Vol. 21

Advances in
Tuberculosis Research

Serology of Tuberculosis and BCG Vaccination

KARGER

Serology of Tuberculosis and BCG Vaccination

Volume Editor
W. Fox, London

5 figures and 24 tables, 1984

S. Karger · Basel · München · Paris · London · New York · Tokyo · Sydney

Advances in Tuberculosis Research

Vol. 18: Editor: G. Canetti, Paris
XIV + 236 p., 57 fig., 40 tab., hard cover, 1972.
ISBN 3-8055-1301-1
Vol. 19: Editor: G. Urbanczik, Basel
VIII + 174 p., 22 fig., 32 tab., hard cover, 1976.
ISBN 3-8055-2215-0
Vol. 20: Editor: G. Urbanczik, Basel
VIII + 112 p., 23 fig., 19 tab., hard cover, 1980.
ISBN 3-8055-2954-6

National Library of Medicine, Cataloging in Publication
Serology of tuberculosis and BCG vaccination/
volume editor, W. Fox.
— Basel; New York: Karger, 1984.
(Advances in tuberculosis research; v. 21)
Bibliography p. 194. Includes index.
1. BCG Vaccine 2. BCG Vaccine – adverse effects 3. Tuberculosis – immunology I. Fox, W.
II. Series
W1 AD883 v. 21 [WF 250 S486]
ISBN 3-8055-3855-3

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The authors and the publisher have exerted every effort to ensure that drug selection and dosage set forth in this text are in accord with current recommendations and practice at the time of publication. However, in view of ongoing research, changes in government regulations, and the constant flow of information relating to drug therapy and drug reactions, the reader is urged to check the package insert for each drug for any change in indications and dosage and for added warnings and precautions. This is particularly important when the recommended agent is a new and/or infrequently employed drug.

Printed in Switzerland by Tanner & Bosshardt AG, Basel
ISBN 3-8055-3855-3

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Introduction

The isolation of the tubercle bacillus by *Robert Koch* in 1882 came at a time when tuberculosis was among the commonest causes of morbidity and mortality throughout Europe, and at a time when both microbiology and immunology were emerging as distinct scientific disciplines. Studies on the humoral immune response had begun with the work of *Behring and Kitasato* on bacterial toxins and antitoxins while *Koch's* work with tuberculin helped to lay the foundations of the subject of cell-mediated immunity. It was, therefore, not very long before *Koch's* tubercle bacillus was being used as an antigen in both skin tests and serological techniques in attempts to diagnose tuberculosis.

There is no simple diagnostic test for tuberculosis. Clinical and radiological signs are notoriously non-specific; and cultural techniques, although providing a definitive diagnosis, are expensive and time-consuming, and vary considerably in their reliability from laboratory to laboratory. Consequently, numerous workers have sought to develop simpler tests, especially serological tests.

When considering any diagnostic test it is necessary to look carefully at its potential role and usefulness. Being an infectious disease, the diagnosis of tuberculosis is of importance both to the individual patient and to the community. In addition, therefore, to its value in reaching a diagnosis in a patient with symptoms, a reliable test would be useful for screening contacts of infected individuals and, in some areas, for detecting infectious cases in the community.

In the more prosperous countries the incidence of tuberculosis is low and is declining, but in many of the poorer countries it continues to be a major health problem. The approach to the disease by the public health authorities in these two different populations must of necessity be quite different.

Although the marked decline in the incidence of tuberculosis in the developed nations is a most welcome occurrence, it has brought problems for the remaining victims of this disease [124]. In the face of such a decline

