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Evaluation of some cell-mediated and humoral responses in dogs experimentally exposed to *Mycobacterium bovis*

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Cynthia Helen Kemper Thomson

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INTRODUCTION

Tuberculosis is one of the oldest and most widespread of the life-threatening infectious diseases still affecting human and animal populations throughout the world (17). Despite years of research, significant improvements in testing methods and effective treatments and vaccination procedures, complete eradication of this persistent disease has not occurred.

Dogs are susceptible to natural infection with Mycobacterium bovis, M. tuberculosis, M. avium and M. avium (MAIS) complex. Some have been infected with various other strains of mycobacteria (28,32,49,62,83,102). The prevalence of tuberculosis in dogs is rare (28,83). However, authorities hold the belief that pet animals possess the capability of transmitting the disease serving as a source of tuberculosis (49). Doubt arises as to whether dogs are truly infected with tuberculosis because the characteristic signs of the disease are not evident (83).

Diagnosing tuberculosis in the dog is sometimes difficult due to unreliable and inconsistent results of tests with dogs. Bacteriological examination is the preferred diagnostic method to confirm infection. Other procedures investigating canine immune responses include tuberculin skin testing, lymphocyte transformation, macrophage migration inhibition assay and also some reports of serological results (13).

Cell-mediated immune responses *in vivo* are characterized by type IV delayed hypersensitivity reactions. This type of hypersensitivity is mediated by cellular reactions that peak between 24-48 hours. The reaction is initiated when sensitized T-lymphocytes contact specific antigen causing these T-cells to undergo blast transformation and proliferation (7,46,57). Previously, tuberculin skin tests for DTH in the dog have been done in the pinna of the ear (89). The DTH test is commonly used to evaluate lymphocyte sensitivity. Sensitized lymphocytes are generally defined as lymphocytes from an animal immunized to the antigen stimulating sensitivity (88).

Lymphocyte blastogenic assay results have been associated with the presence of skin test hypersensitivity (57,66,75,90). The lymphocyte blastogenic assay is one of several widely used *in vitro* test for evaluation of cell-mediated responses (57,74). Antigen-specific induction of T-cell proliferation can be measured *in vitro* by ^{3}H-thymidine incorporation with the lymphocyte blastogenic assay. The lymphocyte blastogenic assay is commonly employed to evaluate lymphocyte responsiveness following specific antigenic stimulation.

Humoral immune responses can be studied by measuring the presence of specific antibodies to a specific antigen. In 1972, Engvall and Perlmann developed a highly sensitive technique for the serodiagnosis of infectious diseases (26).

The enzyme-linked immunosorbent assay (ELISA) procedure has been adapted for the serodiagnosis of mycobacterial diseases (24). A part of this study is designed to explore the use of the ELISA for detecting antibodies in the sera of dogs experimentally exposed to M. bovis.

Previous studies of cell-mediated immunity in the dog have been hampered by lack of assay methods (88). The tests described herein are used to detect mycobacterial infections in various animal species. However, these tests have seldom been used to screen dogs for mycobacterial exposure.

The purpose of this study was to develop and evaluate diagnostic tests for detecting responses in the dog to heat-killed M. bovis. Benefits of the present investigation include 1) a study of DTH reactions in dogs using tuberculin injections 2) an evaluation of lymphocyte responses of dogs to antigen and mitogen induced lymphocyte stimulation and 3) a positive demonstration of specific antibody development.

LITERATURE REVIEW

The importance of mycobacterial diseases throughout history has prompted extensive research studies of tuberculosis. A significant amount of literature has been written on the area. This literature review is intended to provide background information into the areas of investigative research described in this thesis.

Genus Mycobacterium

The mycobacteria belong to the Mycobacteriaceae family and Actinomycetales order (103). In 1896, Lehman and Neuman introduced the genus which has expanded to recognize 54 species of mycobacteria including pathogens, nonpathogens, and environmental saprophytes (103). Robert Koch discovered and isolated the tubercle bacillus in 1882 (52).

Generally, mycobacterial pathogens are small, aerobic bacilli, 0.2-0.6 x 1.0-4.0μ, acid-fast (although sometimes classified as gram positive), non-motile, without endospores or capsules, and characteristic slow growers with slow metabolic rates (50,103). Mycobacteria are stained with carbol fuchsin classifying them as acid-fast bacilli. The high lipid content in their cell walls retains the stain after acid-alcohol washes. This lipid component is composed

primarily of waxy mycolic acids (86).

Commonly referred to as the bovine tubercle bacillus, Mycobacterium bovis causes tuberculosis in cattle (82,103,107). Other animals infected with M. bovis include domestic and wild ruminants, man and other primates, carnivores (including dogs and cats), swine, parrots and other birds, hamsters and mice (103,107). The neotype strain of M. bovis ATCC 19210 (American Type Culture Collection) is microaerophilic and grows on laboratory culture media at 37 C. Based on antigenic structure, M. bovis is closely related to M. tuberculosis, the human tubercle bacillus; however, M. bovis is generally more pathogenic for animals (6,103).

Canine Tuberculosis

Tuberculosis is one of the infections common to many species of animals (49). Dogs are susceptible to M. tuberculosis, M. bovis, M. avium and M. avium complex (MAIS complex) infections (28,32,49,62,83,102). Reported infections in the dog with the avian and atypical strains of mycobacteria have been rare prompting researchers to speculate about the resistance of dogs to these strains (27). According to a world survey, 75% of the strains isolated from naturally infected dogs were M. tuberculosis (85). Early descriptions of tuberculosis in domesticated animals were reported by

Blaine in 1817 and Rayer in 1842, years before Koch discovered the etiological agent (83). Observations noted the correlation between incidences of human or bovine tuberculosis and canine tuberculosis, which suggested a transmission via contact (28,49,83,102). Reports concerning the incidence of canine tuberculosis varied in different areas of the United States depending mainly on prevalence of infected bovine and human sources (28). Speculations about the low prevalence of canine tuberculosis being reported in recent years is attributed to the reduced cases of human tuberculosis (62). The prevalence of canine tuberculosis in New York between 1962 and 1978 was only 0.05% (8 cases/15,272 dogs examined), the lowest of worldwide cities studied. London had the highest incidence in 1938 and 1939 with 4.6% of 543 dogs examined (83).

Dogs in close association with active cases of human and/or bovine tuberculosis represent a high risk group, however, the disease remains rare among companion animals in the United States (83).

Tuberculosis in the dog often appears without clinically characteristic signs (62). The signs and types of tuberculosis in the dog are extremely variable. The thoracic form of the disease is the most common type affecting the dog with a few characteristic clinical signs such as loss of weight, progressive weakness, lethargy, respiratory distress,

and paroxysmal coughing (28,83). Other forms of tuberculosis in the dog may remain localized in a specific organ or tissue or become a generalized progressive disease by disseminating via the lymphatics or the blood. Other types of tuberculosis reported to affect the dog include abdominal, lymphatic, cutaneous, bone, ocular and generalized forms (83). Clinical diagnosis of mycobacterial infection in companion animals is difficult. Most infections are not confirmed until after necropsy by isolation of the organism (102).

Antigenic Preparations of Mycobacteria

Most mycobacterial antigen preparations are obtained as sonic cell extracts or as culture filtrates of broth-grown organisms (21,42). Both preparations contain cell wall proteins and other components associated with these proteins (42). Tuberculin skin test antigens used to detect delayed-type hypersensitive (DTH) reactions are preparations of mycobacteria that are standardized based on biological activity to minimize excessive variation in antigenic concentration. There are 2 types of skin test tuberculin antigens currently used for detecting DTH in animals. They are old tuberculin (OT) and purified protein derivative (PPD).

Koch's Old Tuberculin (KOT) originally prepared in 1891 from *M. tuberculosis* grown in liquid beef broth cultures was

the first prepared tuberculin antigen (21). However, the impurities associated with this material were numerous due to the beef broth proteins which caused non-specific reactions during testing. Old Tuberculin (OT) describes a similar preparation but from mycobacteria cultured on synthetic media to remove some of the impurities. This OT material was a culture filtrate of M. tuberculosis which was heated, concentrated by evaporation and preserved with glycerol (21). Old tuberculins prepared today are not manufactured according to the same procedures used by Koch. The term OT refers to the product of M. tuberculosis or M. bovis organisms killed by heat, filtered, dialyzed and concentrated by various methods (13,107). Because so many products of the growing tubercle bacilli are present, impurities of this material are numerous and standardization is difficult.

In 1932, Florence Seibert experimented with trichloroacetic acid precipitation to isolate purified tuberculoprotein from Koch's OT. Years later, she introduced tuberculin purified protein derivative (PPD) prepared by precipitation with ammonium sulfate at 50% saturation and neutral pH (78). Today, PPD refers to a culture filtrate that has been precipitated with trichloracetic acid or ammonium sulfate, concentrated, dialyzed and standardized based on protein content (21,78,107). A PPD can be prepared from most mycobacterial strains. PPD contains many different proteins

and polypeptides, therefore, it is not considered a pure substance. The number of nonspecific DTH reactions is reduced when using PPD, which is primarily protein, instead of OT, which also contains carbohydrate and nucleic acid (13,107). Immediate hypersensitivity is presumed due to polysaccharide antigens in a vaccine and also in a PPD (18). Numerous investigators have claimed that killed mycobacterial cells, cell walls and cell extracts incorporated into a water-in-oil emulsion will induce a DTH response (18).

The extensive studies of the antigenic composition of mycobacteria has demonstrated at least 20 antigenic components in each of 6 different species including M. tuberculosis, M. bovis, M. smegmatis, M. lepraemurium, M. simiae and M. leprae by crossed immunoelectrophoresis methods (CIE) (43). Tuberculins and extracts derived from mycobacteria are crude preparations containing species-specific and nonspecific complex antigenic components (43). Investigation of the purification procedure for OT and PPD has demonstrated that heating the cultures of mycobacteria causes denaturation and coagulation of many heat-labile antigenic proteins (20,43). Some of these bacterial antigens could possess species-specificity, and the effect of heating may contribute to their variation or destruction. For this reason, considerable work on isolating and purifying mycobacterial antigens has reverted to fractionation of culture filtrates or cell extracts (21).

Modern techniques of fractionation attempt to isolate individual species-specific purified mycobacterial antigenic constituents (20,21). Investigators have prepared soluble extracts of M. bovis ATCC 19210 using an enzyme (lysozyme), a chaotropic salt (potassium chloride), a nonionic detergent (Triton X-100) and an anionic detergent (sodium deoxycholate, DOC) (40,41,42). Detergents have been widely used to increase the solubility of cell membrane and cell wall components of many bacterial species including mycobacterial surface components (40,42). Chaotropic salts have been used to obtain specific antigens by solubilizing prokaryotic membranes (40). Lysozyme dissolves the bond between polysaccharide components cleaving the bacterial cell wall. Mycobacterial cell walls treated with lysozyme release various water-soluble cell wall arabinogalactan-peptidoglycan complexes, polysaccharides and low molecular weight complexes of proteins (41). Previous reports have made comparisons between the potency and specificity of the M. bovis extract preparations to PPD of M. bovis using DTH tests with M. bovis sensitized guinea pigs and indirect ELISA tests with cattle sera after experimental exposure to M. bovis (40,41,42).

Immunoabsorbent affinity chromatography was studied for the purification of mycobacterial protein antigens by using monospecific antisera from goats to prepare immunoabsorbents (22). This method produced a more pure homogenous

mycobacterial protein antigen.

Cell-mediated Immunity (CMI)

The mycobacteria are included in a group of bacteria identified as facultative intracellular parasites. This term describes the unique ability of the bacteria to survive within polymorphonuclear leukocytes and mononuclear phagocytes after phagocytic ingestion. Humoral immune protection was insufficient when researchers examined the immune mechanism against infection by facultative intracellular bacteria. Passive transfer of immunity against facultative intracellular parasites could not be achieved with serum containing specific antibody. Rather, protection against intracellular microorganisms could be transferred with sensitized lymphocytes. Helmholz in 1909 and Bail in 1910 reported the successful transfer of tuberculin hypersensitivity from immune donor to normal host using whole blood (38). In 1925, Zinsser and Mueller were credited with introducing the term "infectious immunity" to describe the nonhumoral response that provided protection and carried the DTH response (38). Landsteiner and Chase showed that the mediators of the DTH response were cellularly derived and could be transferred to nonsensitized animals with cells from sensitized animals (25,38). The term "cell-mediated immunity" (CMI) results from

the work of many investigators that proved the cellular nature of the immune response against infections caused by facultative intracellular bacteria.

Mackaness and co-workers developed the concept describing the cooperation between a specific inducer cell and a nonspecific effector cell in CMI (38,63). This work by Mackaness and colleagues greatly contributed to our understanding today of the classic concept of cellular immunity (38,63). Specific cell types involved are antigen-activated T-lymphocytes and mononuclear phagocytes. The macrophage function is crucial in generating an effective CMI response. Acting as the nonspecific effector cell, macrophages phagocytize and process antigens to facilitate their presentation to T-lymphocytes (57). Previous reports demonstrated that T-cells are the tuberculin reactive lymphocytes (59). Development of antigen sensitized T-cells following exposure is most important to induce CMI response. To generate specifically sensitized T-cells, live bacteria are most efficient. Dead bacteria, culture filtrates or purified cell wall fractions require the use of Freund's complete adjuvant or a water-in-oil emulsion to induce specific T-cell proliferation (38,57).

