Cooperative Studies on the Immunology of Tuberculosis at Airlangga University, Surabaya, Indonesia

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Tuberculosis is a major health problem in many parts of the world. The exact incidence and prevalence of the disease is unknown but, according to estimations by the World Health Organization, about 100 million individuals are infected by the tubercle bacillus annually. From the pool of infected people, between 10 and 20 million individuals become ill each year: about 5 million of these become infectious and about 3 million die.

Despite decades of research, the immune mechanisms in tuberculosis are poorly understood and, as a consequence, few practical benefits have emerged from immunological studies. Thus at the present time there are no useful immunodiagnostic tests for active tuberculosis; the only available vaccine, Bacille Calmette-Guerin (BCG), is far from satisfactory and there are no immunotherapeutic measures that enhance the individual's ability to overcome the infection. Thus the long-term aims of immunological studies of tuberculosis are the development of a simple and reliable test that will replace or supplement microscopy and cultivation of the tubercle bacillus, an effective vaccine that will prevent the emergence of the open or infectious forms of post-primary, disease and immunotherapy that will permit the lengthy and expensive courses of chemotherapy to be significantly shortened.

During the last decade, the Faculty of Medicine at Airlangga University, with the associated Dr. Soetomo Hospital, at Surabaya, Indonesia, has been the centre of a number of studies to elucidate the nature and mechanisms of the immune responses in human tuberculosis in the hope of achieving the goals outlined above. The results of these studies are reviewed in this paper.

Immunodiagnosis

Many attempts have been made to develop serological tests for tuberculosis but, without exception, all have proven to be either too insensitive or too non-specific for clinical use. The problem of sensitivity was overcome by the introduction of enzyme-linked immunosorbent assay (ELISA) which is as sensitive as radioimmunoassay 1 but the problem of specificity has remained. This problem was approached in two ways: first, by studying the humoral immune response in the different classes and subclasses of immunoglobulin and, secondly, by quantitating antibody responses to species-specific epitopes or antigenic determinants rather than preparations of whole tubercle bacilli.

Accordingly, ELISA was used to assay antibodies to ultrasonicates of Mycobacterium tuberculosis in the IgG, IgM and IgA classes. 2 The greatest discrimination between tuberculosis patients and healthy control subjects was found in the IgG class, followed by the IgA class, but levels of specific IgM antibody were similar in the two groups. Although assay of class specific antibody gave better discrimination than assays of total specific antibody, the overlap between patients and controls was still too great to be of any clinical value. It was shown that previous BCG vaccination did not affect the specific antibody levels in any class but that some healthy, tuberculin positive individuals had elevated...

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specific antibody in the IgG class, but not in the other classes, when compared with tuberculin negative healthy subjects. It is, of course, possible that some of the apparently healthy tuberculin positive individuals had undetected foci of active tuberculosis and that others were reacting successfully to a recent infection by virulent tubercle bacilli.

When the necessary monoclonal antibodies became available, antibody response to ultrasonicated *M. tuberculosis* antigen was analyzed in terms of the four subclasses of IgG. This assay also improved the discrimination but was not completely definitive.

*Mycobacterium tuberculosis* contains some antigens unique to that species but also some that are shared with other slowly growing mycobacteria, some that are common to all mycobacteria and some that are even more widely distributed amongst bacteria more generally. An alternative approach therefore, was to look at the antibody response restricted to species-specific antigens. This was done in two ways. First, a differential ELISA was developed in which the levels of antibodies to *M. tuberculosis* and to another slowly growing species, *M. kansasii*, were assayed in parallel. The difference in the antibody response to the two species gave an indirect measure of the specific humoral response to *M. tuberculosis* but, in practice, this test was no more useful than the assay of antibody to *M. tuberculosis* alone. Secondly, antibody responses to specific antigenic determinants or epitopes were analyzed by using a set of monoclonal antibodies to *M. tuberculosis* in a solid phase antibody competition test (SACT). In this test, dilutions of the test serum are added to unfractionated antigen attached to a solid phase, usually a plastic tube. After incubation, a radio- or enzyme-labelled murine monoclonal antibody to one of the epitopes of *M. tuberculosis* is added. This labelled monoclonal antibody will bind to the corresponding epitope only if it is not already bound to the corresponding antibody in the human serum. Thus, the amount of monoclonal antibody binding to the solid phase is inversely proportional to the amount of the corresponding antibody in the patient's serum. This procedure increased the specificity of the test greatly, especially when the results with six different monoclonal antibodies were considered together, but the test still failed to discriminate between all cases of smear positive tuberculosis and healthy subjects.