it is natural that both individual physicians and medical organizations should become less interested in tuberculosis and more concerned with other pulmonary diseases; some of which are caused by, or aggravated by, conditions prevailing in modern industrial societies. Under such circumstances tuberculosis, if it is considered at all, will be seen as one of a number of possible causes of pulmonary symptoms, lung shadows, lymphadenopathy, genito-urinary disease, pyrexia of unknown origin, orthopaedic conditions and many other presenting features. There is no doubt that tuberculosis is often misdiagnosed; many cases are not recognized until a post-mortem examination is performed [32] and cases of renal tuberculosis are often only diagnosed late in the course of the disease [210]. A simple, reliable, inexpensive test could therefore be of great benefit by alerting the clinician to a diagnostic possibility that might otherwise have been overlooked. Such a test would have to be one that could be easily, cheaply and routinely performed alongside other routine laboratory examinations. Its inclusion in a serological screening test for antibodies to common infecting agents would be a possibility. It would be necessary for such a test to diagnose tuberculosis in a high proportion of cases, but also to be consistently negative in other diseases, or clinicians would rapidly lose confidence in the test. It might be possible, however, to perform a quantitative test on all sera found to be positive on a screening test and, in some cases, a very high level of antibody would give a virtually unequivocal diagnosis.

The situation in poor countries with a very high incidence of tuberculosis is quite different. In these countries the main priority is to block the transmission of the disease. This can be achieved by improving the standard of living and by reducing overcrowding both in the home and at work, but this is a social problem rather than a medical one. Transmission may also be reduced by finding *infectious* cases of tuberculosis and by ensuring that they receive a full course of chemotherapy.

It has been shown [255] that tuberculosis is spread by individuals who are excreting enough bacilli in the sputum for these to be detected by microscopic examination, the so-called smear-positive cases. The search for additional cases of tuberculosis by more expensive and complicated methods would only be justifiable if it were also possible to offer curative chemotherapy to all these additional patients. Under the circumstances prevailing in most countries which have a high incidence of tuberculosis, an immunological test would only replace sputum microscopy if it was shown to be cheaper, technically easier and as socially acceptable as the latter, and if it contributed usefully to the tuberculosis control programme.

For a serological test to become widely used, it must therefore be able to withstand a very severe trial of its applicability and effectiveness under several quite different circumstances.

Diena [80] summarized the requirements for the ideal serological test for tuberculosis as follows: (1) It must differentiate between antibody responses due to natural infection and to bacille Calmette-Guérin (BCG) vaccination. (2) It should be a diagnostic aid in case-finding surveys. (3) It should be of use in the monitoring of chemotherapy. (4) It should assist in the diagnosis of other mycobacterial infections. (5) It should be of value in the diagnosis of extrapulmonary tuberculosis in which radiological studies are unhelpful and specimens not easily obtained.

The fact that no serodiagnostic test for tuberculosis is used extensively at the present time is a sure indication that all such tests have so far fallen short of such requirements. It is therefore of relevance to consider why they have proved ineffective. It is of even greater relevance to consider whether it will be possible to develop a near-perfect serological test or whether the basic biological nature of both the disease and the host's immune response renders this an impossibility. In order to reach a logical conclusion it is necessary to take into account the basis of the humoral immune response in general, the antigenic structure of the infecting pathogen and the nature of the host's response to it.

The Antigenic Structure of Mycobacteria

A mycobacterial cell is a complex structure containing many different proteins, sugars and lipids which are often closely associated in macromolecular complexes. The number of different antigenic determinants, or epitopes, occurring in each mycobacterium is unknown but must be enormous. The number of antigens demonstrable in practice depends on the sensitivity of the test and the nature of the bacterial preparation. No serological technique reveals the total set of antigens in a bacterial cell [169]. Thus, immuno-electrophoresis of cytoplasm or concentrated culture filtrates of *M. tuberculosis* reveals 14 distinct antigens [221]; immunodiffusion of bacterial ultrasonicates demonstrates about the same number [283] while crossed immuno-electrophoresis permits the detection of up to 100 such antigens [54, 247]. Yet even this technique probably gives a gross underestimate of the total number.

Many workers have shown that there is a high degree of sharing of antigens between the various mycobacterial species and that some of the

antigens are also shared with other bacterial genera [169]. In the Stanford classification [281, 283] the soluble antigens demonstrable as distinct lines on immunodiffusion analysis are placed into four groups: Group i: antigens detectable in all mycobacterial species. Group ii: antigens restricted to the slowly growing species. Group iii: antigens present in rapidly growing species. Group iv: antigens restricted to individual species.