Measuring the state of T-cell activation to mycobacteria was the next step towards complete understanding of CMI responses. Both _in vivo_ and _in vitro_ tests measuring CMI

responsiveness have been developed. DTH is the classic in vivo test for measuring antigen-specific CMI (10,66,107). In vitro assays that have been developed to measure antigen-specific acquired sensitization include lymphocyte transformation (LT) or blastogenesis (LB) and leukocyte migration inhibition (LMI) or macrophage migration inhibitory factor (MIF) (38,57). Numerous investigators have reported a correlation between these in vivo and in vitro test results (10,66,75,90).

Macrophage migration inhibitory factor (MIF) is an intensely studied lymphokine. This soluble factor described initially by Bloom and Bennett functions in vitro to inhibit the migration of macrophages from a capillary tube into the surrounding area of a dish (10). MIF is produced only by antigen-stimulated sensitized T-lymphocytes and mitogen-activated B and T lymphocytes (38). Speculation about the function of MIF in vivo is to prevent macrophages from leaving the site of cellular immune reactions (25).

Delayed-type Hypersensitivity (DTH)

The tuberculin skin test measuring delayed-type hypersensitivity is a widely practiced in vivo test for measuring cell-mediated immunity (CMI). This type IV hypersensitivity is mediated by a series of cellular reactions

at the injection site. Sensitized T-cells contact the specific antigen, T-cells are stimulated to undergo blast transformation, proliferating T-cells secrete lymphokines and lymphokines enhance macrophage accumulation at the site of antigen administration and infiltration in the dermis (75).

Historically, Koch observed the local immunological reaction that led to his discovery of cell-mediated hypersensitivity in 1891 (37,52,57,107). Koch experimented with the phenomenon by injecting live viable tubercle bacilli subcutaneously into guinea pigs previously inoculated with the organism. This second injection caused a local induration leading to a necrotic reaction at the injection site (37,57,107). Koch observed this hypersensitive reaction in animals reinoculated with not only living but also with killed tubercle bacilli as well as protein fractions from the bacilli (57,107). Clemons von Pirquet elaborated on Koch's phenomenon and received credit for pioneering the development of the tuberculin skin test (37). DTH reactions are important for measuring antigen-specific CMI. Classically, DTH testing has been employed as a diagnostic test to detect exposure to mycobacteria.

Antigen-activated (sensitized) T-lymphocytes and mononuclear phagocytes are identified as the specific cell types involved in cellular immune reactions (38,63). The cellular responses at the tuberculin injection site was made

possible by biopsy procedures. Skin punches and histometric methods facilitated the analysis and study of changes within the dermis at the skin test site (7,8). The distribution of cells that emigrate into the dermis at the antigen injection site are predominantly CD4 lymphocytes (delayed-type hypersensitive T-cells and helper/inducer T-cells) and M3 monocytes (macrophages) (7).

When tuberculin is introduced into a subject that is hypersensitive (sensitized) to this antigen, an acute inflammatory reaction occurs. This reaction is not evident in a nonsensitized subject introduced to the same antigen (107). This acute inflammatory response is delayed in action reaching a maximum response during a 24-72 hour period (107).

Histologically, the succession of events for the tuberculin inflammatory response in DTH testing is not different from the acute inflammatory response induced by nonspecific irritants and injury (107). Ultimately, the skin test site lesion consists predominantly of accumulated macrophages and lymphocytes which results in the indurated appearance of the lesion. The hyperemia observed at the site of a tuberculin skin test is also resultant of the acute inflammatory response process. Maximal hyperemia is seen during a 24-48 hour period due to increased cutaneous blood flow from dilation of arterioles and capillaries and edema in the area is due to degranulation of basophils (7,107).

Researchers have examined punch biopsies from BCG-immunized dogs skin-tested with tuberculin purified protein derivative (PPD) (89). The biopsy results from the 48-hour skin reaction showed dense inflammatory cell infiltrates composed primarily of macrophages and prominent numbers of neutrophils and small lymphocytes.

Reports of DTH testing in dogs indicate the test to be unreliable (83,89). Intradermal skin testing for tuberculosis infection, as used in humans and cattle with Koch's old tuberculin, purified protein derivative and Bacille-Calmette Guérin vaccine, has had variable and unreliable results in small animals (103). The inconsistent reactions of dogs to intradermal injections of tuberculin causes inconclusive diagnosis of infection. Both false positives and false negatives tend to occur (45,83,103). Since the publication of these early reports, extensive studies have been conducted producing more favorable results. Researchers have noticed that M. tuberculosis experimentally infected dogs reacted consistently to intradermal injections of 0.1 ml of 1:100 OT (1.0 mg) or 0.1 ml of PPD (0.01 mg) (19). Previous reports discuss reliable and significant tuberculin skin test results for DTH if a tuberculin with few impurities was used (11). Intradermal testing dosages of 500 IU to 5,000 IU of heat-concentrated synthetic medium tuberculin injected intradermally resulted in fewer nonspecific inflammatory

reactions.

Several investigators have used Bacille Calmette-Guérin (BCG) intradermal injections for the diagnosis of canine tuberculosis (5,58,65). Based on sensitivity and reliability, these tests confirmed that BCG testing had diagnostic advantages over PPD testing. More recent reports have demonstrated a reliable and quantitative skin test method to determine antigen-specific DTH in BCG vaccinated dogs (89). Intradermal injections of PPD and coccidioidin in the pinna of the ear resulted in increased ear thickness at the injection site that correlated with sensitization of the dogs. Reports examining DTH responses in dogs to both old tuberculin (OT) intradermal injections and 2,4-dinitrochlorobenzene (DNCB) skin surface application following injection with a sensitizing dose of BCG or DNCB were investigated (72).

In attempts to produce a more sensitive and more specific skin test antigen, experimental procedures for fractionating and purifying are continuously being tested (20,21,23,40, 41,42). Production of a more pure skin test antigen could minimize some of the unreliable results that are associated with DTH testing. The reliability of the tuberculin skin test is sometimes questioned due to nonspecific skin reactions, inadequate injection amounts, improper injection techniques or inaccurate quantitative measurement of the induration area (101). If purified antigens were isolated, then advantages

such as known composition, easier standardization and uniform antigenicity could be expected (21).

Lymphocyte Blastogenesis

Lymphocyte transformation is an in vitro assay for measuring the CMI response after specific mitogen or antigen stimulation. Park and Good modified conventional methods to micromethod parameters using phytohemagglutinin (PHA) and whole blood (74). Conventional methods used for lymphocyte transformation were time-consuming averaging 7 days, required large blood sample volumes for lymphocyte isolation and were extremely cumbersome. Park and Good determined optimal conditions for PHA concentration, hours of incubation and duration of pulse with ^{3}H-dT by incorporating a broad scale of test points. Since the development of a microtechnique for lymphocyte transformation, assay procedures were reported by several authors that assess cell-mediated immune reactions against tuberculosis in vitro.

Viljanen and Eskola described a simple micromethod to measure the response of lymphocytes to PPD stimulation (101). They reported the use of whole blood from patients vaccinated and boostered at 10-years with Bacillus Calmette-Guérin (BCG). Their results showed equal maximal stimulation responses to 100 and 10 μg/ml PPD at 90 hours of incubation.

Once the assay procedures were understood, researchers explored the technique for evaluating CMI responses in other animal species. Assessing cell-mediated immune responses in the dog via lymphocyte stimulation tests was an important development. The test has proved extremely helpful in the laboratory diagnosis of CMI deficiency in the dog (77). Initially, a comparison of lymphocyte stimulation results was made using canine whole blood versus isolated lymphocytes (80,105). Initial reports about a significant increase in reactivity using whole blood as compared to gradient-isolated lymphocytes were observed (80). For evaluation of the canine cell-mediated immune status, some investigators prefer the whole blood test claiming that it better reflects the _in vivo_ immunologic status of the dog because it includes other blood cells that may interact with lymphocytes (80). However, a 3-step technique to separate canine lymphocytes from whole blood has been described (105). This separation technique recovered 30 per cent of the circulating lymphocytes and contained in the range of 84 to 94 per cent pure lymphocytes (105). Gradient-isolated lymphocytes, most commonly with Ficoll-Hypaque, are currently in wide use for assaying lymphocyte stimulation. To alleviate some variability, cell suspensions are adjusted to a desired concentration of lymphocytes per milliliter (39,53,54,55,56,80,90,92).

Kristensen, Kristensen and other coworkers initiated the

study of canine lymphocyte responsiveness to commonly explored mitogens such as concanavalin A (Con A), phytohemagglutinin (PHA), and poke-weed mitogen (PWM) (54,55,56). The studies examined whether the standard lymphoblast techniques employed for other species were suitable for the dog or whether canine lymphocytes responded with less vitality to mitogens than other species. The necessity for this intense examination was to clarify contradicting reports about low responses of canine lymphocytes to mitogens (3,53,80,81). Previous results suggest that poor viability of canine lymphocytes incubated *in vitro* is one of the major problems (54). Once parameters were established for *in vitro* mitogen-induced lymphocyte transformation in dogs, specific antigen transformations were developed.

Antigen-induced lymphocyte transformations generally indicate existence of CMI due to the presence of antigen sensitized T-lymphocytes. Thilsted and Shifrine established the kinetics and dose responsiveness of lymphocytes to PPD and coccidioidin antigen stimulation compared to PHA mitogen (90,88). PHA responses were maximum at 4 days of incubation where as antigen responses reached maximum at 6 days of incubation. Generally, increasing the antigen or mitogen dose would increase lymphocyte stimulation responses up to a peak point and then plateaued at about 10 μg/ml PHA, 5 μl/ml coccidioidin and 20 μg/ml PPD. Stimulation responses to PHA

mitogen in each dog was high. However, stimulation responses to PPD was much greater in BCG immunized dogs with or without Freund's complete adjuvant (FCA). PPD showed an enhanced stimulatory effect on sensitized lymphocytes versus nonsensitized lymphocytes after day 11 postimmunization (88). The responses of these sensitized lymphocytes increased more at day 18, but decreased by day 32 post immunization only to show a rise again on day 57 in 3 of the 4 dogs. It was also apparent from the study results that dogs immunized with BCG in FCA showed markedly greater responses to PPD than dogs immunized with BCG alone.

Since both delayed-type hypersensitive skin reactions and lymphocyte transformation responses rely on existing sensitized lymphocytes, correlation between these tests is expected and often tested simultaneously. PPD induced lymphocyte transformation demonstrates a significant correlation to tuberculin skin testing as reported by many studies (46,66,75,90,101). Previous investigators have demonstrated a consistent relationship between lymphocyte transformation and skin testing with both low and high doses of PPD (101). Continuous reports of positive correlation studies between DTH and lymphocyte transformation further supports the hypothesis that the lymphocyte blastogenic assay measures CMI *in vitro* (90).

Enhancement of CMI responses with Indomethacin

Another important aspect to consider while measuring activation of lymphocyte cultures was the production of prostaglandins. Prostaglandins (PGs) are hormonelike substances that regulate inflammatory and immune responses (34,64,69). Normal immune reactions result in the production of large quantities of prostaglandin (30). Previous studies have shown macrophages, B-cells and glass-adherent suppressor cells are capable of producing prostaglandins (30,69). The macrophage is the major cell type responsible for synthesis and release of E-type PGs (35,47). Production of these PGEs is in response to specific stimuli. The presence of PGE modifies the secretion of lymphokines by lymphocytes and inhibits lymphocyte activation (35).

Indomethacin is a potent prostaglandin synthetase inhibitor (30,34,35,64,69,70). Indomethacin is used as a nonsteroidal anti-inflammatory drug to enhance immune responses. Suppression of endogenous production of PGs by mononuclear cell cultures restores lymphocyte immune responses. Research has shown that coordinating blastogenic responses can be obtained by separating and removing populations of prostaglandin producing cells or by the addition of indomethacin to unseparated cultures.

Earlier reports have demonstrated that PGE synthesis and

release from unstimulated macrophages exposed to zymosan (induces time-dependent synthesis and release of PGs) could be completely inhibited by indomethacin (47). Review studies on PHA-stimulated lymphocytes have shown maximum levels of PGE in cultures by 48 hours (34). Experimental results revealed that the amount of PGE produced increases with higher concentrations of PHA (34). Investigations have found that PGE inhibits T-cell only and not B-cell mitogenesis (34,70). Results leading to this conclusion demonstrated that PHA and Con A mitogens stimulate T-cells whereas PWM is a B-cell mitogen and PGE levels did not inhibit PWM activation (53).

Enhanced lymphocyte blastogenic responses to purified protein derivative stimulation with both normal and _M. bovis_-sensitized lymphocyte cultures incubated with indomethacin was demonstrated (69). A statistically significant increase in ^{3}H-thymidine uptake caused by addition of indomethacin was apparent from the data (69). The enhancing effects of indomethacin were only noted when the drug was added simultaneously or within hours of mitogen or antigen addition (69). Since PGs are synthesized quickly following antigenic or mitogenic stimulation, indomethacin must be administered according to strict time measures to acquire its enhancing effects.

