in pediatric populations skin tests with more than one standardized mycobacterial skin test antigen could distinguish between tuberculous and nontuberculous mycobacterial infections. In adult populations sensitivity is not as virginal as it is in young children and multiple infections with mycobacteria may cloud interpretability of skin reactions. Properly prepared sonicates represent preparations of mycobacterial extracts in their most undenatured extracellular state. Stanford and colleagues (84,86,87) have investigated sonicates of mycobacterial species (new tuberculins) endemic in various environments and found them to be considerably more specific and promising as skin test reagents for tuberculosis. However, one would have to question whether such enzyme-rich extracts would retain the specific epitopes in intact form or whether specificity would decrease with or without the loss of skin test reactivity (84,86,87). Claims have been made that the usefulness of "new tuberculins" for skin tests is maintained for at least 10 years but data to establish stability and specificity have not been presented.

The problem of specificity has been addressed in a previous section. With regards to skin test reagents results of purified components have been very disappointing. Partial specificity has been reported by Harboe and Nagai (43) with an antigen, MPB 70, which appears to be absent from *M. tuberculosis* but present in many (but not all) strains of BCG and nontuberculous mycobacteria. Such an

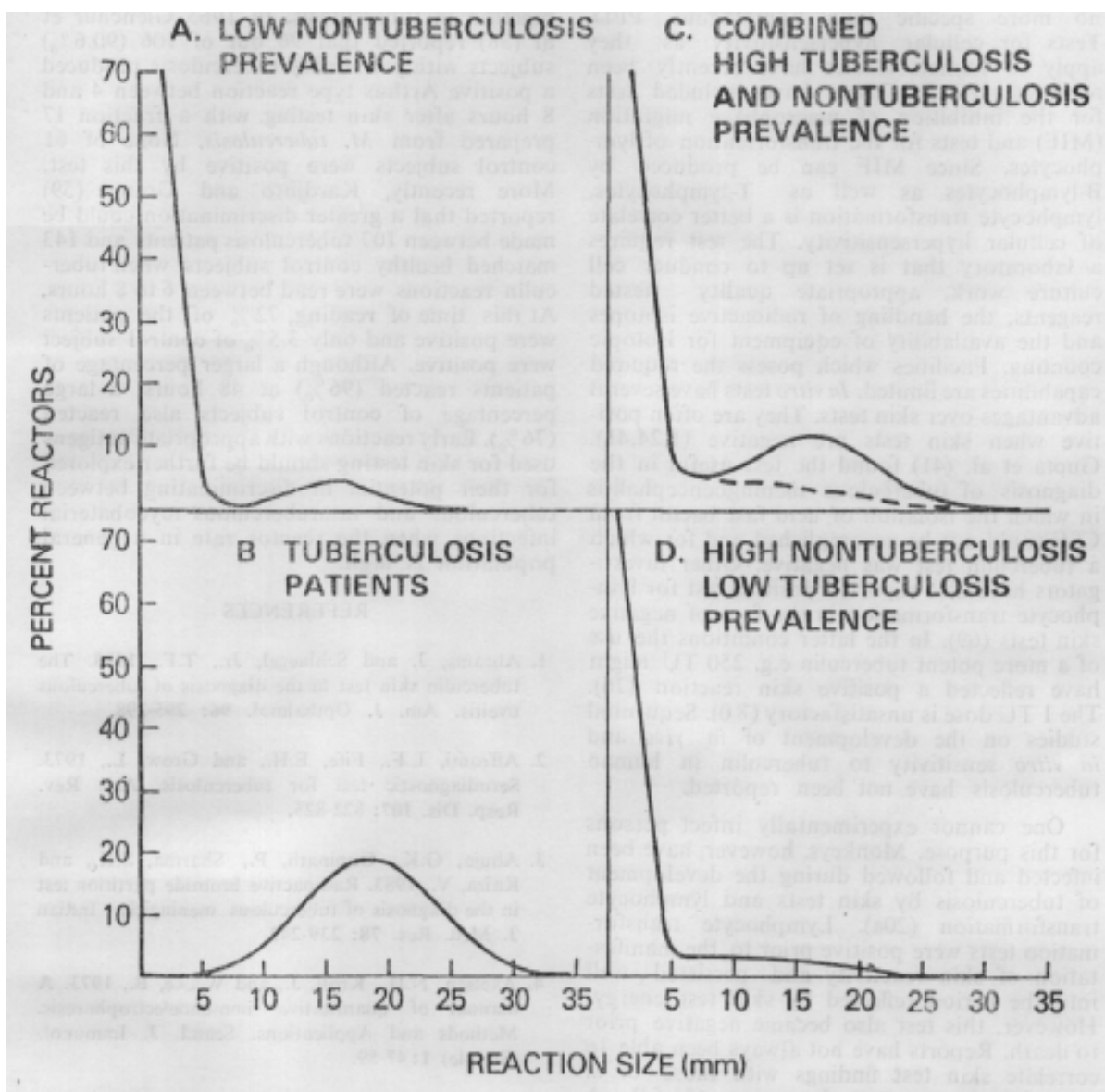


Fig. 2

Distribution of reaction sizes in four different groups.

antigen would be useful in excluding tuberculosis sensitivity from that induced by BCG or species of mycobacteria which contain the antigens. Niculescu et al. (72) prepared what appeared to be antigens specific for BCG relative to *M. tuberculosis* by immunoelectrophoresis. When these were used in skin tests in an effort to distinguish reactivity between BCG vaccinated children compared to those with tuberculosis, specificity was lacking. The preparative counter-current immunoelectrophoresis procedure used apparently did not allow sufficient purification. Furthermore, present on most

protein antigens are not only specific epitopes but also a complement of nonspecific ones which confound specific reactivity in skin testing. It is also quite possible that determinants for cellular hypersensitivity are different from those which cause serologic activity also discussed above.

Antigens 5, 6 and 7 of *M. tuberculosis* which have been purified by Daniel and coworkers (6,29,30,62) are all cross-reactive in sensitized human beings or animals. Antigen 5 which appeared initially to show promise as

a specific skin test reagent was found to be no more specific than homologous PPD. Tests for cellular hypersensitivity as they apply to human studies have recently been reviewed (16,17). These have included tests for the inhibition of macrophage migration (MIF) and tests for the transformation of lymphocytes. Since MIF can be produced by B-lymphocytes as well as T-lymphocytes, lymphocyte transformation is a better correlate of cellular hypersensitivity. The test requires a laboratory that is set up to conduct cell culture work, appropriate quality tested reagents, the handling of radioactive isotope and the availability of equipment for isotopic counting. Facilities which possess the required capabilities are limited. *In vitro* tests have several advantages over skin tests. They are often positive when skin tests are negative (1,24,41). Gupta et al. (41) found that test useful in the diagnosis of tuberculous meningoencephalitis in which the isolation of acid fast bacilli from CSF could not be accomplished and for which a tuberculin test was negative. Other investigators have also reported positive test for lymphocyte transformation in the face of negative skin tests (69). In the latter conditions the use of a more potent tuberculin e.g. 250 TU might have reflected a positive skin reaction (76). The 1 TU dose is unsatisfactory (80). Sequential studies on the development of *in vivo* and *in vitro* sensitivity to tuberculin in human tuberculosis have not been reported.

One cannot experimentally infect primates for this purpose. Monkeys, however, have been infected and followed during the development of tuberculosis by skin tests and lymphocyte transformation (20a). Lymphocyte transformation tests were positive prior to the manifestation of skin reactivity and persisted well into the period reflected by skin test energy. However, this test also became negative prior to death. Reports have not always been able to correlate skin test findings with those of *in vitro* lymphocyte transformation (24,54). A number of factors may account for this. One factor is the fact that peripheral lymphocytes removed from the body or placed *in vitro* are thereby removed from a number of humoral and cellular facilitating or inhibiting influences. For example, autologous serum from tuberculosis subjects can inhibit the transformation of lymphocytes which respond well in the presence of nonautologous serum. The T-helper and T-suppressor cell interaction may operate differently *in vitro* than *in vivo*. Often in culture many of the macrophages which may be suppressor cells have been eliminated. It is not unusual to find *in vitro* tuberculin responsiveness in the absence of skin test reactivity even in clinically normal individuals (1).

A final point to be covered in this

review is the significance of an early skin test reaction in tuberculosis. In 1965 Glenchur et al (38) reported that 96 out of 106 (90.6%) subjects with pulmonary tuberculosis produced a positive Arthus type reaction between 4 and 8 hours after skin testing with a fraction 17 prepared from *M. tuberculosis*. None of 61 control subjects were positive by this test. More recently, Kardjito and Grange (59) reported that a greater discrimination could be made between 107 tuberculosis patients and 143 matched healthy control subjects when tuberculin reactions were read between 6 to 8 hours. At this time of reading, 72 % of the patients were positive and only 3.5% of control subject were positive. Although a larger percentage of patients reacted (96%) at 48 hours, a large percentage of control subjects also reacted (76 %). Early reactions with appropriate antigens used for skin testing should be further explored for their potential in discriminating between tuberculous and nontuberculous mycobacterial infections when the reactor rate in a general population is high.

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SERODIAGNOSTIC TESTS IN TUBERCULOSIS

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Summary: The problem of development of serological tests in detection of tuberculosis is an old one. Techniques involving detection of antibodies have inherent disadvantages. It is often difficult to distinguish normal people with BCG Vaccination from tubercular patients. The more sensitive the assay, the greater is the problem of differentiation. Since antibodies persist for a long time after bacillary clearance, antibody levels cannot differentiate between active disease and healed lesion. Monoclonal antibodies produced to a specific single epitope of the bacillus is the newest entrant in the field. Extensive studies need to be done to establish its efficacy in clinical practice.

The detection of antigen is a better proof of infection than antibody assay. Tests have been developed to detect antigen as well as antibody which are sensitive and specific at least for *M. Tuberculosis* and *M. Bovis*. Antigen could be detected in patients with positive sputum but not in controls; in bronchial aspirates of tubercular patients and in pleural and ascitic fluids of tubercular origin. Antigen detection yield was poor in CSF and in patients with high antibody levels in pleura and ascitic fluids. In such situations specific antitubercular antibody has proved a better diagnostic index to detect disease. Detection of specific tubercular antigen and antibody in circulating immune complexes correlated with presence of infection. Antigen detection is not only useful to identify presence of infection but is also informative regarding the efficiency of treatment.

The methodology of molecular biology has progressed in recent years to the extent that it can now be applied directly to a number of health problems including tuberculosis. Extensive attempts to develop more specific skin tests for diagnosis of infection have been made over the last ten years by the biochemical fractionation of *M. tuberculosis*. However, no component with satisfactory superiority over PPD, with a few exceptions, has been identified. In fact, the picture that has emerged of the antigenic mosaic of mycobacteria is that most, if not all, macromolecules of these organisms contain antigenic determinants (i.e. epitopes) some of which are unique to each strain while others are shared. The chemical characterisation of mycobacterial cell walls and other lipid-containing components has made important progress in recent years. Another area of rapid development of tuberculosis is the preparation of monoclonal antibodies. Claims for species specificity and strain specificity have already been made (Coates et. al. 1981). The development of a serological test using some of the newer techniques mentioned above, particularly if it is able to differentiate active disease from past infection or BCG-vaccination, would revolutionise the epidemiology and control of one of the world's most important problems. The problem of development of serological tests is an age old one. Obviously, there are many difficulties encountered and during the course of the review, some of the problems will be discussed. Serological tests are developed

either to detect the antigen i.e. some component of the mycobacteria or to detect specific antibody produced by the host as a part of its mounting an immunological reaction to the presence of mycobacteria. Most of the techniques which have been reported in the literature involve the detection of antibodies in the host. The detection of antibodies have their inherent disadvantages which will be discussed later. The progressive evaluation of serological tests for detection of antibodies will be discussed. The newer techniques of antigen detection will be extensively described.

The use of whole *M. tuberculosis* antigen

The most logical and simplest approach to the setting up of a diagnostic assay is the use of the whole bacillus as an antigen. It is possible to fix whole bacilli on a slide and react these with fluorescein conjugated antihuman immunoglobulin obtained from patients with tuberculosis. Good results have been claimed by using this test (Nassau and Merrick, 1970) especially if the surface lipids are removed by phenol or organic solvents. However the use of a fluorescent microscope is cumbersome and only antibodies to antigens expressed on the surface can be detected.

Another assay using alkali treated whole bacilli was also used in an agglutination test similar to a Widal test for Typhoid (Nichols, 1975). However the results of the test showed that it could not differentiate patients with active disease from treated cases of tuberculosis.

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Assays using crude culture supernatants or PPD as antigens

Later, attempts were made using crude antigens such as Old Tuberculin or PPD prepared from supernatants of cultures. These contain a broad spectrum of antigens present in *M. tuberculosis*. However many important antigens are lost in these preparations as a result of autolysis before harvesting them from the cultures and subsequent autoclaving to kill viable organisms. The disastrous effect of these manipulations has been reported by several authors (Harboe et al, 1981).

Earlier methods using these antigen preparations used techniques such as agglutination, precipitation or complement fixation. The problems with these methods are that all antibodies are not detected by such an assay. The detection of positive from negative cases is evaluated by determining a "cut off" value which will differ from test to test. For example, relatively large amounts of antibody may be required to give a visible precipitate or agglutinate particles like erythrocytes or latex particles. So a negative result with such tests may be detected while significant quantities of antibodies are still present. Middlebrook and Dubos (1948 and 1950) observed that certain antigenic components from crude supernatants of liquid cultures of *M. tuberculosis* will spontaneously adhere to erythrocytes which after washing could be used in standard haemagglutination or haemolysis assays. Considerable efforts were spent on correlating the clinical status and these tests but no reliable correlations could be established (Popp, 1971). These antibodies were only detecting glycolipids and polysaccharide antigens which could spontaneously adhere to erythrocytes. Daniel and Baum (1969) used the procedure of tannic acid treated erythrocytes which could bind protein antigens instead of polysaccharides and glycolipids. Such assays also failed to distinguish patients from tuberculin positive controls. Others used latex, Kaolin or bentonite particles instead of erythrocytes but with similar results (Kaplan and Chase 1980).

