TUBERCULOPOLYSACCHARIDE AND TUBERCULOPHOSPHATIDE PASSIVE AGGLUTINATION WITH BOVINE AND PORCINE SERUMS

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ABSTRACT

Robinson, Patsy J. (Michigan State University, East Lansing, Michigan). Tuberculopolysaccharide and tuberculophosphatide passive agglutination with bovine and porcine serums. 1964.--Tuberculopolysaccharide and phosphatide specific antibodies were measured by the hemagglutination (HA) and kaolin-phosphatide (KP) tests, respectively. The serums tested were from calves and swine inoculated with mycobacteria and from tuberculin positive cows from gross-lesion and no-gross-lesion herds. Serum titers were 20 or less from all except four of 120 animals prior to experimental infection, and were considered negative. Two cows from a gross-lesion herd had fourfold (anamnestic-like) response in HA and KP titers 3 to 13 days after tuberculin testing and progressive disease at necropsy. Two cows from a no-gross-lesion herd did not respond anamnestically to tuberculin and had no lesions at necropsy. Animals inoculated with pseudochromes, Group IV and Group III organisms (inanimate origin) had few or no lesions and low serum titers. Group III organisms (porcine origin) caused few lesions in calves but the calves responded anamnestically to tuberculin. Swine inoculated with Group III organisms (porcine origin) had fourfold increases in HA and KP serum titers and progressive disease at necropsy. Cattle's serologic responses were

altered quantitatively by the route of administration of Group III organisms (bovine origin), intradermally, intrauterine, or orally. Specific phosphatide extracts prepared from various mycobacteria did not increase the specificity or diagnostic value of the test.

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By

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INTRODUCTION

The need exists to differentiate between tuberculous infection and disease, and between active and closed cases. Since the demonstration of the etiologic agent of tuberculosis, the need of a single diagnostic test has been recognized and investigated. The tuberculin test is one of the most widely used tests for the detection of tuberculosis but it does not distinguish between past and present infection or disease.

Polysaccharide specific, protein specific and phosphatide specific antibodies may be present in the serums of infected individuals and animals. Most of the serologic studies have been made with serums from human cases of tuberculosis. As yet, no one test or tests have proved to be reliably diagnostic or differential.

As the incidence of bovine tuberculosis has been reduced, the problem of the tuberculin positive animal which has no gross lesions at slaughter has become more apparent and acute. Mycobacterium bovis has been isolated from some of the cattle. From far more, mycobacteria have been isolated which are not the classical pathogens, and have been called atypical, anonymous or unclassified mycobacteria for want of a better name. Their role in the tuberculosis or tuberculosis-like disease and in inducing tuberculin

sensitivity is presently under investigation. In conjunction with those investigations, studies were made of the serums from the calves and swine inoculated with the mycobacteria. Polysaccharide specific and phosphatide specific antibodies in serums have been measured by hemagglutination and kaolin particle agglutination. This is a report of the serologic studies.

HISTORICAL REVIEW

Agglutination. Agglutination, a visible reaction which may be observed when a particulate antigen is mixed with serum containing specific antibodies, was reported in 1896 by Gruber and Durham. Since that time applications of the phenomenon have been the most useful serologic technique for the diagnosis of infectious diseases.

Unknown organisms or antigens can be identified by the reaction with specific serums. The presence and relative concentrations of antibodies can be determined by using known antigens. The diagnostic significance of the latter must be determined empirically. Antibodies may occur in serums of healthy individuals not known to have had previous contact with the antigens.

Agglutination tests may be performed on surfaces such as glass slides, in containers such as test tubes, or the reaction may be measured photometrically. Of the three the surface method is the simplest and quickest to perform, is less sensitive, and is generally used qualitatively. The test tube method is used quantitatively by adding antigen to decreasing amounts of serum or serum to decreasing amounts of antigen. The titer is recorded as the reciprocal of serum or antigen which caused detectable agglutination.

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Photometric determination was introduced by Hirst and Pickels (1942) with agglutination of erythrocytes by influenza virus. The virus agglutinates the erythrocytes, and the presence of antibodies in a serum is detected by the specific inhibition of agglutination. Photometric determinations are made after the mixtures begin to settle and three layers are present in the test tube: the top clear layer devoid of erythrocytes, the middle layer of sedimenting cells of uniform density, and the bottom layer of cells which settled more rapidly. The optical density of the middle layer is measured and compared with a control tube.

Initially, suspensions of bacterial cells served as the particulate antigen. Subsequently extracted bacterial antigens have been adsorbed onto the surface of various particles such as erythrocytes (Middlebrook and Dubos, 1948), collodion (Jones, 1927), carbon (Meyer and Pic, 1936), latex (Singer and Plotz, 1956a,b), and kaolin (Takahashi and Adachi, 1960). These coated particles may be agglutinated by specific antibodies. Since the bacterial cells are composed of many antigens, exclusion of those antigens other than the one which is species or strain specific, generally yields a more specific test.

Hemagglutination. Hemagglutination, the agglutination of erythrocytes, was first noted in the 19th century when it was observed that transfusions to human beings of blood

from sheep or other domestic animals resulted in fatal reactions. Landois (1875) found that if human blood was mixed *in vitro* with blood from other animals, the blood cells were lysed or agglutinated. The studies by Landsteiner and others on the blood cell groups of man followed. In 1900 Landsteiner discovered that the blood of human beings could be divided into three distinct groups, A, B, and O, on the basis of hemagglutination reactions. The fourth and rarest group, AB, was discovered in 1902 by von Castello and Sturli. Through extensions of those studies, the complexities, presence of isoantibodies, phylogenetic relations, and many interesting phenomena concerning the blood cells of humans and higher animals have been established (Wiener, 1939).

As a result of the above and other studies, hemagglutination was investigated for use with other antigen-antibody systems. Davidsohn (1927, 1929, 1930, and 1933) demonstrated that sheep erythrocytes were agglutinated by heterophilic agglutinins in serums from individuals who had received injections of materials containing horse serum protein. Paul and Bunnell (1932) employed the Davidsohn technique to determine the increased amounts of heterophilic agglutinins in patients with infectious mononucleosis. A test was developed by Davidsohn (1937) which is now used routinely in the diagnosis of this disease.

Another application of hemagglutination measures

"cold agglutinins," so called because their action occurs only at low temperatures and is reversible when warmed to 37 C (Turner, 1943). Cold agglutinins are found in patients with atypical or virus pneumonia.

Hemagglutination by virus was discovered in 1941. Allantoic fluid from chicken embryos infected with influenza virus agglutinated the embryonic chicken blood. The agglutination was due to the direct action of virus particles on the erythrocytes and required no specific antibodies (Hirst, 1941; McClelland and Hare, 1941).

Neter (1956) described eight types of microbial hemagglutination:

a) the direct microbial hemagglutination reaction. Kraus and Ludwig (1902) observed clumping of erythrocytes in the presence of staphylococci and vibrios. (Later work has shown that many different bacteria possess the ability to agglutinate erythrocytes).

b) the indirect, passive, or conditioned hemagglutination reaction. Keogh, North and Warburton (1947) observed that polysaccharide antigen would adsorb onto the surface of erythrocytes which subsequently was agglutinated by specific antibody added to the mixture. Boyden and Suter (1952) have suggested the term "hemosensitin" for those antigens which adsorb spontaneously onto erythrocytes and render them sensitive to agglutination or lysis by antibody against the adsorbed antigen. The term would exclude and distinguish from those substances which agglutinate erythrocytes directly such as certain hemagglutinating viruses.

c) bacterial hemagglutination of erythrocytes treated with antigen-antibody mixture. Boyden and Anderson (1955) found that untreated erythrocytes exposed to a mixture of tuberculoprotein and homologous antibody resulted in hemagglutination.

d) bacterial hemagglutination of erythrocytes pretreated with tannic acid. Erythrocytes which had been previously treated with tannic acid and the microbial antigen, were agglutinated by specific antibody (Boyden, 1951).

e) bacterial hemagglutination by antibodies to protein antigens which have been attached to erythrocytes by chemical linkages. Pressman, Campbell, and Pauling (1942) devised such a method employing bis-diazotized benzidine as the protein conjugating material. Cole and Farrell (1955) succeeded in attaching to formalinized erythrocytes tuberculin PPD via tetrazotized benzidine.

f) erythrocyte linked antigen hemagglutination test. Non-bacterial protein antigens are attached to incomplete Rh (Coombs, Howard and Wild, 1952), ox red cell (Coombs, Howard and Mynors, 1953), and Forssman antibodies (Coombs and Fiset, 1954). The erythrocyte antibodies then act as Schleppers (carriers) for the protein and when treated with an antigen become agglutinable in the presence of homologous antibody.

g) bacterial panagglutination or the Thomsen-Friedenreich phenomenon. This was first observed by Thomsen in 1927 and extensively studied by Friedenreich in 1930. Enzymatic activity of certain bacteria uncovers a substrate referred to as T receptor or antigen, which reacts with the corresponding T antibody.

h) bacteriogenic hemagglutination. In 1940 Davidsohn and Toharsky reported that certain bacteria produce changes in normal serum which result in a panagglutinating capacity.

Hemagglutination in the study of tuberculosis. Middlebrook and Dubos (1948) used the principle of hemagglutination demonstrated by Keogh, North, and Warburton (1947, 1948) in the study of experimental tuberculosis. Incubation of sheep erythrocytes with culture filtrates of tubercle bacilli sensitized the sheep erythrocytes to agglutination by the serums of tuberculous human beings and experimentally

infected laboratory animals. Methanol extracts of phenol-acetone treated tubercle bacilli adsorbed onto the surface of sheep erythrocytes which were then agglutinated by serums from tuberculous patients and rabbits experimentally infected with an attenuated strain of M. bovis, the Bacillus of Calmette and Guèrin (BCG). The hemagglutination reaction could be inhibited by extracts and fractions of tubercle bacilli added to the serum before the introduction of the sensitized erythrocytes into the system (Middlebrook, 1950a). Fisher and Keogh (1950) modified this system by adding a small amount of complement. Hemolysis, rather than agglutination, occurred.

Modifications of the original Middlebrook-Dubos hemagglutination test have been developed in an attempt to make the test easier, faster to perform, less expensive and more specific. A practical modification of the test was developed which appeared to be equally specific for antibodies produced by the tubercle bacillus. Old Tuberculin (Lederle) four times the standard strength, diluted 1 to 15 in buffered isotonic saline, was substituted for the extract of the tubercle bacillus (Scott and Smith, 1950).

Erythrocytes from species other than the sheep have been used in hemagglutination tests. Gernez-Rieux and Tacquet (1952) compared erythrocytes from sheep, human Group "O," rabbits, guinea pigs, horses, cattle and chickens. Variation in the intensity of agglutination was observed

with erythrocytes from these different species. Human erythrocytes agglutinated less strongly than sheep erythrocytes. Sohier (1952) and Adcock, et al. (1951) compared sheep erythrocytes and human Group "O" erythrocytes and found the human erythrocytes to be effective. In addition, the natural antisheep agglutinins did not have to be adsorbed from the human serum.

A "drop technique" was developed by Rheins, et al. (1954). Drops of the serum and reagents were used instead of the exact amounts delivered from a pipette. A slide modification was devised by Thalhimer and Rowe (1951) which utilized glass slides rather than test tubes. Both techniques proved to be as sensitive as the original test and have the added advantages that small amounts of serum and reagents are required, and the incubation period was shortened to three minutes in the latter test.

During the years 1950 to 1954 many investigators evaluated the hemagglutination test by clinical and experimental observations (Adcock, et al., 1951; Anderson and Platou, 1951; Fleming, et al., 1951; Hall and Manion, 1951; Kirby, et al., 1951; Maillard and Gagliardo, 1951; Rothbard, 1951; Hentel and Guilbert, 1952; Maillard, 1952; Mollov and Kott, 1952; Schwartz, et al., 1952; Spain, et al., 1952; Hollander, et al., 1953; Rabe and Spicer, 1953; Choremis, et al., 1954). According to some of these investigators, the test was of diagnostic value. Others reported it to

be of little or no value. The serums used in the studies were primarily from hospital patients, patients with active tuberculosis and non-tuberculous patients with miscellaneous diseases or from normal individuals before and after vaccination with BCG. Fleming, et al. (1951) studied serums from over 400 individuals. Patients with active tuberculosis had the highest titers, and non-tuberculous patients and normal individuals usually had no or low titers. Titers decreased when the patient was improving or in the terminal stages of the disease (Anderson and Platou, 1951; Rabe and Spicer, 1953). Individuals who were tuberculin negative after repeated tuberculin tests usually had positive hemagglutination titers after vaccination with BCG (Smith and Scott, 1950; Haley, et al., 1952).

What constitutes a significant titer is controversial. Middlebrook (1950b) reported that a positive titer of 8 or higher was presumptive evidence of disease due to tubercle bacilli or to a mycobacterium very closely related in antigenic structure. Kirby, et al., (1951) reported that a large percentage of normal individuals had positive titers of 8 or higher, and for that reason, the test was not reliably diagnostic. Rabe and Spicer (1953) reported the test as being acceptably specific on the basis of a "significantly elevated" titer of 64. With the latter criterion, the test was positive with serum from 90% of thirty tuberculous patients. A titer of 32 ("doubtful significance")

was present in 87% of the tuberculous patients and 4% of the non-tuberculous patients.

Studies have been made of the serums from cattle. The percentage of positive reactions in tuberculous cattle was higher in those animals suffering from visceral tuberculosis than in those animals with isolated lymphatic lesions. Positive reactions were obtained in 85 to 90% of cattle with active visceral tuberculosis (Sohier, et al., 1950; Fisher and Gregory, 1951; Gernez-Rieux and Tacquet, 1952).

Richey, Mack, and Stafseth (1954) studied the hemagglutination reaction of chickens experimentally infected with avian tubercle bacilli. Adult chickens which had positive avian tuberculin tests all had antibody titers of 32 or higher. Of 41 chickens hatched from eggs inoculated with tubercle bacilli, seven reacted to tuberculin at seven months of age and had lesions when sacrificed at eight months of age. The seven had hemagglutination titers of 16 or higher. The remaining 34 chickens varied in response to tuberculin, none showed lesions, and 91% had hemagglutination titers of 4 or higher. Hemagglutination tests performed before and after tuberculin testing on serums from normal adult tuberculin negative chickens showed no increase in hemagglutination titer.

Boyden (1951) treated sheep erythrocytes with tannic acid. These cells then preferentially adsorbed

tuberculoprotein and were agglutinated by specific antibodies. Agglutination could be inhibited by adding culture extracts to the system before the introduction of sensitized erythrocytes.

Takahashi, et al. (1961a,b) used the hemagglutination test to measure three different antibodies, the tuberculoprotein, the tuberculopolysaccharide, and the tuberculophosphatide specific antibodies in serums from patients with tuberculosis and experimental aniamls. Experimental animals were inoculated with M. bovis (BCG) and a virulent M. tuberculosis (strain Nakano). Polysaccharide specific and protein specific antibodies were detectable regardless of the route of inoculation or the virulence of the bacilli. The phosphatide specific antibody was elicited under conditions in which the authors believed there had been in vivo multiplication and destruction of the bacilli. The presence of phosphatide antibody reportedly was indicative of progressive infection.

Takahashi, et al., (1961b) studied the three antibodies with serums from tuberculin positive patients with active tuberculosis and tuberculin positive healthy individuals. The polysaccharide specific antibody was present in all individuals of both groups. Approximately 54% of the tests for the protein specific antibody were positive and 46% were negative. The phosphatide specific antibody was found only in those individuals with active tuberculosis,

but not detectable in 20%. In the healthy tuberculin positive individuals, all had polysaccharide specific antibodies, 44% had protein specific antibodies, and none had phosphatide specific antibodies. Results of tuberculin skin tests, extent of tuberculous lesions, types of disease, existence of cavities, sputum findings and disease activity, and antibody titers were compared. There was no relationship between the titers of the three types of antibodies and tuberculin skin hypersensitivity. In all other instances there was a direct correlation between the phosphatide specific antibody titers and disease.

Although the hemagglutination test and its various modifications are controversial as to their use as aids in the diagnosis of tuberculosis, it has been found useful for other purposes. Investigators used the hemagglutination procedure to study the antigens of the tubercle bacilli (Fisher, 1951; Meynell, 1954; Rheins and Thurston, 1955; Thurston and Rheins, 1956a,b,c,d).

Meynell (1954) demonstrated that the polysaccharide and protein antigens present in a protein precipitate were composed of 88% protein, 5% polysaccharide and 7% nucleic acid. They could be separated by adsorbing the polysaccharide antigen on normal sheep erythrocytes and the protein antigen on tanned sheep erythrocytes. Although polysaccharide antigen would adsorb onto tanned cells, the smaller concentration of protein necessary for adsorption allowed dilution

of the suspension and the polysaccharide portion adsorbed onto the tanned cells was negligible. Sensitization of normal erythrocytes with only polysaccharide was possible with concentrated solutions because protein does not reportedly adsorb onto normal cells under any conditions. Tanned cells are sensitized by protein only by using antigen solution so dilute that the concentration of polysaccharide is too small to produce detectable sensitization.

Polysaccharide and phosphatide antigens are found on the surface of the bacterial cell while the protein antigen is a "deep" antigen. Bacillary suspensions combine with the polysaccharide and phosphatide specific antibodies. They do not combine with the protein specific antibody. The protein specific antibody remains in the supernatant (Meynell, 1954).

Successive adsorption of Old Tuberculin (Lederle) with sheep erythrocytes permitted demonstration of antibodies which probably are directed against antigens not detected by the usual sensitizing procedures (Rheins and Thurston, 1955). In a series of experiments Thurston and Rheins (1956a,b,c,d) studied extract antigens prepared from M. bovis (BCG), M. phlei, and M. tuberculosis (strain H37Rv). Water and saline extracts of the organisms, as well as the extracts prepared by the older, more tedious methods and Old Tuberculin, sensitized sheep erythrocytes to agglutination by specific antibodies. Water and saline extract-treated

sheep erythrocytes exhibited serologic specificities not seen with Old Tuberculin-sensitized erythrocytes. Antigens from aqueous extracts of M. bovis (BCG) and in Old Tuberculin were adsorbed to different sites on the erythrocytes. Tanning of erythrocytes did not result in demonstration of antibodies other than that observed with normal erythrocytes.