Prostaglandins have been shown to suppress immunological responses therefore, indomethacin is recognized as a powerful

immunoenhancing drug. Results from titration experiments showed that increasing concentrations of indomethacin in presence of constant antigen or mitogen resulted in enhanced blastogenic responses (70).

The principles of indomethacin enhanced lymphocyte responses have been applied to canine lymphocytes (30). Lymphocyte blastogenic responses increased with decreasing doses of PHA when a determined optimal indomethacin concentration was added to cultures from dogs (30). Similar results were published with human lymphocytes (34) and bovine lymphocytes (69). Time dependence studies with lymphocyte cultures from dogs correlated with previous results. Addition of indomethacin to lymphocyte cultures was required within the initial 4 hours in order to obtain the drug's effects. Prostaglandin appears to interfere with the initial interaction of the mitogen or antigen and the lymphocyte and delay triggering the blastogenic response is the explanation offered from findings with the canine cell-mediated immune response (30).

Enzyme-linked Immunosorbent Assay (ELISA)

Isolating and identifying mycobacteria from suspected tuberculosis infections is relatively time-consuming. Diagnostic procedures that produce rapid and reliable results

are continuously under research. Many serodiagnostic techniques have been developed and quickly applied to the diagnosis of tuberculosis without much promising success. Surveying humoral antibody responses to tuberculosis has employed numerous microbiologists and test procedures since the early 1900's (61). Arloing and Courmont in 1898 described the basis of the agglutination test applied to tuberculosis. The tests introduced to mycobacteriology include precipitation test, complement fixation tests, sensitized carrier tests (i.e. hemagglutination, bentonite flocculation, latex agglutination), fluorescent antibody tests, radioimmunoassays and enzyme-linked immunosorbent assays (ELISA) (61). In spite of all the intense serodiagnostic testing, studies on diagnosing tuberculosis by humoral antibody testing have been unfavorable. The general difficulty with serodiagnostic testing and mycobacteria is specificity (61). Different mycobacterial species share several common antigens (14,20,21,61). Solving this serodiagnostic problem could be possible with preparations containing purified species-specific antigens. Identification of unique antigens specific for each species is under research by many.

It is generally agreed by investigators that the ideal serodiagnostic test should be highly sensitive and specific (26). Potential use of species-specific purified mycobacterial antigens to examine sera for antibodies produced

during immunological response to mycobacterial infection was the next investigative step. Using currently available mycobacterium extracts and purified preparations, a barrage of serodiagnostic tests was examined for efficiency in detecting tuberculosis infections.

Soluble antigen fluorescent antibody test (SAFA) which measures antibody concentration in serum was a popular serodiagnostic test (1). The SAFA test detected antibodies using various soluble active antigen isolates from mycobacteria fixed on an artificial matrix. The serologic test results were compared with tuberculin skin tests at various stages throughout the progression of tuberculosis infection. Findings from the study suggested that the SAFA test using purified antigens was somewhat more sensitive than intradermal tuberculin skin tests for early detection of infection (1).

Whether natural infection or experimental exposure, circulating antibodies have been identified to various mycobacterial protein and polysaccharide antigens. In 1972, Engvall and Perlmann introduced a highly sensitive and simple method for quantitation of specific antibodies using antigen-coated tubes and enzyme-conjugated anti-immunoglobulins (26).

The enzyme-linked immunosorbent assay (ELISA) builds an antigen-antibody sandwich on a solid polystyrene phase (in a tube or in a well of a microtiter plate). The presence of the

assay sandwich is detected by the reaction of the enzyme label that catalyzes the production of a chromogenic product from an initially colorless substrate. The degree of color change is directly proportional to the amount of enzyme-labeled immunoglobulin conjugate present which specifically binds to the unknown antigen or antibody in the sandwich. In essence, the color change response provides a direct measure of the amount of unknown being evaluated.

Variable applications and procedures of the ELISA demonstrate the usefulness of the test for detecting antigen or antibody. ELISA is most extensively used for identifying specific antibodies to measure the immune status of an individual. The specificity of the ELISA technique is determined largely by the antigen employed (24).

The ELISA techniques are becoming the preferred serodiagnostic test for many infectious diseases, including mycobacterial diseases (24,95). Nassau and coworkers applied the ELISA microtechnique to the serologic diagnosis of tuberculosis (71). Investigators have routinely used a modified ELISA procedure for detecting antibodies to mycobacteria in the sera of a variety of animal species (4,93,94,95,98,99). However, the ELISA test has not been used to evaluate humoral immune responses in dogs exposed to mycobacterium.

Several antigen extracts of mycobacteria have been

evaluated using ELISA (40,41,42). ELISA reactivity of these extracts was compared to PPD of the same species. The DOC and lysozyme extracts responded comparably to PPD during ELISA tests (41,42). However, evaluation of the extracts using DTH tests elicited weak responses compared to the potency of PPD.

Benjamin and colleagues analyzed several mycobacterial antigens for potential diagnostic use in ELISA (9).

The ELISA test possesses several hindrances which may detour accurate results. Proteins, glycoproteins and a few other substances can be adsorbed to polystyrene for ELISA tests (26). Since these antigens are only adsorbed to the solid phase and not covalently bound, stability of the antigen attachment is questioned (26). Optimal antigen concentration is important because too high or too low a concentration prevents detection of low concentrations of antibody. One of the most difficult problems with the ELISA is presence of background color. Excessively high backgrounds can occur when components of the assay steps cross-react. In addition, false positives and false negatives are common. The data and experience available thus far suggest ELISA techniques have not achieved a high sensitivity and specificity standard rating (24). By modifying assay parameters and by using more highly purified mycobacterial antigens, a highly sensitive and specific ELISA test will be possible.

Previous investigators have compared humoral and

cell-mediated immune reactions using serodiagnostic test results (ELISA and SAFA) and DTH skin test results. Currently, it is not known whether the cell-mediated and humoral immune responses are directed against the same or different antigenic components of mycobacteria (43).

MATERIALS AND METHODS

Experimental Animals

Twelve, mixed breed, male dogs weighing between 20 to 30 pounds and negative on tuberculin skin test were used in the study. The animals were obtained from Laboratory Animal Resources, College of Veterinary Medicine, Iowa State University, Ames, IA. The animals were maintained in separate cages at the Animal Resources Station, Iowa State University for the duration of the study. Their diet consisted of Hill's Dry Maintenance Diet pellets.

Sensitization

Six dogs were injected subcutaneously in the brisket with 0.1ml (200mg wet weight) of heat-killed Mycobacterium bovis strain AN-5, a laboratory adapted strain, in mineral oil. (National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA.) Four weeks after injection of M. bovis sensitinogen, the six dogs were administered Benzapin injections and treated orally with Clavamox and Batril to soften and heal subcutaneous granulomas at the injection site.

Tuberculin Skin Tests

Tuberculin skin tests using purified protein derivatives were conducted on the cervical region to measure delayed-type hypersensitivity (DTH) responses (89,90,96). The test area on the cervical region was shaved and cleaned with alcohol. A 0.1ml volume of the PPD tuberculin was injected intradermally with a tuberculin syringe to produce a bleb. The skin thickness was measured at the injection site before and at 24, 48, and 72 hours after injection. Induration and swelling were measured using a dermal thickness gauge. The responses were recorded as the increase in skin thickness (mm) and area $(mm)^2$; cross-sectional measurements of the response site were multiplied to give the area.

Each dog was skin tested with M. bovis PPD (1mg/ml Sr 31 BAL 8701, National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA) 4 weeks before and 15 weeks after injection of sensitinogen. Comparative cervical tuberculin skin tests were conducted on separate sites using M. bovis PPD or M. avium PPD (1mg/ml Sr 31 BAL 8702, National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA) injected on 6 of 12 dogs (animal no. 8770, 8596, 8782, 8721, 8174, 8238). Repeat comparative cervical tuberculin skin tests were conducted at 6 and 12 weeks following injection of sensitinogen. Three of 6 dogs

injected with sensitinogen (animal no. 8770, 8596, 8782) and 3 of 6 control dogs (animal no. 8721, 8174, 8238) were selected at random to receive repeated skin tests (Table 1). One dog (animal no. 8238) developed a rash with skin-thickening and subcutaneous inflammation associated with irritation following shaving of an area of skin.

Skin Biopsies

Biopsy samples were obtained using a 4mm disposable punch 15 weeks after injection of sensitinogen. After administering local anesthesia (Xylocaine), punch biopsies were obtained from the center of the tuberculin skin test sites 48 hours following final tuberculin skin test injection. Biopsy sections from skin test sites and a control site from 2 of 6 dogs injected with sensitinogen (animal no. 8763 and 8745) were examined. Tissues specimens were fixed in 10% neutral buffered formalin immediately after collection. Fixed tissues were trimmed, dehydrated with graded alcohols, embedded in paraffin and sectioned at 6 μm. Sections were stained with hematoxylin and eosin (HE) for light microscopy. Stained slides were examined for differences between control sites and skin test sites.

Table 1. Protocol for sensitinogen and tuberculin injections for 12 experimental dogs

Group Number	Animal Number	*Mycobacterium bovis* sensitinogen	Tuberculin skin Tests
	8770	+	repeat[a]
1	8596	+	repeat
	8782	+	repeat
	8794	+	single[b]
2	8763	+	single
	8745	+	single
	8721	-	repeat
3	8174	-	repeat
	8238	-	repeat
	8563	-	single
4	8718	-	single
	7946	-	single

[a]Skin tests were conducted using *M. bovis* PPD at 4 weeks before and 15 weeks after subcutaneous injection of *M. bovis* sensitinogen. Skin tests were also conducted at 6 and 12 weeks after injection of sensitinogen using *M. bovis* PPD and *M. avium* PPD.

[b]Skin tests were conducted using *M. bovis* PPD at 4 weeks before and 15 weeks after subcutaneous injection of *M. bovis* sensitinogen.

Mycobacterial Antigens

A Mycobacterium bovis ATCC (neotype strain) 19210 culture was grown on Middlebrooks 7H10 medium (Difco Laboratories) containing sodium pyruvate (4g/L, Fisher Scientific) and horse serum (50ml/L, HyClone Laboratories, Inc.) without added glycerol and mycobactin. Cultures were incubated for 8 weeks at 37 C before antigen extractions were conducted as previously described (40).

After incubation, the growth was scraped from the media slants and suspended in a sterile screw-capped flask containing glass beads and distilled water. Cells were heat-killed by autoclaving for 30 minutes at 121 C with 15 PSI. Cells were transferred into a 50ml Oakridge centrifuge tube (Nalgene Labware Co.) and centrifuged at 4 C for 30 minutes at 10,000 RPM. The supernatant was discarded and the cell pellet divided into 2 aliquots. The wet weight of each cell aliquot was determined.

One cell aliquot was resuspended in 3M KCl in 0.01M phosphate buffer (pH 7.5) to a ratio of 2:1 3M KCl/wet weight of cells. The other cell aliquot was resuspended 4:1 in 10% Triton X-100 (Bio-Rad Laboratories), pH 8.4, containing 0.003M ethylenediaminetetraacetic acid (EDTA), 0.1M Tris-HCl, 0.15M NaCl and 0.2% sodium azide (NaN_3). Phenylmethylsulfonyl fluoride (PMSF) dissolved in 2-propanol was added to each

extraction solution to a final concentration of 10mM. The cell suspensions were incubated for 48 hours at 4 C while continuously stirring. The suspension extracts were centrifuged at 4 C for 30 minutes at 10,000 RPM to remove the cells. The supernatants were filter sterilized using a 0.2μ disposable Acrodisc filter units. (Gelman Sciences, Inc.) The supernatant from the KCl extraction was dialyzed for 48 hours at 4 C against phosphate buffered saline (PBS) solution, pH 7.2, containing 2mM PMSF using 6,000-8,000 molecular weight cutoff (MWCO) Spectrapor membrane tubing. (Spectrum Medical Industries, Inc.) The supernatant from the Triton X-100 extraction was dialyzed for 48 hours at 4 C using 6,000-8,000 MWCO membrane tubing against 0.003M EDTA, 0.01M Tris-HCl, 0.1% Triton X-100 (pH 8.4) and PMSF dissolved to a final concentration of 2mM.

After dialysis, the concentrated KCl and Triton X-100 antigen extracts were filter sterilized using 0.2μ disposable Acrodisc filter units. Protein concentrations for each extract were determined using the microtiter plate BCA protein assay (Pierce Chemical Co.) with bovine albumin (Fisher Scientific) as the protein standard.

The antigen extracts were used in the enzyme-linked immunosorbent assay (ELISA) procedure, described later, with limited success. Therefore, a Triton X-100 extract of _M. bovis_ AN-5 (U.S. Department of Agriculture, 8-1983, 11 mg/ml)

was obtained for testing serum samples from experimental dogs.