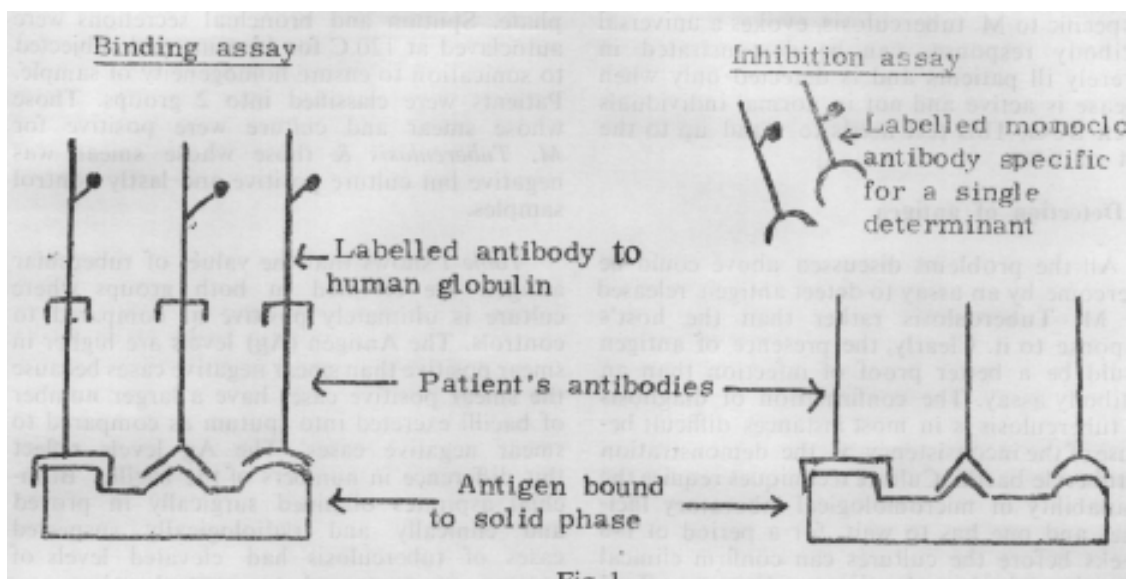
Recently, "binding" assays, that is radioimmunoassays and enzyme related immunoassay have replaced the techniques which employ physical characteristics like agglutination, precipitation etc. These new assays detect the binding of antibody to antigen directly and hence theoretically can detect all kinds of antibodies. The assays can be done in a liquid phase or on a solid support (Samuel et al, 1983). In fluid phase binding assays the antigen is labelled with I^{125} and allowed to react with patients serum. The complexes of antibody antigen labelled with I^{126} are separated either by

precipitation with reagents or with antihuman globulin or adsorbed with staphylococcal proteins A. The amount of labelled precipitate is then proportional to the amount of antibody present in the sample (Bardama et al, 1973; Samuel et al, 1983). In solid phase assays, the antigen is allowed to bind to polystyrene surfaces in the form of tubes, microtitre plate walls or beads. (Fig. 1a) The serum containing antibody is then incubated with the antigen fixed to polystyrene and the bound antibody is detected by added antihuman globulin labelled with I^{125} (radioimmunoassay) or with an enzyme (Enzyme linked immunosorbent assay ELISA) and can hence be quantitated. The ELISA assays are as sensitive as the radioimmunoassays. Both the assays, liquid phase and solid phase are inherently so sensitive that even very minute amounts of antibody can be detected. This increased sensitivity has shown that almost everybody has detectable antibody to *M. tuberculosis*. The apparent absence of antibody tested by agglutination and precipitation methods is because of the high cut off levels. Hence, since binding assays show antibodies in almost all individuals, its diagnostic potential is limited. In fact it was reported (Kardjito, Handayo and Grange, 1982) that in a group of Indonesian patients only 62 % of the patients had antibody levels which were above the level which included 97.5% of tuberculin positive controls. Zeiss et al (1982), on the other hand, showed that no such overlap was present in a group of American subjects.

The possible explanation for the presence of antibodies in normal individuals could be because of the ubiquitous nature of mycobacteria in the environment e.g. water and soil. This exposure to non-pathogenic organisms could be involved in priming appropriate immune responses to BCG or pathogenic mycobacteria. Mycobacteria share antigens with other genera such as corynebacteria (Stanford et al, 1974; Stanford and Grange, 1974) and these may also stimulate antimycobacterial antibody formation.

Purification of antigens

In view of these failures, the next logical step was to purify antigens and identify species specific antigens with a hope to develop more specific tests. Unfortunately, isolation of species specific antigens of *M. tuberculosis* has proved extraordinarily difficult (Daniel and Janicki, 1978). This could perhaps be due to the fact that many separation procedures rely on physical characteristics such as size and charge. By this process products of molecular heterogeneity are obtained so that meaningful physical separation is not possible. The cell wall for example breaks up into an assortment of comp-



(a) The standard solid phase binding assay in which the presence of the patient's antibody bound to antigen is detected by radiolabel or enzyme labelled antibody to human immunoglobulin. Antibody to all available antigenic determinants is detected.

(b) The inhibition assay, used with monoclonal antibodies. The monoclonal, which is labelled will bind to only one available determinant. If the patient has antibody to this determinant, the binding monoclonal is blocked. Antibodies in the patients serum which bind to other determinants, are irrelevant.

lex fragments of various sizes, rather than into discrete reproducible molecules. Molecules even when isolated and carrying species-specific determinants, also tend to carry other determinants which are not species specific: This suggests that an antigen is a whole single molecule and the many different individual determinants or epitopes that this molecule carries are varied in nature.

Monoclonal antibodies

Since separation of species specific antigens has proved such a problem, the real hope lies in the exploitation of monoclonal antibodies. These antibodies are raised by fusing individual antibody secreting cells with myeloma cells, so that the resulting hybrid clone has the immortality of a myeloma cell and the antibody secreting function of the B-cell (Galfre Milstein, 1981). In this way it is possible to generate unlimited supplies of antibody specific for an individual antigenic determinant.

The assay Using a monoclonal antibody is the inhibition assay, a variant of solid phase binding assay (Fig.6) The monoclonal anti-

body which is labelled with I^{126} or enzyme will bind only one of the available determinants. If the patient has an antibody to this same determinant, the binding of the monoclonal is blocked, while antibodies to other determinants in the serum would not interfere.

As these assays are still under trial (Hewitt et al, 1982) their usefulness is yet to be established. However, some problems can be anticipated. The immune responses in man presented with a complex antigenic mixture, as in a natural infection, would be such that only some of the thousands of antigenic determinants would evoke a response. Which of these antigens would evoke a response would be determined by genetic factors and previous priming of the immune cells. When sera from tuberculosis patients (Kaplan and Chase, 1980; Reggiardo et al, 1981) were tested against a range of glycolipids, different individuals developed antibodies to different antigen determinants. This kind of an assay detecting antibodies to an individual determinant may be useful only in a selected group of patients. Hence an inhibition assay using a monoclonal antibody will be useful only if it binds to a determinant which

is specific to *M. tuberculosis*, evokes a universal antibody response, can be demonstrated in severely ill patients and is detected only when disease is active and not in normal individuals given BCG. This test needs to stand up to the test of time.

II Detection of antigen

All the problems discussed above could be overcome by an assay to detect antigen released by *M. Tuberculosis* rather than the host's response to it. Clearly, the presence of antigen would be a better proof of infection than an antibody assay. The confirmation of diagnosis of tuberculosis is in most instances difficult because of the inconsistency of the demonstration of tubercle bacilli. Culture techniques require the availability of microbiological laboratory facilities and one has to wait for a period of 6-8 weeks before the cultures can confirm clinical suspicion of tubercular disease. Can one afford to wait so long, and besides is it ethical to start extensive, prolonged and an expensive chemotherapeutic regime with a clear conscience? It seems that there is an urgent need for newer methods in diagnosis of tuberculosis. Radioimmunoassay (RIA) techniques using radionuclides as tracers have been introduced in medical practice since the late 1950's. These techniques have been shown to have the ability to detect very small concentrations of substances in the ranges of femtograms, nanograms and picograms. This exquisite sensitivity of detection has never been possible by conventional biological and biochemical methods.

In addition to the sensitivity offered by RIA's (because of the inherent properties of the technique) it is also specific to the detection of the substance, and can be a very accurate and precise method. RIA's have found applications in endocrinology, neuroendocrinology, Gastroenterology, renal diseases and a number of other applications. Since infectious and tropical diseases still pose diagnostic problems, we decided to employ the advantages of RIA to develop sensitive and specific methods in study of tuberculosis. We have developed tests for the detection of antitubercular antibody and tubercular antigens from sputum, bronchial aspirates, CSF, pleural and ascitic fluids and have been encouraged by the promising results we have obtained.

Sputum and Bronchial aspirates from patients suffering from pulmonary tuberculosis were collected. Smears were stained according to the Ziehl Neelsen method and scanned for the presence of acid fast bacilli. Cultures were made on LJ solid slants after decontaminating the sputum by a 15 min exposure to a mixture of 30% NaOH and 15% sodium dodecyl sul-

phate. Sputum and bronchial secretions were autoclaved at 120.C for 15 mins and subjected to sonication to ensure homogeneity of sample. Patients were classified into 2 groups. Those whose smear and culture were positive for *M. Tuberculosis* & those whose smear was negative but culture positive and lastly control samples.

Table I shows that the values of tubercular antigen are elevated in both groups where culture is ultimately positive as compared to controls. The Antigen (Ag) levels are higher in smear positive than smear negative cases because the smear positive cases have a larger number of bacilli excreted into sputum as compared to smear negative cases. The Ag levels reflect this difference in numbers of the bacilli. Bronchial aspirates obtained surgically in proved and clinically and radiologically suspected cases of tuberculosis had elevated levels of antigen as compared to control value.

II Pleural and Ascitic fluids

Ascitic and pleural fluids from 41 cases of pleural and peritoneal tuberculosis were obtained. The biochemistry of ascitic and pleural fluids was typical of an exudate with variable numbers of lymphocytic infiltrates and elevated proteins. The diagnosis of peritoneal tuberculosis was established in 3 of the cases by laproscopic demonstration of tubercles on the peritoneum and histopathological confirmation of tubercles on biopsy and in one case by the growth of tubercle bacilli on culture. The remaining 7 cases were diagnosed on the evidence of positive mantoux test with associated lesions like paratracheal gland enlargement, infiltration, cavitation etc. In 29 cases of pleural involvement, diagnosis was based on Mantoux test, positive X-ray lesions and response to antitubercular treatment. Only 3 cases were sputum positive by culture. 38 ascitic and pleural fluids obtained from patients suffering from malignancy, cirrhosis of the liver and hypoproteinaemia were evaluated as controls.

Table II indicates the levels of TB antigen and TB antibody in controls, proved cases of tuberculosis and clinically and radiologically suspected cases. As can be seen from the table, both antigen & antibody levels were significantly elevated ($P < .001$) in proved as well as clinically suspected casts of tuberculosis when compared to control levels.

The presence of either TB antigen, antibody or both could be considered as an index of the presence of disease (*Table III*). In 7 proved cases of peritoneal and pleural tuberculosis, only Ag was detected in 5 and both Ag and antibody (Ab) in 2. There were no false negatives.

TABLE I

Tubercular antigen levels in sputum and bronchial aspirates

	Smear -f-Ve Culture-}- Ve	Smear—Ve Culture+Ve	Control
I. Pulmonary TB (X + SE)	(n=19)	n=(20) (X+SE)	(n = 60) (X + SE)
Tb Agng/ml.	58.5 + 6.7*	26.6+4.3*	1.4+0.2*
II. Bronchial aspirates culture+Ve	Smear + Ve	Clinical & Radio- logical Diagnosis	Controls
	u=14 (X+SE)	n=(39) (X+SE)	n = 19 (X+SE)
TbAgng/ml.	55.5+9.9*	64.1 + 5.6*	4.1+0.75*

*P<001 as compared to respective control values.

TABLE II

Anti TB antibody & TB antigen concentrations in Ascitic & Pleural effusions

	Anti TB antibody Ratio of std/sample	TB antigen ng/ml.
	X + SE	X + SE
Control n=38	0.74+0.03	2,8+0.62
Proved tuberculosis by biopsy or culture n=7	10.22+7.23	75.25+20.55
Clinical and radiological evidence of tuberculosis n=34	29.97+6.89	10.73 + 3.12

TABLE III

Presence of Detectable TB Ag and/or TO Ab in Ascitic and Pleural Effusions

	Only TB Ag detected	Only TB Ab detected	Both Ag and Ab	Neither Ag/Ab detected
1. Control n=38		—	—	38/38
2. TB patients proved by biopsy or culture n=7	5/7	—	2/7	—
3. Clinical and radiological evidence of TB n=34	7/34	20/34	6/34	1/34

In the 34 remaining cases, Ag was detected in 7 only, Ab in 20, both Ag and Ab in 6 and a single case had neither Ag nor Ab. The detectability of Ag and/or Ab probably reflects the stage or progress of the disease and the relative concentrations of Ag and Ab in the exudates.

Tubercular meningitis

For this study, 138 control CSF samples from patients with epilepsy, febrile convulsions, encephalitis and tetany, 9 patients with pyogenic meningitis and 46 of TBM were selected. The diagnosis of pyogenic meningitis was based on clinical signs, history, typical CSF changes and therapeutic response to antibiotics. The cases of tubercular meningitis were diagnosed on the basis of clinical presentation, CSF changes typical of TBM, strongly positive Mantoux test, X-ray evidence of pulmonary TB and strong family history of contact. Gastric lavage was negative in all the patients. AFB was demonstrated in 1 case. Cases of TBM were evaluated in 3 groups viz. untreated, treated for less than a week and treated for a month or more.

Table IV shows that the TB antigen levels in TBM, and pyogenic meningitis were comparable to controls. However, the anti TB antibody levels were elevated in TBM and not in pyogenic meningitis. As the duration of treatment increased, the antibody levels were reduced, indicating that the test is most effective in distinguishing untreated and cases treated for a few days from control and pyogenic meningitis.

Circulating Immune complex characterisation (CIC)

Sera were obtained from adult patients attending a tuberculosis clinic. These patients had undergone detailed investigations which included clinical examination, investigation of family members, radiological and haemato-logical investigations. Sputum was cultured for *M. tuberculosis*. Patients were classified into two groups: group I—radiologically positive for tubercular lung lesions and sputum smear and/or culture positive for *M. Tuberculosis* and group II—radiologically positive with tubercular lesions in the lung but AFB negative by smear and/or culture. Table V shows that TB antigen concentrations of CIC was higher in Gr. I and Gr. II than controls. Both groups were significantly different from each other. The anti-TB antibody levels in Ag Ab complexes again were higher in patient group than in controls, with a ($P < .001$) degree of significance. The total circulating antitubercular antibody levels were also markedly raised in patient group as compared to control values.