Lipids and lipid extracts such as lecithin and cephalin inhibit sensitization of erythrocytes with polysaccharide (Boyden and Grabar, 1954). The polysaccharide is believed to attach to a phospholipid on the erythrocyte surface.

Gerstl, et al., (1955) detected antibody activity in apparently negative serums from tuberculous patients after fractionation of the serums. Serums which were negative by the polysaccharide test caused hemagglutination and complement-fixation after fractionation by the cold ethanol method of Nichols and Deutsch (1948). The fractions were labeled supernatant A, largely albumin; precipitate B, largely beta and $gamma_1$ globulins; precipitate C_2, $gamma_2$ globulins. Serums from eight individuals had hemagglutinins in the C_2 precipitate. Seven of eight serums had complement fixing antibodies in the B precipitate. Serums from non-tuberculous patients did not react in either test before or after fractionation.

Injections of Old Tuberculin into guinea pigs which had previously received tubercle bacilli resulted in the appearance of increased amounts of polysaccharide antibody

in the serum (Boyden and Suter, 1952). The increase appeared within four to seven days with a maximum increase at ten days. By 28 days the increase in antibodies had disappeared in most of the animals. There was no relationship between the amount of skin hypersensitivity and the antibody titers. The amount of Old Tuberculin did not significantly change the antibody response. The route of injection of Old Tuberculin made no difference in antibody response.

Kaolin agglutination. Takahashi and Hukae (1960) substituted kaolin for sheep erythrocytes as the antigen carrier in the passive hemagglutination test. They compared the hemagglutination and the kaolin agglutination tests using phosphatide antigens prepared from M. tuberculosis (strains Nakano, H37Rv, and Aoyama B), and M. bovis (BCG) and serums from patients with pulmonary tuberculosis. The kaolin agglutination test was twofold to fourfold times as sensitive as the hemagglutination test.

Prior to this time kaolin had not been used as an antigen carrier in agglutination tests.

Tuberculopolysaccharides. The polysaccharides of the tubercle bacilli have been studied extensively since Koch first examined extracts of cells and culture filtrates in 1882. They have been primarily of three sources, those associated with the somatic portion of the cell (Chargaff and Schaefer, 1936; Menzel and Heidelberger, 1939; Tennent and Watson, 1942; Siebert, 1950); those associated with the

lipids (Anderson, Lothrop and Creighton, 1930); and those present in culture filtrates (Anderson, Peck, and Creighton, 1940; Suto-Nagy and Anderson, 1947). All fractions were complex in structure, chiefly composed of units of D-arabinose, D-mannose, D-galactose, and L-rhamnose, and inositol in the lipid derivatives (Stacey and Kent, 1948).

Isolations of components from the organisms have been based generally on dilute acid and alkali extraction after the organisms were grown on synthetic media to exclude extraneous carbohydrate material. Polysaccharides from cells or filtrates were of similar nature (Stacey and Kent, 1948).

Siebert (1950) reported that polysaccharides from unheated cells differed from polysaccharides of autoclaved cells, and that fractions from autoclaved cells of the same species varied from lot to lot. Free inosite instead of manninositose was found. Siebert isolated two different serologically specific polysaccharides from a filtrate. Polysaccharide I had a low molecular weight and was composed mainly of mannose and arabinose. Polysaccharide II was electrophoretically homogeneous, contained a small amount of lipid which may have been impurity, and a small amount of amino sugar. It was composed of a minimum of fifteen hexose units and contained no dextran, starch, or glycogen.

Schaefer (1938, 1940) applied Landsteiner's method (1919, 1920) of specific inhibition to study the polysaccharide

specific antibody with an active fraction from mycobacterial cells which was soluble in 50 to 85% methyl alcohol and an inactive fraction which was soluble in 50% methyl alcohol. The polysaccharide specific antibody was removed by excess antigen when the supernatant was measured by the complement fixation test. Antigen suspensions prepared by adding the antigen drop by drop to saline were better than antigen suspensions prepared by adding antigen rapidly to the saline. The polysaccharide antigen was treated in three ways: heated in acid solution, heated in alkaline solution, and unheated. The results of the tests were identical with the antigens which were unheated or heated in acid solution. There was no reaction or a prozone reaction using the antigen heated in alkaline solution.

Meyer and Pic (1936) removed the polysaccharide specific antibodies by adsorption to kaolin particles. The optimal kaolin-antigen complex required to remove the antibodies was determined by mixing antigen with varying amounts of kaolin. There was no adsorption of antigen to the kaolin with less than 10 mg of kaolin/ml of antigen suspension. There was 80% adsorption with 10 mg of kaolin/ml of antigen suspension, 100% adsorption with 50 mg of kaolin/ml of antigen suspension, and nonspecific adsorption with more than 50 mg of kaolin/ml of antigen suspension. With 50 mg of kaolin and 1.0 ml of antigen suspension, 98% of the specific antibodies was removed from the serum by

two adsorptions. To obtain the same results with one adsorption, 8.0 ml of antigen was necessary.

Cross reactions were observed with antigen fractions prepared from M. tuberculosis, M. avium, M. phlei (Bacillus phlei), M. bovis and M. leprae (Stacey and Kent, 1948; Iland, 1951). Tennent and Watson (1942) suggested that the polysaccharides are genus specific rather than species specific.

Some polysaccharides are haptenic (Stacey and Kent, 1948). They react with specific antibodies in dilutions of serum as high as 1:1,000,000 but do not elicit antibody formation. Polysaccharide II, a high molecular weight substance, is reportedly a true antigen (Siebert, 1950). However, Crowle (1958) suggested its antigenicity could be due to being complexed with a lipid. No tuberculoimmunity or delayed hypersensitivity was induced in rabbits inoculated with either polysaccharide I or II (Siebert, 1950). Raffel (1946) inoculated guinea pigs with polysaccharides extracted from culture filtrates and from defatted tubercle bacilli. No tuberculoimmunity or delayed sensitivity was induced.

Tuberculophosphatides. A phosphatide is a lipid, which, when hydrolysed, yields fatty acids, phosphoric acid, an alcohol which is generally glycerol, and a nitrogenous base such as choline or ethanolamine (Hawk, Osser, and Summerson, 1947).

The extensive studies of tuberculophosphatides by Anderson and coworkers in the 1930's and 1940's were reviewed

by Crowle (1958). Anderson (1941) described the tuberculophosphatides as substances which were easily soluble in ether, precipitated from ether solution with acetone, and formed a colloidal solution in water. They contained 2.6 to 3.5% phosphorus in organic combination as glycerophosphoric acid and as a phosphorylated polysaccharide or glyceride, and a small amount of nitrogen. When hydrolyzed, they yielded 33 to 40% water soluble compounds. These compounds yielded a small amount of inorganic phosphoric acid and a larger quantity of organic phosphoric acids similar in composition to glycerophosphoric acid when completely hydrolyzed with dilute sulfuric acid. The hydrolysed solutions also contained inositol, mannose, and a hexose which formed a typical glucosazone on treatment with phenylhydrazine. Fatty acid components varied with each phosphatide. The saturated acids were chiefly palmitic acid. The unsaturated acids were chiefly oleic acid. In addition, every phosphatide contained saturated branched chain acids of higher molecular weight which were oils at room temperature.

Phosphatide fraction from M. phlei (Chargaff, Pangborn, and Anderson, 1931), M. tuberculosis (strain H37Rv) (Anderson, Lothrop, and Creighton, 1930), and M. bovis (Anderson and Roberts, 1930) are very similar.

As the phosphatide content of M. tuberculosis (strain H37Rv), M. avium, M. bovis, and M. phlei decreased respectively, the polysaccharide content increased. The following

results for each organism were reported: M. tuberculosis 6.54% phosphatide, 0.97% polysaccharide; M. avium 2.26% phosphatide, 1.02% polysaccharide; M. bovis 1.53% phosphatide, 1.09% polysaccharide; and M. phlei 0.59% phosphatide and 3.90% polysaccharide (Chargaff, Pangborn, and Anderson, 1931).

The composition of the phosphatides of heated bacilli differed from the composition of unheated bacilli (Siebert, 1950). The major difference was the absence of pure phthioic acid and the presence of a new substance, inositol glycerol diphosphoric acid, in the heated cells. It was not clear whether the differences were due to the use of different strains or to changes caused by heating. No phosphatide or high temperature melting wax was found in human tubercle bacilli grown on Long's modified medium while other lipids seemed to be similar to those grown on standard medium.

It has been the phosphatide from the classic fractionation of an ether-alcohol extract that has been studied chemically. Serologic and immunologic studies have been made of phosphatides prepared from acetone-washed bacilli extracted with methanol. Boquet and Negré (1923) prepared the original methanol extract known as antigène méthylique and used it as an antigen in serologic tests.

The resistance inducing properties of antigène méthylique were reviewed by Crowle (1958). Boquet and Negré (1923), Negré (1952), and other investigators have

proved that antigène méthylique used prophylactically can increase the tuberculoimmunity of guinea pigs and rabbits but they indicate that such resistance is not necessarily equivalent to classic antitubercular immunity.

Atypical mycobacteria. Atypical, anonymous or unclassified mycobacteria exist other than those of the established species. They have been reported (Alvarez and Tavel, 1885; Griffith, 1916; Beaven and Bayne-Jones, 1931) since shortly after the discovery of the tubercle bacillus by Koch (1882). They have been isolated by many investigators. Until recently they have been largely disregarded as inconsequential saprophytes. Youmans (1963) cited several reasons for the tardy recognition of these organisms as causative agents of latent infection and pulmonary disease. First, after the isolation of M. tuberculosis by Koch (1882), the prevalence of tuberculosis was so great that the relatively few cases which may have been caused by the atypical mycobacteria were not apparent. Second, the failure of workers in diagnostic laboratories to cultivate and isolate routinely the acid-fast bacilli present in the sputum or infected tissues of persons with tuberculosis. Third, the lack of pathogenicity for guinea pigs, and fourth, the presence of pigmented and non-pigmented saprophytes found in association with tubercle bacilli in sputum and gastric washings and in sputums of normal individuals.

The atypical mycobacteria have been isolated repeatedly

and shown to be the cause of human disease (Tarshis and Frisch, 1956a,b,c; Wood, et al., 1956; Runyon, 1959). Runyon (1959), jointly sponsored by the Veterans Administration and the National Tuberculosis Association, studied a large number of atypical mycobacteria and grouped them into four groups on the basis of pigment production and rate of growth. Group I, the photochromogens, produce no pigment unless exposed to light. Ten minutes' exposure during the period of active growth activates yellow pigment production during the subsequent incubation in the light or in the dark. Group II, the scotochromogens, produce yellow or orange pigment in both light or dark and are more deeply pigmented if grown in continuous light. Group III, the non-photochromogens, produce little or no pigment in the light or dark, and Group IV, the rapid growers, are generally non-pigmented. Group IV organisms require only three to four days for well isolated colonies to become visible on Lowenstein-Jensen medium at 37 C. The other three groups require ten to twelve days for visible colonies under the same conditions.

Atypical mycobacteria similar to the Runyon groups of human disease have been isolated repeatedly from cattle and swine by members of the Michigan State University Tuberculosis Research Project. The research project, sponsored jointly by the Animal Disease Eradication Division and the Animal Disease and Parasite Research Division of the United States Department of Agriculture, provides for investigating

the questions raised by the fact that many tuberculin positive cattle when slaughtered show no gross lesions at necropsy. Organisms isolated from such cattle have been studied by means of various cytochemical and morphological characteristics, animal infectivity and allergenicity. Some are M. bovis. Most are atypical mycobacteria. They were found to be heterogenous, highly variable and adaptable (Mallmann, Mallmann, and Robinson, 1964). Many of the isolants are classified as Group III organisms. These range in virulence for calves, guinea pigs, chickens and rabbits from none to a virulence almost equal to that of M. bovis. In addition, the Group III organisms isolated from swine tissues have little or no virulence for calves, but produce a disease in swine intermediate of that produced by M. bovis and M. avium.

Among the Group III organisms avirulent for calves, there are those which are believed to be identical to some of the Group III organisms isolated from human disease. These produce only intradermal lesions in calves. Others are undoubtedly non-pathogenic saprophytes.

At present, there is no laboratory test which differentiates the virulent from avirulent Group III organisms.

In the latter group, some strains produce some yellow color when the culture in a liquid medium is exposed unnecessarily to light or upon prolonged incubation. These strains are called pseudochromes (Mallmann, Mallmann, and Robinson, 1964).

MATERIALS AND METHODS

Cultures for inoculation. The cultures used to inoculate experimental animals are listed in Table 1. They were isolated from cattle, swine, soil and feed by members of the Bovine Tuberculosis Research Project, Michigan State University, East Lansing, Michigan.

Cultures are designated by the following coding system. Letters preceding the culture number refer to the method of isolation. "B" refers to the pentane-enzyme method; "M" refers to the pentane method followed by a disinfectant treatment; and no letter refers to the sodium hydroxide method. The letter "X" preceding the culture number indicates the strain came from soil. The letters following the culture number refer to the tissues from which the strain was isolated; A = cervical lymph nodes, B = thoracic lymph nodes, C = mesenteric lymph nodes, D = carcass lymph nodes, E = lung, F = skin lesion, and H = Peyer's patches. The number following the letter refers to the year in which the culture was isolated; 0 = 1960, 1 = 1961, and 2 = 1962.

Preparation of cultures for inoculation. Eight ml Dubos Broth (Difco) with 0.5% dextrose were seeded with organisms and incubated at 35 C for approximately 14 days. The supernatant fluid was removed and the cells were resuspended in

TABLE 1. List of animals inoculated with mycobacteria

Culture No.	Source	Mycobacterium	Calf No.	Swine No.
81-0	Swine	M. bovis[1]	20,81	2-1,2-2 2-4,7-1 7-2,7-3
81-0	Swine	M. bovis (heat-killed)	26	
130-0	Cattle	M. bovis	6	
Lab strain	Chicken	M. avium	21	5-1,5-2 5-3
Lab strain	Chicken	M. avium (heat-killed)	27	
206-0	Swine	M. avium		10-5,10-6
50B-0	Cattle	III	1,66,67, 68,78,79, 80,87,88, 89	
51C-0	Cattle	III	2,42,43, 59,60,61, 62,74,75, 77,84,90, 91	
51C-0	Cattle	III (heat-killed)	29	
62D-0	Cattle	III	3,44	
68C-0	Cattle	III	4,45,63, 64,65,70, 71,73,82, 83,86	
71C-0	Cattle	III	7,38,39	
B78B-0	Cattle	III	10	

[1]Where species is not indicated, organisms are atypical mycobacteria. The Roman numeral indicates the Runyon Group.

TABLE 1.--Continued

Culture No.	Source	Mycobacterium	Calf No.	Swine No.
93C-0	Swine	III	16	
107E-0	Cattle	III	13,40	
$152A_1$-1	Swine	III	19,41	
167C-1	Swine	III	35,37	1-3,3-4
$172C_1$-1	Swine	III	23,30,31	1-1,1-4 6-1,6-2 6-3
173C-1	Swine	III	24	
186C-1	Swine	III	34,36	3-1,3-2
$193C_2$-1	Swine	III	32,33	1-6,2-3
15D	Swine pen	III		161-4
19_2D	Swine pen	III		161-3
X37	Soil	III	48	
X41	Soil	III	49,50	
A	Feed	III	46,47	
112B-0	Cattle	Pseudochrome	17	
128F-0	Cattle	Pseudochrome	8,56	
52H-1	Cattle	Pseudochrome	11,57,58	
61B-1	Cattle	Pseudochrome	14,55	
B117B-0	Cattle	IV	15,52	
B117B-0	Cattle	IV (heat-killed)	28	
B124F-0	Cattle	IV	5	
4F-1	Cattle	IV	9	
M7F-1	Cattle	IV	18,51	
B254F-1	Cattle	IV	25,53,54	

Dubos medium so that the number of mg wet weight of cells injected in an animal was contained in 0.1 ml.

Experimental animals. Experimental animals consisted of eight lots of animals as follows:

Twenty-three calves (Lot I) were inoculated with cultures of M. bovis, M. avium, Group III organisms (bovine origin), Group III organisms (porcine origin), pseudochrome organisms, and Group IV organisms.

Four adult cows (Lot II), two from gross-lesion herds and two from no-gross-lesion herds, were not experimentally infected but were maintained under observation for approximately one year. Numerous tuberculin tests were administered.

Of twenty-two swine (Lot III), three were inoculated with M. bovis, three were fed M. bovis, three were inoculated with M. avium, eight were inoculated with Group III organisms (porcine origin), and three were fed a Group III organism (porcine origin). Two swine were uninoculated.

Two swine (Lot IV) were inoculated with Group III organisms (inanimate origin).

Of thirty-three calves (Lot V), four were inoculated with heat-killed (1 hr, 100 C) cultures of M. bovis, M. avium, a Group III organism (bovine origin) and a Group IV organism. The remaining calves were inoculated with Group III organisms (bovine origin), Group III organisms (porcine origin), Group III organisms (inanimate origin), pseudochrome organisms

and Group IV organisms.

Ten calves (Lot VI) were inoculated with Group III organisms (bovine origin).

Nine calves (Lot VII) were inoculated with the same Group III organisms (bovine origin) as Lot VI.

Nine calves (Lot VIII) were inoculated with the same Group III organisms (bovine origin) as Lots VI and VII.

The experiments were performed over a period of time and were part of other experiments designed primarily to establish pathogenicity for swine (Ray, 1963), pathogenicity for calves (McGavin, 1963), and route by which calves were susceptible to three Group III mycobacteria (McGavin and Goyings, 1963).