Lymphocyte Blastogenic Assay

To evaluate cell-mediated immunity in vitro, a lymphocyte transformation assay technique was developed (39,53,54,56,80, 88,90). Twenty ml of blood was collected from each dog via jugular venipuncture. The whole blood was immediately transferred to a sterile 50ml test tube siliconized with a 1:40 dilution of Sigmacote siliconizing solution. (Sigma Chemical Co.) Each tube contained 3ml of 2X acid-citrate dextrose (ACD) solution consisting of trisodium citrate (44g/L), citric acid (16g/L) and dextrose (50g/L) stored at 4 C. The blood was diluted in siliconized test tubes with an equal volume of phosphate buffered saline solution (PBS) pH 7.2. Four aliquots of 10ml each of the diluted blood were layered over 8ml of sterile ficoll-hypaque. (Histopaque-1077, Sigma Chemical Co.) Tubes were centrifuged at 1400 RPM for 40 minutes at 22 C. The lymphocyte-rich buffy coat interface was pipetted from each of the tubes and washed in 1X Hank's Balanced Salt Solution (HBSS) without calcium and magnesium. (Flow Laboratories, Inc.) Two of 4 tubes were combined, and the cells pelleted by centrifugation at 900 RPM for 20 minutes at 22 C. Supernatants were discarded and the cell pellet was resuspended in 0.95ml of HBSS. A 100μl volume

of the cell suspension was used to determine the mononuclear cell concentration. The white blood cells were diluted 1:500 into cuvettes using a cell diluter (Model 365A, Fisher Scientific) and counting with a Coulter counter. (Model F, Coulter Electronics, Inc.)

The cell suspensions were adjusted to a lymphocyte concentration of 2.5 x 10^6 cells/ml in medium M199 modified with Earle's salts and glutamine (Flow Laboratories, Inc.) supplemented with 1.0% penicillin/streptomycin solution (10,000U penicillin G/ml and 10mg streptomycin/ml, Sigma Chemical Co.), 25mM HEPES (N-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) buffer (Flow Laboratories, Inc.) and 10% heat-inactivated pooled dog serum. Allogeneic and autologous serum was obtained before the dogs were injected with sensitinogen. The serum samples were pooled, heated at 56 C for 30 minutes and stored at -20 C with 50μl 1:10,000 merthiolate/10ml serum added.

Separate tests for lymphocyte stimulation were done with and without addition of 10μl indomethacin/100ml medium M199 with additives. The indomethacin solution was prepared at a concentration of 10 mg/ml in absolute ethanol (Sigma Chemical Co. I-7318).

The adjusted lymphocyte suspensions were stimulated by addition of either mitogen or antigen. Phytohemagglutinin (PHA) mitogen (Bacto-PHA, Difco Laboratories) was prepared in

stock solution with PBS and stored in 0.5ml aliquots at -20 C. PHA was diluted 1mg/ml with medium M199 without additives. A PPD of M. bovis antigen (Sr 31 8002, National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA) was prepared 1mg/ml with medium M199 without the additives. Dilutions of 25 and 10 μg/well of PHA solution and 10, 1.0, and 0.1 μg/well of M. bovis PPD solution were added to the wells.

Cell cultures were set up in triplicate in 96-well flat-bottomed tissue culture plates. (Corning Glass Works) Each adjusted lymphocyte suspension (200μl/well) was added to the microtiter plates containing either mitogen or antigen volumes or neither for a control. Cultures were incubated at 37 C in a 5% CO_2 humidified atmosphere. After an incubation period of 48 hours for PHA and 96 hours for M. bovis PPD, the cell culture were labelled with 50μl/well (0.75 μCi/well) ^{3}H-thymidine (Amersham International) to assess lymphocyte blastogenic response, then incubated for an additional 18 hours.

The cells were harvested onto glass fiber filter pads (Titertek, Flow Laboratories) using a Skatron cell harvester. (Flow Labortories) The filtered samples were washed 5 times with water on the cell harvester to remove the excess radioactivity from the filters. The filter disks were dried and then transferred to 20ml disposable scintillation vials

(Kimble) containing 10ml of Scintiverse BD. (Fisher Scientific) Radioactivity was counted in a liquid scintillation spectrometer (Packard Instrument Company, Inc.) with a 4-minute minaxi program. For each animal sample, the average counts per minute (CPM) from the triplicate wells cultured alone or with the concentration of PHA or M. bovis PPD was calculated. The stimulation index (SI) for each sample was determined from mean CPM values using the following equation:

$$SI = \frac{\text{CPM of stimulated cells (PHA or PPD)}}{\text{CPM of unstimulated cells in media}}$$

Blood was collected from each of 12 dogs 4 and 2 weeks before and at 3, 6, 9, and 12 weeks after injection of M. bovis sensitinogen and assayed for lymphocyte stimulation.

Enzyme-linked Immunosorbent Assay (ELISA)

Blood was obtained from each of 12 dogs 4 and 2 weeks before injection with sensitinogen and at 3-week intervals for 15 weeks after injection. Serum samples were tested for antibodies to M. bovis antigens using ELISA procedure described in previous reports (24,92,93,94,95). Blood (7-10ml) was collected in vacutainer blood collection tubes

(Becton, Dickinson and Co.) and allowed to clot. Tubes were centrifuged at 1500 RPM for 30 minutes at room temperature to separate serum. Serum samples were pipetted into sterile tubes and stored at -20 C with 50μl 1:10,000 merthiolate added.

A modified ELISA procedure was used to assay the serum samples for antibody titer to M. bovis antigens. A carbodiimide (Cyanamide, Sigma Chemical Co.) solution was prepared 1mg/ml in 0.1M Na_2CO_3 pH 9.6. An M. bovis PPD tuberculin antigen (U.S. Department of Agriculture) was diluted 1:100 with 0.1M Na_2CO_3 pH 9.6. Triton X-100 and KCl antigen extracts described previously were screened to determine dilution standards. A Triton X-100 extract of M. bovis AN-5 (U.S. Department of Agriculture, 8-1983) diluted 1:100 with 0.1M Na_2CO_3 pH 9.6 was also tested. Equal volumes of the carbodiimide solution and antigen dilution were mixed and 100μl of the solution added to each well of a 96-well flat-bottomed Immulon 2 microtiter plate. (Dynatech Corp) The plates were placed in a plastic bag and incubated at 4 C for 16 hours.

Following incubation, plates were washed 3 times with phosphate buffered saline solution (PBSS) pH 7.2 and 100μl of 0.1M NH_4Cl was added to each well and incubated for 30 minutes at room temperature. Antigen-coated plates were washed 3 times with ELISA washing solution (phosphate buffer containing

0.5M NaCl with 0.5% Tween 80, pH 7.5). Serum samples were diluted 1:10 with ELISA diluent solution (phosphate buffer containing 0.5M Nacl with 1% Tween 80, pH 7.5). Serial dilutions of each serum sample (1:20 to 1:160) were made in microtiter plate wells by combining 50 µl diluted serum with 50 µl ELISA diluent in each well. The plates were covered with parafilm and incubated on a horizontal shaker at room temperature. The incubation time for serum depended upon antigen preparation and conjugate (Table 2).

After serum incubation, plates were washed 8 times with the ELISA wash solution. Horseradish-peroxidase labeled Protein A conjugate (Kirkegaard and Perry Laboratories, Inc.) was diluted 1:5000 with ELISA diluent solution or affinity purified goat antibody to dog IgG (H+L), labeled with horseradish-peroxidase (Kirkegaard and Perry Laboratories, Inc.) was diluted 1:500 with ELISA diluent. A 50µl volume of the prepared conjugate was added to each well. Plates were covered with parafilm and incubated on a horizontal shaker at room temperature for the optimal time determined in pretest screening (Table 2). The plates were washed 8 times with the ELISA wash solution and inverted to dry.

The substrate solution was prepared using 2-2' azino-di-(3-ethyl benthiozaline-6-sulfonate), ABTS, (National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, IA) in 0.05M citric acid, pH 4.0 and 3%

Table 2. ELISA protocol for antigen dilutions and serum/conjugate incubation times

	PPD of *M. bovis* (1:100)	Triton X-100 extract of *M. bovis* AN-5 (1:100)
Serum	8 minutes	60 minutes
Protein A (1:5000)	30 minutes	60 minutes
Serum	8 minutes	15 minutes
Anti-species (1:500)	15 minutes	30 minutes

hydrogen peroxide. After plates were dry, 100μl of the substrate solution was added to each well. The color intensity was measured at 30, 60, 120 and 180 minutes using a Vmax Kinetic Microplate Reader (Molecular Devices Corp.) with absorbance at 405nm.

Statistical Analysis

Statistical significance was tested by the Students T-test for grouped comparisons.

RESULTS

Delayed-type Hypersensitivity (DTH) Skin Tests

Skin thickness at the injection site was measured before and at 24, 48 and 72 hours after intradermal injection of *M. bovis* PPD and/or *M. avium* PPD in the cervical region of dogs exposed to killed *M. bovis* and controls. Skin test responses are reported as increase in skin thickness at the injection site in Tables 3, 4 and 5. Also, cross-sectional diameter of the indurated and erythematous area was measured at 24, 48 and 72 hours following intradermal injection *M. bovis* PPD or *M. avium* PPD and these responses (mm^2) are reported in Tables 6, 7 and 8.

No tuberculin responses at 24 or 48 hours were observed in each of 12 dogs tested with *M. bovis* PPD 4 weeks before sensitization. Positive tuberculin reactions to *M. bovis* PPD were observed in 5 dogs at 15 weeks following injection with killed *M. bovis*; one dog (animal no. 8794) injected with killed *M. bovis* was euthanatized after 12 weeks. The increase in skin thickness (mean value ± SEM) at 24 hours was 7.0mm ± 0.8; the area of response (mean value ± SEM) was 289.2mm^2 ± 49.9. The increase in skin thickness mean response was 7.8mm ± 0.6 and 294.2mm^2 ± 47.4 at 48 hours. No detectable skin test responses were observed at 48 hours to *M. bovis* PPD at 15

Table 3. Tuberculin skin test responses (increase in skin thickness) at 24 hours following intradermal injection of *Mycobacterium bovis* PPD or *Mycobacterium avium* PPD in dogs experimentally exposed to killed *M. bovis* cells in oil

		Weeks Pre-exposure and Post-exposure					
		-4	6		12		15
Group	Animal No.	*M. bovis*	*M. bovis*	*M. avium*	*M. bovis*	*M. avium*	*M. bovis*
1	8770	NR [a]	8 [b]	3	14	8	6
	8596	NR	8	2	8	5	10
	8782	NR	5	1	9	4	7
2	8794	NR	NT [c]	NT	NT	NT	-- [d]
	8763	NR	NT	NT	NT	NT	5
	8745	NR	NT	NT	NT	NT	7
3	8721	NR	NR	NR	NR	NR	3
	8174	NR	NR	NR	NR	NR	NR
	8238	NR	NR	NR	NR	NR	NR
4	8563	NR	NT	NT	NT	NT	NR
	8718	NR	NT	NT	NT	NT	NR
	7946	NR	NT	NT	NT	NT	NR

a NR = No response.
b Increase in skin thickness (mm) 24 hours following injection of PPD.
c NT = No test conducted.
d Dog euthanized after 12 weeks.

Table 4. Tuberculin skin test responses (increase in skin thickness) at 48 hours following intradermal injection of *Mycobacterium bovis* PPD or *Mycobacterium avium* PPD in dogs experimentally exposed to killed *M. bovis* cells in oil

		Weeks Pre-exposure and Post-exposure					
		-4	6		12		15
Group	Animal No.	*M. bovis*	*M. bovis*	*M. avium*	*M. bovis*	*M. avium*	*M. bovis*
1	8770	NR a	13 b	6	16	7	7
	8596	NR	12	6	10	5	10
	8782	NR	8	4	7	4	7
2	8794	NR	NT c	NT	NT	NT	-- d
	8763	NR	NT	NT	NT	NT	8
	8745	NR	NT	NT	NT	NT	7
3	8721	NR	NR	NR	NR	NR	NR
	8174	NR	NR	NR	NR	NR	NR
	8238	NR	NR	NR	NR	NR	NR
4	8563	NR	NT	NT	NT	NT	NR
	8718	NR	NT	NT	NT	NT	NR
	7946	NR	NT	NT	NT	NT	NR

a NR = No response.
b Increase in skin thickness (mm) 48 hours following injection of PPD.
c NT = No test conducted.
d Dog euthanatized after 12 weeks.

Table 5. Tuberculin skin test responses (increase in skin thickness) at 72 hours following intradermal injection of *Mycobacterium bovis* PPD or *Mycobacterium avium* PPD in dogs experimentally exposed to killed *M. bovis* cells in oil

		Weeks Pre-exposure and Post-exposure					
		-4	6		12		15
Group	Animal No.	*M. bovis*	*M. bovis*	*M. avium*	*M. bovis*	*M. avium*	*M. bovis*
1	8770	NM a	17 b	6	7	9	NM
	8596	NM	13	5	4	4	NM
	8782	NM	6	3	6	3	NM
2	8794	NM	NT c	NT	NT	NT	-- d
	8763	NM	NT	NT	NT	NT	NM
	8745	NM	NT	NT	NT	NT	NM
3	8721	NM	NR e	NR	NR	NR	NM
	8174	NM	NR	NR	NR	NR	NM
	8238	NM	NR	NR	NR	NR	NM
4	8563	NM	NT	NT	NT	NT	NM
	8718	NM	NT	NT	NT	NT	NM
	7946	NM	NT	NT	NT	NT	NM

a NM = No measurement.
b Increase in skin thickness (mm) 72 hours following injection of PPD.
c NT = No test conducted.
d Dog euthanatized after 12 weeks.
e NR = No response detected.