Table VI presents an analysis to show the prevalence of Ag and/or Ab in the CIC in the 2 groups of patients. In those patients where sputum was positive for AFB 56% had both Ag and Ab in the complexes, 21 % only Ag, 19% only Ab and 4 % showed absence of Ag and Ab. In patients with sputum negative for AFB, 36% showed presence of both Ag and Ab, 6% only Ag and 25 % only Ab while 17% were negative for both.

TABLE IV

Tubercular antigen and Antitubercular antibody levels in CSF

	TB antigen conc, ng/ml lowest detectable std. titre/	Anti TB antibody Radio of unknown titre.
	X+SE	X+SE
1. TBMn=28	4.16+3.7	50.5+2.5*
2. TBM treated less than 1 week n=7	nd	18.2+6.5*
3. TBM treated less than 1 month n=11	nd	3.2+0.5**
4. Pyogenic meningitis n=9	4.2+2.8	1.56+0.3
5. Controls n=138	3.1+1.2ng/ml	1.5+0.5

* $P < .001$ significantly different from control values.

** $P < .05$ significantly different from control values.

TABLE V

Antigen and Antibody Concentrations of CIC in various groups of patients

Disease State	TB antigen det. from CIC ng/ml	TB antibody det. from CIC	Circulating total TB antibody from serum
	X+SE control's sample	Ratio of titres X + SE	Ratio of litres X + SE
Pulmonary TB sputum+ve for M. tuberculosis x-ray lesions seen (n=57)	19.1+2.3	11.7 + 1.48	14.5 + 62.2
Pulmonary TB sputum — ve for M. tuberculosis	9.9. + 1.9*	5.1 + 1.5*	72.5+34.7**
3. Controls (n=40)	2.2 + 0.3°	0.6 + 0.1°	1.4 + 0.2°

*=P<.001 controls significantly different from sputum +^{ve} and sputum—ve cases

*=P<0.001 sputum -j-ve and sputum —ve cases **=P<0.001 sputum -j-ve and sputum —[^]ve cases.

TABLE VI

Relative frequency of Antigen Antibody detection in CIC in sputum positive and sputum negative patients

Disease State	Both TB Ag +Ab detected in CIC % of cases	Only TB Ag detected in CIC %	Only TB Ab detected in CIC %	Both TB Ag +TB Ab not detected %
1. Pulmonary TB sputum - -ve for				
M. tuberculosis x-ray lesions seen.	56	21	19	4
2. Sputum — ve for				
M. tuberculosis	36	6	25	17

Table VII shows the effect of antitubercular chemotherapy on the Ag/Ab components of CIC. It indicates that in those cases where sputum is persistently positive for AFB either because of drug resistance or inadequate treatment, there is no significant reduction in the levels of tubercular Ag or antitubercular antibody. In patients who were sputum positive and became negative on chemotherapy and those originally sputum negative but who showed a good therapeutic

response, the TB antigen concentrations decreased progressively. The anti-TB antibody levels, however, showed a progressive elevation.

The detection of mycobacteria by identification and measurement of specific bacterial associated material represents a new approach to the diagnosis of tuberculosis. The decreasing reliability of acid fast smear techniques for detection of tuberculosis and the expensive and

TABLE VII

Effect of Chemotherapy on Ag/Ab components of CIC

	Untreated cases		Anti TB treated for less than 12 weeks		Anti TB treatment given for 12 weeks — 2 years	
	CICAg	CICAb	CICAg	CICAb	CICAg	CICAb
	ng/ml	Ratio titre control/sample	ng/ml	Ratio titre control/sample	ng/ml	Ratio of titre control sample
	X+SE	X + SE	X+SE	X+SE	X + SE	X + SE
1. Pulm. TB sputum-)- ve for tuberculosis X-ray lesions seen. (n=16) M.	19.2 -f 5.4	(n=16)	(n=14)	(n=14)	(n=23) 22.1	(n=27)
		8.3+4.1	18.2+2.1	9.5+5.3	+6.0**	14.8+5.7
2. Pulm. TB sputum— ve for M. tuberculosis X-ray lesions seen. ((n=8)*	14.8+5. S	(n=8)	(n=13)f	(n=13)	(n=16)	(n=16)J
		1.1+0.4	11.4+4.2	4.9+2.6	6.2+1.7	7.85+3.1
3. Controls (n=40)	2.2+0.3°	0.6+0.1°	—	—	—	—

* P< 0.001 Controls different from sputum +ve and sputum —ve cases.

* P<0.01 difference between sputum —ve untreated and treated more than 12 weeks (CIC Ag)

** P<0.01 difference between sputum +ve and sputum —ve cases treated for more than 12 weeks (CIC Ag)

† P<0.05 difference (CIC Ab) between sputum —ve cases untreated and treated for less than 12 weeks

†† P<0.05 difference (CIC Ab) between sputum —ve cases given treatment for < 12 weeks and >>12 weeks.

time-oriented culture methods do not afford a quick diagnostic facility.

The sensitivity of our assay is 1.0 ng/ml. The levels of antigens rise *pari passu* with increase in the number of whole organisms ranging from 1.5×10^6 to 50×10^6 cells/ml, with minimum detectability of 1×10^5 organisms/ml.

A sputum smear by Ziehl-Neelsen stain can be demonstrated as positive if 50×10^3 or more organisms/ml are present. Since our assay can detect the presence of 1×10^5 organisms/ml it is possible to detect antigen directly even when smear does not show acid fast bacilli. As seen from the results of sputum analysis the antigen levels are higher in samples which are correspondingly lower in samples which are negative on smear but are positive on culture later. However, in patients on extensive treatment, if the number of non-viable bacilli outnumber

viable ones, correlation may not exist as the assay detects non-viable bacilli also. In the method described by Strauss and Yalow (1980), sputum samples were cultured for 2-3 week prior to estimation of PPD from culture filtrate suggesting that the sensitivity of the assay is not sufficient to detect small number of organisms.

The initial aim of the study was to validate the laboratory based technique in clinical studies. Encouraged by our preliminary attempts to evaluate antigen detection in sputum, we proceeded to study different tubercular disease manifestations. The study of circulating antigens in blood was the most logical proposal as blood is the most easily obtainable material. We obtained sera of patients suffering from pulmonary tuberculosis. However we found that even in normal subjects, a certain concentration of antitubercular antibodies was detec-

table. This observation has been corroborated by several other studies wherein antitubercular antibody levels were being measured as an aid to diagnosis of active tubercular disease. Since normal subjects can and do have a certain level of antitubercular antibodies, two problems were encountered. The first was that the detection of tubercular antigen in the presence of antibody was an impossibility. This was because of the inherent properties of the technique itself. Secondly antitubercular antibodies were not an evidence of active disease, since antibody levels remained elevated several months after treatment was discontinued. The only positive evidence of active disease was rising titres of antibody. Antibody detection was abandoned in favour of TB antigen detection as an index of active disease. However, antigens could not be detected when antibody was present. The solution was to get rid of antibody or else to devise measures for detection of antigen and antibody specific to the disease. We decided to standardise sensitive, specific techniques for detection of antigen and antibody and proceeded to study them in different disease states.

The problem of antitubercular antibody present in normal CSF, ascitic and pleural fluids was not encountered, unlike the presence of these antibodies in blood. In our study we could demonstrate that in CSF, ascitic and pleural fluids of nontubercular diseases there were no antitubercular antibodies. Antitubercular antibodies were only present in tubercular disease conditions. Whenever antitubercular antibodies could not be detected, TB antigen was demonstrable in these secretions or fluids so that either antigen or antibody detection indicated that tuberculosis was active and present. In 7 cases of peritoneal and pleural tuberculosis proved by laparoscopy or positive culture, not a single case was missed and both Ag and/or Ab was detected. The yield of positive cultures being very low, the true/false positive rate is difficult to evaluate. The other cases were presumed to be of tubercular origin by indirect evidence used in day to day clinical practice. Hence the reliability for diagnosis of exudates, be it pleural or peritoneal is greatly enhanced and confirmed. One can treat such patients with confidence.

Since normal levels of circulating antibodies are present in blood, it was thought that in active disease conditions, there must be some bacteria in circulation and consequently antigens would be present in circulation. The host responds by developing humoral antibodies (as also CMI responses) and therefore circulating antigen/antibody complexes (CIC) should be formed in blood. We thought that the evaluation of CIC as a diagnostic technique for any tubercular infection should primarily be validated

in pulmonary tuberculosis, as definitive diagnosis in such conditions is relatively easy. Circulating immune complexes were isolated, dissociated and TB antigen and antitubercular antibody components identified. The study revealed that when patient had active disease, the TB antigen levels were elevated and once treatment was effective, the antigen levels decreased while antibody levels persisted. Now that it is possible to detect antigen by this technique, active disease becomes distinguishable. We intend in future to study CIC in renal, osseous, meningeal and other forms of tuberculosis. Will this simple aid single technique be the only method for diagnosis replacing the older, conventional and inadequate methods available?

Tuberculosis of the meninges is one of the bugbears of pediatric diagnosis. Cultures of CSF and gastric lavage are invariably negative. A question is often asked by the clinicians. Is this IBM or is it not? In fact when we first standardised the method of RIA of TB Ag, we thought we had at last found the solution to the problem of TBM. Our preliminary studies however were depressing. TB antigen could not be detected in CSF. Why not? Was it possible that the amount of bacterial antigen was very minute, beyond the sensitivity of the assay technique? Was it that meningitis was the result of an immune reaction because of cell mediated immune responses? Was it because presence of antibody prevented the detection of antigen? It was at this stage of our study that we decided to develop an RIA for the detection of antitubercular antibodies. The technique standardised could measure antibody in dilutions of 1:1000,000, equivalent to less than $1\text{ ng}/1\text{ ml}$ of immunoglobulin. We started detecting antitubercular antibodies in TBM and found that they were not detectable in controls and in pyogenic meningitis. The only drawback is that once treatment is instituted, especially the administration of steroids, there is a suppression of antibody formation. The levels drop to almost normal levels and the chances of false negative results are increased. It seems that the antitubercular antibodies in CSF are most probably secreted by the local meningeal lymphocytes. This hypothesis is surmised from the data obtained by us showing that in children with progressive primary complex but no meningitis, although the circulating blood antitubercular antibodies are elevated, the CSF antitubercular antibodies are within normal limits. Secondly, in cases of pyogenic meningitis & viral meningitis, where there is an alteration of the blood brain barrier, there does not appear to be any transfer of the circulating antitubercular antibodies into CSF, as these latter are not raised in CSF. However, definite

direct experimental evidence is still to be obtained.

In conclusion, radionuclide techniques i.e. RIA for the detection of TB antigen and anti-tubercular antibodies have been evaluated in a variety of tubercular diseases. The presence of antigen or antibody is indicative of the presence of active disease processes. It is not only useful in distinguishing tuberculosis from other normal and diseased states but it has been useful to monitor the effectiveness of treatment. It has also been seen that detection of antibodies in blood has poor diagnostic potential since the methods available are either too sensitive or too insensitive for detection. Normal individuals also carry small amounts of circulating antibodies and these persist in treated individuals for long periods of time. The answer lies in the detection of antigen in blood and antigen and antibody in other body fluids.

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IMMUNOLOGICAL SPECTRUM IN TUBERCULOUS CHILDREN

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Summary: Tuberculosis is a classic example of disease that is controlled entirely by cell mediated immunity. The latter is essentially a local phenomenon carried out by macrophages that are activated locally at the site of infection by lymphokines secreted by specific subset of T. lymphocytes. The polymorphonuclear cells only engulf the bacteria but are incapable of destroying them. The immune spectrum in respect of some tuberculous manifestations has been described and need for more immunological studies in children as in adults has been brought out.

A. Problem of Tuberculosis in children

Tuberculosis in adults, as a major health hazard, has been well recognised in developing countries like India. The prevalence of active disease in the population is 15 to 25 per 1000 population. Of the 15 to 20 million active cases of tuberculosis, 4 to 5 million are bacillary. With such a vast magnitude of the problem of infectious adults, the prevalence of infection in the child population is naturally very high. 40 % of the children by the age of 6 years and nearly 80% by the age of 15 years can be considered as infected if 6 mm induration is considered as the cut-off point (Udani et al, 1982). Gothi et al (1979) have revealed that increasing the size of induration from 10 to 12 mm made only a difference of 2% in the positive tuberculin rates.

With an annual rate of fresh infections in children as 4,% in India (Raj Narain, 1980) 3.64 million children in the age group of 0-4 years are infected annually (Udani, 1963). Primary focus in the lungs usually heals and enlargement of the mediastinal lymph nodes may be the only evidence of disease. The problem of neuro-tuberculosis, specially tubercular meningitis, in children has been highlighted by Udani (1983) on the basis of hospital data. Rao et al (1982) have shown that the risk of death in tuberculous meningitis is three times more than in intrathoracic tuberculosis in children under 5 years and 1.5 times more in the age group of 5-14 years. The factors which determine the outcome of primary infection are (i) age at which infection occurs, (ii) immunological mechanisms, (iii) degree of malnutrition, (iv) number of infecting bacilli, (v) associated infection such as measles etc. and (vi) the genetic make up of the child. In the presence of these unfavourable factors, primary infection may progress to post primary disease resulting in severe haematogenous tuberculosis like miliary tuberculosis, multiple disseminated foci in

different organs, tuberculous meningitis or severe disease of the lungs. The other consequence of the primary infection is the development of protection against subsequent infection by tubercle bacilli. In developing countries like India, with adverse environmental conditions, morbidity and mortality is high if primary infection occurs early in life. Genetic factors play an important role in many diseases and tuberculosis is no exception. Differences exist in the susceptibility of various organs to tuberculosis. However, the growth of bacilli in each organ is controlled presumably by identical macrophages arriving at the site from blood stream indicating that both genetic and local factors control the course of infection. Tubercle bacillus is such a dangerous organism that once it invades the human body at any age, its progeny is likely to survive throughout the remaining life of that individual (Myers, 1952, 1962). Natural resistance to tuberculous infection varies greatly among animal species. Lurie (1964) was able to breed resistant species of rabbits and showed that though tubercle bacilli disseminated just as well in the resistant rabbits, their multiplication within the tissues was inhibited. He concluded that this susceptibility was due to their inability to mount an effective immune response and was genetically controlled. Singh et al (1983) demonstrated a marginal increase in DR2 and a concurrent significant decrease in DRW6 in adults with tuberculosis in the major histocompatibility system. The predominance of DR2 locus in families with more than two siblings suffering from tuberculosis has also been demonstrated by Singh et al (1983).