The antigens demonstrable by immunodiffusion in groups i, ii and iii, number 5, 3 and 4, respectively, while the group iv antigens vary in number from 2 to 8 depending on the species. 6 group iv antigens are detectable in ultrasonicates of *M. tuberculosis* [283] and minor variations in the distribution of the species-specific antigens occur in many species including *M. tuberculosis*.

In many instances more than one antigenic determinant is present on a single molecule. While some molecules, such as certain polysaccharides, consist of repeating subunits with identical antigenic properties, others, notably proteins, bear many different epitopes. As evolutionary changes may occur only in certain parts of a protein molecule, a single protein could possess both species-specific and shared epitopes. Immunodiffusion analyses of mycobacterial catalase [318] and superoxide dismutase enzymes [160] from different species have shown that these proteins possess both common and species-specific antigenic determinants. *Daniel and Janicki* [76] have also shown that widely distributed mycobacterial epitopes may occur on the same molecule as epitopes of more limited distributions. In view of this molecular linkage of antigenic determinants, *Chaparas* [49] considers it highly unlikely that monospecific reagents for use in the diagnosis of mycobacterial disease will be forthcoming.

An additional problem encountered in the purification of mycobacterial antigens is the occurrence of a particular antigenic determinant on a range of molecules of differing physicochemical properties. *Kniker and LaBorde* [157] showed that culture filtrates of *M. tuberculosis* could be eluted as 12 fairly distinct fractions from an ion-exchange column (diethylaminoethyl cellulose, DEAE-cellulose) but 22 of the 23 demonstrable antigens occurred in more than 1 of the fractions and 1 antigen was present in 8 of the peaks. Similar results were obtained by *Pickett* et al. [223] who used ammonium sulphate fractionation in addition to ion-exchange chromatography. *Nassau and Nelstrop* [200] found that 1 antigen specific for *M. tuberculosis* was present on 4 distinct protein bands separable by acrylamide gel electrophoresis. This phenomenon is probably due, in part, to the uneven degradation of macromolecular complexes in culture filtrates by autolytic enzymes.

The earliest serious attempts to separate the soluble components of *M. tuberculosis* were reported by *Seibert* [267] in 1949 and *Affronti and Seibert* [4]. 2 polysaccharide fractions (I and II) and 3 protein fractions (A, B and C) were separated by precipitation by various concentrations of acetic acid and ethanol. Subsequent antigenic analysis of these fractions by *Daniel and Affronti* [70] revealed that the polysaccharide fractions I and II contained 2 and 1 distinct antigens, respectively, while the protein fractions contained many antigens, and there was considerable sharing of these antigens between the fractions.

Subsequent to *Seibert's* pioneering studies, many investigators attempted to fractionate the antigens of *M. tuberculosis* by more sophisticated techniques, including differential precipitation, gel-filtration, ion-exchange chromatography and preparative electrophoresis but, in general, the results [reviewed in ref. 76] were disappointing. Somewhat better results were obtained by the combination of several separative methods. *Yoneda and Fukui* [335] isolated 2 antigens from culture filtrates of *M. tuberculosis* by a combination of ammonium sulphate precipitation, preparative electrophoresis and ion-exchange chromatography. The β-antigen was detected in various strains of *M. tuberculosis* while the α-antigen was present in a number of slowly growing species but not in rapidly growing species. These 2 antigens would therefore belong to groups iv and ii, respectively, in the Stanford classification.

Polysaccharides are readily separable from proteins by differential precipitation [267], ion-exchange chromatography [81] and by continuous flow electrophoresis [141].