The failure of serological studies to yield a satisfactory test for the diagnosis of tuberculosis is due to four main factors. First, the antibody response in tuberculosis is not a very strong one relative to the background level of antibody to all species of mycobacteria. Secondly, the comparative ELISA showed that almost all the antibody response was directed towards those antigens common to all mycobacteria. Thirdly, the use of SACT and immunoelectrophoresis revealed that the range of epitopes eliciting antibody responses varied considerably from one individual to another. The reason for this is unknown: one possibility is that it is the result of genetic polymorphism affecting the presentation of antigens to lymphocytes. Thus, a single, purified, species-specific antigen would only be expected to give a positive diagnosis in a proportion of patients with tuberculosis. Fourthly, there is an as yet unexplained occurrence of elevated levels of antibody to species-specific antigens of one mycobacterial species in patients with disease due to another species. Thus, use of SACT revealed that many patients with leprosy had elevated antibody levels to specific epitopes of *M. tuberculosis*, despite having no clinical or radiological evidence of tuberculosis.

### Tuberculin reactivity

The positive tuberculin test is regarded as the classical example of Type IV, cell-mediated or delayed hypersensitivity (DHS) reaction. Its relationship to protective cell mediated immunity (CMI) has been the subject of considerable debate and great confusion for decades. Some workers have claimed that DHS and CMI reactions are essentially the same while others claim that they are distinct and unrelated phenomena.

It is now evident that a positive tuberculin test is due to a number of different immune reactions. Thus there are qualitative differences in the appearance of the reaction at 48 hours and in the way that the reactions evolve over this time. For example it was demonstrated that some, but not all, tuberculin positive individuals have a distinct erythematous reaction visible 6 to 8 hours after testing and that this reaction is seen much more frequently in patients with active tuberculosis than in healthy tuberculin positive individuals. This phenomenon was subsequently demonstrated in an English hospital, with the additional observation that nurses caring for patients with tuberculosis often had very extensive early erythematous reactions. This new finding was confirmed in a study of the staff of Dr. Soetomo Hospital, Surabaya. Almost all of these individuals had very extensive early reactions, significantly more extensive than the patients with tuberculosis, while workers in a nearby factory had little or no early reactivity, despite the fact that most were tuberculin positive at 48 hours. Skin testing with a range of mycobacterial sonicate antigens showed that the erythematous reaction was elicited selectively by antigens specific to *M. tuberculosis*: the molecular nature of these antigens is unknown and they are not necessarily the same as those eliciting the 48 hour indurating reaction.
As this early reaction is common in healthy hospital staff who are regularly exposed to patients with open tuberculosis, it may be of relevance to protective immunity. The nature of the reaction is unknown. The timing suggests an Arthus reaction, due to antigen-antibody complexes, but the size of the reaction does not correlate with levels of antimycobacterial antibody in the IgG, IgA and IgM classes. Another possibility is that it is a late IgE mediated reaction. To investigate this possibility, a radio-allergosorbent test (RAST) for the assay of antibodies to \textit{M. tuberculosis} in the IgE class was developed. This test revealed that patients with tuberculosis had higher levels of specific IgE antibody than healthy control subjects, but the overlap in levels was too great to be of diagnostic value. Patients with leprosy had similarly elevated levels of IgE antibody to \textit{M. tuberculosis}, suggesting that the response in this immunoglobulin class is also to the common mycobacterial antigens. Of more relevance was the finding that the hospital workers with the extensive early erythematous reactions had similar specific IgE antibody levels to factory workers who did not generally react in this manner. Thus, the cause of this 6-8 hour reaction remains unknown and further studies are required.

Little is known about the antigens involved in the induction of a positive tuberculin test. Skin testing with a set of four ultrasonicate antigens ('New Tuberculin') prepared from different mycobacterial species showed that healthy tuberculin positive individuals mostly reacted to all antigens while patients with active tuberculosis tended to react to the reagent prepared from \textit{M. tuberculosis} and to a variable number of the others. This suggests that sensitized but healthy individuals recognize common mycobacterial antigens (and probably specific ones as well) whilst recognition by patients is often restricted to species-specific antigens. (A similar phenomenon is seen amongst leprosy patients and controls in Nepal.) This finding could be very important as there is increasing evidence that the 'protective' antigens of mycobacteria are to be found amongst those common to all mycobacteria rather than amongst the species-specific ones. Thus, an immune mechanism that switches off recognition of the common antigens may play a central role in predisposing infected individuals to the development of active mycobacterial disease.