Calves 1 through 6 from Lot I were injected intradermally at four sites, 0.1 or 1.0 mg per site, with a total of 2.2 mg wet weight of organisms. One mg was injected proximal to the carpus on the medial side of the left foreleg and 1.0 mg was injected into the skin just distal to the hock on the medial side of the right hindleg. One tenth mg was injected distal to the carpus on the medial side of the left foreleg and 0.1 mg was injected proximal to the hock on the medial side of the right hindleg. Calves 7 through 25 from Lot I were injected intradermally with 1.0 mg wet weight of organisms into the lateral side of the left foreleg just proximal to the carpus.

The calves from Lot I died or were euthanized 37 to 86 days post inoculation.

Calves 26 through 58 from Lot V, with the exception of 47 and 50, were injected intradermally with 1.0 mg wet weight of organisms into the lateral side of the left foreleg just proximal to the carpus. Calf 47 and Calf 50 were inoculated with a total of 10.0 mg wet weight of organisms by five routes. Two mg were given by each of the following routes: orally, intradermally at the same site as the other calves in Lot V, subcutaneously behind the right elbow, intramuscularly into the right gluteal mass and intraperitoneally into the right paralumbar fossa.

Calves 26 through 58 were euthanized or died 43 to 73 days post inoculation.

Calves 59 through 69 were injected intradermally (ID) in the same manner as calves 26 through 58 with 1.0 mg wet weight of organisms.

Calves 59 through 69 were divided into three groups of three animals. One calf of each group was euthanized two months after inoculation, two calves were euthanized four months after inoculation and one calf from each group was euthanized six months after inoculation. One calf died three months after inoculation.

Nine heifers, 70, 71, 73 through 75, and 77 through 80, were inoculated with Group III organisms (bovine origin). One mg weight of organisms was placed in a disposable

artificial insemination tube and a 10 ml syringe containing 5.0 ml bovine semen was attached. The organisms and semen were introduced into the uterine cavity (IU) of heifers in induced estrus.

The heifers were divided into three groups of three animals. Heifers 73, 74 and 78 were euthanized two months after inoculation, 70, 75 and 79 were euthanized four months after inoculation, and 71, 77 and 80 were euthanized six months after inoculation.

Nine calves, 82 through 84 and 86 through 91, were exposed to an aerosol (AE) of Group III organisms (bovine origin). A calf was placed in an aerosol chamber and organisms, suspended in 9.1% bovine albumin and 0.1% Tween 80 were sprayed into the chamber with an atomizer. Fifteen ml of culture medium containing approximately 1×10^8 organisms/ml were sprayed into the chamber during a one hour exposure period for each calf.

Three calves, one from each group, were to have been euthanized two months after inoculation, three at four months after inoculation and three at six months after inoculation but, due to the severity of the disease, calves 82, 83, 86, 90, 84, and 91 were euthanized prior to the scheduled time. Calf 89 was euthanized two months after inoculation, Calf 87 was euthanized four months after inoculation and Calf 88 was euthanized six months after inoculation.

Twenty-five swine were inoculated or fed 2.0 mg wet weight of organisms as follows:

Type of Culture	Swine Numbers	Route
M. bovis	2-1, 2-2, 2-4	ID
	7-1, 7-2, 7-3	Oral
M. avium	5-1, 5-2, 5-3	ID
	6-1, 6-2, 6-3	Oral
III	1-1, 1-3, 1-4	ID
	3-4, 1-6, 2-3	ID
	3-1, 3-2	ID
	161-4, 161-3	ID
	6-1, 6-2, 6-3	Oral

They were injected intradermally on the lateral surface of the left anterior leg just distal to the carpus or were fed ration containing the organisms.

Swine were killed 39 to 224 days post inoculation.

At necropsy animal tissues were collected to be examined histologically. Results were recorded as follows: No Significant Lesions--no gross or microscopic lesions or only a granuloma at the site of inoculation; Primary Complex--lesion in the lymph node draining the inoculation site, may or may not have a skin lesion because skin lesion may have healed; Generalized--lesions anywhere beyond the primary complex, i.e., disseminated in general circulation and generally in the lymph nodes draining the lungs.

Attempts were made at determining whether the lesions were progressive or not on the basis of the criteria laid down by Feldman (1943) for guinea pigs. He stated that the signs of progressive lesions were:

a) peripheral extension of the disease with daughter tubercles
b) confluence of morbid tissue
c) conglomerate tubercles
d) slight to extensive necrosis

Non-progressive lesions were characterized by:

a) peripheral encapsulation without daughter tubercles in the peripheral zone of the capsule
b) presence of noncaseating tubercles
c) evidence of the transition of epithelioid cells to fibroblasts
d) fibrosis
e) calcification or bone where there are no signs of progressive tuberculosis

Cattle and swine were tuberculin tested at intervals during the course of the experiments. The days post inoculation the cattle were tuberculin tested are recorded on Tables 5, 6, 7, and 8.

Cattle were tested with 0.1 ml mammalian tuberculin* injected into the caudal fold and 0.1 ml mammalian tuberculin, 0.1 ml avian tuberculin,** and 0.2 ml Johnin*** injected into the cervical region. Results were read at 48 and 72 hr.

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*Tuberculin, mammalian, intradermic produced for the Agricultural Research Service, U.S. Department of Agriculture.
**Tuberculin, avian, intradermic, produced for the Agricultural Research Service, U.S. Department of Agriculture.
***Johnin, intradermic, produced for the Agricultural Research Service, U.S. Department of Agriculture.

Swine which were inoculated with Group III organisms (porcine origin) and M. bovis and uninoculated swine were tested with 0.1 ml avian tuberculin undiluted, 0.1 ml avian tuberculin diluted 1:5, 0.1 ml avian tuberculin diluted 1:10, 0.1 ml mammalian tuberculin undiluted, 0.1 ml mammalian tuberculin diluted 1:5, and 0.1 ml mammalian tuberculin diluted 1:10. Swine which were inoculated with M. avium, fed M. bovis and a Group III organism (porcine origin) and inoculated with Group III organisms (inanimate origin) were tested with 0.1 ml avian tuberculin and 0.1 ml mammalian tuberculin. Avian tuberculin was injected into one ear, and mammalian tuberculin was injected into the opposite ear. Results were read at 48 and 72 hours.

Collection of serum. Blood was collected prior to inoculation and at different post inoculation times, as indicated in Tables 2, 5, 6, 7, and 8. After clotting, serum was removed and stored at -20 C.

Hemagglutination (HA) reagents and test.

Saline: A stock solution of saline (final pH 7.2) contained

NaCl	170.00 g
KH_2PO_4	2.78 g
Na_2HPO_4	11.30 g
H_2O q.s.ad.	2000.00 ml

For use the stock solution was diluted 1:10 with distilled water.

Collection and sensitization of sheep erythrocytes: Sheep blood was obtained aseptically and put directly into

an equal volume of modified Alsever's solution. The mixture was stored at 4 C and used from one to ten weeks after collection. The erythrocyte suspension was removed from the flask, centrifuged, and was washed three times with buffered saline. Centrifugation was in an International Centrifuge Model UV at 550 X G for 15 min.

Modified Alsever's solution contained

Dextrose	16.40 g
Sodium citrate	6.40 g
NaCl	3.36 g
Citric acid	0.44 g
H_2O q.s.ad.	800.00 ml

The solution had a pH of 6.1. It was dispensed in 200 ml quantities into 500 ml Erlenmeyer flasks and sterilized at 110 C for 10 min.

Mammalian Tuberculin, License No. 107, Serial Numbers 77 and 91, was supplied by the Animal Disease Eradication Division of the United States Department of Agriculture.

Old Tuberculin was diluted 1:15 in buffered saline, and washed sheep erythrocytes were added, 0.1 ml per 6.0 ml of diluted OT. The suspension was incubated in a water bath at 37 C, 2 hr. After incubation, the suspension was centrifuged at 550 X G for 4 min, and the supernatant fluid discarded. The cells were washed three times with buffered saline, and after the third washing, the cells were suspended in buffered saline at 0.5% concentration. The sensitized-erythrocyte suspension was not used beyond 24 hr.

Inactivation and adsorption of serums: Serums were

inactivated by heating in a water bath, 56 C for 30 min. Washed sheep erythrocytes equal to 1/10 the volume of each serum was added and the mixture incubated in a water bath, 37 C for 30 min. After incubation the cells and serum were separated by centrifugation, the serum transferred to a clean tube, and the adsorption was repeated. The adsorbed serums were stored at -20 C.

Serum dilutions: Beginning with dilutions of 1:2, 1:5, or 1:20, twofold serial dilutions of the adsorbed serum were made with buffered saline in 12 x 75 mm tubes. Serum and saline necessary to make the initial dilution in a total volume of 1.0 ml were placed in the first tube. After mixing the serum and saline no less than 10 times with a pipette, 0.5 ml was transferred to the second tube containing 0.5 ml buffered saline. This procedure was repeated through the remaining tubes using a clean pipette after each transfer. The last dilution of 0.5 ml, from which further dilutions could be made if an end point were not reached, was stored in the refrigerator overnight. Controls of positive and negative serums and saline with sensitized and unsensitized cells were included.

Hemagglutination (HA) test: After three drops of 0.5% sensitized erythrocyte suspension were added to each of the serum dilutions, the tubes were shaken vigorously. Preliminary results indicated the most easily reproducible results were obtained by incubating in a water bath at 37 C 2 hr,

removing from the water bath and allowing to stand at room temperature for 2 hr and then at 4 C overnight. The tubes were removed from the refrigerator and allowed to warm to room temperature for 30 min. The tubes were swirled gently and observed under a 60 watt light bulb and against a white background. The reciprocal of the highest dilution of serum which caused agglutination was recorded as the HA titer.

Kaolin-phosphatide (KP) reagents and test.

TME buffer solution: The TME buffer solution (Takahashi, 1962) was obtained from Takahashi for use in tests employing the Takahashi phosphatide-methanol solution. Subsequently, TME buffer solution was made for other tests by the following formula:

Tris (hydroxymethyl) amino methane	121.10 g
Maleic acid (anhydrous)	9.80 g
EDTA (disodium ethylene diamino-tetracetate)	5.63 g
Tween 80	0.05 g
NaOH	3.75 g
H_2O q.s.ad.	1000.00 ml

The pH was adjusted to 6.6 by adding 1N NaOH.

The buffer solution was dispensed to glass ampules. The ampules were fire-sealed and sterilized by heating at 100 C for 15 min. They were incubated overnight at 37 C, heated again in the same manner, and stored at room temperature. The resulting suspension was usable for at least one year after preparation.

Preparation of TME buffered saline: Buffered saline solution was prepared by adding one volume of TME buffer

to nine volumes physiological saline. The solution was good for one week.

Preparation of phosphatide-methanol antigen: Phosphatide-methanol solution obtained from Takahashi (1962) had been prepared from M. bovis (BCG) and M. tuberculosis (strains H37Rv and Nakano). The organisms had been grown for eight weeks on Sauton medium and 60 g of bacilli was treated two times with 600 ml of acetone at 40 C, 3 hr each time. After drying the cellular residue was treated two times at the same temperature with 600 ml methanol, 5 hr each time. The methanol extracts were combined, filtered in a Seitz filter, and evaporated in a nitrogen atmosphere to 200 ml. Two volumes of acetone were added, and the precipitate was collected by centrifugation, redissolved in warm methanol, and again centrifuged to eliminate impurities. Reprecipitation by addition of acetone was followed by drying in vacuo. Five-tenth ml amounts of methanol solution containing 0.05 mg/ml of phosphatide from each strain of organism and 0.05 mg/ml of ovolecithin were distributed in brown ampules which were sealed and sterilized in the same manner as the TME buffer and kaolin suspension.

A saline emulsion of phosphatide was used to treat the kaolin suspension. The saline suspension contained 7.5 gamma of phosphatide/ml. One part of phosphatide methanol solution was added dropwise by means of a 1.0 ml pipette to 19 parts of buffered saline previously placed

in a flask and under constant agitation by means of a magnetic stirrer.

Preparation and sensitization of kaolin: Kaolin obtained from Takahashi (1962) had been treated with 5% HCl and kept under constant agitation by means of a magnetic stirrer for 24 hr. The acid was removed and the kaolin washed repeatedly with deionized water. The kaolin-water suspension was filtered, and the kaolin was dried in a hot air oven at approximately 120 C and stored at room temperature. Approximately 10.0 g of kaolin was heated in a crucible at 800±10 C 2 hr. After cooling the kaolin was mechanically pulverized 2 hr in a porcelain mortar, 20 ml of H_2O was added, and the suspension pulverized for 30 min. Sufficient water was added to yield a thick suspension which was then distributed in equal amounts into five 15 x 120 mm tubes. Water was added to the tubes to a height of 10 cm, and the mixture allowed to stand 15 to 20 hr at room temperature. The supernatant fluid was transferred to a centrifuge tube and centrifuged for 15 min at 1240 X G. The supernatant fluid was decanted and discarded. The precipitate was resuspended in water, 1.0 mg/ml. The kaolin suspension was dispensed into glass ampules, 6.0 ml/ampule, and sterilized by the same method as the TME buffer and phosphatide-methanol antigen.

To sensitize the kaolin, one part of the standard kaolin suspension was added to two parts of the saline

suspension of antigen. The mixture was shaken thoroughly and incubated at 37 C for 30 min, during which time it was shaken every 15 min. The sensitized kaolin suspension was used only on the day of preparation.

Inactivation and dilutions of serums: Serums were inactivated by chemical inactivation with EDTA (disodium ethylene diaminotetracetate) (Eastman Organic Chemicals). EDTA contained in the TME buffered saline used for the dilution of serums was sufficient to inactivate the complement.

Beginning with initial dilutions of 1:10 and 1:20, twofold serial dilutions were made in TME buffered saline in the same manner as described for the hemagglutination test.

Kaolin-phosphatide (KP) agglutination test: A modification of the technique of Takahashi (1962) was followed. The sensitized kaolin suspension was added to each of the serum dilution and control tubes, 0.1 ml per tube. The rack of tubes was thoroughly shaken and incubated 24 hr in a 37 C incubator in a plastic bag with a piece of dampened cheesecloth. Agglutination was observed with a concave magnifying mirror with the aid of a 60-watt bulb. The reciprocal of the highest dilution of serum which caused agglutination was recorded as the KP titer.

Preparation of experimental phosphatide extracts: Phosphatide fractions were extracted from the following strains of organisms: M. bovis, M. avium, and M. phlei;

human origin P4 and P8 (Group I), P15 (Group II), P39 (Group III), and RIV (Group IV); Group III of animal origin, 186C-1 and 172C_1-1 (porcine), 51C-0 (bovine), 266-1 (semen); and a Group III of soil origin, X9. With the exception of the M. bovis strain, grown in Dubos broth with 0.5% dextrose, all organisms were grown in Modified Sauton's Medium. The Modified Sauton's Medium was prepared according to the following formula and dispensed in 50.0 ml amounts in 250 ml Erlermeyer flasks.

Asparagine	6.00 g
Citric acid	2.00 g
K_2HPO_4	1.50 g
Ferric ammonium citrate	0.05 g
$MgSO_4$	0.25 g
Glycerol	30.00 ml

The ingredients were dissolved in distilled water, and the solution adjusted with concentrated NH_4OH to pH 6.2. Water was added to a final volume of one liter. The solution was sterilized at 121 C, 15 min. An 80% glucose solution, slightly acidified with citric acid, was sterilized by filtration and 12.5 ml added aseptically to the first solution.

The kaolin-phosphatide reagents and test were completed as previously described with the following exceptions:

The Seitz-filtered methanol solution was evaporated in a Buchler flash-evaporator under vacuum. Nitrogen was introduced into the evaporator and flask containing the methanol solution to remove the residual oxygen present. The final product was air dried and stored in the freezer at -20 C until used at which time it was dissolved in

methanol at a concentration of 0.15 mg/ml of methanol. Extracts prepared from M. avium, 186C-1, and 51C-0 evaporated to approximately 0.25 ml prior to sealing of ampules.

The oven-dried kaolin was heated in a furnace at 700 C 2 hr, and the heated kaolin was ground as finely as possible using an agate mortar and pestle. It was then suspended in a 15 x 120 mm test tube, mixed and allowed to settle overnight (Takahashi and Ono, 1958).

RESULTS

Preliminary determinations of the HA test. Serum titers were two to fourfold higher when incubated for 2 hr at 37 C, room temperature for 2 hr and overnight at 4 C than when incubated for 2 hr at 37 C and overnight at 4 C; room temperature for 2 hr and overnight at 4 C; 1 hr at 37 C and overnight at 4 C; or overnight at 4 C. Readings were made at the end of each incubation period, i.e., 1 hr, 2 hr, 4 hr, and the following morning.

When mammalian OT, avian OT, PPD-S, and Weybridge PPD (mammalian and avian) were used as antigens, mammalian OT yielded the highest titers with the positive control serum.

Sheep erythrocytes collected in Alsever's solution in our laboratory were less fragile than those commercially obtained.

Serum HA and KP titers of uninoculated animals. Hemagglutination tests of serums collected from the calves and swine were not always zero. Calves 5, 13, 15, 16, 17, 20, 36, 40, 43, 44, 45, 48, 50, 53, 71, 74, 75, and Swine 2-4, 2-3, 5-1, 5-2, 5-3, 6-1, 6-3, 7-1, and 7-2 had serum HA titers ranging from less than 5 to 20. Titers of 20 or less were considered negative. Four calves, 60, 67, 70, and 79, had pre-inoculation HA serum titers greater than 20.

Calves 1 through 25 (Lot I). Serial HA serum titers of

23 calves inoculated with M. bovis, M. avium, Group III organisms (bovine origin), Group III organisms (porcine origin), pseudochrome organisms, and Group IV organisms are presented in Table 2. Since the serums on which HA tests were performed were collected at different intervals from different calves after the calves were inoculated, serial titers are not always comparable.

Initial or pre-inoculation and final HA serum titers, initial and final KP serum titers, and histopathologic findings are presented in Table 3.

The following results are presented in groups inoculated with a given type of organism. All calves had negative HA serum titers prior to inpculation with organisms. All calves had negative KP serum titers except calves 14 and 15. Calves 14 and 15 had pre-inoculation KP serum titers of 40 and 80 respectively.