B. Immunology of Tuberculosis

The immunology of tuberculosis is a subject which has attracted tremendous interest in recent years. Still there are many aspects which remain poorly understood. Tuberculosis remains the classic example of a disease that is

controlled entirely by cell mediated immunity involving the macrophage as the effector cell and the lymphocyte, (especially the T-cell) as the immuno-responsive cell (Mackness, 1968). This type of immunity is also called acquired cellular resistance. Cell-mediated immunity is essentially a local phenomenon carried out by macrophages that are activated locally at the site of infection by lymphocytes and their lymphokines. It is intimately linked with cellular hypersensitivity.

Mitchison (1983) designed a greatly simplified model of some of the immunological events in tuberculosis. When a previously unexposed child is infected by *M. tuberculosis*, the small clump of bacilli begins to multiply at the site of infection. These are then phagocytosed. Some of the phagocytes stay at the original site and others migrate to local lymph nodes and still others to more distant parts of the body. The polymorphonuclear (PMN) cells engulf the bacteria but are incapable of destroying them. Antigen carried by monocytes stimulates T-cells. The latter activate macrophages either by cell-to-cell contact when the two types of cells have the same HLA-antigens or via lymphokines which are not HLA restricted. The activation of macrophages prevents early dissemination of bacilli by the formation of non-immune granuloma before the onset of specific immune response. The specific immune granuloma of tuberculosis is called high turnover granuloma in contrast to the non-immune or low turnover granuloma. Peptidoglycan layer of the bacterial cell wall or its synthetic derivative muramyl dipeptide is responsible for this macrophage activation and granuloma formation.

The specific immune response leading to macrophage activation and to granuloma formation is cell mediated and cannot be passively transferred with humoral antibodies. Following stimulation by the appropriate antigen, thymus dependent lymphocytes (T-cells) proliferate and synthesize a number of hormone like molecules termed lymphokines. Adams (1982) revealed that the nature and proportion of the T-cell subsets involved in the immune response differ from pathogen to pathogen and the ultimate effect on the macrophage is not quite similar in each case. The T-cells do not themselves effect the cell-mediated antibacterial immunity; rather they act indirectly through lymphokines which act via macrophages. The lymphokines attract macrophages to the site of infection and activate them to kill bacteria that these cells ingest.

Tuberculo-immunity can be transferred in mice by T-cells and can be abrogated by dep-

leting T-cells and restored by agents that restore T-cells (Lefford, 1975). Youmans (1979) was the first to succeed in assembling the complete model of tuberculo-immunity *in vitro* using mouse immune spleen cells (the T-cells) to make lymphokines and then being able to inhibit ingested virulent tubercle bacilli in macrophages.

Crowle et al, (1983) has summarized the following characteristics of tuberculo-immunity:

1. It is cell-mediated.
2. It is expressed by appropriately activated macrophages.
3. The macrophages are appropriately activated by an immune lymphokine.
4. The immune lymphokine is made by immune T-lymphocytes which are activated by immunizing antigen of the tubercle bacillus.

Hence immunizing antigen, immune T-lymphocytes, macrophages and tubercle bacilli can be made to interact *in vitro* to reproduce specific expression of tuberculo-immunity.

B.I. Macrophages

When tubercle bacilli infect an animal or man, they are readily phagocytosed first by the PMN cells in which they multiply. The killing of these organism is by macrophages, first described by Lurie (1943). Human tuberculo-immunity as summarized by Crowle et al (1983) has mechanisms similar to mouse tuberculo-immunity. The necessary human cells can be readily obtained and made to function analogously *in vitro*. Bergholtz and Thorsby (1979) and McCalmon and Kirkegaard et al (1980) have demonstrated specific production of lymphokines by incubating peripheral blood monocytes, T-lymphocytes drawn from a tuberculin positive individual and PPD. Macrophage function can also be elicited *in vitro* by 3 to 4 days' cultivation of human blood monocytes into macrophages using micro culture techniques (Johnson et al, 1977, Crowle, 1981). At the beginning of infection, the tubercle bacillus destroys PMN cells which have ingested bacilli. The bacteria continue to multiply slowly but regularly in macrophages that come to the site of infection where the PMN cells have failed. Some of the infected macrophages reach the draining lymph nodes where they present tubercle bacillus antigen to T-lymphocytes. Specifically responsive clones of T-cells become activated and replicate, then leave the lymph node in large numbers and circulate in the blood

throughout the body where they give the infected subject two hallmarks of tuberculosis infection:-

- Tuberculin hypersensitivity (T-cells reactive to tuberculo-proteins).
- Tuberculo-immunity (T-cells reactive to immunizing antigen).

Dannenberget al (1982), have shown that many macrophages are killed in the process of pathogen-macrophage interaction in tuberculosis and hence the ability of the host to overcome the infection depends on the speed and effectiveness of the activation of new macrophages entering the lesion. It is not the alveolar macrophages, but the tissue or blood borne monocytes that effectively handle tubercle bacilli. Grange (1983) emphasized that studies on the pathogen macrophage interaction are rendered very difficult by the fact that these cells are divisible into sub-populations with widely different properties depending on their location within the body. However, Crowle et al (1981) effectively used an *in vitro* assay system using the cultured macrophages from peripheral blood monocytes and tubercle bacilli in the presence of lymphokines to illustrate tuberculo-immunity in human beings.

Following ingestion, the basic mechanism by which macrophages kill the organisms is the fusion of the phagosomes with the lysosomes. The microbicidal enzymes in the latter kill the organisms. *M. tuberculosis* can survive in the host tissue by (i) resistance to the lysosomal contents (ii) escape from the phagosome into the cytoplasm and (iii) inhibition of the fusion of lysosomes with the phagosomes. It has been postulated by Lowrie (1981) that phagosome-lysosome fusion may inhibit the growth but not kill *M. tuberculosis*. This may be relevant to the establishment of dormant bacilli that are responsible for relapse or reactivation of disease. Hence, it can be summarized that virulence of the bacilli, effectiveness of the macrophage-activating mechanism and microbicidal power of the activated macrophages determine the outcome of primary infection in children. If these mechanisms of immune response take the upper hand as usually happens in children by the time tuberculin conversion occurs about 4 weeks later, the primary lesion i.e. local lymph nodes and the more distant foci resolve and heal by fibrosis.

B.2. T-cells

Events following infection are dominated by antigen stimulating T-cells. Monocytes must present the antigen to the T-cell acting in a helper capacity. Suppressor T-cells and suppressor macrophages suppress the T-cell

function. The balance between helper and suppressor effects of T-cells is fundamental in the immune system of tuberculosis.

B.3. Lymphokines

There is very strong evidence that CMI in tuberculosis is due to the activation of macrophages by soluble factors (lymphokines) liberated by antigen-specific helper T-cells. Rook (1983) postulated that these activated macrophages may be further stimulated to secrete a factor or factors that cause tissue necrosis by a second signal which would be a lymphokine from a distinct set of T-cells. This lymphokine might merely prime the macrophages to react in this way in response to bacterial products.

Various lymphokines are:—

(i) Macrophage Activation Factor (MAF) which induces a marked increase in the metabolic activity of the macrophages which in turn leads to enhanced phagocytosis and intracellular killing.

(ii) Migration Inhibition Factor (MIF)

(iii) Macrophage Fusion Factor (MFF) which contributes to the formation of the characteristic granuloma or tubercle, containing giant cells.

(iv) Growth Inhibition Factor (GIF).

The macrophages are capable of killing the bacilli in the presence of GIF. If the GIF is absent, there is no expression of immunity and bacilli multiply in the macrophages and kill them. Crowle (1983) postulated the presence of a bacillary Growth Enhancing Factor called GEF isolated from mouse model of tuberculo-immunity which is probably responsible for liquifying caseous lesions and reactivation of quiescent foci.

Crowle et al (1983) have also put forward the hypothesis that the hypersensitivity T-cells probably are mainly destructive to the subject and produce GEF predominantly in addition to causing tissue damage and tubercle formation. The immune T-cells are mainly helpful inducers of tuberculo-static or tuberculo-cidal macrophages. At the site of infection, reactive T-cells respond to the infecting tubercle bacilli and cause inflammation and macrophage accumulation. The immune lymphokine GIF activates the macrophages to kill ingested tubercle bacilli. The bacilli appear to be most easily killed if they are replicating and are spared if they are not replicating. The pathogenic lymphokine

GEF may to a certain extent have a beneficial effect of stimulating bacillary replication and thus keep the bacilli susceptible to killing by activated macrophages. However, it will promote the growth of bacilli in caseous lesions where macrophages can not reach. Progression or regression of tuberculosis depends upon the balance between the production of predominantly GEF or GIF producing antigen responsive T-cells.

The use of pure antigens obtained from tubercle bacilli or culture filtrates by electrophoresis is a great advance in explaining the immune reactions in tuberculosis. Prostaglandin E₂ inhibits and indomethacin stimulates the presentation of antigen to T-cells by monocytes. Main effect of chemotherapy in the early stages is against actively dividing microorganisms. Immunopharmacological agents could be of benefit by counteracting the suppression existing in the active disease and thus increasing the overall bactericidal action of chemotherapeutic agents.

B.4. Tuberculin test and delayed hypersensitivity

Though Tuberculin test is the most frequently performed clinical test, its immunological basis is poorly understood. Grange (1983) has rightly concluded that not only cell mediated immunity (CMI) and delayed hypersensitivity (DH) are separate phenomena but DH is antagonistic to protection if the reaction is excessive. Though DH and CMI are both induced by lymphocytes, these cells are divisible into subsets with quite different functions in response to different components of mycobacterial antigens. High levels of DH have an adverse effect on tuberculosis in children (Udani, 1982 b). The tuberculin test is usually read after 48 to 72 hours. Grange (1983) has suggested that some patients develop an immediate or Type I reaction and this reaction may reduce the eventual 48 hour reaction because it assists the removal of antigen from the infection site by vasodilation. The second is Arthus or type III DH reaction which develops at 4 hours and reaches its maximum at 8 hours. It does not attain the size of the 48 hour reaction and is common in adults with active tuberculosis but not in healthy individuals, who are tuberculin positive at 48 hours. This needs to be explored in childhood tuberculosis. The third reaction is maximum at 24 hours. A small peptide isolated from the ribonucleo-proteins of *M. tuberculosis* could elicit a good response at 24 hours but not the 48 hours' reaction in 76% of children with primary tuberculosis (Grange, 1983). A high percentage of healthy BCG vaccinated children under the age of 7 did not respond to this peptide. The final reaction is the classical tuberculin reaction

which causes induration and necrosis although the latter may not be microscopically evident.

B.5. Delayed hypersensitivity in tuberculosis

Delayed hypersensitivity is responsible for the rapid accumulation of lymphocytes and macrophages which become activated wherever tubercle bacilli and their tuberculin like antigens exist in the tissues. The reaction causes accelerated tubercle formation. This accelerated tubercle formation causes the destruction of inhaled tubercle bacilli that are not destroyed by alveolar macrophages. Similarly, accelerated tubercle formation often stops the progression of many small lesions of haematogenous origin in the lungs, spleen, liver and kidney thereby controlling endogenous infection. DH development to small number of bacilli in the tissue site is beneficial but not so when large doses of bacilli and their antigen are present. In the latter situation, the hypersensitivity reaction itself causes cell death and tissue destruction resulting in caseation, liquefaction and tuberculous bronchopneumonia. This latter reaction is produced by the cells and tissues of the host namely cytotoxic lymphocytes, macrophages and granulocyte products.

Accumulation of a large number of macrophages and lymphocytes at the site of tuberculin injection is responsible for large tuberculin reaction and is a protective effort on the part of the body. A large tuberculin reaction has no prognostic value (Dannenberg, 1982). Energy in extensive and serious disease is probably due to the fact that most of the available lymphocytes and macrophages are infiltrating the site of infection. The other postulations are that either there is saturation of the antigen receptor sites on lymphocytes or stimulation of suppressor lymphocytes. The hematogenous spread will progress if the accumulating blood-borne macrophages cannot limit the growth of the bacilli and regress if the accumulating macrophages become activated enough to stop such bacillary growth.

Accelerated tubercle formation is the mechanism by which cell-mediated immunity is produced (Mackness, 1968). It is the ineffective activation of blood borne macrophages which instead of killing organisms get themselves killed causing progression of disease. Macrophages in active caseous tuberculous granuloma have a rather high turnover. Over 90% of them are replaced every 10 days. During the phase of development of tuberculin hypersensitivity, the presence of a sufficient amount of antigen leads to more mobilisation of macrophages at the site of the lesion in comparison to when DH is weak. Local acquired cellular resistance is the accumulation of large number of activated

macrophages in the lesion. These activated cells ingest and kill bacilli released from dying, ineffective macrophages.

B.6.a. Immunogens of BCG

A variety of the constituents of the tubercle bacilli induce resistance to tuberculous infection when injected in experimental animals. The immunogen located predominantly in the cell wall of tubercle bacilli induces a low grade immune response to tubercular infection. The other immunogen is quite labile and is found in the ribosomal or RNA preparation. This labile fraction induces a much greater degree of specific immunity to tuberculous infection. The discovery that the major immunogen in *M. tuberculosis* is located in the ribosomal and RNA preparation leads to the belief that there is a relationship between the nature of the antigen and the type of immune response in tuberculosis (Youman, 1965). Hewitt and Coats (1983) recently isolated seven monoclonal antibodies (MABS) to *M. tuberculosis* which are being used to develop serodiagnostic tests for active tuberculosis and for the isolation of specific mycobacterial antigens. In a recent memorandum (1983) by WHO, the areas demarcated for research in the immunology of tuberculosis are Molecular Biology, Monoclonal Antibodies, Immuno-regulation in Human Tuberculosis, Experimental Immunology of Tuberculosis and Cloning of Mycobacteria. Use of appropriate antigens purified by monoclonal antibodies in specific skin tests or *in-vitro* tests of CMIR will be able to establish infection with a specific organism. These antigens might be of great value in differentiating exposure to *M. tuberculosis* from exposure to BCG in humans. They could also be useful in exploring

the relationship between immunization and previous contact with environmental mycobacteria, which may determine the efficacy of vaccine.