The cells involved in the tuberculin reaction may now be studied in great detail by labelling the various cell types in histological sections by means of specific monoclonal antibodies. Such histometric studies have shown that there are three main characteristics of the positive tuberculin test: first, an inflammatory exudate of blood-derived white cells into the pericapillary and perappendicular regions of the skin; secondly, a migration of some cells from the inflammatory foci into the intervening dermis, particularly in the subdermal region and, thirdly, the clinically evident edema and induration. Patients with tuberculosis or leprosy showed the same general pattern of histological response when tested with tuberculin, leprosin A or reagents prepared from environmental mycobacteria. Thus direct and cross reactions provoked the same general pattern of histological response although the extent of migration of cells into the intervening dermis tended to be greater in the direct reactions.

The intensity of the inflammatory response at the centre of the reaction was found to bear no relation to the diameter of the induration, indeed some clinically negative tuberculin tests showed that up to 40% of the dermis at the test site was composed of inflammatory cellular foci much more than was seen in some tests with extensive induration. There is evidence that some individuals, including healthy subjects and those with tuberculosis, fail to respond with clinically evident induration to standard doses of tuberculin and that this is under genetic control. The extent of the inflammatory response is influenced by the mode of preparation, being relatively less with sonicate antigens ('New Tuberculin') than with the more denatured purified protein derivative of tuberculin (PPD).

The histological characteristics of the 48 hour tuberculin reaction in hospital workers exposed to patients with tuberculosis, and with large 6-8 hour erythematous reactions, and factory workers have been compared. The number of cells of the monocyte/macrophage series diffusing from the perivascular foci into the dermis was significantly greater in the hospital workers than in the factory workers. This more extensive recruitment of phagocytic macrophages may be an important contribution to protection against overt disease in the occupationally exposed hospital workers.

The tuberculin test in man is analogous to the necrotic Koch phenomenon in the guinea-pig. For clinical use, tuberculin is diluted so that, although inducing a measurable reaction, it does not usually induce overt necrosis, although this does occasionally happen. The mechanism for the induction of necrotic component of delayed hypersensitivity, and the generation of cavities in the lung, has long been a mystery. Studies on the capillary blood flow in tuberculin reactions has been made possible by the technique of laser Doppler velocimetry. In most tuberculin reactions, the blood flow is higher relative to that of the normal surrounding skin and reaches a maximum at the centre of the test site. This increased blood flow compensates for the greatly increased cellularity of the skin due to the inflammation. In some reactions, however, there is a distinct relative slowing of the blood.
flow at the centre of the test site which could well lead to tissue anoxia, aci­
flow at the centre of the test site which are not known but a very likely candidate is macrophage-derived tumour necrosis factor: this is the same sub­
stance as cachectin which is responsible for the extreme wasting seen in advanced post-primary tuberculosis.

Serum proteins and other factors in tuberculosis

In addition to the specific cell­
mediated and humoral immune res­
ponses in tuberculosis, there are many non-specific changes detectable in the blood. These include changes in the levels of immunoglobulin and of a group of proteins known as acute-phase reactants hemoglobin levels and of the circulating white cells.

Total immunoglobulin levels in tuberculosis have been studied in several countries and in most, but not all, cases there has been a rise in levels in the IgG and IgA classes but not in the IgM class. Indonesian patients show this change in immunoglobulin levels and, during these studies, it was noted that healthy Indonesians tend to have high levels of IgA, relative to levels found in other South-east Asian countries. The changes in immunoglobulin levels in the IgG and IgA classes are unrelated to the appearance of specific anti-mycobacterial antibody in these classes and are probably the result of non-specific adjuvant activity.

The possible relation between vitamin D and protective immunity in tuberculosis has long been a subject of interest. Cod liver oil was first used to treat tuberculosis in the 18th century and vitamin D was an effective remedy for skin tuberculosis. Recently it has been shown that a sudden drop in vitamin D levels due to migration from a hot and sunny country to a cold and often cloudy one (such as England) may lead to reactivation of tuberculosis and it has also been shown that metabolites of this vitamin are involved in macro­
phage activation. In Indonesia about a quarter of a studied population had very high levels of vitamin D and that those within this group who developed tuberculosis tended to do so later in life and to develop less extensive pulmonary disease than those with lower levels. At one time it was believed that vitamin D was therapeutically effective in tuber­
culosis as it led to hypercalcemia, thereby promoting calcification of tuberculous lesions. The serum calcium levels in tuberculosis have been the subject of some controversy but, in Indonesia, no differences in calcium levels between patients and controls were found. However, patients had higher serum phosphorus levels than controls.