Table 6. Tuberculin skin test responses (diameter of skin response) at 24 hours following intradermal injection of *Mycobacterium bovis* PPD or *Mycobacterium avium* PPD in dogs experimentally exposed to killed *M. bovis* cells in oil

		Weeks Pre-exposure and Post-exposure					
		-4	6		12		15
Group	Animal No.	*M. bovis*	*M. bovis*	*M. avium*	*M. bovis*	*M. avium*	*M. bovis*
1	8770	NR a	192 b	96	1591	320	210
	8596	NR	320	168	204	132	399
	8782	NR	210	154	837	120	288
2	8794	NR	NT c	NT	NT	NT	--- d
	8763	NR	NT	NT	NT	NT	150
	8745	NR	NT	NT	NT	NT	399
3	8721	NR	NR	NR	NR	NR	48
	8174	NR	98	35	NR	NR	NR
	8238	NR	NR	NR	NR	NR	NR
4	8563	NR	NT	NT	NT	NT	NR
	8718	NR	NT	NT	NT	NT	NR
	7946	NR	NT	NT	NT	NT	NR

a NR = No response.
b Cross-sectional diameter (mm^2) of indurated and erythematous area 24 hours following injection of PPD.
c NT = No test conducted.
d Dog euthanatized after 12 weeks.

Table 7. Tuberculin skin test responses (diameter of skin response) at 48 hours following intradermal injection of _Mycobacterium bovis_ PPD or _Mycobacterium avium_ PPD in dogs experimentally exposed to killed _M. bovis_ cells in oil

		Weeks Pre-exposure and Post-exposure					
		-4	6		12		15
Group	Animal No.	_M. bovis_	_M. bovis_	_M. avium_	_M. bovis_	_M. avium_	_M. bovis_
1	8770	NR a	405 b	210	1540	357	400
	8596	NR	440	99	340	121	418
	8782	NR	315	168	460	72	204
2	8794	NR	NT c	NT	NT	NT	--- d
	8763	NR	NT	NT	NT	NT	209
	8745	NR	NT	NT	NT	NT	240
3	8721	NR	NR	NR	NR	NR	NR
	8174	NR	NR	NR	NR	NR	NR
	8238	NR	NR	NR	NR	NR	NR
4	8563	NR	NT	NT	NT	NT	NR
	8718	NR	NT	NT	NT	NT	NR
	7946	NR	NT	NT	NT	NT	NR

a NR = No response.
b Cross-sectional diameter (mm^2) of indurated and erythematous area 48 hours following injection of PPD.
c NT = No test conducted.
d Dog euthanatized after 12 weeks.

Table 8. Tuberculin skin test responses (diameter of skin response) at 72 hours following intradermal injection of *Mycobacterium bovis* PPD or *Mycobacterium avium* PPD in dogs experimentally exposed to killed *M. bovis* cells in oil

		Weeks Pre-exposure and Post-exposure					
		-4	6		12		15
Group	Animal No.	*M. bovis*	*M. bovis*	*M. avium*	*M. bovis*	*M. avium*	*M. bovis*
1	8770	NM [a]	928 [b]	110	1558	255	NM
	8596	NM	330	156	240	154	NM
	8782	NM	195	99	270	100	NM
2	8794	NM	NT [c]	NT	NT	NT	--- [d]
	8763	NM	NT	NT	NT	NT	NM
	8745	NM	NT	NT	NT	NT	NM
3	8721	NM	NR [e]	NR	NR	NR	NM
	8174	NM	NR	NR	NR	NR	NM
	8238	NM	NR	NR	NR	NR	NM
4	8563	NM	NT	NT	NT	NT	NM
	8718	NM	NT	NT	NT	NT	NM
	7946	NM	NT	NT	NT	NT	NM

a NM = No measurement.
b Cross-sectional diameter (mm^2) of indurated and erythematous area 72 hours following injection of PPD.
c NT = No test conducted.
d Dog euthanatized after 12 weeks.
e NR = No response detected.

weeks in each of 6 control dogs.

Increase in skin thickness results of comparative DTH skin test responses at 6 and 12 weeks with M. bovis PPD and M. avium PPD on 3 of 6 dogs exposed to M. bovis and 3 of 6 control dogs are shown in Tables 3-5. Six weeks post-sensitization, the increase in skin thickness (mean value ± SEM) to M. bovis PPD at 24 hours was 7.0mm ± 1.0, at 48 hours was 11.0mm ± 1.5, and at 72 hours was 12.0mm ± 3.2. Increase in skin thickness responses to M. avium PPD in M. bovis exposed dogs were 2.0mm ± 0.6 at 24 hours, 5.3mm ± 0.7 at 48 hours and 4.7mm ± 0.9 at 72 hours.

Tuberculin skin test responses (mean increase in skin thickness) at 12 weeks to M. bovis PPD in dogs exposed to killed M. bovis were 10.3mm ± 1.9 at 24 hours, 11.0mm ± 2.6 at 48 hours and 5.7mm ± 0.9 at 72 hours. The skin test responses (mean value) to M. avium PPD at 12 weeks in the same dogs were 5.7mm ± 1.2 at 24 hours, 5.3mm ± 0.9 at 48 hours and 5.3mm ± 1.9 at 72 hours.

Results from comparative DTH skin test responses measuring cross-sectional diameter (mean square area) are shown in Tables 6-8. At 6 weeks, mean tuberculin skin test responses to M. bovis PPD in dogs exposed to M. bovis were observed to be 240.7mm^2 ± 40.0 at 24 hours, 386.7mm^2 ± 37.2 at 48 hours and 484.3mm^2 ± 225.2 at 72 hours. Lower skin test mean responses were observed with M. avium PPD compared to M.

bovis PPD in M. bovis exposed dogs at 6 weeks. Responses to M. avium PPD were $139.3mm^2 \pm 22.0$ at 24 hours, $159.0mm^2 \pm 32.4$ at 48 hours and $121.7mm^2 \pm 17.5$ at 72 hours. Mean responses observed to M. avium PPD at 12 weeks were $190.7mm^2 \pm 64.8$ at 24 hours, $183.3mm^2 \pm 88.0$ at 48 hours and $169.7mm^2 \pm 45.4$ at 72 hours. Higher responses to M. bovis PPD were observed at 12 weeks in dogs exposed to M. bovis compared to M. avium PPD. Responses were $877.3mm^2 \pm 400.9$ at 24 hours, $780.0mm^2 \pm 381.6$ at 48 hours and $689.3mm^2 \pm 434.4$ at 72 hours.

No increase in skin thickness responses were observed to M. bovis PPD or to M. avium PPD in the control dogs at 6 or 12 weeks. Minimal responses were detectable by cross-sectional diameter at 24 hours in animals no. 8721 and 8174.

Histopathologic examination of skin biopsy sections showed an infiltration of cells into the dermis of the skin test site on sensitized dogs (Figures 1 and 2); this was not observed in the skin test site of a control dog (Figures 3 and 4). The cellular infiltrate consisted primarily of mononuclear cells (lymphocytes, monocytes, plasma cells). Examination of the skin test site of the sensitized dog detected separation of dermal collagen fibers in the connective tissue. The separation was attributed to edema.

Figure 1. Skin biopsy section from cervical skin test site of *M. bovis* sensitized dog 48 hours following injection of *M. bovis* PPD. There is infiltration by mononuclear cells and edema separating the collagen fibers in the dermal connective tissue.

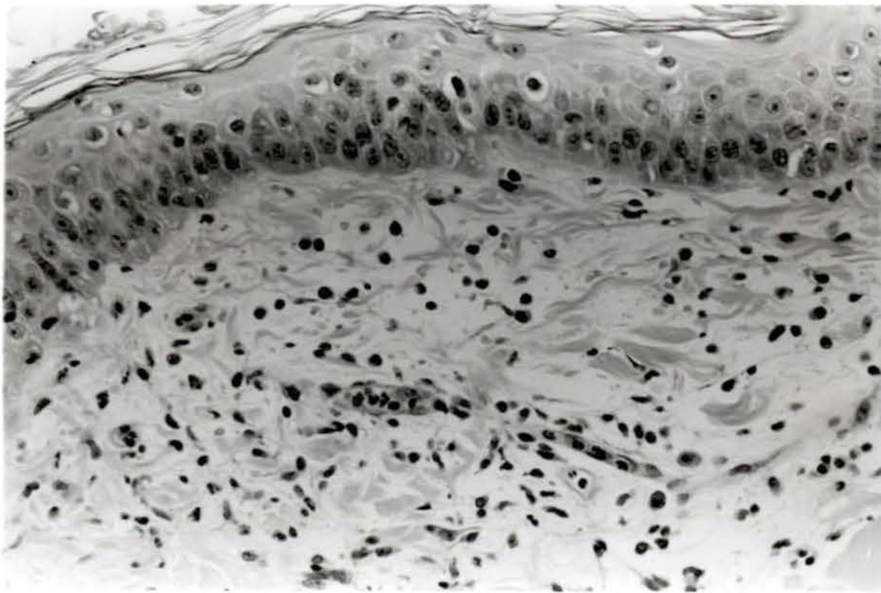


Figure 2. Skin biopsy section from cervical skin test site of *M. bovis* sensitized dog 48 hours following injection of *M. bovis* PPD. There is infiltration by mononuclear cells and edema separating the collagen fibers in the dermal connective tissue.

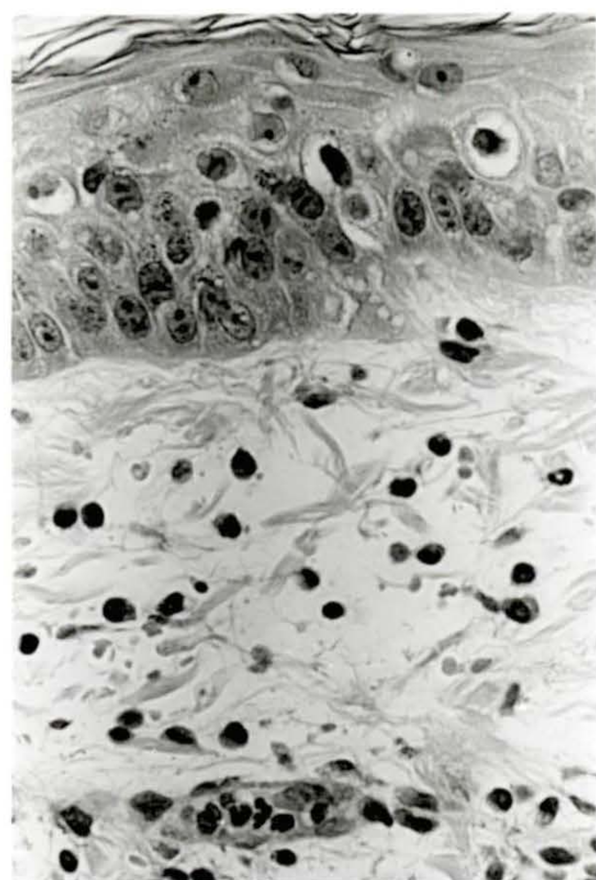
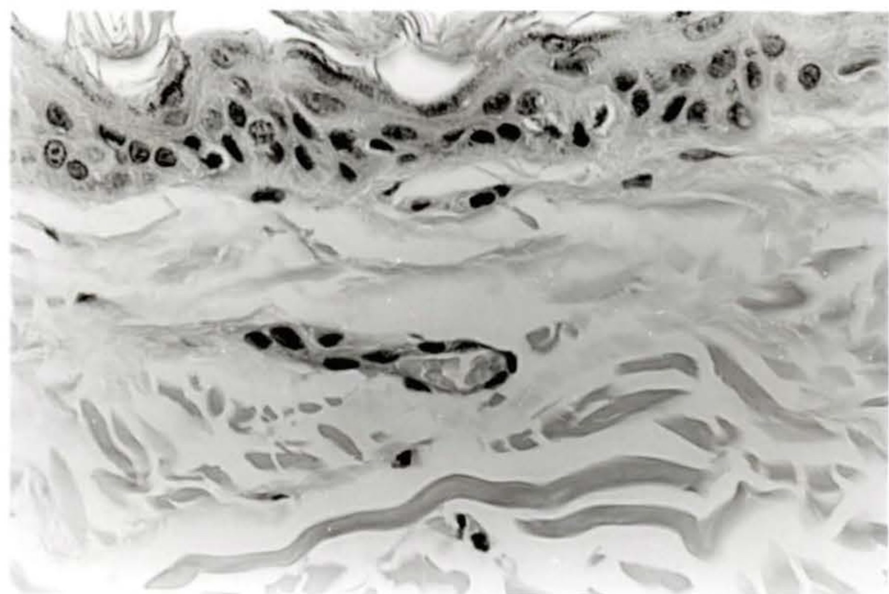


Figure 3. Skin biopsy section from cervical skin test site of a control dog 48 hours following injection of *M. bovis* PPD. Neither cellular infiltration nor edema were observed in the dermis. Note: collagen bundles are not fragmented as in Figures 1 and 2.

Figure 4. Skin biopsy section from cervical skin test site of a control dog 48 hours following injection of *M. bovis* PPD. Neither cellular infiltration nor edema were observed in the dermis. Note again that collagen bundles are not fragmented as in Figures 1 and 2.