Strain to strain variation in the immunizing potential of the vaccine, local genetic factors, natural immunization by tubercle bacilli of low virulence and a programming of the immune system by prior contact with mycobacteria that live freely in the man's environment are some of the factors that are responsible for wide variations in the protective value of BCG.

Stanford et al (1981) have postulated that some environmental mycobacteria tend to direct the immune response towards protective macrophage-activating responses while others direct it more towards the antagonistic hypersensitivity reactions. The administration of BCG thus tends to boost the pre-existing tendencies with useful, indifferent or even harmful results. Adenitis after BCG vaccination is probably an indication of development of a severe degree of hypersensitivity. Hence BCG should be administered before the environmental mycobacteria can exert their untoward effects. Kathipari et al, (1983) have demonstrated that full-term, normal weight newborns are capable of evoking an immune response after BCG vaccination.

B.6.b. Delayed type hypersensitivity after BCG

DTH tested after BCG in relation to nutritional status demonstrated that percentage of cases having different extent of induration ranging from 5-9, 10-14 and ≥ 15 mm in vaccinated children was comparable in the three grades of nutrition (Table-1) (Seth, 1984 a). A similar

TABLE I
Post-vaccination Tuberculin reaction in relation to nutritional status

Nutritional status	Size of Tuberculin reaction (mm)		
	5-9	10-14	≥ 15
a. Normal 61	14(33.3)	12(28.6)	16(38.1)
b. Undernourished 69	12(27.9)	14(32.5)	17(39.6)
c. Severe P—E.M. 24	3(33.3)	2(22.2)	4(44.5)
P value a x b NS b X c <0.05 a X c <0.02	a X b	b x c	a x c =NS

Bold numerals indicate number of cases.
Figures in parentheses indicate percentages.

trend has also been reported by Raj Narain et al (1979). Kielmann et al (1976) in a study of Indian children after BCG also showed no significant difference in the mean size of induration. He suggested that sensitivity to tuberculin following BCG develops on all or none basis. One must keep in mind while interpreting DTH as a measure of T-cell function, that it is a composite of several sequential steps in sensitization, antigen recognition, proliferation and elaboration of lymphokines by T-cells and vascular changes and migration of inflammatory cells to the local site. Hence reduced cutaneous reactivity may be the result of interference with one or more of these processes.

B.7. Immune spectrum in Tuberculosis

Clinical, morphological and immunological studies of human tuberculosis have demonstrated the existence of a spectrum of immune response in tuberculosis. At one extreme, the infection is subclinical and merely leads to tuberculin hypersensitivity and at the other extreme is a progressive disseminated disease of the nature of miliary and meningeal tuberculosis. The presence of an immune spectrum in tuberculosis was first suggested by Skinsness, (1968).

Lenzini (1977) described an immune spectrum with two polar forms, reactive and unreactive tuberculosis (RR & UU). The reactive form (RR) is characterized by localized lesions with lymphocytes and epithelioid cells and by a marked early response to antitubercular drugs. Immunologically, this form shows evidence of active cell mediated immunity with little or no antibody response. In particular, the reaction of PPD is that of a typical delayed hypersensitivity response and is also reflected in the positive cellular response *in vitro*. The unreactive form (UU) is characterized by rapid diffusion of the lesions within the chest and to other organs and a poor response to treatment. This group shows immunologically a very poor or an absent cell mediated immune response, resulting in both Tuberculin test and Leucocyte Migration Inhibition Test (LMIT) being negative with abundant antibody response. In between these two polar forms is an intermediate reactive group (IR) showing characteristics of the two extreme polar groups RR and UU.

A study was designed to investigate the radiological spectrum and its correlation with the immune parameters in childhood tuberculosis (Seth et al 1984b, 1984c). The following profile has emerged using the above criteria:

- (1) Tuberculin positive, no manifest tuberculin lesion.

- (2) Pulmonary Primary Complex (PPC) which is of three types (i) Nodal, (ii) Parenchymal and (iii) Parenchymal and Nodal.
- (3) Progressive Primary Tuberculosis (PPT)
- (4) Miliary Tuberculosis (MTB)
- (5) Meningeal Tuberculosis (TBM)
- (6) Tubercular Lymphadenitis (TBL) with or without pulmonary lesion of the nature of Nodal, Parenchymal or Nodal with Parenchymal.

Data in respect of SMP, PPC and TBL only are being presented here. An induration of ≥ 10 mm to P.P.D. has been taken as a positive tuberculin reaction and LMIT. was considered as positive if the index was less than 0.8 (Seth et al, 1981).

LMIT. was positive only in 64% of Tuberculin positive children with no obvious tuberculous lesion (SMP). In PPC group, 72.8% were tuberculin positive and 42.8% LMIT. positive. In TBL group, the immune profile was somewhat similar to PPC showing 80% tuberculin positive and 44% LMIT. positive (Table 2). Tuberculin reaction was positive in significantly larger number of children ($P < 0.01$) in the SMP group as compared to PPC. However, the difference in the SMP and TBL groups was not significant.

Distribution of cases according to the size of tuberculin reaction was comparable in the three types of lesions (Table 3). The extent of inhibition in LMIT positive cases in the three groups was also similar (Table 4). Percentage of T-cells was significantly lower in TBL group in comparison to PPC and SMP. However, the total lymphocyte and absolute T-cell counts were comparable in the three groups. In miliary and meningeal tuberculosis reported earlier (Seth et al, 1981) only 13.3% were tuberculin positive as against 93.3% LMIT positive, the difference being highly significant ($P < 0.001$).

It is quite baffling that no correlation was found between the tuberculin and LMIT positivity in the three clinical presentations of SMP, PPC and TBL in childhood tuberculosis. Skyor and Trnka (1979) observed that in active tuberculosis, there is, on an average, a decreased proportion of lymphocytes, competent for cellular immunity and the more extensive the tuberculous process, the greater the decrease in T-cells. However, in the present study the absolute T-cell counts were comparable irrespective of the type of tuberculosis. In severe

TABLE 2

Tuberculin Reaction and LMIT in various manifestations of Tuberculosis

Type of lesion	Positive Tuberculin Reaction	Positive LMIT
a. SMP25	25(100)	16(64.0)
b. PPC70	51(72.8)	30(42.8)
c. TBL25	20(80.0)	11(44.0)
P value a x b	<0.01	NS
b X c	NS	NS
a x e	NS	NS

Bold numerals indicate number of cases
 Figures in parentheses indicate percentages.

TABLE 3

Degree of Positive Tuberculin Reaction in various manifestations of Tuberculosis

Type of lesion	Size of Tuberculin reaction (mm)		
	10-14	15-19	>20
a. SMP 25	3(12)	11(44)	11(44)
b. PPC70	8(15.7)	20(39.21)	23(45.12)
c. TBL 25	5(25.0)	4(20.0)	11(55.0)
P value a X b	<0.01	NS	
b X c	NS	NS	
a x e	NS	NS	

Bold numerals indicate number of cases
 Figures in parentheses indicate percentages.

cases of pulmonary tuberculosis in adults, the T-cell unresponsiveness has been attributed to antigen load. Lenzini (1977) demonstrated that in the intermediate forms of tuberculosis, the LMIT positivity could be induced with a larger dose of antigen in the *in vitro* system.

In the present study, the dose of antigen used was 15 ug/ml standardised in a tuberculin positive child who had not received BCG and had no evidence of active disease radiologically or bacteriologically. It is possible that PPC and TBL groups are some what similar to the

TABLE 4

Tcell percentage and LMIT Index in various tuberculous manifestations

Type of lesion	T-cell %	Total lymphocyte count	Absolute T-lymphocyte count	LMIT Index
a. SMP 25	62.6 ± 7.85	2719.28 ±1053.09	2462.48 ±1796.51	0.65 ±0.13
b. PPC 70	58.93 ± 9.06	4030.07 ±2103.29	2086.54 ±1178.34	0.64 ±0.12
c. TBL 25	48.76 ±10.42	3709.92 ±1838.24	1864.92 ± 912.37	0.70 ±0.08
P value a x b	NS			
b X c	p<0.001	NS	NS	NS
a X c				

Bold numerals indicate number of cases.

intermediate category of immune spectrum of tuberculosis described by Lenzini (1977). These may be requiring a large dose of antigen to have an optimum amount of secretion of specific MIF lymphokine necessary for LMIT positivity. It is possible that in PPC and TBL groups, the lesion gets localised mostly by the formation of non-immune granuloma before the onset of specific immune response. The direct activation of macrophages by bacterial cell wall prevents early dissemination of bacilli (Grange, 1983). This type of macrophage activation and granuloma formation is induced by peptidoglycan layer of the bacterial cell wall and also by its synthetic derivative muramyl dipeptide. This probably is the mechanism by which the local host response is sufficient to fight the infection locally in the lungs. The specific cell mediated immune response induced by thymus dependent lymphocytes activates the macrophages for their antibacterial action through a lymphokine called Macrophage Activating Factor (MAP). It is possible that in PPC and TBL groups this amount of specific immune response by its action through macrophage activation by MAP is sufficient to localize the infection at the local site. The other lymphokine MIF which is necessary for the LMIT positivity is probably secreted in lesser quantities as there may be lesser number of a subset of immune competent T-cells due to a low antigen load in well-localized lesions of PPC and TBL. In SMP, the host defense mechanisms attempt to localise the infection

by preventing the migration of leucocytes by secreting differentially a larger amount of MIF. Probably different stimuli or signals induce secretion of different lymphokines by different subpopulations of T-cells. Gatner et al, (1980) have demonstrated that the amount of Leucocyte Migratory-Inhibitor Factor (LIP) secreted in adults with pulmonary tuberculosis is comparable to the value among non-tuberculosis subjects. It substantiates our hypothesis that there is differential secretion of various lymphokines by different immune-competent T-cells and probably MAP has an upper hand in localising the infection. Hence in addition to the detailed studies of subpopulation of T-cells, assay of different lymphokines *in vitro* is needed to unravel the mechanisms of tuberculoimmunity in children.

Kramer et al (1976) demonstrated in animals that the T-cells from malnourished guinea pigs secrete a larger amount of MIF to low doses of antigen in comparison to the T-cells of well nourished animals. Keeping this in view the nutritional status was also assessed. It is clear from Table 5 that the percentage of children having grade III malnutrition was very low in SMP, PPC and TBL in comparison to miliary and meningeal tuberculosis. This may be responsible for lower response in LMIT positivity in these three clinical situations. In an earlier study by Seth et al, (1982), where CMIR was measured by tuberculin reaction and LMIT after BCG in

TABLE 5

Nutritional status in various Tuberculous manifestations

Type of lesion	Nutritional Status		
	Normal nutrition	Under-nutrition I & II	PEM III & IV
SMP25	12 (48)	12(48.0)	1(4)
PPC70	30(42.9)	36(51.4)	4(5.7)
TBL 25	6(24)	17(68.0)	2(8)
Miliary & TBM 15	2(13.3)	8(53.4)	5(33.3)

Bold numerals indicate number of cases.
 Figures in parentheses indicate percentages.

TABLE 6

Cell mediated immune response after BCG in pre-school children in relation to nutritional status

Nutritional status	Tuberculin		LMIT +ve	
	No	%	No	% -
a. Normal 61	42	(68.8)	34	(55.7)
b. Undernourished 69	43	(62.3)	51	(73.9)
c. Severe P-E.M. 24	9	(37.5)	20	(83.3)
P value a X b	NS		<0.05	
b X c	<0.05		NS	
a X c				

Bold numerals indicate number of cases.
 Figures in parentheses indicate percentages.

relation to the nutritional status, it was demonstrated that LMIT positivity had a direct relationship with the degree of malnutrition. The severe the malnutrition, the higher the positivity of LMIT (Table 6). Khomenko et al (1983) have demonstrated lesser extent of inhibition in LMIT in pulmonary tuberculosis of adults after three months' treatment. In the

present study most of the children' had not received any antitubercular drugs. A few had received chemotherapy upto 2 weeks. It is possible that mere presence of the drug *in vivo* differentially activates the macrophage system much more, both directly and through MAP and there is less secretion of MIF.

In conclusion, the comparison of immune mechanisms in some of the clinically encountered types of tuberculosis in children has raised many queries. There is an urgent need for carrying out prospective studies on immunology of tuberculosis in adults and children as per WHO recommendations to provide some guide-lines regarding management of tuberculosis in children. Research in the areas of (i) determination of phenotype and function of T-cells, (Suppressor or Helper), lymphokine production, assessment of macrophage function, monocyte/macrophage activation for killing of *M. tuberculosis* and chemotaxis would be of immense help in the understanding of a very wide clinical spectrum of tuberculosis in children.

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SERUM C3, ITS IMMUNOELECTROPHORETIC FORMS (p1C AND p1A) AND SERUM HAEMOLYTIC ACTIVITY OF PATIENTS WITH PULMONARY TUBERCULOSIS

A. MAHEN, R.S. KELKAR, K.C. MOHANTY AND S.S. KELKAR

Summary: Fifty cases of pulmonary tuberculosis were classified into unreactive (3 cases), unreactive intermediate (26 cases), reactive intermediate (18 cases) and leactive (3 cases) cases. Sera were studied for haemolytic activity by a microtiter system, C3 by single radial immunodiffusion, and the pi C/ β 1A proportions by two dimensional crossed immunoelectrophoresis (2DCIF.P). The haemolytic activity and the C3 levels were significantly raised in patients. Similarly, the conversion of piC to pi A was significantly greater in patients than in controls. There was no significant intergroup difference in the classes of patients.

Introduction:

The Koch phenomenon, described at the turn of the century in guinea pigs, indicated the importance of immunological mechanisms in the pathogenesis of tuberculosis. Caseous necrosis of secondary tuberculosis represents bystander cell injury—a type IV b hypersensitivity reaction.

Recently Skinness (1981) suggested host immunological responses to be important in determining the pattern of human infections. This classification was like the immunological spectrum accepted for leprosy caused by another mycobacterium. Lenzini et al (1977) elaborated this spectrum and classified cases into UU (unreactive), UI (unreactive intermediate), RI (reactive intermediate) and RR (reactive).

The complement system is an important non-specific mediator of immune responses. In this cascading system C3 plays the pivotal role. Ganguly (1977) studied serum C3 levels in tuberculosis and found them to be normal. The assay was a method of immune adherence. In our laboratory, on the other hand, Yemul et al (1984) found serum C3 levels to be significantly raised in cases of pulmonary tuberculosis. Another study from our laboratory showed that C3 occurred in two electrophoretically distinctive forms. The β 1C form was active and pi A form the inactive variety (Yemul, Jad and Kelkar 1983).