Small amounts of individual protein antigens have been obtained by acrylamide gel electrophoresis. The position of protein bands in the gel were ascertained by staining the gel, and slices corresponding to the stained bands were cut out of a parallel gel. Serological analysis of eluted antigens showed that a rapidly migrating component was probably specific for *M. tuberculosis* [22, 253]. A species-specific antigen was also demonstrated by *Nassau and Nelstrop* [200]. These authors selected sera from patients with tuberculosis which were considered to contain antibodies to a species-specific antigen only of *M. tuberculosis*. The lines of precipitation formed by the pooled sera and concentrated culture filtrates of *M. tuberculosis* H37Rv in immunodiffusion plates were excised and used as an antigen to raise antisera in rabbits. The resulting rabbit serum gave a single precipitin line on electrophoresis against the H37Rv antigen and was used to detect proteins containing this antigenic determinant after polyacrylamide gel elec-

trophoresis. Similarly *Harboe* et al. [133] raised antibodies to a single component of BCG obtained by cutting out an antigen-antibody line from a countercurrent immuno-electrophoresis gel.

As noted above, minor antigenic variations occur within the group iv antigens and such variation has been reported in *M. tuberculosis* [283]. An antigen present in human strains of *M. tuberculosis* but not in bovine strains has been isolated and purified by *Hirai* et al. [138] by differential salting out with ammonium sulphate followed by gel filtration. Likewise *Stavri* et al. [286] reported the isolation of antigens from *M. tuberculosis* H37Rv that were not present in BCG by the technique of preparative countercurrent immuno-electrophoresis. The antigens, used as a skin testing reagent, distinguished between guinea-pigs infected with *M. tuberculosis* or vaccinated with BCG.

The use of a set of 7 murine monoclonal antibodies enabled *Coates* et al. [55] to demonstrate antigenic differences between human and bovine strains of *M. tuberculosis* and between strains within these two groups.

Affinity chromatography is a powerful technique for the isolation of antigens and utilizes the natural affinity between certain molecules such as antigens and antibodies, or certain sugars and a class of plant proteins termed lectins. One of the pair of compounds is immobilized by attaching it to solid particles such as agarose beads. When crude preparations containing several antigens are passed down a column containing such particles, the relevant antigen binds to the antibody while all other antigens are eluted. The retained antigen is subsequently eluted by altering the physical conditions so that the antigen-antibody complex is dissociated. The use of lectins, such as concanavalin A, in affinity chromatography permits the isolation of polysaccharides and also of certain glycoproteins which bind to the lectin through their sugar moieties [71]. Although the use of this technique isolates antigens irrespective of the size of the molecule to which they are linked, it will not separate antigens from others which are situated on the same molecule. Consequently reagents prepared by this method are not necessarily monospecific.

Affinity chromatography has also been used to remove shared antigens from mycobacterial preparations. In such a technique, crude preparations are passed down a column containing antibodies to a heterologous strain or species so that shared antigens are retained but specific antigens pass directly through. This technique was used by *Wayne and Sramek* [319] to demonstrate that resting cells of *M. tuberculosis* in the sediments of cultures contained an antigen not present in actively replicating cells.

It may be concluded that, in general, polysaccharide antigens are easier to isolate and purify than protein antigens. Polysaccharides are readily separable from proteins and from each other and tend to be antigenically distinct as they often consist of repeating subunits. In contrast, proteins often contain several antigenic determinants of differing specificities and distribution.

In all studies on antigens of mycobacteria and their interactions with human sera, the antigens have been obtained from bacilli grown in vitro. It is, however, very likely that antigens from such bacilli differ both quantitatively and qualitatively from those grown in vivo. *Wayne and Sramek* [319] have stressed the need to consider this possibility when antigens are to be used to study host-pathogen interactions.

Boyden and Sorkin [29], in a useful review of the early studies on mycobacterial antigens, suggested that tubercle bacilli might produce special adaptive enzymes when growing in vivo and that these might elicit the formation of antibodies which, by neutralizing the enzymes, could play a protective role. Such antibodies would not, obviously, be detectable by the use of antigens from in vitro-grown bacilli.

Analysis and Nomenclature of the Soluble Mycobacterial Antigens

In the early studies of *Stanford* and his colleagues [282], mycobacterial antigens detected by immunodiffusion were numbered, but this practice was subsequently abandoned as it served no useful purpose. Instead, the antigens were simply classified according to their distribution within the genus as described above.