Lymphocyte Blastogenesis

Lymphocyte blastogenic (LB) responses were evaluated using 0.1, 1.0 and 10 μg concentrations of M. bovis PPD for lymphocytes from M. bovis exposed and control dogs; results are shown in Tables 9, 10 and 11.

No significant differences in stimulation indices were detected between lymphocyte responses of dogs to each of 3 concentrations of M. bovis PPD at 4 and 2 weeks before sensitization. No significant differences in stimulation indices to M. bovis PPD concentrations were detected between lymphocytes from M. bovis exposed and from control dogs at 3 and 6 weeks following sensitization. Significant differences were detected at 9 weeks post-exposure between M. bovis exposed and control dog lymphocyte responses with 0.1 and 10 μg M. bovis PPD ($p \leq .1$) and 1.0 μg M. bovis PPD ($p \leq .05$). Statistically significant ($p \leq .1$) differences in stimulation indices between responses of M. bovis exposed and control dogs were detected at 12 weeks post-sensitization.

Higher stimulation indices (mean values) were detected with lymphocytes from M. bovis exposed dogs than from control dogs at 3, 6, 9 and 12 weeks for each of the 3 M. bovis PPD concentrations. Stimulation indices (mean values) at 3 weeks post-exposure were 5.23 ± 1.09, 4.65 ± 0.93 and 4.26 ± 0.77 with 0.1, 1.0 and 10 μg M. bovis PPD respectively for

Table 9. Results of lymphocyte blastogenic assays using 0.1 μg/well *Mycobacterium bovis* PPD and lymphocytes from dogs experimentally exposed to *M. bovis* and controls

	Weeks Pre-exposure and Post-exposure					
M. bovis exposed	-4	-2	3	6	9	12
8782	NT a	0.68 b	8.55	3.84	7.00	14.20
8745	NT	1.85	3.07	1.69	8.12	4.26
8596	NT	0.65	3.11	1.60	15.89	1.14
8770	NT	0.39	5.32	2.30	9.60	8.88
8794	NT	2.05	2.93	1.64	48.99	0.67
8763	NT	0.32	8.40	0.48	5.92	0.75
Controls						
8563	NT	0.86	4.13	0.24	2.19	1.49
8721	NT	0.67	1.70	0.99	1.85	0.92
8718	NT	0.88	3.08	2.35	3.62	1.85
8174	NT	1.05	5.14	0.31	3.26	0.80
8238	NT	NT	1.48	0.85	3.26	0.89
7946	NT	0.70	NT	1.16	1.23	1.21

a No test results.
b Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of *M. bovis* PPD stimulated cultures/ counts per minute of nonstimulated cultures.

Table 10. Results of lymphocyte blastogenic assays using 1.0 μg/well Mycobacterium bovis PPD and lymphocytes from dogs experimentally exposed to M. bovis and controls

M. bovis exposed	Weeks Pre-exposure and Post-exposure					
	-4	-2	3	6	9	12
8782	NT [a]	1.33 [b]	6.37	4.69	8.46	9.77
8745	NT	2.45	2.67	3.20	5.87	5.12
8596	NT	1.55	3.09	1.25	19.89	0.88
8770	NT	0.82	3.64	1.14	8.85	5.71
8794	NT	2.43	3.64	1.62	41.51	0.71
8763	NT	0.97	8.46	0.56	8.84	0.87
Controls						
8563	NT	1.33	3.82	0.59	1.76	1.03
8721	NT	1.61	0.94	0.93	1.53	0.87
8718	NT	1.26	3.25	3.70	2.97	1.76
8174	NT	1.03	3.84	0.72	2.73	0.81
8238	NT	NT	1.28	0.97	2.91	1.09
7946	NT	0.83	NT	0.61	1.88	1.96

a No test results.

b Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of M. bovis stimulated cultures/ counts per minute of nonstimulated cultures.

Table 11. Results of lymphocyte blastogenic assays using 10.0 µg/well *Mycobacterium bovis* PPD and lymphocytes from dogs experimentally exposed to *M. bovis* and controls

M. bovis exposed	Weeks Pre-exposure and Post-exposure					
	-4	-2	3	6	9	12
8782	2.21 a	1.37	7.17	7.52	4.80	6.86
8745	0.22	1.77	2.08	3.00	2.40	4.25
8596	4.76	1.16	2.95	1.00	24.03	0.99
8770	0.83	0.71	4.86	1.17	8.46	10.53
8794	1.32	0.80	3.09	0.73	11.67	1.05
8763	1.24	0.92	5.39	1.03	3.93	0.88
Controls						
8563	1.14	0.77	4.53	0.69	1.33	1.14
8721	0.54	2.46	0.68	1.87	2.32	0.72
8718	0.58	1.02	2.42	1.67	3.91	0.61
8174	2.18	1.06	4.88	0.63	2.44	1.20
8238	4.36	NT b	0.72	1.19	3.81	1.03
7946	1.27	0.54	NT	1.15	1.64	1.77

a Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of *M. bovis* stimulated cultures/ counts per minute of unstimulated cultures.
b No test results.

lymphocytes from dogs injected with M. bovis. Lymphocyte responses of control dogs at 3 weeks had mean stimulation indices of 3.11 ± 0.70, 2.63 ± 0.63 and 2.65 ± 0.90 for 0.1, 1.0 and 10 μg M. bovis PPD.

At 6 weeks, lymphocyte responses of M. bovis exposed dogs to 0.1, 1.0 and 10 μg M. bovis PPD were observed to have stimulation indices (mean values) of 1.93 ± 0.45, 2.08 ± 0.64 and 2.41 ± 1.08 respectively. Lymphocyte responses of control dogs to 0.1, 1.0 and 10 μg M. bovis PPD observed at 6 weeks were of 0.98 ± 0.31, 1.25 ± 0.49 and 1.20 ± 0.20.

Maximum stimulation indices were observed at 9 weeks following exposure to M. bovis with each of 3 concentrations of M. bovis PPD. Stimulation indices (mean values) of 15.92 ± 6.77, 15.57 ± 5.56 and 9.22 ± 3.27 were detected with 0.1, 1.0 and 10 μg of M. bovis PPD respectively. Stimulation indices (mean values) of 2.57 ± 0.39, 2.30 ± 0.26 and 2.58 ± 0.44 were observed at 9 weeks to 0.1, 1.0 and 10 μg M. bovis PPD for lymphocytes from control dogs.

Stimulation indices (mean values) at 12 weeks post-exposure were 4.98 ± 2.25, 3.84 ± 1.50 and 4.09 ± 1.62 to 0.1, 1.0 and 10 μg M. bovis PPD respectively for lymphocytes from M. bovis exposed dogs. Lymphocyte responses of control dogs at 12 weeks to 0.1, 1.0 and 10 μg M. bovis PPD were observed to be 1.19 ± 0.17, 1.25 ± 0.20 and 1.08 ± 0.17.

Significant increases in lymphocyte responses of M. bovis

exposed dogs were observed at 3 ($p \leq .01$) and 9 ($p \leq .1$) weeks to 0.1 μg M. bovis PPD. Control dogs also exhibited a significant ($p \leq .01$) increase in lymphocyte responses to 0.1 μg M. bovis PPD at 3 and 9 weeks. Significant increases in lymphocyte responses of M. bovis exposed dogs were detected with 1.0 μg M. bovis PPD at 3 ($p \leq .01$) and 9 ($p \leq .05$) weeks. Lymphocyte responses of control dogs demonstrated a significant increase to 1.0 μg M. bovis PPD at 3 ($p \leq .1$) and 9 ($p \leq .02$) weeks. Statistically significant increases in lymphocyte responses of M. bovis exposed dogs were detected to 10 μg M. bovis PPD at 3 ($p \leq .01$), 9 ($p \leq .01$) and 12 ($p \leq .05$) weeks. A significant increase was observed at 9 weeks ($p \leq .1$) with lymphocytes from control dogs.

Lymphocyte blastogenic responses to 10 and 25 μg concentrations of PHA in dogs exposed to killed M. bovis and controls are shown in Tables 12 and 13. No statistically significant differences in stimulation indices were detected at 4 and 2 weeks before sensitization with 10 or 25 μg PHA. Significant differences in stimulation indices were not detected between M. bovis exposed and control dog lymphocyte responses at 3,6,9 and 12 weeks using 10 or 25 μg PHA.

Increased responses were observed at 3 and 9 weeks ($p \leq .05$) to 10 μg PHA with dogs exposed to M. bovis. No significant increase in lymphocyte responses of control dogs was detected to 10 μg PHA. Significant increases in

Table 12. Results of lymphocyte blastogenic assays using 10 μg/well phytohemagglutinin (PHA) and lymphocytes from dogs experimentally exposed to *M. bovis* and controls

	Weeks Pre-exposure and Post-exposure					
M. bovis exposed	-4	-2	3	6	9	12
8782	4.86 a	37.87	10.99	21.22	81.50	17.70
8745	10.83	12.96	5.27	12.72	20.63	93.88
8596	1.18	23.50	178.92	3.68	297.56	1.33
8770	4.09	3.21	6.22	2.23	28.95	9.74
8794	1.04	58.20	182.46	4.24	5.75	1.45
8763	1.05	41.03	64.69	21.19	107.23	1.73
Controls						
8563	2.74	11.89	28.65	12.82	1.32	NT b
8721	86.10	23.19	NT	7.96	93.66	12.00
8718	3.20	5.21	4.31	11.32	11.99	NT
8174	1.13	148.84	23.63	5.38	22.85	5.49
8238	1.39	3.09	0.73	0.77	22.52	6.16
7946	0.93	17.00	NT	0.74	101.95	5.54

a Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of PHA stimulated cultures/ counts per minute of nonstimulated cultures.
b No test results.

Table 13. Results of lymphocyte blastogenic assays using 25 μg/well phytohemagglutinin (PHA) and lymphocytes from dogs experimentally exposed to *M. bovis* and controls

	Weeks Pre-exposure and Post-exposure					
M. bovis exposed	-4	-2	3	6	9	12
8782	3.41 [a]	19.73	10.72	38.08	158.15	29.50
8745	14.81	18.40	13.46	24.27	45.97	126.53
8596	1.05	13.78	137.36	6.42	445.46	1.20
8770	5.06	3.01	13.64	1.28	64.29	14.14
8794	0.41	52.85	237.38	6.53	7.34	1.40
8763	0.78	40.97	74.72	18.04	193.88	2.36
Controls						
8563	0.79	9.71	55.60	15.90	1.99	NT [b]
8721	101.93	15.06	NT	21.09	194.95	45.05
8718	7.09	11.71	7.14	22.39	28.11	NT
8174	1.47	129.06	32.95	13.68	35.89	9.80
8238	1.11	4.92	1.75	3.87	43.35	21.62
7946	1.92	19.76	NT	0.67	207.60	5.13

a Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of PHA stimulated cultures/ counts per minute of nonstimulated cultures.
b No test results.

lymphocyte responses were observed at 3 ($p \leq .02$) and 9 ($p \leq .01$) weeks to 25 μg PHA in dogs exposed to M. bovis. A significant increase in lymphocyte responses of control dogs to 25 μg PHA was detected at 9 weeks ($p \leq .1$).

Mean stimulation indices for dogs exposed to M. bovis were higher than for control dogs at 3,6,9 and 12 weeks with both 10 and 25 μg PHA. Stimulation index responses (mean values) for lymphocytes from M. bovis sensitized dogs using 10 μg PHA were 74.76 ± 34.71, 10.88 ± 3.59, 90.27 ± 44.38 and 20.97 ± 14.82 at 3, 6, 9 and 12 weeks respectively. Lymphocyte responses of control dogs to 10 μg were 14.33 ± 6.93, 6.50 ± 2.10, 42.38 ± 17.85 and 11.95 ± 4.81 at 3, 6, 9 and 12 weeks.

Stimulation index responses (mean value) to 25 μg PHA for lymphocytes from dogs exposed to M. bovis observed at 3, 6, 9 and 12 weeks post-exposure were 81.21 ± 37.29, 15.77 ± 5.65, 152.52 ± 65.26 and 29.19 ± 19.98. Mean stimulation indices of 24.36 ± 12.44, 12.93 ± 3.64, 85.32 ± 37.15 and 20.40 ± 8.92 were observed with lymphocytes from control dogs to 25 μg PHA at 3, 6, 9 and 12 weeks respectively.

Lymphocyte Blastogenesis with Indomethacin

The enhancing effect of indomethacin in lymphocyte cultures was evaluated with varied dosages of *M. bovis* PPD and PHA at 4 and 2 weeks before and 3, 6, 9 and 12 weeks after experimental exposure to *M. bovis*. Lymphocyte blastogenic responses to 0.1, 1.0 and 10 μg concentrations of *M. bovis* PPD with indomethacin added to lymphocyte cultures from dogs exposed to killed *M. bovis* and controls are shown in Tables 14, 15 and 16.

No significant increase in stimulation indices was detected at 4 and 2 weeks before sensitization with each of 3 concentrations of *M. bovis* PPD with indomethacin added to lymphocyte cultures.