We describe here a study of cases of tuberculosis classified into the immunological spectrum of Lenzini et al (1977). Sera from these patients were assayed in a microtiter system for total haemolytic activity. At the same time they were also assayed for total serum C3 concentrations and in two dimensional crossed immunoelectrophoresis (2 DCIEP) for the β 1C and pi A fractions.

Materials and Methods

Cases: Fifty cases of pulmonary tuberculosis admitted at the Sir J.J. Group of Hospitals, Bombay, were studied. They were classified into Lenzini et al (1977) immunological types, on the basis of the X-ray of the chest, result of tuberculin test and presence and number of acid fast microorganisms in the sputum as follows:

1. Reactive (RR) X-ray picture showing micronodular (lesion less than 3 cm diameter) localised lesion, a tuberculin induration in excess of 25 mm diameter and sputum showing no organisms.

2. Reactive Intermediate (RI): X-ray showing a nodular (lesion greater than 3 cm in diameter) localised or a micronodular localised lesion associated with cavitation; a tuberculin test induration of 18-24 mm diameter and no organisms in the sputum.

3. Unreactive Intermediate (UI): X-ray showing a nodular chronic diffuse lesion 01 micronodular chronic diffuse with cavitation and fibrosis; a tuberculin test induration of 11-17 mm diameter and a few organisms in the sputum.

4. Unreactive (UU): X-ray picture of miliary tuberculosis, a weak tuberculin reaction (6 to 10 mm diameter) and many organisms in the sputum.

In each case a sample of blood was collected and assayed within 2 his for haemolytic activity and pi C/ β 1A. The residual sample was stored at -20°C and studied for total C3.

Controls: Sera from 25 normal healthy individuals were collected and processed like sera from patients.

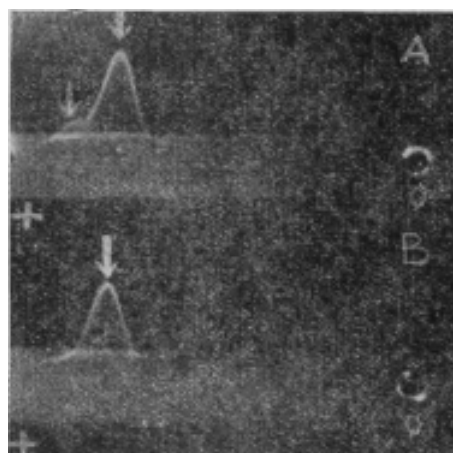
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Serum Haemolytic activity: Sera were diluted in doubling dilutions of 0.025 ml using a microtiter system. To this was added an equal volume of 2 percent sheep red blood cells sensitised with 10 minimum haemolytic doses of amboceptor (Haffkine Biopharmaceuticals, Bombay). After mixing, plates were incubated at 37°C for 30 minutes and kept in a refrigerator overnight. The highest dilution showing complete haemolysis was the end point titer. This was scored in units as follows: neat=1, 1 in 2= 2; 1 in 4=3; 1 in 8=4 and so on (Kotwal and Kelkar, 1974).

Serum C3 estimation: This was done by single radial immunodiffusion (Mancini et al, 1965 and Pole, 1984). Monospecific antiserum was obtained from Immunodiagnostics, New Delhi and a standard from Hyland Laboratories, USA.

(31C, pi A Proportions: This was done by 2DCIEP (Laurell and Lundh, 1967 and Yemul, 1984). The separations were photographed and enlargement tracings made on paper. These were now cut out and weighed to obtain relative proportions.

Results: The 50 cases included 50 males and 10 females. The mean age was 36.5 years (range 10-62 years). The 25 controls included 23 males and 2 females. The average age was 25 years (range 19-49 years).



Figure

Photograph of a two dimensional crossed-immunoelectrophoretic separation of two sera, on one gel plate. 0 = origin. A=serum of a patient with tuberculosis. It shows two peaks. The tall one is (31C (thick arrow) and the low one (31A (thin arrow). B = Serum from a control. Note the single peak of [31C (thick arrow).

Table 1 summarises the results. The standard error (S.E.) of difference between two means was used to determine the statistical significance between controls and all cases together. Haemolytic activity, total serum C3 and inactive

TABLE 1

Summary of results of serum haemolytic activity, total C3 and [31 C (active) and (31A (inactive) fractions of C3

Group	Number	Haemolytic	C3	pi C%	pi A %
	activity (units)		mg./dL		
Control	25 (3—5)	3.8±0.7	129 ±41 (69—248)	89	11
All cases	50 (4—6)	5.0±0.6	166±72 (44—360)	83	17
RR	3 (5)	5.0±0.0	166±125 (44—294)	85	15
RI	18 (4—6)	4.9±0.7	146 ±62 (60—256)	79	21
UI	28 (4—6)	5.0±0.5	179±76 (72—360)	86	14
UU	3 (4—6)	5±1	151 ±35 (111—171)	84	16

Figures in brackets indicate the range.

form (β 1A.) of C3, all showed significantly higher values in the cases together (>2 S.E. of difference between two means). There were no differences when the groups of immunologically classified cases were compared with each other.

Discussion : Normal C3 levels vary widely in different laboratories and in different geographical areas (Fleetwood, Maier, Lewis 1981). Indian reports for C3 in normal individuals show a wide range—Ganguly et al 1977 and 1978 (132 ± 24 , 134 ± 32 mg/dL respectively), Saha et al 1979 (108 ± 25 mg/dL), Jagdeesan and Reddy 1979 (120 ± 16 mg/dL) and Jad et al 1983 (114 ± 42 mg/dL). Yemul et al (1984) obtained a mean of 114 ± 42 mg/dL. These results were obtained in the same laboratory using the same reagents and standards and are quite comparable to the values obtained in the present study.

Determination of haemolytic activity is a broad based assay which is affected by any of the several factors taking part in the complement cascade. The results of the present study show a significant increase in the haemolytic activity of patients with tuberculosis indicating a gearing up of this system to the chronic insult and injury by *Myc. tuberculosis*.

Measurement of C3, which is a pivotal substance in the complement cascade, showed a similar significant increase in serum C3 levels in cases of pulmonary tuberculosis. These results are comparable with a previous study, reported from our laboratory (Yemul, Jad and Kelkar, 1984). The mean serum C3 levels in cases was 162 ± 32 mg/dL. This rise in C3 may be attributed to the chronicity of the disease stimulating this non-specific mediator of the immune response. It may also be on account of a compensation to the decrease in cell mediated immunity or even a compensatory hypersynthesis on account of utilisation of complement in the pathogenesis of the local tubercular lesion. Lastly, it may be a compensation to utilisation of complement by circulating immune complexes.

In a previous study from our laboratory it was shown that inactivation of a sample of serum by heating or other means led to loss of total haemolytic activity without significant changes in the total serum C3 levels (Yemul, Jad and Kelkar, 1983). The same study, however, showed that the electrophoretic forms of C3 changed from the β 1C to β 1A forms. Determination of these active and inactive proportions were also attempted in the present study with rewarding results. Cases showed, in fresh sera,

studied immediately, the presence of more inactivated (β 1A) form of C3 than in controls. This means that the C3 was being utilised and inactivated by the disease process. It indicated utilisation of complement. Presence of excess β 1A C3 may also be attributed indirectly, to the occurrence of circulating immune complexes. Brostoff et al (1981) did find an increase in immune complexes in sera of patients with pulmonary tuberculosis. Interestingly enough they observed an increase in immune complexes as one moved across the immunological spectrum from RR to UU. The present study did not show any such differences. Further Brostoff et al (1981) and Joshnson et al (1981) have suggested that a rising titer of immune complexes might indicate a failure of the host immune responses or even the appearance of bacterial drug resistance.

A word about the immunological classification of tuberculosis might be appropriate. The criteria used in the present study appeared adequate. There was little difficulty in ascribing the cases into the four broad categories on the basis of X-ray findings and acid fast bacilli in sputum. As far as tuberculin response goes, Lenzini et al (1977) have described three main varieties.

1. A typical delayed hypersensitivity reaction, where the induration is present at 24 hours, peaking at 48 hours and persisting upto 72 to % hours.
2. Early reaction: There is a rapid development of induration which persists upto 24 hours and gradually decreases at 48 hours.
3. Biphasic response: Here, there is an early phase of oedema within 3—24 hours which gradually disappears at 48 hours. This is followed by persistent induration and at times necrosis at 72 hours (See Table 2).

In the present study, Lenzini's method was not used. Preference was given to the more precise classification of Rao (1981). The reaction was classified into 4 grades, according to the response to ITU of PPD. The resulting induration was measured in millimeters (See Table 2).

Besides the tuberculin test Lenzini et al (1977) used other criteria to determine the cell mediated immunity in cases of tuberculosis. These included changes in the paracortical and cortical regions of a biopsied lymph node and presence of humoral anti-PPD antibodies. These tests are difficult to perform and may not be so appropriate in our environment.

TABLE 2

Comparison of the methods of studying immune response to tuberculin used by Lenzini et al (1977) and Rao (1981)

	RR	RI	UI	UU
Lenzini et al Typical delayed hypersensitivity	100%	30%	5%	—
Early reaction	—	13%	15%	—
Mixed reaction	—	80%	57%	—
Rao's classification	(+)	(++)	(+++)	++++
	6—1 Omni	11—12mm	21—30mm	30mm
Present study	0—9mm	10—17mm	18—25mm	25mm

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LEUCOCYTE MIGRATION INHIBITION ACTIVITY AND DELAYED TYPE OF HYPERSENSITIVITY IN PULMONARY TUBERCULOSIS

M. VENKATA REDDY* AND T. PRABHU**

Summary: Leucocyte migration inhibition (LMI) activity and delayed type of hypersensitivity (DTK) were studied in active, relapse and treated cases of pulmonary tuberculosis. Active and relapse cases had significant decrease in leucocyte migration inhibition activity, and delayed type of hypersensitivity. A close correlation between leucocyte migration inhibition activity and delayed type of hypersensitivity was observed. Allergic patients had abnormal *in vitro* cellular immune responses.

Introduction

Immunity in tuberculosis is cell mediated and delayed type of hypersensitivity is an essential part of cell mediated immunity (CMI) (Mackness 1968). The relationship between delayed type of hypersensitivity and acquired cellular immunity in tuberculosis has not yet been resolved (Editorial, A.R.R.D., 1975).

Macrophage migration inhibition in the presence of antigen was found to be an *in vitro* correlate of DTH (George and Vaughan 1962), Bloom and Bennett, 1968, Rocklin et al., 1970. Leucocyte migration inhibition test with peripheral blood leucocytes has also been employed as an indicator of cellular immune response and DTH (Bendixen and Soborg, 1969, Rosenberg and David, 1970, Federlin et al., 1971).

In our previous study (Prabhu and Reddy, 1983) reduced levels of circulating T-lymphocyte subpopulations were observed in active and relapse cases of pulmonary tuberculosis. In the present study, leucocyte migration inhibition activity and its relation with the extent of lung abnormalities and DTH were studied in different groups of patients with pulmonary tuberculosis.

Material and Methods

The subjects of the study included patients with bacteriologically confirmed pulmonary tuberculosis, having varying degrees of lung involvement, admitted to S.D.S Sanatorium, Bangalore.

Active cases—patients who were excreting tubercle bacilli, who never had the disease earlier, and were not treated at the time of the study, (b) Treated cases i.e. patients who were bacteriologically positive and became negative after more than 2 months treatment, (c) Relapse

cases i.e. patients who were discharged from the hospital after recovery from the disease (showing bacteriological negativity and radiological improvement) and were readmitted due to relapse (became bacteriologically positive irrespective of radiological findings) after varying periods of quiescence.

Normal healthy persons served as controls.

All the patients and controls were given 5 TU of PPD intradermally to study their tuberculin hypersensitivity reactions, immediately after the collection of blood samples and the readings were taken after 48 and 72 hours.

Leucocyte migration inhibition test was done according to Federlin et al's (1970) method with slight modifications. The migration chambers used were locally made Mackness type of chambers. Heat killed H37Rv strain grown in Dubos liquid medium, suspended in phosphate buffered saline was used as the antigen in the test, at the final concentration of 200 μ g per ml in tissue culture medium 199.

After overnight incubation of the chambers at 37°C, the area of migration was measured with the help of a projection microscope, and percent migration inhibition was calculated as follows:

Percent migration inhibition =

$$\frac{100 - \text{area of migration with antigen}}{\text{area of migration without antigen}}$$

Results

The mean percent leucocyte migration in

1. Active cases showed significantly low migration cases and there was a gradual

TABLE 1

Leucocyte migration inhibition test in patients and controls

Clinical types	No. of subjects	Mean percent migration inhibition \pm SD
Active cases	48	24.2 \pm 10.68*
Relapse cases	26	25.7 \pm 12.73
Treated cases	32	30.8 \pm 14.12**
Controls	19	18.1 \pm 13.64

*P<0.05 when compared with treated cases

**P<0.002 when compared with controls

increase in migration inhibition activity from active cases to relapse and treated cases.

Distribution of migration inhibition in patients (fig. 1) shows that larger proportion of active cases and relapse cases (33.3 percent and 34.6 percent respectively) had negative LMI test compared to treated cases (18.7 percent).

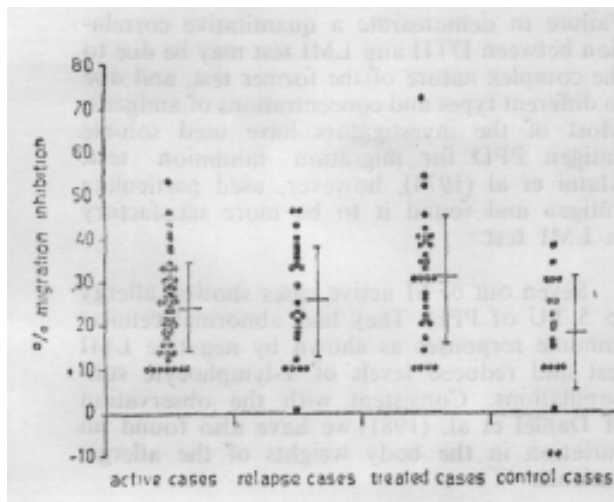


Fig. 1

Distribution of Leucocyte migration test results in patients and controls

Delayed hypersensitivity reaction to 5 TU of PPD in patients and controls is shown in table 2. Active cases had significantly low levels of delayed hypersensitivity reaction when compared with treated cases. However, not much variation in tuberculin reaction in different age groups of patients was observed.