11 major antigens of *M. tuberculosis* demonstrable by electrophoresis have been numbered by *Janicki* et al. [142], and standard ultrasonicate antigens and antisera are available for reference purposes [75]. Information concerning the physicochemical nature of these antigens, and their distribution within the genus, is being acquired. Antigens 1, 2 and 3 are polysaccharides and have been identified as arabinomannan, arabinogalactan and a macromolecular glucan, respectively [76]; they are common to all mycobacteria and occur in some other bacterial genera. Antigens 6, 7 and 8 were also found to be widely distributed throughout the genus although the molecules bearing antigens 6 and 7 also carried determinants of more limited distribution. Antigen 5 has been identified as a glycoprotein with antigenic specificities apparently restricted to *M. tuberculosis* [77].

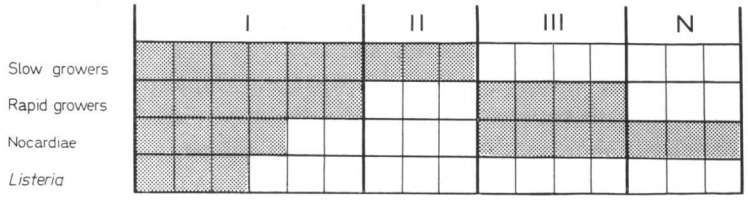

Fig. 1. The distribution of the group i,ii,iii and N antigens among the slow growing mycobacteria, the rapid growing mycobacteria, nocardiae and *Listeria*.

Closs et al. [54] studied the antigenic composition of concentrated culture filtrates of BCG (Copenhagen strain) by crossed immuno-electrophoresis. A commercially available anti-BCG serum was used and 31 distinct and stable precipitation lines were numbered for reference purposes. Many of these antigens were absorbed by antisera to other mycobacteria and 5 were absorbed by an antiserum to *Nocardia asteroides*.

The arabinomannan and arabinogalactan antigens of mycobacteria are shared with the genera *Nocardia* and *Corynebacterium* [333] and arabinogalactan also occurs in the cell walls of *Thermomonospora* and *Micropolyspora* [123]. It is also likely that the arabinogalactan antigen is the same as the α-haemosensitin of *Boyden and Sorkin* [29, 169]. The β-haemosensitin is, by contrast, a protein with some degree of species specificity. Other widely distributed antigens are associated with ribosomes. *Baker* et al. [15] have shown that ribosomal antigens constitute an important part of the precipitinogens of mycobacterial cytoplasm and culture filtrates. *Ridell* et al. [243, 244] isolated an antigen, termed the β-antigen, from the core particles of mycobacterial ribosomes. This antigen was also present in the genera *Nocardia*, *Corynebacterium*, *Rhodococcus*, *Kurthia* and *Arthrobacterium*. A ribonucleoprotein isolated from *M. tuberculosis* was found to react with sera from patients with lepromatous leprosy [327], and the ribosomal β-antigen of *Ridell* et al. [243] also reacted with sera from patients with this disease. A ribonucleoprotein prepared from *M. tuberculosis* by the method of *Wilhelm and Sellier* [326] was found to be one of *Stanford's* group i antigens [*Grange*, unpublished data].

Stanford and Wong [285] found that mycobacteria and nocardiae shared the common mycobacterial (group i) antigens and also the antigens present in rapidly growing but not slowly growing species (group iii). Thus, there is a very close antigenic similarity between the nocardiae and the rapidly growing mycobacteria. The nocardiae possess a group of antigens which do not

occur in the mycobacteria and are termed the N antigens. The antigenic relationships of the rapidly growing and slowly growing mycobacteria and the nocardiae are shown in figure 1. *Rajki* et al. [236] also found a marked sharing of antigens between mycobacteria and nocardiae. Thus, on crossed immuno-electrophoresis, the 4 most prominent antigens in cytoplasmic extracts of BCG were also present in *Nocardia asteroides.*

Lipid Antigens

The mycobacterial cell wall is probably the most complex of all the cell walls throughout nature (for reviews see *Ratledge and Stanford* [237]; *Barksdale and Kim* [20]). The cell wall contains a number of peptides, polysaccharides, lipids, glycolipids and peptidoglycolipids which bear antigenic determinants.