Significant increases in blastogenic responses were detected at 3 weeks when indomethacin was added to lymphocyte cultures from dogs exposed to killed *M. bovis* with *M. bovis* PPD concentrations of 0.1 μg ($p \leq .05$), 1.0 μg ($p \leq .02$) and 10 μg ($p \leq .05$). No significant increases in stimulation indices were detected at 3 weeks in control dog lymphocyte cultures when indomethacin was added with *M. bovis* PPD concentrations.

Significant increases in lymphocyte responses from dogs exposed to killed *M. bovis* and controls were detected when indomethacin was added at 6 weeks with 0.1 μg ($p \leq .01$) and

Table 14. Results of lymphocyte blastogenic assays using indomethacin and 0.1 μg/well *Mycobacterium bovis* PPD added to lymphocyte cultures from dogs experimentally exposed to *M. bovis* and control dogs

M. bovis exposed	Weeks Pre-exposure and Post-exposure -4	-2	3	6	9	12
8782	NT a	1.70 b	8.84	2.96	6.24	2.00
8745	NT	0.66	10.94	25.35	5.44	6.16
8596	NT	1.69	7.39	1.05	1.00	0.62
8770	NT	1.17	11.02	0.39	49.42	19.06
8794	NT	0.96	4.68	0.22	17.33	1.25
8763	NT	0.76	7.96	0.88	5.08	0.97
Controls						
8563	NT	2.10	2.64	3.19	2.38	0.81
8721	NT	0.41	NT	2.83	1.10	2.07
8718	NT	1.38	3.83	1.82	6.35	1.21
8174	NT	1.14	1.48	1.40	10.27	3.28
8238	NT	NT	0.86	2.15	1.85	1.48
7946	NT	2.86	0.77	2.68	1.41	1.15

a No test results.

b Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of *M. bovis* stimulated cultures/ counts per minute of nonstimulated cultures.

Table 15. Results of lymphocyte blastogenic assays using indomethacin and 1.0 μg/well *Mycobacterium bovis* PPD added to lymphocyte cultures from dogs experimentally exposed to *M. bovis* and control dogs

M. bovis exposed	Weeks Pre-exposure and Post-exposure					
	-4	-2	3	6	9	12
8782	NT a	1.79 b	9.00	3.36	7.82	2.80
8745	NT	0.70	9.64	24.79	5.69	6.83
8596	NT	1.41	5.58	1.52	1.38	1.01
8770	NT	1.47	11.13	0.53	57.37	15.67
8794	NT	1.01	5.29	0.49	15.84	2.30
8763	NT	0.89	9.49	1.19	8.01	1.00
Controls						
8563	NT	1.20	2.51	2.29	2.40	0.90
8721	NT	0.33	NT	4.54	1.48	1.87
8718	NT	1.52	4.50	1.83	6.18	1.23
8174	NT	1.07	1.57	5.71	6.82	2.50
8238	NT	NT	1.37	1.02	1.97	2.06
7946	NT	2.40	0.93	2.40	2.04	1.23

a No test results.

b Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of *M. bovis* stimulated cultures/ counts per minute of nonstimulated cultures.

Table 16. Results of lymphocyte blastogenic assays using indomethacin and 10.0 μg/well *Mycobacterium bovis* PPD added to lymphocyte cultures from dogs experimentally exposed to *M. bovis* and control dogs

	Weeks Pre-exposure and Post-exposure					
M. bovis exposed	-4	-2	3	6	9	12
8782	1.03 a	3.72	9.54	2.52	5.16	2.31
8745	0.78	3.29	7.23	9.62	2.62	5.91
8596	11.46	2.17	6.34	0.99	0.97	0.94
8770	2.11	0.77	14.78	1.96	51.36	18.93
8794	1.17	0.79	3.51	0.49	5.95	2.37
8763	1.69	2.41	7.25	0.78	3.13	0.88
Controls						
8563	2.05	2.34	2.70	1.13	4.68	0.92
8721	0.99	1.68	NT b	3.17	1.26	2.85
8718	2.60	6.87	4.42	1.40	5.88	2.95
8174	1.80	1.66	1.63	6.38	5.24	4.58
8238	NT	NT	1.07	0.59	1.45	1.71
7946	1.02	1.20	0.65	1.31	1.50	1.32

a Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of *M. bovis* stimulated cultures/ counts per minute of nonstimulated cultures.
b No test results.

1.0 μg ($p \leq .1$) M. bovis PPD.

No significant increase in blastogenic responses were detected for lymphocytes from M. bovis exposed or control dogs when indomethacin was added to M. bovis PPD concentrations at 9 or 12 weeks with one exception. A significantly ($p \leq .05$) increased stimulation index was detected with 10 μg M. bovis PPD at 12 weeks in lymphocyte cultures from control dogs when indomethacin was added.

Lymphocyte blastogenic responses to 10 and 25 μg concentrations of PHA with indomethacin added to lymphocyte cultures from dogs exposed to M. bovis and controls are shown in Tables 17 and 18. No significant increase in stimulation indices was detected to 10 or 25 μg PHA with indomethacin compared to cultures without indomethacin. A single exception was a significant increase ($p \leq .02$) detected with 10 μg PHA at 6 weeks for dogs exposed to killed M. bovis.

No statistically significant differences in lymphocyte blastogenic responses were detected between M. bovis exposed dogs that received tuberculin skin tests at 6 and 12 weeks and dogs not skin tested. No significant differences were detected in lymphocyte blastogenic responses of control dogs skin tested at 6 and 12 weeks and control dogs not skin tested.

Table 17. Results of lymphocyte blastogenic assays using indomethacin and 10 µg/well phytohemagglutinin (PHA) added to cultures of lymphocytes from dogs experimentally exposed to M. bovis and control dogs

	Weeks Pre-exposure and Post-exposure					
M. bovis exposed	-4	-2	3	6	9	12
8782	12.36 a	42.96	29.12	32.60	79.45	111.25
8745	49.74	14.50	11.83	20.14	62.63	42.91
8596	1.37	47.29	135.91	24.57	168.67	184.88
8770	1.66	12.41	18.30	16.74	57.56	30.48
8794	1.69	14.73	226.81	25.03	11.56	507.18
8763	3.24	61.58	60.53	13.74	146.67	1.49
Controls						
8563	0.59	21.92	12.63	8.57	35.31	NT b
8721	78.85	22.41	NT	18.50	1.63	47.99
8718	14.05	5.43	29.47	9.44	19.82	30.55
8174	0.85	52.94	NT	17.04	68.33	35.82
8238	NT	19.38	2.37	1.31	38.02	7.28
7946	3.26	23.74	NT	17.90	60.04	3.19

a Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of PHA stimulated cultures/ counts per minute of nonstimulated cultures.
b No test results.

Table 18. Results of lymphocyte blastogenic assays using indomethacin and 25 μg/well phytohemagglutinin (PHA) added to cultures of lymphocytes from dogs experimentally exposed to *M. bovis* and control dogs

	Weeks Pre-exposure and Post-exposure					
M. bovis exposed	-4	-2	3	6	9	12
8782	10.12 a	23.92	24.30	35.60	130.66	267.76
8745	37.07	12.90	16.66	44.33	108.74	99.69
8596	0.55	31.53	101.11	18.29	251.36	291.55
8770	0.53	10.48	29.21	13.17	113.70	60.14
8794	0.81	15.39	247.04	32.24	14.58	904.06
8763	0.54	45.83	59.94	19.51	216.36	1.22
Controls						
8563	2.63	23.78	25.52	8.97	74.06	NT b
8721	22.84	20.78	NT	36.63	1.40	84.50
8718	16.67	9.88	43.20	15.29	32.73	57.10
8174	1.10	42.44	NT	4.48	97.34	63.76
8238	NT	11.49	1.12	1.20	52.27	13.56
7946	7.06	47.17	NT	23.47	91.73	4.90

a Data expressed as stimulation index (SI) mean value from duplicate or triplicate cultures. SI = counts per minute of PHA stimulated cultures/ counts per minute of nonstimulated cultures.

b No test results.

Enzyme-linked Immunosorbent Assay (ELISA)

Antigen extracts of M. bovis ATCC 19210 (neotype strain) prepared using Triton X-100 and KCl were examined. A detectable level of protein concentration was calculated for each extract. However, weak reactions on ELISA with the extracts failed to detect differences between positive and negative results. Therefore, an alternative Triton X-100 extract of M. bovis AN-5 was used for ELISA to examine sera samples from dogs.

Results of ELISA evaluated using M. bovis PPD or Triton X-100 extract of M. bovis antigen and Protein A or goat anti-dog conjugates are shown in Figures 5-8.

No significant differences in ELISA reactions were observed with either antigen or conjugate combination at 4 and 2 weeks before injection of sensitinogen into experimental dogs. Significant ($p \leq .01$) increases in ELISA reactions were detected at 3, 6, 9, 12 and 15 weeks in the sera of dogs injected with killed M. bovis. Increased ELISA reactions were not observed in the sera from control dogs at 3, 6, 9, 12 or 15 weeks to any M. bovis antigen or conjugate combination.

Results of ELISA conducted using M. bovis PPD and goat anti-dog conjugate with sera from M. bovis exposed and control dogs are shown in Figure 5. Significant differences in ELISA reactions were detected in sera from M. bovis exposed and

Figure 5. Results of enzyme-linked immunosorbent assays (ELISA) using a PPD of *Mycobacterium bovis* and goat anti-dog IgG as conjugate. The results shown are mean ± SEM values (3 animals/group). The ELISA tests were conducted on sera collected from *M. bovis* exposed and control dogs at 4 and 2 weeks before and at 3, 6, 9, 12 and 15 weeks following exposure to *M. bovis*. A 1:80 dilution of serum was used. Serum was incubated for 8 minutes, conjugate incubated for 15 minutes and color change was measured at 2 hours.

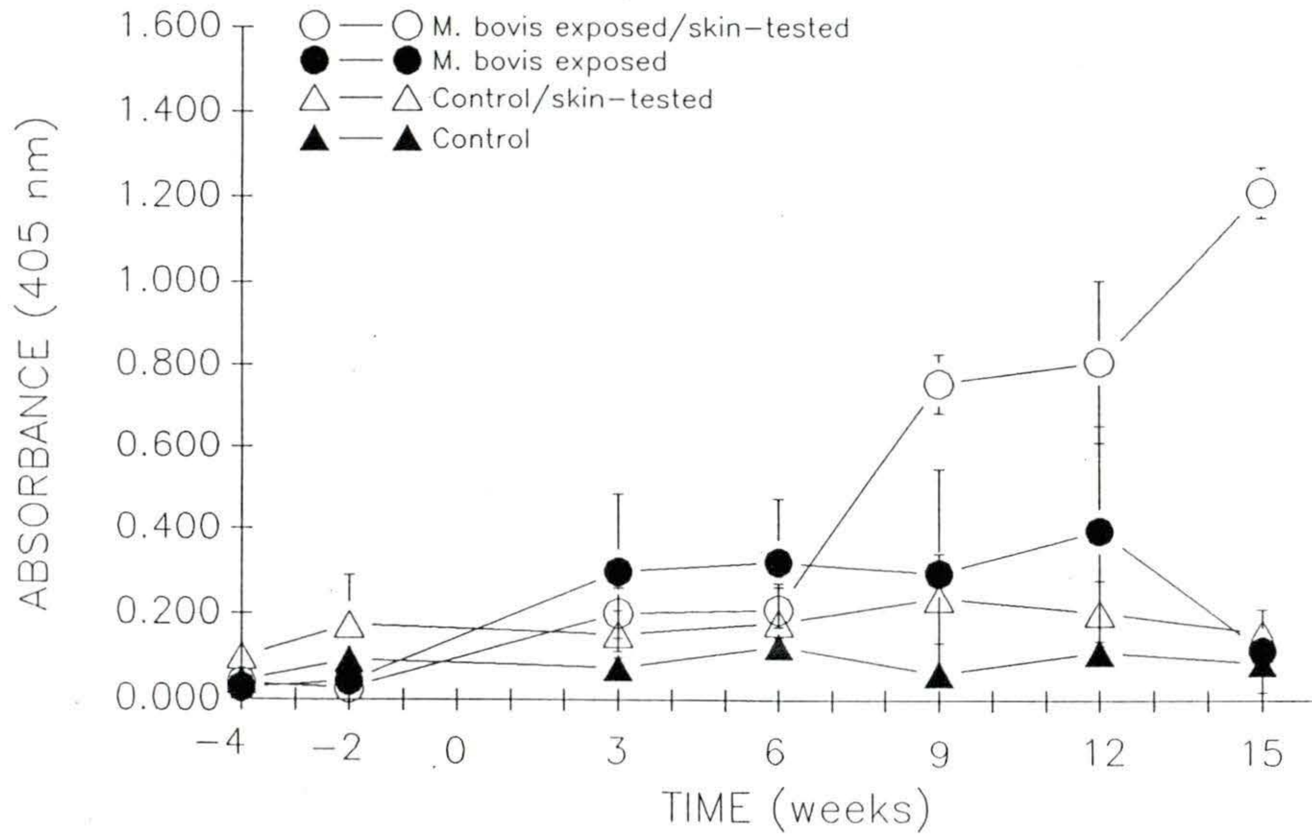

M. bovis exposed/skin-tested
M. bovis exposed
Control/skin-tested
Control
ABSORBANCE (405 nm)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-4
-2
0
3
6
9
12
15
TIME (weeks)

Figure 6. Results of enzyme-linked immunosorbent assays (ELISA) using a PPD of *Mycobacterium bovis* and Protein A conjugate. The results shown are mean ± SEM values (3 animals/group). The ELISA tests were conducted on sera collected from *M. bovis* exposed and control dogs at 4 and 2 weeks before and at 3, 6, 9, 12 and 15 weeks following exposure to *M. bovis*. A 1:80 dilution of serum was used. Serum was incubated for 8 minutes, conjugate incubated for 30 minutes and color change was measured at 2 hours.