TABLE : 2

Tuberculin hyper sensitivity in patients and controls

Clinical type	No. of subjects	Mean diameter of induration \pm S D	Range
Active cases	61	16.7 \pm 7.2*	0—27
Relapse cases	32	18.8 \pm 4.49	8—25
Treated cases	34	19.5 \pm 4.54	10—26
Controls	27	9.4 \pm 6.75	0—24

*P<0.05 when compared with treated cases

Distribution of the diameter of induration in patients and controls shows (fig 2) that 7

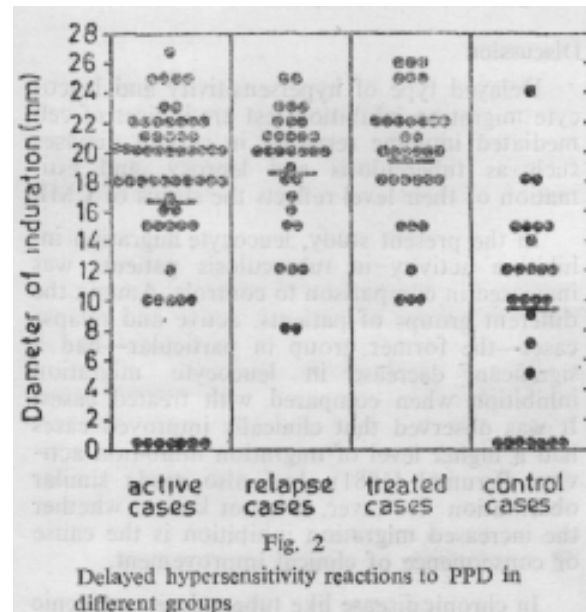
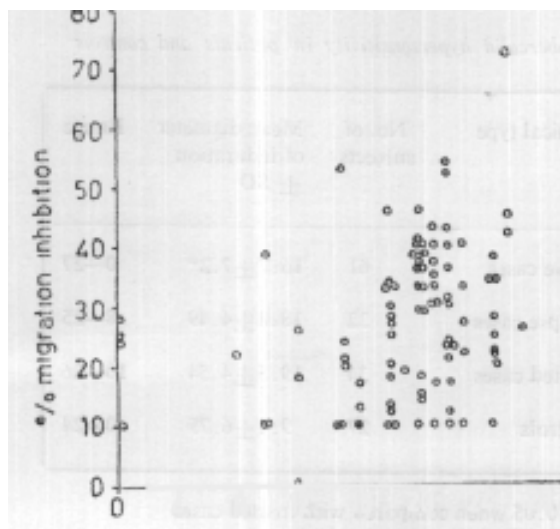


Fig. 2

Delayed hypersensitivity reactions to PPD in different groups

cases with active tuberculosis were allergic to 5 TU of PPD and 7 control subjects failed to react to the same concentration of PPD. All the allergic patients had abnormal cell mediated immune response as reflected by negative LMI and reduced levels of T-lymphocyte subpopulations.

There was a significant correlation between the extent of leucocyte migration inhibition and intensity of delayed hypersensitivity reaction in patients with tuberculosis, with a correlation coefficient of $r=0.346$ and $P<0.01$ (fig. 3).



Diameter of induration (mm)

Fig. 3

Relationship between PPD reactions and percent migration inhibition in patients

Discussion

Delayed type of hypersensitivity and leucocyte migration inhibition test are indices of cell mediated immune response in certain diseases such as tuberculosis and leprosy, and estimation of their level reflects the status of CMI.

In the present study, leucocyte migration inhibition activity in tuberculosis patients was increased in comparison to controls. Among the different groups of patients, active and relapse cases—the former group in particular—had a significant decrease in leucocyte migration inhibition when compared with treated cases. It was observed that clinically improved cases had a higher level of migration inhibition activity. Perumal (1981) had also made similar observation. However, it is not known whether the increased migration inhibition is the cause or consequence of clinical improvement.

In chronic disease like tuberculosis, antigenic load influences the cellular immune response. With the reduction of bacterial load following treatment, immune response dominates. Trnka and Skvor (1979) have observed a relation between leucocyte migration inhibition and sputum conversion. Circumstances in which CMI fails to increase even after the elimination of bacilli from sputum may probably result in relapse. The patients with relapse had decreased LMI activity and reduced levels of T-lymphocytes

An increase in leucocyte migration inhibition activity was observed with the increase in the extent of radiological lung abnormalities'

Rieger et al. (1979) also found a similar association

In our previous study (Prabhu and Reddy 1983) we had found that the treated cases had significantly higher levels of "active" and "total" E-rosette forming subpopulations of T-lymphocytes when compared with active cases and relapse cases. It was also observed that patients with far-advanced lung changes had decreased proportion of T-lymphocytes. We support the opinion of Skvor and Trnka (1979) that the far advanced cases, despite reduced T-lymphocyte levels, had increased migration inhibition activity, which is probably due to the production of separate clones of T-lymphocytes with enhanced ability to produce migration inhibition.

Delayed hypersensitivity to 5 TU of PPD is decreased in active and relapse cases when compared with treated cases. We could show a correlation between the extent of leucocyte migration inhibition and the intensity of delayed type of hypersensitivity reaction. Though with the use of particulate antigen leucocyte migration inhibition test correlates well with skin test, a number of investigators have failed to show correlation (Reiger et al. 1979, Lockshin 1969, Szabo et al. 1976). However, Federlin et al. (1971) Rosenberg and David (1970) have shown correlation between the two tests. Failure to demonstrate a quantitative correlation between DTH and LMI test may be due to the complex nature of the former test, and due to different types and concentrations of antigens. Most of the investigators have used soluble antigen PPD for migration inhibition test. Maini et al (1974), however, used particulate antigen and found it to be more satisfactory in LMI test.

Seven out of 61 active cases showed allergy to 5 TU of PPD. They had abnormal cellular immune responses as shown by negative LMI test and reduced levels of T-lymphocyte subpopulations. Consistent with the observation of Daniel et al. (1981) we have also found no variation in the body weights of the allergic patients.

Allergy has been attributed to malnutrition (Stead 1972) and severity of the disease (Rooney et al. 1976), presence of suppressor monocytes (Elmer 1979), and sequestration of lymphocytes in tuberculous lesions (Rook et al. 1976). Bhatnagar et al. (1977) and Daniel et al. (1981) have reported that the patients with allergy to tuberculin have had high levels of circulating antibodies to mycobacterial antigens, and had an immune spectrum resembling that of leprosy. They have found an inverse relation between cellular and humoral responses.

Acknowledgement

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Dr. H. B. DINGLEY

Dr. H. B. Dingley—the popular veteran died suddenly on 22-11-1984.

Born on 17th September, 1917, Dr. Dingley graduated from K.E.M. Medical College, Lahore in 1941 and obtained post-graduation in Tuberculosis and Chest Diseases from U.M.T. Sanatorium, Madanapalle and Medical College, Madras in 1944. The same year he joined the Tuberculosis Association of India as a Medical Officer in the New Delhi TB Centre. After a short stint there, he shifted to the Lady Linlithgow Sanatorium, Kasauli where he worked till 1953 when the Tuberculosis Association of India started the Lala Ram Sarup Tuberculosis Hospital, Delhi, of which institution he was appointed as the first Medical Superintendent. He retired after a meritorious service of 38 years in September 1982 and since then he had been working as a senior consultant in ESI Hospital, Delhi and practising privately.

Dr. Dingley had a very distinguished career. He was the recipient of the fellowships of the Rockefeller Foundation of USA in 1950 and the Chest and Heart Association, U.K. in 1964. He was a Fellow of the National College of Chest Physicians. In recognition of his outstanding services in the field of tuberculosis, in general, and the Tuberculosis Association of India, in particular, he was awarded the Association's Gold Medal in 1982.

Starting as a physician he trained himself in major thoracic surgery and was, thus, not only a highly competent physician but an equally competent thoracic surgeon and a paediatrician. During his stewardship of the L.R.S. TB Hospital, it grew from a modest hospital of about 100 beds to 400 beds with a full-fledged thoracic surgery unit and a ward for treatment of tuberculosis children at the time of his retirement. By virtue of sheer hard work, dedication and efficiency, he made this institution one of the best in the whole country.

He was a teacher in tuberculosis for the undergraduates of All India Institute of Medical Sciences and the University Medical College, Delhi and post-graduate students of AIIMS and Delhi University and also examiner for post-graduate qualifications of several universities in the country. He conducted several research studies and contributed chapters on Tuberculosis in various textbooks meant for undergraduates and post-graduates. He was the author of a number of original scientific papers published in national and international journals. He attended several international conferences in tuberculosis and chest diseases where his contributions were highly valued.

He had a long and close association with the Tuberculosis Association of India. He had been a member of almost all important committees of the Association, viz. Standing Technical Committee, Local Advisory Committee and the Research Committee and the Editorial Board of the Indian Journal of Tuberculosis. He was the Chairman of the Standing Technical Committee for 1975-76 and presided over the 30th National Conference in Hyderabad in 1975. There was hardly a Tuberculosis and Chest Diseases Conference in India which he missed. In fact, his very amiable and pleasant personality will be missed in future conferences for a long time.

In Dr. Dingley, the country, the medical profession, the Tuberculosis Association of India and the discipline of tuberculosis and chest diseases have lost a stalwart. He will be remembered long by his innumerable friends, colleagues, students and above all his patients. May God grant eternal peace to the departed soul

BOOK REVIEWS

Modern Drug Treatment in Tuberculosis by J.D. Ross and N.W. Home; published by Chest, Heart and Stroke Association, London; 6th Edition, 1983, PP. 123, £3.00.

That this small book has gone into its 6th edition within the course of a few years is a proof of its popularity and usefulness. This new edition updates the knowledge about chemotherapy, particularly, short-course chemotherapy and the various drug regimens in use.

A busy practitioner wants a ready answer to which regimen to prescribe for an uncomplicated case and how to modify a regimen in special situations such as co-existing diabetes or deranged function of liver, kidney, etc. This information is provided in the book in very clear and concise terms, in addition to the adverse reactions of various drugs and their management. All unnecessary details have been excluded and this is a very welcome feature of the book. The short chapter of steroids will also be very useful and helpful.

According to its title, the book concerns chemotherapy only, but brief chapters on tuberculin test and BCG and control of tuberculosis are also included even though they are not strictly relevant to the subject. This is, ostensibly, to increase the utility of the book for students. However, since tuberculin test and BCG are fairly controversial subjects, oversimplification for the sake of brevity of presentation is likely to cause confusion. Some statements have been made which are not in accordance with policy and practice in some countries where the book will be read. It would have been better if these chapters had not been included in the book.

All in all, the book, like its previous editions, is well-written and well-produced. It is indispensable for students and busy general practitioners.

—S.P.P.

Advances in Tuberculosis Research; Volume 21; Serology of Tuberculosis and BCG Vaccination; S. Karger, Basel; 1984, PP. 252. SFr. 165/-or U.S.I 99.00.

The volume consists of three sections; the first dealing with the immune response in Tuberculosis, its nature, biological role and diagnostic usefulness; the second with the current view on the protective value of BCG

vaccination, particularly the recent South Indian Trial, and the third with complications of BCG.

In the first section, the author Prof. J.M. Grange of London after a brief account of the antigenic structure of the mycobacterium discusses extensively the various diagnostic tests based on serology. It is very well brought out that although the results of various tests such as agglutination, Elisa, Safa, Radio-immunoassay, etc. are promising when carried out on pre-selected sputum positive patients, their specificity and sensitivity gets considerably reduced when patients with other chest diseases which mimic Tuberculosis are included in the test. Furthermore, an acceptable test must not only be capable of discriminating between infection, arrested and active tuberculous disease but should also be simple in technique, should not require highly sophisticated equipment and must be capable of being carried out even at the periphery where largest number of cases are found at least in developing countries. All available tests fall short of these requirements.

H.G. Ten Dam analyses the results of the recent South Indian trial on the protective value of BCG in the second section of the volume. Factors likely to be responsible for the disparity in the results obtained from various studies carried out heretofore are dealt with. The various hypotheses to explain the results of the South Indian Trial, viz. Virulence of the South Indian variant of *M. tuberculosis*, possibility of exogenous re-infection, high level of infection with environmental Mycobacteria, etc. have been critically examined. The author reiterates the WHO recommendation that BCG vaccination should continue as at present.

The third section on BCG complications, written by Dr. A. Lotte and associates, is based on a retrospective study of the frequency of complications of BCG vaccination, from trivial like excessive local reaction to serious ones like death, carried out recently under the auspices of the International Union Against Tuberculosis. A questionnaire issued to 187 countries where BCG vaccination was being given was responded to by only 79 countries, mostly European. All complications of BCG numbering nearly 10,000, amongst 1500 million vaccinations during 1949-1974, have been analysed. The complications have been dealt with very exhaustively and help to put this important aspect of Tuberculosis Control in its proper perspective.

The volume, like its predecessors, is very well compiled and produced. It includes lot of useful information. Bibliography is so comprehensive that it is unlikely that any significant and relevant study in any part of the world on

these subjects is not mentioned in this volume. No medical library, particularly those of Tuberculosis and Chest Institutions, should be without this book.

—S.P.P.

REGIONAL CONFERENCE OF THE IUAT

The XIV Eastern Region Conference of the International Union Against Tuberculosis will be held in Kathmandu (Nepal) from 24th to 29th November, 1985. The subjects selected for discussion at the Conference are : Epidemiology and Evaluation, TB Control (Pulmonary and extra-pulmonary), Bacteriology and Laboratory, Chemotherapy in TB Control Programme, Non-TB Respiratory Diseases, B.C.G., Health & Smoking and Health Education and Community participation. Those who intend to present papers at this Conference may send abstracts, in triplicate, to the Secretary-General, Tuberculosis Association of India, 3. Red Cross Road, New Delhi-1 before 28.2.1985. For details regarding the Conference, Secretary, XIVth Eastern Region TB Conference, Nepal Anti-TB Association, P.B. No. 1494, Kalimati, Kathmandu (Nepal), should be contacted.