Calves inoculated with M. bovis: Calf 6 (inoculated with culture 130-0 which produced a local ulcer, disseminated lesions, progressive disease) had HA serum titers greater than 20 after inoculation except the 21st day, at which time the titer was 20. The maximum HA serum titer was 80. The final KP serum titer was 80.

Calf 20 (inoculated with culture 81-0 which produced a local ulcer, disseminated lesions, progressive disease) had HA serum titers of 32 or greater 37 days after inoculation with the culture. The maximum HA serum titer was 64.

TABLE 2. Serial tuberculopolysaccharide specific hemagglutination (HA) serum titers of calves (1-25) inoculated with mycobacteria

Culture number	50B-0[1]	51C-0[1]	62D-0[1]	68C-0[1]	B124F-0[2]	*M. bovis*
Calf number	1	2	3	4	5	6
Days[3]						
0	-	-	20	-	5	-
7	64	80	40	-	-	80
14	128	80	40	5	-	80
21	32	40		10	-	20
28	64	80	20	10	-	80
35	32	40	10	10	-	
36						40
42	128	80	20	10	-	
54			10			
57	128	80		10	-	
63	256					
64		80				
72					-	
76				10		

[1]Group III--bovine origin.
[2]Group IV--bovine skin lesion origin.
[3]Days after inoculation of calves intradermally on the forelegs, 1.0 mg and 0.1 mg wet weight at each of two sites per each amount.

TABLE 2.--Continued

Culture number	*M. bovis*	*M. avium*	71C-0[1]	B78B-0[1]	107E-0[1]	93C-0[5]	152A_1-1[5]
Calf number	20	21	7	10	13	16	19
Days[4]							
0	16	-	-	-	20	5	-
30	16	80	10		20	80	5
37	64	20	20	80	10	80	10
45	64		20	80	10	40	-
52	64	80	20	80	10	20	10
59	32	80	5	80	-	10	
70	64	80	10		10	10	10
87					5		
88	64						
93			10				
95				80			
99							
100							
101						10	
107		80					10

[4]Days after inoculation of calves intradermally on the foreleg with 1.0 mg wet weight at one site.

[5]Group III--swine.

TABLE 2.--Continued

Culture number	B117B-0[2]	4F-1[2]	M7F-1[2]	128F-0[6]	52H-1[6]	61B-1[6]	112B-0[6]
Calf number	19	9	18	8	11	14	17
Days[4]							
0	-		-	-	-	-	10
30	5	10	-	10	10	-	40
37	10	20	-	20	20	20	80
45	-	20	-	10	40	5	80
52	10	20	-	20	20	20	20
59		20	-	-	40	10	20
70	10	10	-	-	20	10	20
87		-					
91		20					
92				-			
94					80		
100						-	
101							40
107	10						
108			-				

[6]Pseudochrome mycobacteria.

TABLE 2.--Continued

Culture number	$172C_1$-1[5]	173C-1[5]	B254F-1[2]
Calf number	23	24	25
Days[4]			
0	-	-	-
6	-	-	-
24	-	-	10
46	-	-	20
55	-	-	-
62	-	-	-
67	-	-	-
69		-	5

TABLE 3. Hemagglutination (HA) and kaolin-phosphatide (KP) serum titers and pathology reports of calves (1-25) inoculated[1] with mycobacteria

Culture type	Culture number	Calf number	HA titer		KP titer		Pathology[2] report	Progressive[3] disease
			Initial	Final	Initial	Final		
M. bovis	130-0	6	-	40	-	40	Gen	+
	81-0	20	16	64	-	160	Gen	+
M. avium	Lab strain	21	-	80	20	40	F & A	-
III-bovine origin	50B-0	1	-	256	-	40	Gen	-
	51C-0	2	-	80	-	160	Gen	+
	62D-0	3	20	10	-	40	Gen	-
	68C-0	4	-	40	-	-	Gen	-
	71C-0	7	-	10	-	80	PC	-
	B78B-0	10	-	80	-	10	NSL	-
	107E-0	13	20	20	20	-	NSL	-

[1]Inoculated intradermally on the forelegs, 1.0 mg and 0.1 mg wet weight at each of two sites/each amount.

[2]Pathology report: Gen = generalized lesions anywhere beyond the primary complex; PC = lesions in the lymph node draining the inoculation site; NSL = no gross or microscopic lesions or only a granuloma at the site of inoculation; F & A = lesions at site of inoculation and in head lymph nodes only; A & C = lesions in head and mesenteric lymph nodes only.

[3]Progressive disease: + = generalized disease; ± = histopathology inconclusive for disease progression; - = encapsulation of lesion if present.

TABLE 3.--Continued

Culture type	Culture number	Calf number	HA titer Initial	HA titer Final	KP titer Initial	KP titer Final	Pathology[2] report	Progressive[3] disease
III-Swine	93C-0	16	5	10	10	10	PC	-
	$152A_1$-1	19	-	10	-	10	PC	+
	$172C_1$-1	23	-	-	10	10	NSL	-
	173C-1	24	-	-	10	20	NSL	-
Pseudo-chrome	128F-0	8	-	20	-	10	A & C	±
	52H-1	11	-	20	-	-	NSL	-
	61B-1	14	-	10	40	20	NSL	-
	112B-0	17	10	40	10	10	NSL	-
IV	B124F-0	5	-	20	-	-	NSL	-
	4F-1	9		20	-	-	NSL	-
	B117B-0	15	5	20	80	80	NSL	-
	M7F-1	18	-	-	10	20	NSL	-
	B254F-1	25	-	5	20	10	NSL	-

The final KP serum titer was 160.

Calf inoculated with M. avium: Calf 21 (inoculated with a laboratory strain of M. avium which produced a local ulcer, and lesions in the cervical lymph nodes, no progressive disease) had HA serum titers of 80 after inoculation except 37 days at which time the titer was 20. The final KP serum titer was 40.

Calves inoculated with Group III (bovine origin) mycobacteria: Calves 1, 2, 3, 4, 7, 10, and 13 (inoculated with Group III organisms of bovine origin) varied widely in serologic and histopathologic responses. The results are presented for each calf in numerical sequence.

Calf 1 (inoculated with 50B-0 which produced a local ulcer, lesions in the pool of the anterior and posterior mediastinal and left and right bronchial lymph nodes, no progressive disease) had HA serum titers of 32 or greater after inoculation of the organism. The maximum HA serum titer was 40. The final KP serum titer was 40.

Calf 2 (inoculated with 51C-0 which produced a local ulcer, disseminated lesions in many lymph nodes, progressive disease) had HA serum titers of 40 or greater after inoculation of the organism. The maximum titer was 80. The final KP serum titer was 160.

Calf 3 (inoculated with 62D-0 which produced a local ulcer, lesions in the right popliteal, right internal inguinal, and left prescapular lymph nodes, no progressive disease)

had maximum HA serum titers of 40 which occurred at 14 and 21 days after inoculation of the organism. Subsequent titers were negative. The final KP serum titer was 40.

Calf 4 (inoculated with 68C-0 which produced a local ulcer, lesions in the pool of the anterior and posterior mediastinal and right and left bronchial lymph nodes, no progressive disease) had negative HA serum titers except the final serum. The final HA serum titer was 40. The final KP serum titer was negative.

Calf 7 (inoculated with 71C-0 which produced a local ulcer, lesions in the left prescapular lymph node, no progressive disease) had only negative HA serum titers. The final KP serum titer was 80.

Calf 10 (inoculated with B78B-0 which did not produce lesions or disease) had HA serum titers which were 80 at all times. The final KP serum titer was negative.

Calf 13 (inoculated with 107E-0 which did not produce lesions or disease) had HA serum titers which were negative at all times.

Calves inoculated with Group III (porcine origin) mycobacteria: Serologic and histopathologic responses varied in calves 16, 19, 23, and 24 which were inoculated with Group III (porcine origin) organisms.

Calf 16 (inoculated with 93C-0 which produced a local ulcer, lesions in the left prescapular lymph node, no progressive disease) had HA serum titers of 80 at 30

and 37 days and of 40 at 40 and 45 days after inoculation. Subsequent HA serum titers were negative. The final KP serum titer was negative.

Calf 19 (inoculated with $152A_1$-1 which produced a local ulcer, lesions in the left and right medial retropharyngeal lymph nodes, progressive disease) had HA serum titers which were negative at all times after inoculation. The final KP serum titer was negative.

Calves 23 and 24 (inoculated with $172C_1$-1 and 173C-1 respectively which did not produce significant lesions or disease) had HA and KP serum titers which were negative at all times.

Calves inoculated with pseudochrome mycobacteria: In general, few or no lesions occurred in calves inoculated with the pseudochromes and serum titers were lower.

Calf 8 (inoculated with 128F-0 which produced lesions in the cervical lymph nodes and in the mesenteric lymph nodes, histopathologic evidence for disease progression inconclusive) had HA and KP serum titers which were negative at all times.

Calves 11, 14, and 17 (inoculated with 52H-1, 61B-1 and 112B-0 respectively which did not produce lesions or disease) had HA serum titers which varied. Calf 11 had HA serum titers of 40 at 45 and 59 days and of 80 at 94 days after inoculation. The remaining titers were negative. Calf 14 had only negative HA serum titers. Calf 17 had

HA serum titers of 40 at 30 and 101 days and of 80 at 37 and 45 days after inoculation. Other HA serum titers were negative. Final KP serum titers were all negative.

Calves inoculated with Group IV mycobacteria: Calves 5, 9, 15, 18, and 25 (inoculated with B124F-0, 4F-1, B117B-0, M7F-1, and 254F-1 respectively which produced no lesions or disease) had HA serum titers which were negative at all times.

Adult cows (Lot II). Serial HA titers of serums collected from cows 96, 97, 98, and 99 at one and two days prior and 1, 2, 9, and 10 days after administration of tuberculin, pre and post tuberculin KP serum titers and histopathologic findings, are presented in Table 4. Cows 96 and 97, obtained from a no-gross-lesion herd, did not have fourfold increases in HA serum titers after tuberculin testing. Cows 98 and 99, obtained from a gross-lesion herd, had fourfold increases in HA serum titers after tuberculin testing.

KP serum titers performed on one serum collected prior to injection of tuberculin and one serum collected after injection of tuberculin were similar to the HA serum titers. Cows 96 and 97 did not have fourfold increases, cows 98 and 99 did have fourfold increases.

At necropsy cows 96 and 97 did not have lesions or disease, and cows 98 and 99 did have lesions and disease.

Swine (Lots III and IV). Serial HA serum titers of 11 swine inoculated with M. bovis and Group III (porcine origin)

TABLE 4. Hemagglutination (HA) and kaolin-phosphatide (KP) serum titers and pathology reports of four cows before and after tuberculin testing

Cow number	96[1]	97[1]	98[2]	99[2]
Days[3]		HA titer		
-2	20	10	10	40
-1	20	5	20	80
0	20	20	20	40
3		20	20	160
4	20	10	20	
5	10	10	80	160
12	20		80	160
13	10	20	160	80
		KP titer		
-2	20	20	10	20
5	20	20	40	80
Pathology report[4]	NSL	NSL	Gen	Gen
Progressive disease[5]	-	-	+	+

[1]Tuberculin positive cow obtained from no-gross-lesion herd.
[2]Tuberculin positive cow obtained from gross-lesion herd.
[3]0 = day of caudal fold tuberculin test, 0.1 ml mammalian tuberculin.
[4]Pathology report: Gen = generalized lesions anywhere beyond the regional lymph nodes draining the inoculation site; NSL = no gross or microscopic lesions or only a granuloma at the site of inoculation.
[5]Progressive disease: + = generalized disease; - = encapsulation of lesion if present.

organisms, three swine inoculated with M. avium, three swine fed a Group III (porcine origin) organism, three swine fed M. bovis and two uninoculated swine are presented in Table 5.

The HA and KP serum titers were negative in all animals prior to the injection of organisms. After the injection of organisms, the HA serum titers of the swine were higher than the HA serum titers found in calves. Calves had relatively low HA serum titers and had little disease after inoculation with Group III (porcine origin) organisms. All of the swine inoculated with these organisms except 2-3 had lesions and HA serum titers which were at least 640 at some time during the testing period.

Since serial HA serum tests were performed at varying intervals of time, correlation between histopathology and HA and KP serum titers are presented on the basis of a fourfold increase in HA and KP serum titers after tuberculin testing. In general there was an increase in HA serum titers after tuberculin testing with decreases in titers during the intervals between tuberculin tests.

Pre and post tuberculin HA serum titers, pre and post tuberculin KP serum titers, and histopathologic findings of 14 swine from Lot III, pre and post tuberculin HA serum titers, histopathologic findings of eight swine from Lot III and two swine, which did not have serial HA serum titers, inoculated with Group III (inanimate origin) organisms from Lot IV, are presented in Table 6.

TABLE 5. Tuberculopolysaccharide specific hemagglutination (HA) serum titers of swine inoculated with mycobacteria

Culture No.	*M. bovis*			$172C_1$-1[1]		167C-1	
Swine No.	2-1	2-2	2-4	1-1	1-4	1-3	3-4
Days[2]							
0	-	-	20	-	-	-	-
8	40	20	20	20	40	20	40
15	-	10	10	-	20	20	20
22	-	10	5	20	20	20	20
35[3]	40	20	10	20		20	160
38	80	320		20	80		80
44	160	20	320	320	640	640	640
52	160	160	160	640	160	320	1280
57		320					640
65	160				320		
78			80	160		80	
92			40	80		320	
99[3]			80	160		160	
100			20	80		160	
101			80	40		80	
102			20	20		40	
108						320	
112			320	640			

[1]Where species is not indicated, organisms are atypical mycobacteria, Group III organisms (swine).
[2]Days after inoculation of swine intradermally on the foreleg with 2.0 mg wet weight of *M. bovis* and Group III organisms (swine).
[3]Days after inoculation swine were tuberculin tested.

TABLE 5.--Continued

Culture number	193C$_2$-1		186C-1			Uninoculated controls	
Swine number	1-6	2-3	3-2	3-2		4-1	4-2
Days[2]					Days		
0	-	10	-	-	0	10	10
8	10	20	80	20	8	10	20
15	-	10	80	10	19	-	-
22	10	10	5	10	32	40	20
35[3]	10	10		10	47	-	-
38	40	20	10	20	61[3]	40	5
44	640	320	640	640	70	40	20
52	1280	320	640		105	80	40
65		160			117	40	10
66				80	131[3]	80	80
78	160		80		140	80	80
92	160		40		161	80	80
99[3]	160		80		181	80	80
100	160		40		196	40	40
101	80		40		208[3]	40	20
102	80		80		216	80	80
108			160				
113	1280						

TABLE 5.--Continued

Culture number	M. avium		
Swine number	5-1	5-2	5-3
Days[4]			
0	10	5	20
8	40	640	10
19	10	80	20
32	20	160	20
47	10	80	10
61[3]	20	80	20
70	1280	1280	640
77			160
105	160	160	
117	80	80	
131[3]	160	80	
138		5120	
140	1280		
161	320		
181	160		
196	40		
208[3]	80		
216	640		

[4]Days after inoculation of swine intradermally on the foreleg with 2.0 mg wet weight of M. avium at one site.

TABLE 5.--Continued

Culture number	172C$_1$-1			*M. bovis*		
Swine number	6-1	6-2	6-3	7-1	7-2	7-3
Days[5]						
0	5	-	10	10	5	-
22	40	20	20	80	80	80
36	10	5	20	40	40	160
59	20	20	40	10	20	40
72[3]	40	80	1280	320	80	160
78	1280	640	1280	640	1280	320
92		640	2560		320	320
108		160	320		160	320
136[6]		40			160	
143			640			1280
168			640			320
177			320			160
196[3]			160			160
204			320			1280

[5]Days after swine were fed Group III organism (porcine origin) and *M. bovis*.
[6]Swine were tuberculin tested 3 days previously.

TABLE 6. Hemagglutination (HA) and kaolin-phosphatide (KP) serum titers and pathology reports of swine inoculated with mycobacteria

Culture type	Culture number	Swine number	Days PI[1]	HA titer Pre[2]	HA titer Post[2]	KP titer Pre	KP titer Post	Pathology report[3]	Progressive disease[4]
M. bovis (intradermally)	81-0	2-1	35	40	160	-	80	Gen	+
		2-2	35	20	160	-	80	Gen	+
		2-4	35	10	320	20	160	Gen	+
			99	80	320				
III-Swine (intradermally)	$172C_1$-1	1-1	35	20	320	10	640	PC	+
			99	160	640				
		1-4	35	20	640	40	320	Gen	+
	167C-1	1-3	35	20	640	40	320	Gen	+
			99	160	320				
		3-4	35	160	640	20	160	Gen	+

[1] = Days post inoculation swine were tuberculin tested.
[2] Pre = serum collected on day of tuberculin testing prior to injection of tuberculin; Post = serum collected 7-14 days post tuberculin testing.
[3] Pathology report: Gen = generalized lesions anywhere beyond the primary complex; PC = lesions in the lymph node draining the inoculation site; NSL = no gross or microscopic lesions or only a granuloma at the site of inoculation.
[4] Progressive disease: + = generalized disease; ± = histopathology inconclusive for disease progression; - = encapsulation of lesion if present.