Acute-phase reactants are pro­
teins, mostly macrophage-derived and with various functions, that are elevated in tissue damaging conditions including infections. The levels of eight acute phase proteins in sera from 107 patients with tuber­
culosis and 144 healthy adults were quantitated by laser nephelometry, a technique that measures micro­
precipitates formed by antigen-anti­
body complexes. Levels of α1 antitrypsin, α1-acid glycoprotein, α2-macroglobulin, ceruloplasmin, haptoglobin, C-reactive protein and the third complement component (C3) were all elevated in tuberculosis but levels of tranferrin were lowered. These acute phase reactants showed little correlation with clinical, radi­
ological or hematological features of disease except for haptoglobin which showed a negative correlation with the peripheral blood lymphocyte count, suggesting an immunoregu­
ulatory role. In a subsequent study a correlation was found between haptoglobin levels and the extent to which sera from patients with tuber­
culosis suppressed the mitogen-driven activation of normal human lympho­
cytes in vitro.

Serum angiotensin converting enzyme (SACE) may also be classed as an acute phase reactant. This protein is frequently elevated in serum from patients with sarcoidosis who, characteristically, have non-necrotic granulomas and often fail to react to skin testing with tuberculin. Only a minority of patients with tuberculosis have elevated SACE levels but a low and significant inverse correlation between the levels of this protein and the diameter of the tuberculin test, suggesting that SACE may have a regulatory function in delayed type hypersensitivity reactions. In a later study, a weak but significant inverse relation between SACE levels and the radiological extent of pul­
monary tuberculosis was found. This suggests that SACE may inhibit the necrotic, cavity-forming, component of immune reactivity in tuberculosis and this could have therapeutic impli­
cations if the mechanism of this inhibi­tion can be established. In general, the roles of acute phase reactants and other serum factors in tuber­
culosis are poorly understood at the present time and require further study.

Lymphocyte subsets in tuberculosis

In recent years, there has been great interest in the numbers of CD4 (putative helper/inducer) and CD8 (putative suppressor/cytotoxic) T lymphocytes, and the ratio of these, in the peripheral blood. This interest has been brought about by the advent of the acquired immune deficiency syndrome (AIDS) pandemic as a lowered number of CD4 cells, in absolute numbers and relative to CD8 cells, is a characteristic feature of this syndrome. It has also been shown that some patients with pul­
monary tuberculosis likewise had a CD4 (T4) lymphopenia but it was suggested that this was a result of the tuberculous infection rather than of an underlying immune defect. This study has proved to be a key one, leading to several investigations by other workers in which the CD4
lymphopenia was confirmed and shown to return to normal after successful chemotherapy.

The behaviour of the CD4 and CD8 lymphocyte subsets in tuberculin test biopsies have also been studied. The CD4/CD8 ratios of the T cells in the perivascular foci and in the peripheral blood are very similar. By contrast, CD4 cells are relatively more numerous in the interstitial dermis and, although their absolute number diminishes with increasing dermal depth, their preponderance over the CD8 cells increases. The relative preponderance of the CD4 subset may be relevant to protective immunity and it may be therefore be of significance that the increase of the CD4/CD8 ratio with dermal depth was less evident in patients with tuberculosis than in healthy subjects. In this context: it is noteworthy that the CD4/CD8 ratio is much lower in lesions of lepromatous leprosy than in those of tuberculoid leprosy and that this CD4 deficit may be corrected by the intradeposal injection of gamma interferon.

Immunogenetics

Another area of considerable and growing interest is the genetic control of immune responsiveness, particularly by the D loci of the major histocompatibility complex (MHC) which controls the presentation of epitopes to the lymphocytes. In Indonesia, HLA-DR2 and DQw1 occurred significantly more frequently in patients with pulmonary tuberculosis (56% and 72% respectively) than in healthy controls (31% and 54% respectively; attributable risks 36% and 39% respectively) while DQw3 was more frequent in the control group (86%) than in the patients (65%; preventive fraction 57%). In addition, levels of antibody to species-specific epitopes on a 38 kilo-dalton protein of M. tuberculosis were significantly higher in individuals of DR2 type than in those of other types.

Additional genetic factors may also affect the immune response in tuberculosis. Thus differences in immunoglobulin allotypes between tuberculosis patients and healthy subjects have been found. The Km1 allotype occurred significantly more frequently amongst controls (57%) than patients (32%), while phenotypes lacking G1m(17) and G3m(21), as well as Km1, were more frequent in patients (37%) than controls (6%).

Conclusions

The studies reported here serve to illustrate the complexity of the immune responses in human tuberculosis. Although contributing much to our understanding, they have raised many important and fascinating questions. They also show that the immune response is two edged sword, contributing to both protection of the host and the pathology of the disease. It is clear that many specific and non-specific cell-mediated and humoral factors are involved in the overall immune responsiveness in mycobacterial disease and these are all interrelated in their effects. There may well be a 'grand unifying theory' enabling the outcome of any mycobacterial infection to be predicted but it is evident that much more basic work is required before theory can be translated into practical benefits for those afflicted by such infections.

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REFERENCES