NEWS & NOTES

35TH TB SEAL CAMPAIGN

As already reported in the previous issue of this Journal, the 35th TB Seal Campaign was inaugurated on 2nd October, 1984—Gandhi Jayanti Day—by Giani Zail Singh, President of India and Patron, Tuberculosis Association of India, at a special function held at Rashtrapati Bhawan, New Delhi. The Campaign was inaugurated in the States by high dignitaries such as Governors, Ministers and other distinguished personalities. The details are as under:

In Andhra Pradesh, the Campaign was inaugurated by the Chief Justice of Andhra Pradesh High Court, Shri P. Chenna Keshava Reddy, and the function was presided over by Sri Narendra Luther, IAS, Principal Secretary to Government, Medical & Health Department and Vice-Chairman of the Association. In Assam, the Campaign was inaugurated by organising a public meeting at the L.G.B. Chest Hospital Campus, Gauhati, Dr. J.N. Bhuyan, Honorary Secretary of the Association, gave a talk on All India Radio on the occasion. In Bengal, the Campaign was inaugurated by Shri Jibendra Nath Biswas, Chairman and Chief Executive Officer of the United Industrial Bank Limited and the function was presided over by Dr. A. L. Goswami, Director-General, Railway Health Services, Indian Railways. In Goa, Daman & Diu, the Campaign was inaugurated by Shri Pratap Singh Rane, the State Chief Minister and the function was presided over by Shri Shaikh Hassan Haroon, Minister for Law. In Gujarat, Shri B. K. Nehru, Governor of Gujarat inaugurated the Campaign at a special function held at the Association's premises. In Jammu & Kashmir, the Campaign was inaugurated by Dr. Ali Mohd. Jan, Chairman, State Tuberculosis Association, who also made a token purchase of 400 TB Seals. In Karnataka, the Campaign was inaugurated by Shri A.N. Banerjee, the State Governor at a special function held at the Raj Bhawan with Dr. H.L. Thimmegowda, the State Minister for Health in the Chair. In Kerala, the Campaign was inaugurated by the Ex-Chief Minister of Kerala, Shri C. Achutha Menon, at the Dist. Hospital Hall at Trichur on 29th October 1984. Catholic Bishop Dr. Poulouse Mar Poulouse presided over the function. In Maharashtra, the Campaign was inaugurated by Shri Pran Lai Bhogi Lai, President, Indian Merchants Chamber. Shri V.Y. Kotwal, Suburban Railway Manager and Dr. R.C. Chandrikapure, Dean, Grant Medical College & Sir JJ. Group of Hospitals were the Guests of

Honour on the occasion. In Meghalaya, the Campaign was inaugurated by Shri Bhishma Narayan Singh, Governor of Assam and Meghalaya and the function was presided over by Shri D.D. Lapang, the State Minister of Finance, Home and Planning. In Orissa, Shri B.N. Pandey, the State Governor, inaugurated the Campaign and Shri J.K. Patnaik, the State Minister for Health & Family Welfare, presided over the function. In Pondicherry, the Campaign was inaugurated by Thiru T.P. Tewary, Lt.-Governor of Pondicherry. In Tamil Nadu, the Campaign was inaugurated by the Collector of Madras at a special function presided over by the Director of Medical Services and Family Welfare. In Uttar Pradesh, the Campaign was inaugurated by Dr. S.S. Misra, the Director of Medical Education & Training, U.P. at a special function held at the Vigyan Bhawan of Balrampur Hospital, Lucknow. The function was presided over by Shri Rani Lila Ram Kumar Bhargava.

REFRESHER COURSES

Andhra Pradesh: The Krishna District Tuberculosis Association, Machilipatnam, in collaboration with the Indian Medical Association, Vijayawada Branch and Bezwada Medical Association, Vijayawada, organised a refresher course for general practitioners in Vijayawada on 18th November, 1984. It was sponsored by M/s. Alembic Chemical Works Company Limited.

Kerala: Three refresher courses were held on 4th and 26th August and 30th September, 1984 at Ernakulam, Manjeri and Trichur respectively. The courses were organised by the respective District Associations in collaboration with the local branches of the Indian Medical Association and these were sponsored by M/s. Alembic Chemical Works Company Ltd.

Madhya Pradesh: Two refresher courses were held on 2nd and 23rd September, 1984 in Betul and Burhanpur respectively. The courses were organised by the respective District TB Associations and these were sponsored by M/s Alembic Chemicals.

Maharashtra: The Maharashtra State Anti-TB Association in collaboration with the respective IMA branches organised three courses on 23rd and 30th September and 28th October, 1984 in Kopargapn, Thane and Khed (Dist. Ratnagiri) respectively. The Kopargoan course

was sponsored by M/s. Pharmaceutical Company of India.

Rajasthan: The Departments of TB & Chest Diseases of the Ajmer, Bikaner and Udaipur Medical Colleges in collaboration with their respective IMA branches organised courses on 30th September, 9th December and 2nd December 1984 respectively. The Ajmer and Bikaner courses were jointly sponsored by M/s Pharmaceutical Company of India and the Ajmer branch of the IMA and the Udaipur Course was sponsored by the National Academy of Medical Sciences under their Continuing Medical Education Programme.

Tamil Nadu: Three courses were held on the 15th and 30th September and 14th October, 1984 at Tiruppur and Tiruchirapally and Nagercoil respectively. The Trichy course was organised by the District TB Association and it was sponsored by M/s Star Laboratories, Bombay. Nagercoil course was sponsored by M/s Pharmaceutical Company of India.

Uttar Pradesh: The Department of Tuberculosis and Chest Diseases, Moti Lai Nehru Medical College, Allahabad, in collaboration with the Allahabad Medical Association, organised a course on 6th September, 1984.

The total number of courses held in various parts of the country under the intensified programme of the Tuberculosis Association of India and its State affiliates during the year has reached 57.

39TH NATIONAL CONFERENCE

The 39th National Conference on Tuberculosis and Chest Diseases scheduled to be held in Cuttack from 9th to 12th November, 1984, had to be postponed due to the dastardly assassination of our late Prime Minister—Smt. Indira Gandhi. The Conference will now be held in the S.C.B. Medical College Auditorium, Cuttack (Orissa) from the 28th to 31st January, 1985.

STATE CONFERENCES

The VIIIth Bengal State TB and Chest Diseases Worker's Conference was held in Calcutta on the 16th December, 1984 in the auditorium of the Association. The Conference was inaugurated by Swami Lokeshwaranandaji Maharaj and it was presided over by Dr. N.M. Sinha. The Programme included four Orations in memory of Drs. B.C. Roy, P.K. Ghosh, K.N. De and Anandilall Podder. The Dr. A.C. Ukil Memorial Gold Medal was presented by Swami Lokeshwaranandaji Maharaj

to the candidate who stood first in the TDD Examination held in 1983.

MULTI-CHECK UP CAMP

A multi-check-up Camp jointly organised by the Maharashtra State Anti-TB Association, the Indian Medical Association and Lions Club was held at Khed, Dist. Ratnagiri (Maharashtra) on 28th October, 1984. 220 persons were screened, 160 children given BCG, 800 persons examined for eye problems and free spectacles were given to 463 persons, 50 persons examined for skin trouble, 15 E.C.G. taken of which 13 were abnormal.

CHANCHAL SINGH MEMORIAL AWARD, 1985

The Tuberculosis Association of India will award a cash prize of Rs. 1,000/- to a medical graduate below 45 years of age and working in tuberculosis, for an original article not exceeding 30 double-spaced foolscap typed pages (approximately 6000 words) excluding charts and diagrams on a subject relating to TB. Article or papers already published or based on work of more than one author will not be considered for this award. Papers may be sent in quadruplicate, to reach the Secretary-General, Tuberculosis Association of India, 3, Red Cross Road, New Delhi-1, before the 31st July, 1985.

ESSAY COMPETITION, 1985

The Tuberculosis Association of India awards every year a cash prize of Rs. 500/- to a final year medical student in India for an original essay on Tuberculosis, adjudged best by a Special Committee of the Association. The subject selected for the 1985 competition is "Management of Tuberculous Meningitis". The essay should be written in English typed in foolscap size, double-spaced and should not exceed 15 pages (approximately 3000 words, excluding tables, diagrams, etc.) Four copies of the manuscript should be forwarded through the Dean or Principal of College/University to reach the Secretary-General, TB Association of India, 3, Red Cross Road, New Delhi-110 001, before 31st July 1985.

HEALTH VISITORS' COURSE

The 1985-86 TB Health Visitors' Course will commence in July 1985. The course will be of nine months' duration and will be held at the New Delhi TB Centre. The minimum qualification for admission to this course is Higher Secondary/Pre-University with Science or Hygiene and Physiology in Matriculation. Application forms for admission to this course

NEWS & NOTES

Can be had from the Secretary-General, Tuberculosis Association of India, 3, Red Cross Road, New Delhi-110 001. The last date for receipt of applications is 30th April, 1985.

XIV EASTERN REGION CONFERENCE

The XIV Eastern Regional Conference of the IUAT will be held in Kathmandu (Nepal) from 24th to 29th November, 1985. The subjects selected for discussions at the Conference are: Epidemiology and Evaluation, TB Control (Pulmonary & Extra Pulmonary), Bacteriology and Laboratory, Chemotherapy in TB Control Programme, Non-TB respiratory diseases, B.C.G., Health and Smoking and Health Education and Community participation. Those desirous of presenting papers at this Conference should send abstracts of such papers, in triplicate, to the Tuberculosis Association of India before 31.3.1985. For further details about the Conference, please contact the Secretary-General, Nepal TB Association, P.B. No. 1494,, Kalimati, Kathmandu (Nepal).

IUAT WORLD CONFERENCE

The XXVIth World Conference on Tuberculosis and Respiratory Diseases will be held in Singapore under the joint auspices of the I.U.A.T. and Singapore Anti-TB Association from the 2nd to 7th November, 1986.

Dr. N.C. Sen-Gupta, Director, Singapore Anti-TB Association and President of the I.U.A.T. will preside over the Conference. This is only the third time that this prestigious Conference will be held in Asia. The programme Committee, under the direction of the IUAT, is making every effort to ensure that current scientific development and recent advances in tuberculosis and respiratory diseases feature prominently in the programme. Further details about the conference can be had from the Secretary-General, Tuberculosis Association of India, 3, Red Cross Road, New Delhi-110 001.

MEMBERSHIP OF THE IUAT

The Tuberculosis Association of India is enrolling individual members on the International Union Against Tuberculosis, Paris, for the year 1985. The member will receive from the Union, free of cost, copies of its quarterly Bulletin, Newsletter, WHO publications dealing with tuberculosis and respiratory diseases, etc. The annual subscription is FF 400 ptr member (equivalent to about Indian Rs. 535.00). Those who wish to enroll themselves as members of the Union for 1985, may kindly remit to the Secretary-General, Tuberculosis Association of India, 3, Red Cross Road, New Delhi-1, the annual subscription of Rs. 535.00 to enable him to recommend their names to the IUAT for its membership.

IUAT WORLD CONFERENCE

The XXVIth World Conference on Tuberculosis and Respiratory Diseases of the International Union Against Tuberculosis will be held in Singapore from the 2nd to 7th November, 1986, Those who wishes to attend the Conference and/or present papers may contact the Secretariat, XXVIth IUAT World Conference, on TB & Respiratory Diseases, C/o S.A.T.A., 267, Cantonment Road, Singapore 0208.

The Indian Journal of Tuberculosis

ABSTRACTS

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Immunological Research in Tuberculosis: WHO Memorandum

Bulletin of the World Health Organization; 1982, 60: 723-727.

This Memorandum discusses the application of modern immunological techniques to various problem areas in tuberculosis. The recent isolation of highly purified mycobacterial antigens will have important application in providing specific skin-test reagents for diagnosis, classification and epidemiological investigations, and agents for use as immunogens and adjuvants. The development of monoclonal antibodies obtained by immunization of susceptible animals with partially purified antigens is considered a most promising approach to the identification and isolation of antigens.

In vitro studies of the mechanisms of immunity and the effects of immunization in tuberculosis are needed. Several methods have been proposed recently which require further validation, e.g. through correlation of *in vivo* resistance with results *in vitro*, and comparison of different antigens in the *in vitro* tests.

The specific antigens as well as the *in vitro* tests of tuberculosis immunity would be readily applicable in clinical investigations of immunological parameters and the effects of immunization. New serological tests using purified antigens would also be of value in this regard. *In vitro* tests for cell-mediated immunity could be used to study the effect of various BCG vaccines in different populations, in order to investigate the role of genetic and environmental factors in determining the response to immunization. Specific antigens and serological tests should prove useful in the diagnosis of different forms of extrapulmonary disease, especially in children. A test that could distinguish between infection with *Mycobacterium tuberculosis* and sensitivity induced by BCG immunization or environmental mycobacteria would be very useful both in diagnosis and in epidemiological studies. Investigations are needed on the mechanisms of endogenous reactivation of

disease and the breakdown of apparently quiescent disease, in order to try to identify high-risk groups.

Immunological Studies with Sulpholipids of Mycobacteria

O.K. Khuller, et al, Tubercle, 1982, 107-111.

Antibodies to sulpholipids of mycobacteria were produced in rabbits when injected as sulpholipid-MBSA complexes and were detected by kaolin agglutination and double diffusion techniques. Sulpholipid antibodies did not cross react with any other lipids of mycobacteria except cord-factor. The antigenicity of sulpholipids appears to be due to—D-trehalose and sulphate groups. Guinea pigs immunized with sulpholipid-MBSA complexer showed partial protection against tuberculous infection with *M. tuberculosis* H₃₇ Rv, as revealed by mortality rate and score of lesions.

Use of Phospholipid Antigen in the Serodiagnosis of Pulmonary Nocardiosis.

S.C. Sehgal, et al, IRCS Medical Sciences: 1979; 7; 20.

A close similarity exists between the phospholipids of *Nocardia* and mycobacteria and nocardial phospholipids have been found to cross react with those of mycobacteria. This cross reaction between the antigens of these two organisms limits the use of serological techniques in the serodiagnosis of nocardiosis. However, in cases where the tuberculous infections are ruled out after proper investigations or in the geographical areas where the incidence of tuberculosis is negligible, the use of phospholipid antigen will serve a useful purpose in the serodiagnosis of nocardiosis. In addition, it has been reported that antibodies to phospholipids are present in the active stages of tuberculosis indicating thereby the severity of the disease. Hence, it is assumed that the presence of antibodies to nocardial phospholipids will also reflect the nature of the disease.