TABLE 6.--Continued

Culture type	Culture number	Swine number	Days PI[1]	HA titer Pre[2]	HA titer Post[2]	KP titer Pre	KP titer Post	Pathology report[3]	Progressive disease[4]
	$193C_2$-1	1-6	35	160	640	80	640	PC	+
			99	160	1280				
		2-3	35	10	320	20	320	PC	+
	186C-1	3-1	35	5	640	80	640	PC	+
			99	80	160				
		3-2	35	10	640	20	640	PC	+
M. avium (intradermally)	Lab	5-1	61	20	1280	20	160	PC	-
			130	80	1280				
			208	80	640				
		5-2	61	80	1280	20	160	PC	-
			130	80	5120				
		5-3	61	20	640	10	80	PC	-
Swine III (orally administered)	$172C_1$-1	6-1	72	40	1280			PC	+
		6-2	72	80	640			PC	+
			133	160	40				
		6-3	72	1280	1280			Gen	+

TABLE 6.--Continued

Culture type	Culture number	Swine number	Days[1] PI	HA titer Pre[2]	HA titer Post[2]	KP titer Pre	KP titer Post	Pathology[3] report	Progressive[4] disease
			133	320	640				
			196	160	320				
M. bovis (orally administered)	81-0	7-1	72	320	640			Gen	+
		7-2	72	80	1280			Gen	+
		7-3	72	40	160			Gen	+
			133	320	1280				
			196	160	1280				
III-pen floor (intradermally)	15W	161-3	48	-	-			NSL	-
	19_2W	161-4	48	5	20			NSL	-
Controls		4-1	61	40	40			NSL	-
			130	80	80				
			208	40	80				
		4-2	61	5	20			NSL	-
			130	10	80				
			208	20	80				

Swine inoculated ID with M. bovis: Swine 2-1, 2-2, and 2-4 (inoculated with 81-0 which produced disseminated lesions, progressive disease) had fourfold increases in HA serum titers after a single tuberculin test. Swine 2-4, tuberculin tested two times, had fourfold increases in HA serum titers after both tests. All three swine had fourfold increases in KP serum titers after tuberculin testing.

Swine fed M. bovis: Swine 7-2 and 7-3 (fed 81-0 which produced disseminated lesions, progressive disease) had fourfold increases in HA serum titers after a single tuberculin test. Swine 7-3, tuberculin tested three times, had fourfold increases in HA serum titers after all three tests.

Swine 7-1 (fed 81-0 which produced disseminated lesions, progressive disease) did not have a fourfold increase in HA serum titer after a single tuberculin test.

Swine inoculated ID with M. avium: Swine 5-2 and 5-3 (inoculated with a laboratory strain of M. avium which produced lesions in the prescapular lymph node, no progressive disease) had fourfold increases in HA serum titers after both of two tuberculin tests. Swine 5-1 (inoculated with a laboratory strain of M. avium which produced lesions in prescapular lymph node, no progressive disease) had a fourfold increase in HA serum titer after one tuberculin test. All three swine had fourfold increases in KP serum titers after tuberculin testing.

Swine inoculated ID with Group III (porcine origin) mycobacteria: Swine 1-1 and 1-4 (inoculated with $172C_1$-1 which produced lesions in prescapular lymph node, progressive disease in Swine 1-1, and disseminated lesions, progressive disease in 1-4) had fourfold increases in HA serum titers after tuberculin tests. Swine 1-4 had one tuberculin test, Swine 1-1 had two tuberculin tests.

Swine 1-3 and 3-4 (inoculated with 167C-1 which produced disseminated lesions, progressive disease) had fourfold increases in HA serum titers after one tuberculin test. Swine 1-3, which had two tuberculin tests, did not have a fourfold increase after the second test.

Swine 1-6 and 2-3 (inoculated with $193C_2$-1 which produced lesions in the prescapular lymph node, progressive disease) had fourfold increases in HA serum titers after tuberculin tests. Swine 1-6 had two tuberculin tests, and Swine 2-3 had one tuberculin test.

Swine 3-1 and 3-2 (inoculated with 186C-1 which produced lesions in the prescapular lymph node, progressive disease) had fourfold increases in HA serum titers after one tuberculin test. Swine 3-1, which had two tuberculin tests, did not have a fourfold HA serum titer increase after the second tuberculin test.

Swine fed Group III (porcine origin) mycobacteria: Swine 6-1, 6-2, and 6-3 (fed $172C_1$-1 which produced disseminated lesions, progressive disease in Swine 6-3, lesions in prescapular lymph node, and progressive

disease in Swine 6-1 and 6-2) had one, two, and three tuberculin tests respectively. Swine 6-1, tuberculin tested once, had a fourfold HA serum titer increase. Swine 6-2, tuberculin tested twice, had a fourfold HA serum titer increase only after the first tuberculin test. Swine 6-3, tuberculin tested three times, did not have fourfold HA serum titer increases after any tuberculin test. Kaolin-phosphatide serum titers were not determined on these animals.

Swine inoculated ID with Group III (inanimate origin) mycobacteria: Swine 161-3 (inoculated with 15W, which did not produce lesions or disease) and 161-4 (inoculated with 19_2W, which did not produce lesions or disease) did not have fourfold increases in HA serum titers after one tuberculin test. These animals did not have KP serum titers determined.

Uninoculated control swine: Swine 4-1 and 4-2 (uninoculated controls) had three tuberculin tests. Swine 4-1 did not have fourfold increases in HA serum titers after any of the three tests. Swine 4-2 did not have a fourfold increase in HA serum titer after the first tuberculin test. There were fourfold increases after the second and third tests.

Calves 26 through 58 (Lot V). Pre and post tuberculin HA serum titers, pre and post tuberculin KP serum titers, and histopathologic findings of calves from Lot V are presented in Table 7.

TABLE 7. Hemagglutination (HA) and kaolin-phosphatide (KP) serum titers and pathology reports of calves (26-58) inoculated with mycobacteria

Culture type[1]	Culture number	Calf number	Days PI[2]	HA titer Pre[3]	HA titer Post[3]	KP titer Pre	KP titer Post	Pathology report[4]	Progressive disease[5]
M. bovis (killed)	81-0	26	46	-	160	-	20	NSL	-
M. avium (killed)	Lab	27	46	40	160	-	20	NSL	-
IV (killed)	B117B-0	28	46	-	20	-	20	NSL	-
III-Bovine origin (killed)	51C-0	29	46	-	80	-	20	NSL	-
III-Swine	$172C_1$-1	30	44	40	640	-	20	NSL	-
		31	44	40	160	-	20	NSL	-
	$193C_2$-1	32	44	-	1280	-	20	NSL	-
		33	44	-	160	-	20	NSL	-

[1]All calves inoculated intradermally in the foreleg with 1.0 mgm wet weight. Exceptions: Calf 47 and Calf 50 received 10.0 mgm.
[2]Days post inoculation calves were tuberculin tested.
[3]Pre = serum collected on day of tuberculin testing prior to injection of tuberculin; Post = serum collected 3 to 31 days post tuberculin testing.
[4]Pathology report: Gen = generalized lesions anywhere beyond the primary complex; PC = lesions in the lymph node draining the inoculation site; NSL = no gross or microscopic lesions or only a granuloma at the site of inoculation.
[5]Progressive disease: + = generalized disease; ± = histopathology inconclusive for disease progression; - = encapsulation of lesion if present.
[6]Organism reisolated from calf in Lot I.

TABLE 7.--Continued

Culture type[1]	Culture number	Calf number	Days PI[2]	HA titer Pre[3]	HA titer Post[3]	KP titer Pre	KP titer Post	Pathology report[4]	Progressive disease[5]
	186C-1	34	37	-	-	-	20	NSL	-
		36	48	-	80	-	20	NSL	-
	167C-1	35	48	40	320	-	20	NSL	-
		37	48	20	160	-	20	NSL	-
	$152A_1-1$[6]	41	48	-	80	-	20	NSL	-
III-Bovine origin	71C-0	38	48	-	20	-	20	NSL	-
	71C-0[6]	39	48	-	20	20	-	NSL	-
	107E-0[6]	40	48	40	40	20	20	NSL	-
	51C-0	42	52	20	160	-	20	PC	-
	51C-0[6]	43	52	160	160	20	20	Gen	±
	62D-0[6]	44	52	40	160	20	20	Gen	±
	68C-0[6]	45	52	80	640	-	20	Gen	+
III-Feed	A	46	42	160	160	20	20	NSL	-
	A	47	42	160	160	20	20	NSL	-
III-Soil	X37	48	42	20	40	-	20	NSL	-
	X41	49	42	20	20	20	20	NSL	-
	X41	50	42	20	80	20	-	NSL	-
IV	M7F-1	51	49	5	10			NSL	-
	B117B-0	52	49	40	5			NSL	-
	B254F-1	53	49	5	20			NSL	-

TABLE 7.--Continued

Culture type[1]	Culture number	Calf number	Days PI[2]	HA titer Pre[3]	HA titer Post[3]	KP titer Pre	KP titer Post	Pathology report[4]	Progressive disease[5]
	B254F-1[6]	54	49	80	20			NSL	-
Pseudo-chrome	61B-1	55	49	80	80			NSL	-
	128F-0	56	49	-	40			NSL	-
	52H-1	57	49	10	40			NSL	-
	52H-1[6]	58	49	5	10			NSL	-

Calves 26 through 50 had KP serum titers which were negative at all times. Kaolin-phosphatide serum titers were not determined for calves 51 through 58.

Calves inoculated with heat-killed mycobacteria: Calves 26, 27, 28, and 29 (inoculated with heat-killed cultures of M. bovis (81C-0), M. avium (laboratory strain), a Group III of bovine origin (51C-0), and a Group IV (117B-0), which had been used to inoculate calves in Lot I) did not have lesions or disease. Calves 26, 27, and 29 had fourfold increases in HA serum titers after tuberculin testing. Calf 28 did not have a fourfold increase.

Calves inoculated with Group III (bovine origin) mycobacteria: Calves 38, 39, 43, 44, and 45, which were inoculated with organisms reisolated from calves 7, 13, 2, 3, and 4 respectively had the following results:

Calf 45 (inoculated with 68C-0 which produced disseminated lesions, progressive disease) had a fourfold HA serum titer increase after tuberculin testing.

Calf 43 (inoculated with 51C-0 which produced disseminated lesions, histopathologic evidence inconclusive for disease progression) did not have a fourfold increase in HA serum titer after tuberculin testing.

Calf 44 (inoculated with 62D-0 which produced lesions in left prescapular lymph node, skin at site of inoculation, left and right bronchial lymph nodes, and anterior and posterior mediastinal lymph nodes, histopathology

inconclusive for disease progression) had a fourfold increase in HA serum titer after tuberculin testing.

Calf 39 (inoculated with 71C-0 which did not produce lesions or disease) did not have a fourfold increase in HA serum titer after tuberculin testing.

Calf 40 (inoculated with 107E-0 which did not produce lesions or disease) did not have a fourfold increase in HA serum titer after tuberculin testing.

Calf 38 (inoculated with 71C-0 which did not produce lesions or disease) did not have a fourfold increase in HA serum titer after tuberculin testing.

Calves inoculated with Group III (porcine origin) mycobacteria: Calves 30 through 37, inoculated with $172C_1$-1, $193C_2$-1, 186C-1, 167C-1, two calves/culture, and Calf 41, inoculated with $152A_1$-1, had no lesions or disease. Eight of the nine calves had fourfold increases in HA serum titers after tuberculin testing. Calf 34, which was inoculated with $172C_1$-1, the same organism with which Calf 36 was inoculated, did not have a fourfold increase in HA serum titer after tuberculin testing.

Calves inoculated with Group III (inanimate origin) mycobacteria: Five calves, inoculated with Group III organisms from soil and feed, did not have significant lesions or disease.

Calves 46 and 47 (inoculated with a Group III organism isolated from feed, 1.0 and 10.0 mg respectively) did

not have fourfold increases in HA serum titers after tuberculin testing.

Calves 48 and 49 (inoculated with 1.0 mg soil isolants X37 and X41 respectively) did not have fourfold increases in HA serum titers after tuberculin testing.

Calf 50 (inoculated with 10.0 mg feed isolant X41) had a fourfold increase in HA serum titer after tuberculin testing.

Calves inoculated with pseudochrome mycobacteria: Four calves inoculated with pseudochromes did not have significant lesions or disease.

Calf 55 (inoculated with 61B-1) and Calf 58 (inoculated with 52H-1, reisolated from Calf 11 in Lot I) did not have fourfold increases in HA serum titers after tuberculin testing.

Calves 56 and 57 (inoculated with 128F-0 and 52H-1 respectively) did have fourfold increases in HA serum titers after tuberculin testing.

Calves inoculated with Group IV mycobacteria: Four calves, inoculated with Group IV organisms did not have significant lesions or disease.

Calves 51, 52, and 53 (inoculated with M7F-1, B117B-0, and B254F-1 respectively) and Calf 54 (inoculated with B254F-1 reisolated from Calf 25 in Lot I) did not have fourfold increases in HA serum titers after tuberculin testing.

Calves inoculated intradermally (ID) (Lot VI). Pre and

post tuberculin titers and histopathologic findings from 10 calves inoculated with Group III organisms (bovine origin) 51C-0, 68C-0, and 50B-0 are presented in Table 8.

Calves 59, 60, 61, and 62 inoculated with 51C-0: Calf 61 (which had disseminated lesions, histopathologic evidence inconclusive for disease progression) had a fourfold HA serum titer increase after a single tuberculin test. Calf 60 (which had disseminated lesions, histopathologic evidence inconclusive for disease progression) had fourfold HA serum titer increases after both of two tuberculin tests. Calf 59 (which had disseminated lesions, no progressive disease) had fourfold HA serum titer increases after three tuberculin tests. Calf 62 died with generalized lesions and progressive disease prior to tuberculin testing.

Calves 63, 64, and 65 inoculated with 68C-0: Calf 65 died before collection of the post tuberculin serum with disseminated lesions and progressive disease. Calf 63 (which had disseminated lesions, progressive disease) had a fourfold HA serum titer increase after one tuberculin test. Calf 64 (which had disseminated lesions, no progressive disease) had fourfold HA serum titer increases after only the first and third of three tuberculin tests.

Calves 66, 67, and 68 inoculated with 50B-0: Calves 66, 67, and 68 (which did not have significant lesions or disease) had varying HA serum titer results after tuberculin testing.

TABLE 8. Hemagglutination (HA) serum titers and pathology reports of calves inoculated intradermally (ID), intra-uterine (IU) or exposed to an aerosol (AE) of Group III mycobacteria of bovine origin

Route	Culture number	Calf number	Days[1] PI	HA titer Pre[2]	HA titer Post[2]	Pathology[3] report	Progressive[4] disease
I.D.	51C-0	62	none			Gen	+
		61	46	-	640	Gen	±
		60	46	80	640	Gen	±
			102	40	320		
		59	46	-	80	Gen	-
			102	40	160		
			165	40	320		
	68C-0	63	46	40	160	Gen	+
		65	46	40		Gen	+
		64	46	40	160	PC	-

[1]Days post inoculation animals were tuberculin tested.
[2]Pre = Serum collected on day of tuberculin testing prior to injection of tuberculin; Post = Serum collected 7-14 days post tuberculin testing.
[3]Pathology report: Gen = generalized lesions anywhere beyond the primary complex; PC = lesions in the lymph node draining the inoculation site; NSL = no gross or microscopic lesions or only a granuloma at the site of inoculation; D Node = prescapular lymph node only; C Node = mesenteric lymph node only.
[4]Progressive disease: + = generalized disease; ± histopathology inconclusive for disease progression; - = encapsulation of lesion if present.

TABLE 8.--Continued

Route	Culture number	Calf number	Days[1] PI	HA titer Pre[2]	HA titer Post[2]	Pathology[3] report	Progressive[4] disease
			102	160	160		
			165	40	160		
	50B-0	68	46	40	160	NSL	-
		67	46	-	80	NSL	-
			102	160	640		
		66	46	40	160	NSL	-
			102	160	160		
			165	160	320		
I.U.	51C-0	74	45	20	80	PC	+
		75	45	40	320	Gen	+
			108	160	640		
		77	45	20	40	Gen	+
			108	40	160		
			165	160	160		
	68C-0	73	45	-	160	Gen	+
		70	45	40	80	Gen	+
			108	80	160		
		71	45	40	320	Gen	+
			108	160	640		
			165	640	1280		

TABLE 8.--Continued

Route	Culture number	Calf number	Days[1] PI	HA titer Pre[2]	HA titer Post[2]	Pathology[3] report	Progressive[4] disease
	50B-0	78	45	-	80	D Node	-
		79	45	40	40	C Node	-
			108	40	80		
		80	45	20	40	NSL	-
			108	40	160		
			165	40	160		
A.E.	51C-0	90	none			PC	+
		84	41	20	-	Gen	+
		91	41	20	20	Gen	+
	68C-0	82	42	-	80	Gen	+
		83	42	80	40	Gen	+
		86	42	40	160	Gen	+
	50B-0	89	43	-	40	NSL	-
		87	43	-	20	NSL	-
			91	-	-		
		88	43	-	-	NSL	-
			91	-	-		
			174	-	40		

Calf 66 had a fourfold HA serum titer increase only after the first of three tuberculin tests. Calf 67 had fourfold HA serum titer increases after two tuberculin tests. Calf 68 had a fourfold HA serum titer increase after one tuberculin test.

Heifers inoculated intrauterine (IU) Lot VII). Pre and post tuberculin HA titers of serums and histopathologic findings of nine heifers, inoculated with Group III organisms (bovine origin) 51C-0, 68C-0, and 50B-0 are presented in Table 8.

Heifers 74, 75, and 77 inoculated with 51C-0: Heifer 74 (which produced lesions in the mesenteric nodes, uterus, and intestine, progressive disease) had a fourfold HA serum titer increase after one tuberculin test. Heifer 75 (which had disseminated lesions, progressive disease) had fourfold HA serum titer increases after two tuberculin tests. Heifer 77 (which had disseminated lesions, progressive disease) had a fourfold HA serum titer increase only after the second of three tuberculin tests.

Heifers 70, 71, and 73 inoculated with 68C-0: Heifers 70, 71, and 73 (which produced disseminated lesions, progressive disease) had varying HA serum titer increases after tuberculin testing.

Heifer 70 had less than fourfold HA serum titer increases after two tuberculin tests. Heifer 71 had fourfold HA serum titer increases only after the first and second

of three tuberculin tests. Heifer 73 had a fourfold HA serum titer increase after one tuberculin test.

Heifers 78, 79, and 80 inoculated with 50B-0: Heifer 78 (which had a microscopic lesion in the body lymph node, no progressive disease) had a fourfold serum HA titer increase after one tuberculin test. Heifer 79 (which had a microscopic lesion in the mesenteric lymph nodes, no progressive disease) did not have fourfold HA serum titer increases after two tuberculin tests. Heifer 80 (which did not have significant lesions or disease) had fourfold HA serum titer increases after the second and third of three tuberculin tests.