Takahashi et al. [295] divided the antibodies detectable in human tuberculosis into antiprotein, antipolysaccharide and antiphosphatide. The 'phosphatide' was a methanol extract of acetone-washed mycobacteria [26, 203] and was subsequently used in serodiagnostic tests (see p. 19). Analysis of this methanol extract showed that it contained phosphatidyl inositol, at least 3 phosphatidyl inositol mannosides and a polyglycerophosphatidic acid similar to the cardiolipin used in the Wasserman test for syphilis [211]. These studies were continued by *Reggiardo and Middlebrook* [239, 240] who isolated 3 classes of serologically active glycolipids and cardiolipin. The latter compound cross-reacted with beef heart cardiolipin (the antigen used in the Wasserman test for syphilis) and the importance of freeing antigens from this compound in order to avoid 'false' positive results was stressed. The 3 glycolipids are termed A (glycolipid without phosphorus), B (phosphatidylinositol dimannoside) and C (a mixture of phosphatidyl inositol pentamannosides). These 3 glycolipids have been used as antigens in the haemagglutination test and the enzyme-linked immunosorbent assay (ELISA) (see pp. 17 and 28).

Mycosides are a class of peptidoglycolipids situated on the surface of mycobacterial cells. They were originally considered to have species-specific differences in their structure but this is now known not to be the case. Some species of mycobacteria which form smooth colonies, notably *M. avium*, are divisible into a number of agglutination serotypes [262], and the responsible antigens have been shown to be mycosides [31]. Sera from some patients infected with environmental mycobacteria agglutinate the homo-

logous organism at a high titre [79] suggesting that such bacteria elicit a marked antimycoside humoral response in the host. Strains of *M. tuberculosis* are rough, and tend to auto-agglutinate, and for this reason relatively few studies have been made on the antigenicity of the surface components of this organism. *Shepard and Kirsh* [272] found that whole cells of *M. tuberculosis* were stained much less efficiently by fluorescein-linked antiserum than disrupted bacilli and therefore suggested that the surface layer of this organism contained little or no antigen. On the other hand, antibody has been shown to bind to *M. tuberculosis* by agglutination and agglutination-inhibition tests [329] and by an antiglobulin fixation test [85]. *Mudd* [197] observed that normal mycobacterial cells entered the oil phase at an oil/water interface while those 'sensitized' with antiserum remained in the water phase, indicating that a considerable amount of hydrophilic antibody became attached to the cell surface.

Methods Used in Serological Studies of Tuberculosis

There are many ways by which the binding of an antibody to an antigen may be detected and quantitated. Not only have almost all of the various technologies been applied from time to time to the study of antimycobacterial antibodies, but in several cases workers in this field have made important contributions to the methodology and to an understanding of the basic immunological principles. In particular, *Boyden* [28] pioneered the attachment of protein antigens to erythrocytes by treating the latter with tannic acid, and *Middlebrook* [186] introduced the haemolytic modification of the haemagglutination reaction thereby showing that antibody binding to foreign antigens attached to red cells, rather than just to the cells' own antigens, could cause cell lysis by activating complement.

The continuing challenge posed by the desire to develop a reliable diagnostic test for tuberculosis may have been a powerful driving force in the development and improvement of the technical methods used in serology. Many eminent workers in the field of immunodiagnosis have, at one time or another, turned their attention to tuberculosis. *Wasserman* [317], for example, studied the complement fixation reaction in tuberculosis as well as in syphilis. Consequently, it can be fairly said that the absence of a reliable serodiagnostic test for tuberculosis at the present time is certainly not due to a lack of effort, initiative or competence on the part of the many workers who have attempted to develop such a test.