Calves of aerosol exposure (AE) (Lot VIII). Pre and post tuberculin HA titers of serums and histopathologic findings from nine calves, inoculated with Group III organisms (bovine origin) 51C-0, 68C-0, and 50B-0, are presented in Table 8.

Calves 84, 90, and 91 inoculated with 51C-0: Calf 90 died prior to collection of the post tuberculin serum. Calf 84 (which had disseminated lesions, progressive disease) and Calf 91 (which had lesions in the right and left bronchial lymph nodes, and anterior mediastinal lymph nodes, progressive disease) did not have fourfold HA serum titer increases after one tuberculin test.

Calves 82, 83, and 86 inoculated with 68C-0: All three calves had disseminated lesions with progressive disease. Calves 82 and 86 had fourfold HA serum titer increases after

one tuberculin test. Calf 83 did not have a fourfold HA serum titer increase after one tuberculin test.

Calves 87, 88, and 89 inoculated with 50B-0: Significant lesions or disease was not produced in any of these calves. Calf 89 had a fourfold HA serum titer increase after one tuberculin test. Calf 87 did not have fourfold HA serum titer increases after two tuberculin tests. Calf 88 had a fourfold HA serum titer increase only after the third of three tuberculin tests.

Kaolin-phosphatide serum titers of calves and swine inoculated with mycobacteria using phosphatides extracted from various mycobacteria. Preliminary titrations were performed with reagents prepared in this laboratory and with Takahashi's reagents using serums from a cow inoculated with M. bovis and a pig inoculated with M. avium. Comparable results were obtained with all combinations of reagents. Sample results are shown in Table 9.

Kaolin-phosphatide titers of serums from four swine and five calves which were tested with phosphatide extracts of the following strains of organisms, M. avium, M. bovis, M. phlei, 186C-1, $172C_1$-1, 266-1, P39, P4, P8, P15, X9, and RIV, are presented in Table 10.

Serums from Swine 10-5 and 10-6, which had been inoculated with M. avium (206-2) had the following results: KP serum titers of Swine 10-5 were equal to or greater than 80 with six of the 13 extracts. Titers of 40 were obtained with two of the extracts. Titers with the remaining five

TABLE 9. Comparison of kaolin-phosphatide (KP) serum titers using Takahashi's reagents and prepared reagents

Reagents	Bovine serum	Swine serum
T Kaolin*		
T Saline	160	160
T Antigen		
R Kaolin**		
T Saline	160	160
T Antigen		
R Kaolin		
R Saline	160	160
T Antigen		
T Kaolin		
R Saline	160	160
T Antigen		
R Kaolin		
R Saline		40
R *M.avium* Antigen		
T Kaolin		
R Saline		40
R *M. avium* Antigen		

*T = Takahashi.
**R = Robinson.

TABLE 10. Kaolin-phosphatide (KP) serum titers of calves and swine inoculated with mycobacteria using phosphatides extracted from various mycobacteria

Culture type	*M. bovis*			*M. avium*			III		
Culture number	81-0			Lab	206-2		$172C_1$-1	51C-0	50B-0
Animal number	2-4	20	81	21	10-5	10-6	6-1	59	66
Phosphatide:	KP titer								
Takahashi[1]	160	160		40	160	80			
M. bovis	80	-	80	-	20	20	10	80	40
M. avium	-	-	20	10	10	20	-	20	20
186C-0-Swine III	-	-	20	20	20	-	40	40	40
$172C_1$-1-Swine III	-	10	10	-	-	-	-	10	10
51C-0-Bovine III	-	80	80	-	20	-	-	80	-
266-1-Semen III	-	-	20	10	40	-	10	-	-
P39-Human III	80	40	20	40	80	80	40	10	10
X9-Soil III	80	40	40	40	80	80	40	40	80

[1]Takahashi antigen prepared from *M. bovis* (BCG) and *M. tuberculosis* (strains H37Rv and Nakano).

TABLE 10.--Continued

Culture type	*M. bovis*			*M. avium*			III		
Culture number	81-0			Lab	206-2		$172C_1$-1	51C-0	50B-0
Animal number	2-4	20	81	21	10-5	10-6	6-1	59	66
Phosphatide:	KP titer								
P4-Human I	80	40	20	40	80	80	40	10	10
P8-Human I	80	80	80	20	80	80	80	80	20
P15-Human II	-	80	80	20	40	10	10	10	10
M. phlei-saprophyte	80	10	20	20	80	40	40	20	10
RIV-Human IV	80	80	80	20	80	80	40	80	40

extracts were negative. A titer of 160 was obtained with Takahashi's antigen. The KP serum titer with the homologous M. avium extract was only 10. Swine 10-6 had KP serum titers equal to or greater than 80 with five of the extracts and Takahashi's antigen. Titers with the seven remaining extracts were negative. The KP serum titer with the homologous M. avium extract was 20.

Serum from Swine 6-1, which had been fed $172C_1$-1, had a KP titer of 80 with the P8 extract, titers of 40 with six extracts, and negative titers with six extracts. The KP serum titer with the homologous extract was negative.

Serum from Swine 4-2, inoculated with M. bovis (81-0), had titers equal to or greater than 80 with the homologous M. bovis extract and six other extracts. Titers with the six remaining extracts were negative. With Takahashi's antigen the KP serum titer was 160.

Calf 21, inoculated with M. avium, had KP serum titers of 40 with three of the extracts and with Takahashi's antigen. The KP serum titer with the homologous M. avium extract was 20, with the other nine extracts negative.

Calf 20, inoculated with M. bovis (81-0), and Calf 81, inoculated subcutaneously with M. bovis (81-0), had KP serum titers of 80 with three and four extracts respectively. Calf 20 had a KP serum titer of 20 with the homologous extract. Calf 81 had a KP serum titer of 80 with the homologous extract. Serum from Calf 20 had KP serum

titers of 40 with three extracts and negative titers with seven extracts. Serum from Calf 81 had a KP serum titer of 40 with one extract and negative titers with eight extracts. A titer of 160 was obtained for serum from Calf 20 with the Takahashi antigen. The test with serum from Calf 81 was not performed with the Takahashi antigen.

Calf 59, inoculated with 51C-0, had KP serum titers of 80 with the homologous extract and three other extracts, 40 with two extracts and negative titers with seven extracts. Calf 66, inoculated with 50B-0, had a KP serum titer of 80 with one extract, 40 with two extracts, and negative titers with 10 extracts. The KP serum titer with the homologous extract was negative.

Kaolin-phosphatide serum titers of calves 30 through 50 inoculated with mycobacteria. Kaolin-phosphatide titers of serums from calves 30 through 50 with phosphatides extracts prepared from cultures of M. bovis, M. avium, 186C-0, $152C_1$-1, 51C-0, 266-1, P39, X9, and Takahashi's antigen are presented in Table 11.

Maximum KP serum titers equal to or greater than 160 were obtained for serum from Calf 36 with extract prepared from 266-1 (semen origin) and for serum from Calf 44 with extract prepared from X9 (soil origin). These organisms were identified as avirulent Group III organisms. Calf 36 had been inoculated with a Group III (porcine origin) organism (186C-1), which did not produce significant lesions or disease. Calf 44 had been inoculated with a Group III

TABLE 11. Kaolin-phosphatide (KP) serum titers of calves inoculated with mycobacteria using phosphatides extracted from various mycobacteria

Culture type	Culture number	Calf number	Phosphatide preparation								
			T[1]	M. bovis	M. avium	186C-1	172C$_1$-1	51C-0	266-1[2]	P39[3]	X9[4]
III-Swine	172C$_1$-1	30		20	-	20	20	20	80	80	20
		31	20	20	-	20	20	-	80	80	40
	193C$_2$-1	32	20	20	20	20	20	-	40	20	80
		33	20	20	-	20	20	-	40	40	40
	186C-1	34	20	40	40	40	40	40	40	40	40
	167C$_1$-1	35	20	20	20	20	20	-	-	80	40
	186C-1	36	20	20	-	-	20	-	160	80	40
	167C$_1$-1	37	20	20	-	-	20	20	20	40	-
	152A$_1$-1[5]	41	20	40	-	20	-	20	40	40	20

[1]Takahashi antigen prepared from M. bovis and M. tuberculosis (strains H37Rv and Nakano).
[2]Group III--semen origin.
[3]Group III--human origin.
[4]Group III--soil origin.
[5]Organism reisolated from calf in Lot I.

TABLE 11.--Continued

Culture type	Culture number	Calf number	Phosphatide preparation								
			T[1]	M. bovis	M. avium	186C-1	172C$_1$-1	51C-0	266-1[2]	P39[3]	X9[4]
III-Bovine origin	71C-0	38	20	40	20	20	20	-	20	20	20
	71C-0[5]	39	20	40	20	-	-	20	20	20	-
	107E-0[5]	40	20	40	20	-	-	20	20	20	-
	51C-0	42	20	-	-	20	20	20	20	80	20
	51C-0[5]	43	20	20	20	20	20	20	20	80	40
	62D-0[5]	44	20	-	20	20	20	20	20	80	160
	68C-0[5]	45	20	80	-	40	20	20	20	80	80
III-Feed	A	46	20	20	20	20	20	20	40	40	20
	A	47	20	20	20	20	20	40	20	80	80
III-Soil	X37	48	20	20	20	20	-	20	-	40	-
	X4	49	20	20	20	20	20	20	20	20	20
	X4	50	20	20	-	-	-	-	20	-	40

organism (bovine origin) (62D-0), which produced generalized lesions with inconclusive evidence of disease progression.

Five calves from calves 30 through 37 and 41, inoculated with Group III organisms (porcine origin), had maximum KP serum titers of 80 with two extracts, P39 and X9. With P39 extract, calves 30, 31, 35, and 36 had KP serum titers of 80. With X9, Calf 32 had a KP serum titer of 80. With P39, calves 33, 37, and 41 had KP serum titers of 40. With X9, calves 31, 33, 35, and 36 had a KP serum titer of 40. With M. bovis extract, Calf 41 had a KP serum titer of 40. Calf 34 had KP serum titers of 40 with all extracts. Kaolin-phosphatide serum titers were negative with all other extracts.

Five calves from calves 38 through 40 and 42 through 45 had maximum serum titers of 80 with M. bovis, P39 and X9 extracts. With P39 extract, calves 42, 43, and 45 had KP serum titers of 80. With M. bovis and X9 extracts, Calf 45 had KP serum titers of 80. Calf 45 had a KP serum titer of 40 with 186C-1 extract. Kaolin-phosphatide serum titers with the remaining extracts were negative.

One calf from calves 46 through 50, inoculated with Group III organisms (soil and feed origin), had maximum KP serum titers of 80 with P39 and X9 extracts. Calf 47 had KP serum titers of 80. With 51C-0, Calf 47 had a KP serum titer of 40. With 266-1, Calf 46 had a KP serum titer of 40. With P39, calves 46 and 48 had KP serum titers of 40,

and with X9, Calf 50 had a KP serum titer of 40. Kaolin-phosphatide titers with the remaining extracts were negative.

In general, the specificity or diagnostic value of the KP test was not increased by the use of extracts from the organisms used.

DISCUSSION

At the beginning of the research project on bovine tuberculosis in 1960, serums were collected from cattle which were found to be sensitive to tuberculin during routine field testing. At the time of slaughter, tissues were collected for bacteriologic and histopathologic examination. The serums were examined by numerous tests which included complement fixation, hemagglutination tests for polysaccharide specific and protein specific antibodies, the hemolytic modification of the hemagglutination test, the tube, ring and gel diffusion tests for precipitins, and inhibition of mycobacterial growth. No apparent correlation existed among the results of the serologic tests, the results of the tuberculin tests, the presence or absence of gross or microscopic lesions, or the kind of mycobacteria isolated from the tissues. It had been hoped that some test would indicate if the sensitivity, detected by the tuberculin, was induced by M. bovis or other mycobacteria, or that a test would differentiate between gross-lesion and no-gross-lesion cattle. Neither the tuberculin test nor the serologic tests differentiated between infections with M. bovis or other mycobacteria as evidenced by the isolation of the atypical mycobacteria from gross-lesion and no-gross-lesion cattle.

Mycobacterium bovis, *M. avium* and atypical mycobacteria were administered by various routes to cattle and swine. Serums were collected from the animals and tested for tuberculopolysaccharide and tuberculophosphatide specific antibodies. However, any evaluation of the results must encompass a number of factors whose influence on the results is not known. Some of the factors are: a) the uncertainty of the role of the atypical mycobacteria in tuberculosis or tuberculosis-like disease, b) the inability to differentiate conclusively infection, progressive or non-progressive disease, or delayed sensitivity, c) the biologic variability of animals in their responses to foreign substances such as mycobacteria, d) serologic effects of tuberculin testing, e) collection of serums and f) determinations of "normal" titer and "meaningful" titer. Each point will be discussed briefly.

Although atypical mycobacteria have been recognized for many years (Griffith, 1916; Beaven and Bayne-Jones, 1931), they had been regarded largely as inconsequential saprophytes. The incidence of infection and disease caused by *M. tuberculosis* has decreased. Increasingly, the atypical mycobacteria have been isolated from cases of human disease indistinguishable from tuberculosis.

The habitat and mode of transmission of the atypical mycobacteria have not been established. They are commonly found in the soil and are often isolated from the

upper respiratory tract of man. Organisms that were classified as Group III organisms have been isolated from tissues from gross-lesion and no-gross-lesion cattle. Some of these organisms experimentally produce disease similar to tuberculosis. Others may produce little or no disease.

The tuberculin test has been used to detect delayed sensitivity induced by the pathogenic mycobacteria but it does not differentiate infection, progressive or non-progressive disease. Takahashi, et al. (1961b) detected tuberculopolysaccharide specific antibodies in active and closed cases of tuberculosis. They were of no prognostic or diagnostic value. On the other hand, tuberculophosphatide specific antibodies were present only in active cases. If the latter were found to be true in cattle, some tuberculin positive cattle might not need to be slaughtered.

Repeated tuberculin tests may cause local sensitization. Tuberculin testing at the same site elicited an "intermediate" reaction (reaction time intermediate of an immediate and a delayed sensitivity) in tuberculin negative individuals (Freebe and Mount, 1963; Knight, et al., 1963). Some reactions were sufficiently severe and persistent that they would have been erroneously interpreted as a positive reaction if observed only at 48 or 72 hr after administration of tuberculin.

The variation in responses to an antigenic stimulus may vary within and between species of animals and within

any one animal. However, certain patterns may be apparent if the results are compared on the basis of lots of animals and the type of organism used for experimental inoculation.

There are few reports concerning the effects of tuberculin on the results of serologic tests for tuberculosis. Smith and Scott (1950) reported that tuberculin testing caused a change in the antipolysaccharide titer of tuberculin positive healthy individuals. Serums from cows from gross-lesion herds had increases in antipolysaccharide and antiphosphatide titers after the administration of tuberculin, as noted in Results.

Another factor which may vary results of serologic tests is the collection of blood and the handling of the serum. Blood was collected from animals at weekly intervals, at two weekly intervals, and from some only before and after tuberculin testing. Blood from calves 26 through 58 was collected after tuberculin testing, but the days varied from 3 to 31 days rather than the desired 7 to 14 days. Although there are differences in the number of days post-inoculation of organisms for the collection of serum, the collection dates for any one lot of cattle (except Lot I) and swine are constant. Results should be examined from each lot of animals rather than all animals together.

According to Middlebrook (1950b), a human serum titer of 8 or higher was presumptive evidence of disease due to Mycobacterium sp. Rabe and Spicer (1953) considered

a "significantly" elevated titer to be 64. Undiluted serums from many of the cattle and swine caused agglutination of sensitized erythrocytes. Of the 120 animals studied only four calves, 60, 67, 70 and 79, had pre-inoculation titers of more than 20. Therefore, titers of 20 or less were considered negative.

There appeared to be a correlation of HA and KP serum titers and the pathogenicity of strains of M. bovis, M. avium and the atypical mycobacteria in calves from Lot I. There was an increase in HA and KP serum titers of calves in which gross lesions with histopathologic evidence of disease was found at necropsy.

When there was no evidence of disease, there generally was no increase in HA and KP serum titers. The calves inoculated with Group IV organisms did not respond with increased titers at any time. Only one calf inoculated with a pseudochrome had a HA serum titer above 20. All of these calves (except Calf 8) had no lesions at necropsy. Calf 8 had lesions with histopathologic results inconclusive for progressive disease.

When HA and KP serum titers were determined for serums from adult cows (Lot II), the titers did not clearly distinguish between the animals obtained from gross-lesion and no-gross-lesion herds. However, a fourfold or greater increase in titer occurred after administration of tuberculin in both tests in the cows from the gross-lesion herd.

No increase in titer occurred in the cows from the no-gross-lesion herd. The fourfold increase in serum titers was present at 5 through 13 days after administration of tuberculin. It is not known how long the titers remained elevated. The cows from the gross-lesion herd had lesions and progressive disease at necropsy, and the cows from the no-gross-lesion herd had no lesions. An anamnestic-like response was elicited by the tuberculin in the gross-lesion animals. Subsequently, serums collected before and after tuberculin testing from the other experimental animals were examined to determine if the anamnestic-like response could indicate if tuberculin positive cattle had active tuberculosis.

It was not and is not known what fraction of the tuberculin elicits the anamnestic-like response, or how specific the response may be. Tuberculin is generally reported as non-antigenic. There are conflicting reports in the literature of desensitization and of sensitization with single, multiple, or repeated tuberculin tests. Tuberculin is a filtrate from heat-killed cultures. It contains proteins, polysaccharides, and any other substances elaborated by the organism which diffuse into the medium during the organism's growth and those which may be released from the organism during heat inactivation. Although denatured to a degree during heating, the proteins retain enough of their original characteristics to elicit a delayed

type skin reaction in a sensitized individual or animal. Some degree of specificity is present since tuberculin prepared from mammalian strains generally elicits a greater response in an animal sensitized with M. bovis than with M. avium.

It is also not known whether the anamnestic-like response is the direct or indirect effect of tuberculin. It may be that other substances can also cause increases in polysaccharide specific antibodies.

The occurrence of the anamnestic-like response is supported by the histopathologic evidence from calves inoculated with atypical mycobacteria. The calves were euthanized two months after inoculation. When the calves were killed soon after the 24 hr reading of the intradermal tuberculin test, the lymph nodes were readily palpated due to their enlargement and firmness. As the interval between the tuberculin test and necropsy increased, the lymph nodes were less easily found. Histologic observations indicated the enlarged firm lymph nodes had numerous cortical follicles containing many mitotic figures. Plasma cells were plentiful in the medullary sinuses. As the time period between the tuberculin test and necropsy increased, there was a progressive reduction in the number of mitotic figures seen in the follicles. The histologic changes occurred in most of the lymph nodes throughout the body whether they drained the site of tuberculin injection or not. Lymph nodes of

calves of the same age which had not been inoculated with mycobacteria had few or sometimes no follicles in the lymph nodes. The histologic and serologic changes noted previously support that an anamnestic-like reaction is elicited by tuberculin.

A test requiring two serum samples is never as desirable as a single serum test. However, no single unequivocably diagnostic test for tuberculosis exists. The increase in titer in the gross-lesion cows after tuberculin testing and the lack of an equal response in the no-gross-lesion cows justified an investigation of serums from calves and swine.

Calves from Lot V which were inoculated with Group III organisms (bovine origin), a Group III organism (porcine origin), a pseudochrome, and a Group IV organism reisolated from calves in Lot I had results similar to the results of the calves from Lot I.

When Group III organisms (bovine origin) were reisolated from calves and injected into other calves, the disease produced by the reisolants differed in three of five calves from that caused by the original cultures. The HA and KP serum titers did not reflect the increase or decrease in virulence of the cultures. Calf 45, which was inoculated with 68C-0 reisolated from Calf 4, had progressive lesions, whereas Calf 4 had had non-progressive lesions. Calf 44, which was inoculated with 62D-0 reisolated

from Calf 3, had lesions with inconclusive evidence for progressive disease. Calf 3 had had lesions which were not progressive. Calf 42, which was inoculated with 51C-0 reisolated from Calf 2, had lesions which were not progressive, whereas Calf 2 had had lesions which were progressive. Calf 39, which was inoculated with 71C-0 reisolated from Calf 7, and Calf 40, which was inoculated with 107E-0 reisolated from Calf 13, did not have lesions. Calf 7 had had lesions in the prescapular lymph node which were not progressive, and Calf 13 had had no lesions. Calf 41, which was inoculated with a Group III organism (porcine origin) ($152A_1$-1) reisolated from Calf 19, did not have lesions. Calf 19 had had a skin lesion at the inoculation site and progressive lesions in the right and left medial retropharyngeal lymph nodes. Increases in HA and KP serum titers varied in calves from both lots.

Hemagglutination serum titers and histology were negative for Calf 54, which was inoculated with the pseudochrome (52H-1) reisolated from Calf 11. This was true also of Calf 58, which was inoculated with the Group IV organism (B254F-1) reisolated from Calf 25. Passage through an experimental animal did not detectably increase the virulence of these two organisms and the serologic tests were negative.

The HA and KP serum titers of calves from Lots I and V, which were inoculated with Group III organisms (porcine origin), could not be correlated to the virulence of

the organisms. In general, the titers of the calf serums were lower than those of the swine, and the organisms did not produce lesions in calves. However, seven of eight calves in Lot V had fourfold increases in HA serum titers after tuberculin testing. Kaolin-phosphatide titers were negative.

Hemagglutination serum titers varied and KP serum titers were negative for the remaining calves in Lot V. The calves were inoculated with Group III organisms (bovine origin), Group III organisms (inanimate origin), pseudochrome, and Group IV organisms, and had no detectable lesions.

The lack of virulence of Group III organisms isolated from soil and other inanimate sources used in calves and swine support the occurrence of saprophytic, non-pathogenic Group III organisms. While they cannot be differentiated from other Group III organisms by growth or cytochemical tests, their virulence and ability to elicit antibodies differ. Further serologic studies may afford differentiation which is less expensive and more facile than animal inoculations.

Three strains of Group III organisms (bovine origin) were used for inoculums for cattle in each of Lots VI, VII and VIII. Regardless of the route of inoculation, culture 50B-0 caused no disease in any of the cattle. Six of the nine animals had the anamnestic-like fourfold or greater increase in HA serum titers after at least one

tuberculin test. Eight of nine animals, which were inoculated with 68C-0, had lesions which were progressive. Six had the anamnestic-like response. Of the 10 animals, which were inoculated with 51C-0, seven had lesions which were progressive and two had lesions with histopathologic evidence inconclusive for progressive disease. Six had the anamnestic-like response in HA serum titer. Of the four calves in Lot VII which were inoculated with 51C-0 (except Calf 62 which died prior to tuberculin testing), all had fourfold increases in HA serum titers after tuberculin testing. However, as the time was increased between inoculation and necropsy, there was histologic evidence that suggested the disease was regressing. It would have been pertinent if these calves could have been examined over a longer period of time to determine if phosphatide specific antibody decreased as disease activity decreased, as Takahashi, et al. (1961a,b) reported in studies on human patients with tuberculosis and on experimental animals. This reemphasizes the uncertainty of degree of pathogenicity of the atypical mycobacteria and differences in what may occur in natural and experimental infections.

The route by which the organisms enter may alter the degree or kind of response. The calves which were exposed to organisms by aerosol did not have as great HA serum titer increases following administration of tuberculin as did the calves inoculated intradermally or into the uterus.

The number of organisms which entered the respiratory tract, in what area they lodged, and what size of droplet or number of organisms in a particular droplet size is not known. All would influence the probability of infection, disease, and sensitivity. The calves inoculated intradermally had greater HA serum titer increases than the calves which had organisms introduced into the uterus.

The route of inoculation caused little or no difference in serologic response in swine whether they were inoculated intradermally or fed cultures. All of the swine which had lesions had increased HA serum titers after tuberculin testing.

When heat-killed M. bovis, M. avium, a Group III organism (bovine origin) and a Group IV organism were injected into calves, three of the four calves had fourfold increases in HA serum titers after tuberculin testing. Calves 26, 27, and 29, which were inoculated with M. bovis (81-0), M. avium (lab strain), and the Group III organism (51C-0) respectively, had the fourfold increase in HA serum titers. Calf 28, which was inoculated with the Group IV organism (B117B-0), did not have an increase. None of these calves had lesions at necropsy. Calves inoculated with the same organisms which were not heat-killed had similar serologic results. The three calves inoculated with M. bovis, M. avium, and the Group III organism had lesions and tuberculopolysaccharide specific antibodies. The calf inoculated

with the Group IV organism did not have a positive HA titer or lesions. The volume of cells injected was constant. Factors which contribute to virulence and to elicitation of antibody are associated with the M. bovis, M. avium, and Group III organism, but not the Group IV organism. When cultures are heated, the virulence factors obviously are destroyed. The antibody eliciting factors are not destroyed. The relatively small amounts of living or heat-killed organisms injected elicited antibodies. The latter was not dependent upon multiplication of the organism in vivo.

Swine had fourfold or greater increases in HA and KP serum titers when lesions were present. Swine which were inoculated with Group III organisms (inanimate origin) or uninoculated swine did not have HA serum titer increases or lesions, with the exception of one uninoculated animal which had fourfold increases after the second and third of three tuberculin tests. The effect of repeated tuberculin tests has not been determined. The fourfold increase after tuberculin testing clearly and consistently occurred in infected and diseased animals. The results of the swine, which were inoculated with Group III organisms (inanimate origin), support the evidence from the calves that these organisms are probably saprophytes.

The question arises as to the degree of specificity of the anamnestic-like response elicited by tuberculin.

If it were entirely non-specific, more of the cattle and swine without lesions or progressive disease could be expected to respond. The results of the four cows in Lot II and the swine indicate that the tuberculin negative animal generally does not respond anamnestically to the tuberculin. The tuberculous animals respond more frequently. The lack of reaction in some of the experimentally infected calves may not be due to lack of reaction but lack of a method to detect the reaction. If other substances are elaborated, some may interfere with detection of the antibodies involved. There is some evidence to support this interference. First, Siebert (1960) proposed a theory of tuberculoimmunity in which there are interactions of tuberculopolysaccharides, free and cell-bound, with the polysaccharide specific antibodies and lysozyme. Second, Turcotte, Freedman and Sehon (1963) reported separation by column chromatography of an $alpha_2$ serum fraction containing tuberculoprotein specific antibodies from another fraction which also contained tuberculoprotein specific antibodies. The former fraction was present only in patients with active disease; the latter was present in all tuberculin positive individuals. Third, other studies have indicated that bovine serum is more difficult to separate and characterize than swine serum.

In general, HA and KP serum titers and histopathology varied in calves inoculated with Group III organisms (bovine

origin). These organisms are heterogenous as measured by infectivity tests on calves. When the significance of these organisms to bovine tuberculosis and tuberculin sensitivity is known, the results of the serologic tests may have more meaning than they appear to have now.

Takahashi's report that phosphatide specific antibodies in humans were a reliable indication of active tuberculosis was not found to be true of bovine disease induced by mycobacteria. Further, phosphatides extracted from different strains did not increase the value of the test. While phosphatides are not credited with the specificity or potential diversity of protein, there have been some differences in the phosphatides obtained from different mycobacteria (Crowle, 1958). Due to the exceedingly slow growth of most of the mycobacteria, a great deal of time is necessary to obtain the volume of cells required for extraction. This impedes pilot or preliminary studies.

There appears to be little specificity associated with the phosphatide extract reactions with serums from four swine and five calves inoculated with M. bovis, M. avium, and Group III organisms. Serums from swine and a calf which had been inoculated with M. avium had higher titers with other than the homologous extract. The same was true of the Group III extracts. Serums from two of three animals which were inoculated with M. bovis had serum titers of 80 with the homologous extract but they also had the same

serum titers with eight other phosphatide extracts. The titers of the serums from all nine animals were negative with the M. avium extract. Titers of 80 were obtained with six serums with the phosphatide extract from P8, a Group I organism (human origin). Titers of 80 were also obtained with seven serums with the phosphatide extract from RIV, a Group IV organism.

Many Group III organisms appear to be closely related to M. bovis. Others resemble M. avium. To determine if the phosphatides were detectably different in their serologic reactions, 8 of 13 phosphatide extracts, prepared from M. bovis, M. avium, and Group III organisms of bovine, porcine, human, and inanimate origin were used to determine KP serum titers of calves 30 through 50, which were inoculated with Group III organisms of bovine, porcine, and inanimate origin. The reactions were not differential for the organism from which the phosphatide was extracted or used as animal inoculum.

Higher serum titers were obtained with the phosphatide extract from P39, a Group III organism (human origin), than with any of the other extracts. Serums from two calves had titers of 160 or greater with one extract, but each had negative titers with homologous extracts. Calf 36, which was inoculated with 186C-0, and Calf 44, which was inoculated with 62D-0, had titers of 160 with 266-1 and X9 respectively. Culture 266-1 is a Group III organism isolated

from porcine semen. X9 is a Group III organism isolated from soil. Both are avirulent for experimental animals.

The preparation of phosphatide extracts from the organisms used does not increase the diagnostic value or specificity of the KP test.

A single hemagglutination test or phosphatide test, as used in this study, has little differential value. The relative amounts of antibodies as detected by these tests may be altered by an animal's anamnestic response to tuberculin. It may be possible that the anamnestic-like response can be utilized to obtain a higher level of antibodies in serums of tuberculin positive cattle with lesions. Fractionation of serums may reveal antibodies actually present in "negative" serums as measured by the present techniques. If one or both of the possibilities can be utilized to differentiate progressive and non-progressive tuberculosis or tuberculosis-like disease and sensitivity, and the need for differentiating is established, those animals that are of no public health hazard can be detected and need not be destroyed.

SUMMARY

Polysaccharide specific antibodies and phosphatide specific antibodies, as determined by the hemagglutination (HA) test and the kaolin-phosphatide (KP) test, were elicited in calves by experimental infections with Mycobacterium bovis and M. avium. At necropsy, the calves inoculated with M. bovis had lesions and progressive disease. The calf inoculated with M. avium had lesions and non-progressive disease.

Calves inoculated with pseudochrome and Group IV atypical mycobacteria did not have increased HA and KP serum titers. At necropsy, no lesions or disease were detected.

Calves inoculated with Group III atypical mycobacteria (bovine and porcine origin) had varying HA and KP serum titers. Group III organisms (bovine origin) had a range of virulence from none to that almost equal to M. bovis and those of porcine origin produced few or no lesions in calves. The HA and KP serum titers could not be consistently correlated with disease.

Two cows from a gross-lesion herd had fourfold or greater increases in HA and KP serum titers after administration of tuberculin. Two cows from a no-gross-lesion herd did not exhibit the anamnestic-like response. At necropsy the former cows had lesions and progressive disease,

the latter cows did not have lesions or disease.

Swine which were inoculated with M. bovis, M. avium, and Group III organisms (porcine origin) had lesions at necropsy and fourfold increases in HA and KP serum titers after administration of tuberculin. Swine which were inoculated with Group III organisms (inanimate origin) had no lesions at necropsy and did not have fourfold increases in HA serum titers after administration of tuberculin. Two uninoculated swine had no lesions or anamnestic-like reaction after the first tuberculin test.

Three of four calves, which were inoculated intradermally with heat-killed organisms, had fourfold increases in HA serum titers. None of the four calves had lesions or disease.

The route by which some of the Group III organisms (bovine origin) were administered to cattle altered the serologic response. When inoculated intradermally, all calves had a fourfold or greater increase after the first tuberculin test. The post-tuberculin titers were from 80 to 640.

When the three strains of Group III organisms were introduced into the uterus, four of the nine heifers did not have a fourfold increase after the first tuberculin test. Two of the heifers were in the lot inoculated with a relatively avirulent strain. This lot had lower titers in general than the other two lots, both inoculated with

virulent Group III organisms.

When the three strains of Group III mycobacteria were administered in an aerosol, the HA serum titers were considerably lower. The post tuberculin titers were from 0 to 160. There was a fourfold increase in only two animals.

Specific phosphatide extracts prepared from a representative group of known and atypical mycobacteria did not increase the specificity of the kaolin-phosphatide test.

Generally, swine and cattle with lesions exhibited the anamnestic-like response to the first tuberculin test. The anamnestic-like response in tuberculin positive animals may afford a means of insuring a higher level of antibodies. With some combination of more specific antigens or fractionation of serums a differential test may be devised.

LITERATURE CITED

Adcock, J., R. R. Haley, and W. N. Davey. 1951. The Middlebrook hemagglutination test for tuberculosis. An evaluation in normal subjects and in patients with tuberculosis. J. Lab. Clin. Med. 38:736-741.

Alvarez and Tavel. 1885. Rescherches sur le bacille de lustgasten. Arch. Physiol. Pathol. 6:303-308. (Cited by Mallmann, Mallmann, and Ray, 1962).

Anderson, H. W., and R. V. Platou. 1951. Middlebrook-Dubos hemagglutination reaction. Study of the test in children. Pediatrics. 84:498-505.

Anderson, R. J. 1941. Structural peculiarities of acid-fast bacterial lipids. Chem. Rev. 29:45-55.

Anderson, R. J., W. C. Lothrop, and M. M. Creighton. 1930. The chemistry of the lipids of tubercle bacilli. LIII. Studies on the phosphatide of the human tubercle bacillus. J. Biol. Chem. 125:299-308.

Anderson, R. J., R. L. Peck, and M. M. Creighton. 1940. The polysaccharide of the phosphatide obtained from cell residues in the preparation of tuberculin. J. Biol. Chem. 136:211-227.

Anderson, R. J., and E. G. Roberts. 1930. The chemistry of the lipids of tubercle bacilli. XIX. Concerning the composition of the phosphatide fraction isolated from the bovine type of tubercle bacilli. J. Biol. Chem. 89:599-610.

Beaven, P. W., and S. Bayne-Jones. 1931. Mycobacterium (Sp?), Ryan strain, isolated from pleural exudate. J. Inf. Dis. 49:399-409.

Boquet, A., and L. Negré. 1923. Sur les propriétés biologiques des lipoïdes du bacilli tuberculeux. Ann. Institt. Pasteur. 37:787-805. (Cited by Crowle, 1958).

Boyden, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exper. Med. 99:107-120.

Boyden, S. V., and M. E. Anderson. 1955. Agglutination of normal erythrocytes in mixtures of antibody and antigen, and haemolysis in the presence of complement. Brit. J. Exptl. Pathol. 36:162-170.

Boyden, S. V., and P. Grabar. 1954. Rôle des lipides dans la sensibilisation des érythrocytes par les constituants de la tuberculine. Ann. Institt. Pasteur. 87:257-267.

Boyden, S. V., and W. E. Suter. 1952. Stimulating effect of tuberculin upon production of circulating antibodies in guinea pigs infected with tubercle bacilli. J. Immunol. 68:577-589.

Chargaff, E., M. C. Pangborn, and R. J. Anderson. 1931. The chemistry of the lipids of tubercle bacilli. XXIII. Separation of the lipid fractions from the timothy bacillus. J. Biol. Chem. 90:45-55.

Chargaff, E., and W. Schaefer. 1935. A specific polysaccharide from the bacillus Calmette-Guerin (BCG). J. Biol. Chem. 112:393-405.

Choremis, C. B., and K. N. Anastasea. 1954. The application of the Middlebrook-Dubos hemagglutination test in tuberculosis of children. Amer. Rev. Tuberc. 70:139-148.

Cole, L. R., and V. R. Farrell. 1955. A method for coupling protein antigens to erythrocytes. I. Description of method. J. Exptl. Med. 102:631-645.

Coombs, R. R. A., and M. L. Fiset. 1954. Detection of complete and incomplete antibodies to egg albumin by means of a sheep red cell-egg albumin antigen unit. Brit. J. Exptl. Pathol. 35:472-477.

Coombs, R. R. A., A. N. Howard, and L. S. Mynors. 1953. A serologic procedure theoretically capable of detecting incomplete or non-precipitating antibodies to soluble protein antigens. Brit. J. Exptl. Pathol. 34:525-534.

Coombs, R. R. A., A. N. Howard, and F. Wild. 1952. Titration of antisera to soluble proteins on the basis of an agglutination reaction. Brit. J. Exptl. Pathol. 33:390-397.

Crowle, A. J. 1958. Immunizing constituents of the tubercle bacillus. Bact. Rev. 22:183-203.

Davidsohn, I. 1927. Heterophilic antigens and antibodies. Arch. Path. and Lab. Med. 4:776-806.

Davidsohn, I. 1929. Heterophilic antibodies in serum sickness. J. Immunol. 16:259-273.

Davidsohn, I. 1930. Further studies on heterophilic antibodies in serum sickness. J. Immunol. 18:31-49.

Davidsohn, I. 1933. Heterophilic antibodies in serum disease. Third Report. J. Infect. Dis. 53:219-229.

Davidsohn, I. 1937. Serologic diagnosis of infectious mononucleosis. J. Amer. Med. Assoc. 108:289-294.

Davidsohn, I., and B. Toharsky. 1940. The production of bacteriogenic hemagglutination. J. Inf. Dis. 67: 25-41.

Decastello, A., and A. Sturli. 1902. Uber die Isoagglutinine im Serum gesunder und kranker Menschen. Münch. Med. Woch. 49:1090-1095. (Cited by Wiener, 1939).

Feldman, W. H. 1943. A scheme for numerically recording of tuberculous changes in experimentally infected guinea pigs. Amer. Rev. Tuberc. 48:248-255.

Fisher, S. 1951. Antigenic relationships of erythrocyte adsorbable fractions of some mycobacteria. Aust. J. Exptl. Biol. Med. Sci. 29:1-8.

Fisher, S., and T. S. Gregory. 1951. Aust. Vet. J. 27: 25-34. (Cited by Middlebrook, 1954).

Fisher, S., and E. V. Keogh. 1950. Lysis by complement of erythrocytes which have adsorbed a bacterial component and its antibody. Nature. 165:248.

Fleming, J. W., E. H. Runyon, and M. M. Cummings. 1951. An evaluation of the hemagglutination test for tuberculosis. Amer. J. of Med. 10:704-710.

Freebe, S., and F. W. Mount. 1963. Evidence of booster effect in serial tuberculin testing. (Abst.) National Tuberculosis Association. Denver.

Friedenreich, V. 1930. The Thomsen hemagglutination phenomenon. Lenin and Munksgaard, Copenhagen, Denmark. (Cited by Neter, 1956).

Gernez-Rieux, C., and A. Tacquet. 1952. Prog. Explor. Tuberc. 5:66. (Cited by Middlebrook, 1954).

Gerstl, B., W. E. Davis, Jr., A. G. Hollander, M. Barbieri, and S. B. Weinstein. 1955. Detection of apparently absent circulating antibodies in tuberculous sera. Amer. Rev. Tuberc. 72:345-355.

Goyings, L. 1963. (Personal communication).

Griffith, A. S. 1916. Investigations of strains of tubercle bacilli derived from sputum. Lancet. 1:721.

Gruber, M., and H. E. Durham. 1896. Munchen Med. Wchnschr. 43:206. (Cited by Smith and Connant, 1960).

Haley, R. R., W. N. Davey, J. Adcock, and C. R. Owen. 1952. Hemagglutination reaction following BCG vaccination in human subjects. Amer. Rev. Tuberc. 66:58-62.

Hall, W. H., and R. E. Manion. 1951. Hemagglutinins and hemolysins for erythrocytes sensitized with tuberculin in pulmonary tuberculosis. J. of Clin. Invest. 30:1542-1546.

Hawk, P. B., B. L. Osser, and W. H. Summerson. 1947. Practical Physiological Chemistry. 12th edition. The Blakiston Company, Philadelphia and Toronto. pp. 87 and 254-255.

Hentel, W., and G. D. Guilbert. 1952. Results in the hemagglutination test for tuberculosis. J. Lab. Clin. Med. 39:426-428.

Hirst, G. K. 1941. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. Science. 94:22-23.

Hirst, G. K., and E. G. Pickels. 1942. A method for the titration of influenza hemagglutinins and influenza antibodies with the aid of a photoelectric densitometer. J. of Immunol. 45:273-283.

Hollander, A. G., M Frobisher, Jr., and K. Kalisch. 1953. Clinical evaluation of the hemagglutination reaction. Amer. Rev. Tuberc. 67:497-502.

Iland, C. N. 1951. The serology of polysaccharides from the tubercle bacillus. J. Pathol. & Bacteriol. 63:735-741.

Jones, F. S. 1927. Agglutination by precipitin. J. Exptl. Med. 46:303-314.

Keogh, E. V., E. A. North, and M. F. Warburton. 1947. Haemagglutinins of the Haemophilus group. Nature. 160:63.

Keogh, E. V., E. A. North, and M. F. Warburton. 1948. Adsorption of bacterial polysaccharides to erythrocytes. Nature. 161:687-688.

Kirby, W. M. M., J. M. Burnell, and B. O'Leary. 1951. Evaluation of the hemagglutination reaction in the diagnosis of active tuberculosis. Amer. Rev. Tuberc. 64:71-76.

Koch, R. 1882. Berl. Klin. Wschr. 19:221. (Cited by Mallmann, Mallmann, and Robinson, 1964).

Kraus, R., and S. Ludwig. 1902. Über bacteriohämagglutinine und antihamagglutine. Wien. Klin. Wochschr. 15:120-121. (Cited by Neter, 1956).

Knight, R. A., M. E. Kabakjian, H. W. Harris, and D. W. Lenin. 1963. An investigation of the influence of PPD-B hypersensitivity on the booster effect associated with multiple tuberculin tests with PPD-S. (Abst.) National Tuberculosis Association. Denver.

Landois. 1875. Die Transfusion des Blutes. Leipzig. (Cited by Wiener, 1939).

Landsteiner, K. 1900. Zentralbl. f. Bakteriol., Parasitenk. u. Infectionskrankh. 27:35. (Cited by Wiener, 1939).

Landsteiner, K. 1919. Bioch. Zeitschr. 93:117. (Cited by Schaefer, 1938).

Landsteiner, K. 1920. Bioch. Zeitschr. 104:280. (Cited by Schaefer, 1938).

McClelland, L., and R. Hare. 1941. The adsorption of influenza virus by red cells and a new method of measuring antibodies for influenza virus. Canad. Pub. Health J. 32:530-538. (Cited by Rivers and Horsfall, 1959).

McGavin, M. D. 1963. (Personal communication).

McGavin, M. D., and L. Goyings. 1963. (Personal communication).

Maillard, E. R. 1952. Further studies of a new serologic test in tuberculosis. Amer. J. Pub. Health. 42: 175-181.

Maillard, E. R., and F. J. Gagliardo. 1951. A new serologic test for tuberculosis. Preliminary report. Amer. Rev. Tuberc. 89:252-258.

Mallmann, V. H., W. L. Mallmann, and J. A. Ray. 1962. Classical and atypical pathogenic mycobacteria of human and animal origin. (Abst.) 8th Annual International Congress for Microbiology. Montreal.

Mallmann, V. H., W. L. Mallmann, and P. J. Robinson. 1964. Relationship of atypical bovine and porcine mycobacteria to those of human origin. Health Lab. Sci. 1:11-20.

Menzel, A. E. O., and M. Heidelberger. 1939. Specific and nonspecific cell polysaccharides of a bovine strain of tubercle bacillus. J. Biol. Chem. 127: 221-236.

Meyer, K., and A. Pic. 1936. De l'isolement des anticorps par fixation a un système adsorbant-antigène et dissociation consécutive. Ann. Instit. Pasteur. 56:401-412.

Meynell, G. G. 1954. The antigenic structure of Mycobacterium tuberculosis, var. hominis. Jour. Path. and Bact. 67:137-150.

Middlebrook, G. A. 1950a. A hemolytic modification of the hemagglutination test for antibodies against tubercle bacillus antigens. J. Clin. Invest. 29: 1480-1485.

Middlebrook, G. A. 1950b. The hemagglutination test in tuberculosis. Editorial. Amer. Rev. Tuberc. 62: 223-226.

Middlebrook, G. A. 1954. Laboratory aids to diagnosis and therapy. Ann. Rev. Med. 5:339-348.

Middlebrook, G. A., and R. J. Dubos. 1948. Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. J. Exptl. Med. 88: 521-528.

Mollov, M., and T. J. Kott. 1952. A comparative study of the hemagglutination test for antibodies and its hemolytic-modification in tuberculosis. Amer. Rev. Tuberc. 65:194-200.

Negré, L. 1952. Résistance antituberculeuse sans allergie conférée aux animaux de laboratoire par l'antigène méthylique. Étude de sa durée et de l'action préventive de ce produit associée à celle du BCG. Ann. Instit. Pasteur. 83:429-436. (Cited by Crowle, 1958).

Neter, E. 1956. Bacterial hemagglutination and hemolysis. Bact. Rev. 20:166-188.

Nichol, J. C., and H. F. Deutsch. 1948. Biophysical studies of blood plasma proteins. III. Separation of gamma globulin from the sera of various animals. J. Am. Chem. Soc. 70:80.

Paul, J. R., and W. W. Bunnell. 1932. Presence of heterophilic antibodies in infectious mononucleosis. Am. J. Med. Sci. 138:90-104.

Pressman, D., D. H. Campbell, and L. Pauling. 1942. The agglutination of intact azo-erythrocytes by antisera homologous to the attached groups. J. Immunol. 44:101-105.

Rabe, E. F., and W. S. Spicer, Jr. 1953. An evaluation of the Middlebrook-Dubos hemagglutination test. J. Lab. Clin. Med. 41:98-107.

Raffel, S. 1946. The relationship of acquired resistance, allergy, antibodies, and tissue reactivities to the components of the tubercle bacillus. Amer. Rev. Tuberc. 54:564-573.

Ray, J. A. 1963. (Personal communication).

Rheins, M., and J. R. Thurston. 1955. Additional tuberculous antibodies demonstrated with erythrocytes sensitized by successive adsorptions of old tuberculin and further studies on the adsorptive capacities of tanned cells. Amer. Rev. Tuberc. 79:210-217.

Rheins, M., J. R. Thurston, N. Newton, and M. C. Dodd. 1954. Evaluation of drop techniques for the detection of hemagglutinins and hemolysins in tuberculous sera. Amer. J. of Med. Tech. 20:254-258.

Richey, D. J., W. N. Mack, and H. J. Stafseth. 1954. The hemagglutination reaction in avian tuberculosis. Poultry Sci. 33:345-350.

Rivers, T. M., and F. L. Horsfall, Jr. 1959. Viral and Rickettsial Diseases of Man. 3rd edition. J. Lippincott, Philadelphia and Montreal. p. 105.

Rothbard, S. 1951. A serological test in tuberculosis. Experimental and clinical observations. Med. Clin. N. A. 35:603-614.

Runyon, E. H. 1959. Anonymous mycobacteria in pulmonary disease. Med. Clin. N. A. 43:273-290.

Schaefer, W. 1938. La méthode de l'inhibition spécifique appliquée a la séparation des anticorps antilipoïdiques et antipolyosidiques des sérums antituberculeux. Compt. Rend. Soc. Biol. 129:543-546.

Schaefer, W. 1940. Sur la séparation des anticorps lipoïdiques, polysaccharidiques et protéidiques des sérums tuberculeux au moyen de la réaction de l'inhibition spécifique. Ann. Institut. Pasteur. 64:301-315.

Schwartz, B., T. Mandelbaum, L. J. Spitz, P. P. Schmidt, and B. A. Battaglia. 1952. Comparative evaluation of the hemagglutination test and the hemolytic modification in tuberculosis. Amer. Rev. Tuberc. 66:594-600.

Scott, N. B., and D. T. Smith. 1950. A simple modification of the Middlebrook-Dubos hemagglutination test for serum antibodies to products of the tubercle bacilli. J. Lab. Clin. Med. 35:303-306.

Siebert, F. 1950. Constituents of mycobacteria. Ann. Rev. Micro. 4:35-52.

Siebert, F. 1958. The interplay of an immune substance with tuberculopolysaccharide and its antibody in tuberculosis. J. Infect. Dis. 103:52-60.

Singer, J. M., and C. M. Plotz. 1956a. The latex fixation test. I. Application to the serodiagnosis of rheumatoid arthritis. Amer. J. Med. 21:888-892.

Singer, J. M., and C. M. Plotz. 1956b. The latex fixation test. II. Results in rheumatoid arthritis. Amer. J. Med. 21:893-896.

Smith, D. T., and N. F. Connant. 1960. Zinnser Microbiology. 12th edition. Appleton-Century-Crofts, Inc., New York. p. 142.

Smith, D. T., and N. B. Scott. 1950. Clinical interpretation of the Middlebrook-Dubos hemagglutination test. Amer. Rev. Tuberc. 62:121-127.

Spain, D. M., W. G. Childress, and C. Rowe. 1952. The hemagglutination test in tuberculosis. Amer. J. Clin. Path. 22:86-88.

Sohier, R., I. Trimberger, and J. Juillard. 1952. Réaction d'hémagglutination type Middlebrook-Dubos réalisée avec les hématies humaines 0 et la nouvelle tuberculine I. P. 48. Ann. Instit. Pasteur. 79:347-350.

Stacey, M., and P. W. Kent. 1948. The polysaccharides of Mycobacterium tuberculosis. Adv. in Carb. Chem. 3:311-336.

Suto-Nagy, G. I., and R. J. Anderson. 1947. Chemistry of the lipids of tubercle bacilli. LXXV. Further studies on the polysaccharides of the phosphatides obtained from cell residues in the preparation of tuberculin. J. Biol. Chem. 171:749-765.

Takahashi, Y. 1962. Specific serum agglutination of kaolin particles sensitized with tubercle phosphatide and its clinical evaluation as a serodiagnostic test for tuberculosis. Amer. Rev. Respir. Dis. 85:708-719.

Takahashi, Y., and K. Adachi. 1960. Activité de la tuberculose pulmonaire humaines et réactions de kaolinoagglutination par le phosphatides du Mycobacterium tuberculosis. Compt. Rend. Soc. Biol. 154:1129-1131.

Takahashi, Y., and H. Hukae. 1958. Etude clinique de la réaction d'hemagglutination par le phosphatide du bacilli tuberculeux. Compt. Rend. Soc. Biol. 152: 690-692.

Takahashi, Y., K. Mochizuki, and Y. Nagayma. 1961b. The behavior of three different kinds of antibodies in tuberculosis: Antiprotein, antipolysaccharide, and antiphosphatide: II: Human tuberculosis. J. Exper. Med. 114:569-579.

Takahashi, Y., and K. Ono. 1958. Réactions d'hémagglutination par les phosphatides du Mycobacterium tuberculosis. Compt. Rend. Soc. Biol. 152:380-383.

Takahashi, Y., T. Onodera, and K. Yamamoto. 1961a. The behavior of three different kinds of antibodies in tuberculosis: Antiprotein, antipolysaccharide, and antiphosphatide: I: Experimental tuberculosis. J. Exper. Med. 114:555-567.

Tarshis, M. S., and A. W. Frisch. 1952a. Chromogenic acid-fast bacilli from human sources. I. Cultural studies. Amer. Rev. Tuberc. 65:278-288.

Tarshis, M. S., and A. W. Frisch. 1952b. Chromogenic acid-fast bacilli from human sources. II. Pathologic studies. Amer. Rev. Tuberc. 65:289-300.

Tarshis, M. S., and A. W. Frisch. 1952c. Chromogenic acid-fast bacilli from human sources. III. Hypersensitivity studies. Amer. Rev. Tuberc. 65:301-315.

Tennent, D. M., and D. W. Watson. 1942. Molecular kinetic analyses and serological specificities of polysaccharides isolated from filtrates of the human tubercle bacillus and related species. J. of Immunol. 45:179-186.

Thalhimer, W., and C. Rowe. 1951. A slide test modification of the hemagglutination test for antibodies against tubercle bacilli. Amer. Rev. Tuberc. 63: 667-671.

Thomsen, O. 1927. Ein vermehrungsfähiges Agens als Veränderer des isoagglutinatorischen Verhaltens der roten Blutkörperchen, eine bisher unbekannte Quelle der Fehlbestimmung. Z. Immunitatsforsch. 52:85-107. (Cited by Neter, 1956).

Thurston, J. R., M. S. Rheins, and T. Huziwara. 1956a. Serologic investigations of mycobacterial antigens. I. The specificities of aqueous and saline extracts of BCG, M. phlei, and H37Rv. Amer. Rev. Tuberc. 73:563-570.

Thurston, J. R., and M. S. Rheins. 1956b. Serologic investigations of mycobacterial antigens. II. Specificities of antigens obtained by successive extraction of BCG and M. phlei. Amer. Rev. Tuberc. 73:571-575.

Thurston, J. R., and M. S. Rheins. 1956c. Serologic investigations of mycobacterial antigens. III. Direct detection of homologous antibodies by BCG extract antigens in serums of rabbits injected with BCG and challenged with Mycobacterium bovis. Amer. Rev. Tuberc. 74:756-763.

Thurston, J. R., and M. S. Rheins. 1956d. Serologic investigations of mycobacterial antigens. IV. Further serologic characterization of BCG extract antigens. Amer. Rev. Tuberc. 74:764-772.

Turcotte, R., S. O. Freedman, and A. H. Sehon. 1963. A new hemagglutination procedure for the diagnosis of active tuberculosis. Amer. Rev. Respir. Dis. 88:725-728.

Turner, J. C. 1943. Development of cold agglutinins in atypical pneumonia. Nature. 151:419-420.

Wiener, A. 1939. Blood Groups and Blood Transfusion. 2nd edition. Charles C. Thomas. Springfield, Ill. pp. 1-2.

Wood, L. E., M. B. Buhler, and A. Pollak. 1956. Human infection with the "yellow" acid-fast organisms. Ann. Rev. Tuberc. 73:917-929.

Youmans, G. P. 1963. The pathogenic "atypical" mycobacteria. Ann. Rev. Micro. 17:473-491.