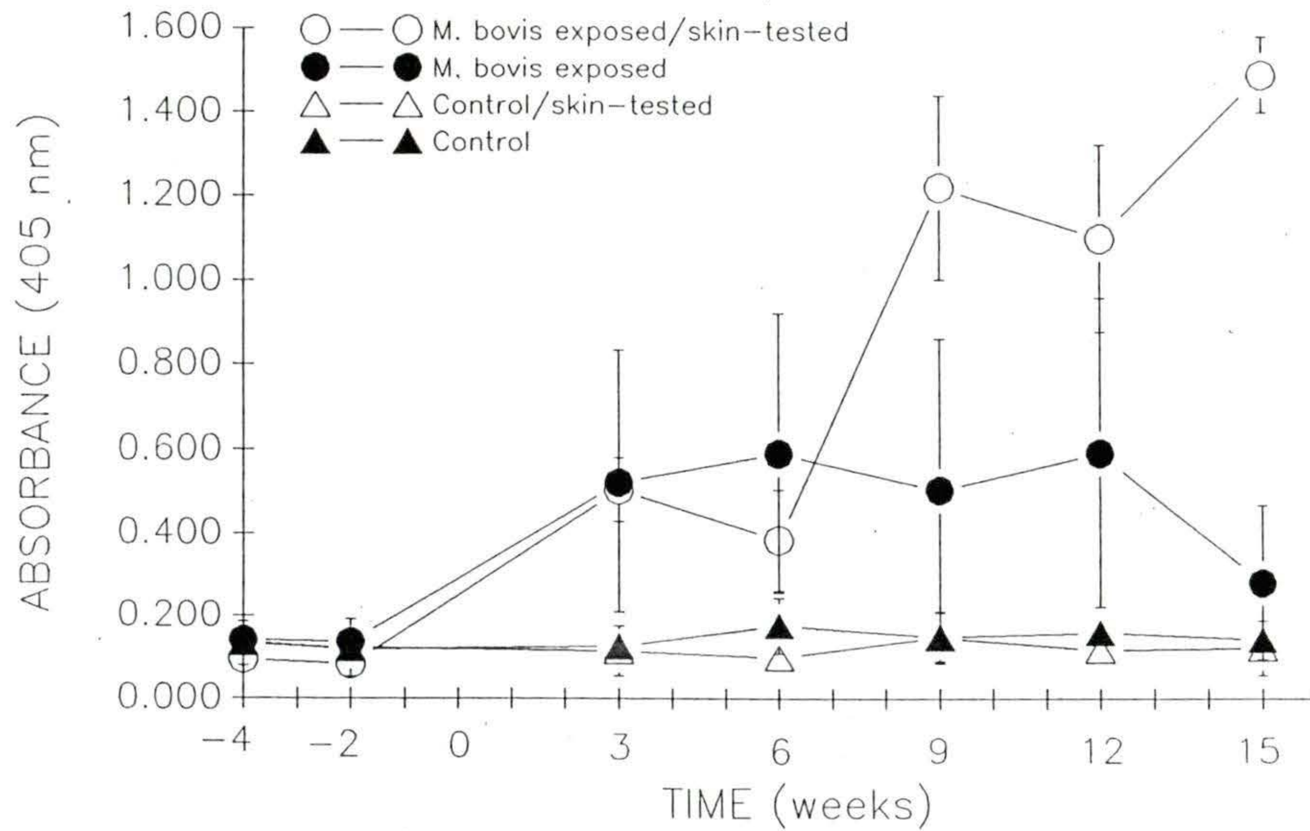

M. bovis exposed/skin-tested
M. bovis exposed
Control/skin-tested
Control
ABSORBANCE (405 nm)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-4
-2
0
3
6
9
12
15
TIME (weeks)

Figure 7. Results of enzyme-linked immunosorbent assays (ELISA) using a Triton X-100 extract of *Mycobacterium bovis* and Protein A conjugate. The results shown are mean ± SEM values (3 animals/group). The ELISA tests were conducted on sera collected from *M. bovis* exposed and control dogs at 4 and 2 weeks before and at 3, 6, 9, 12 and 15 weeks following exposure to *M. bovis*. A 1:80 dilution of serum was used. Serum was incubated for 60 minutes, conjugate incubated for 60 minutes and color change was measured at 3 hours.

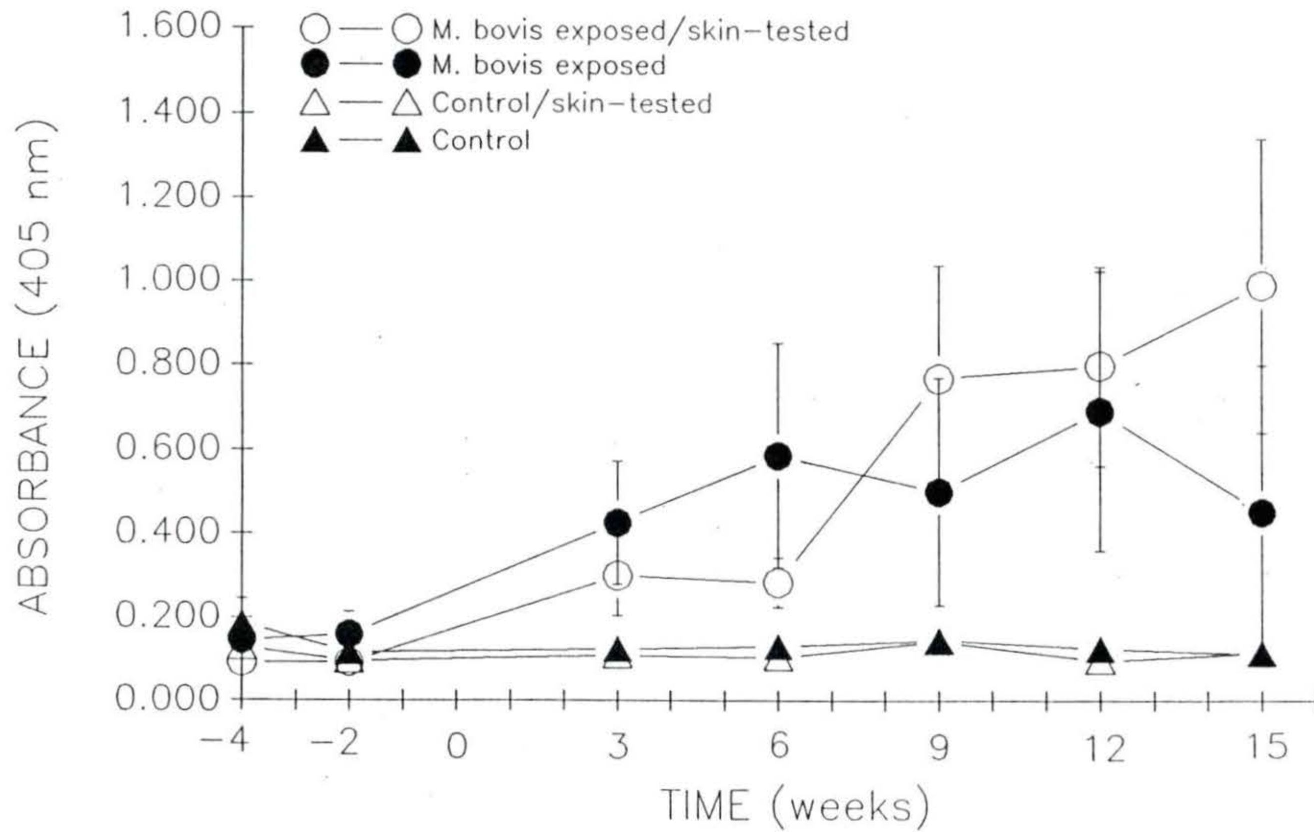
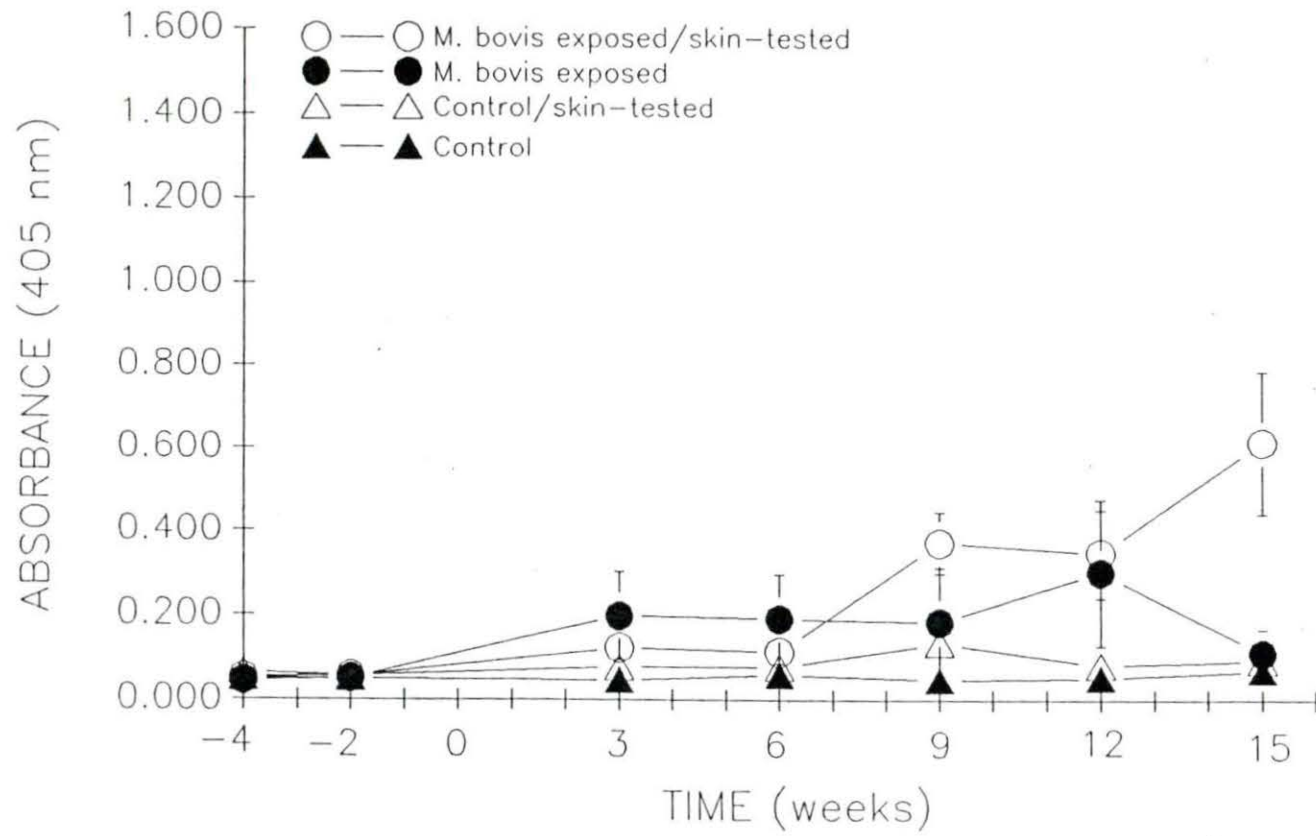

M. bovis exposed/skin-tested
M. bovis exposed
Control/skin-tested
Control
ABSORBANCE (405 nm)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-4
-2
0
3
6
9
12
15
TIME (weeks)

Figure 8. Results of enzyme-linked immunosorbent assays (ELISA) using a Triton X-100 extract of *Mycobacterium bovis* and goat anti-dog IgG conjugate. The results shown are mean ± SEM values (3 animals/group). The ELISA tests were conducted on sera collected from *M. bovis* exposed and control dogs at 4 and 2 weeks before and at 3, 6, 9, 12 and 15 weeks following exposure to *M. bovis*. A 1:80 dilution of serum was used. Serum was incubated for 15 minutes, conjugate incubated for 30 minutes and color change was measured at 3 hours.


M. bovis exposed/skin-tested
M. bovis exposed
Control/skin-tested
Control
ABSORBANCE (405 nm)
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-4
-2
0
3
6
9
12
15
TIME (weeks)

control dogs at 3 weeks post-sensitization ($p \leq .05$). Significant differences in ELISA reactions were also detected at 6 ($p \leq .1$), 9 ($p \leq .02$), 12 ($p \leq .01$) and 15 ($p \leq .02$) weeks.

Significant differences ($p \leq .05$) in ELISA reactions were detected in sera of M. bovis exposed and control dogs using M. bovis PPD and Protein A at 9, 12 and 15 weeks post-sensitization (Figure 6). No significant difference in ELISA reactions were observed in sera M. bovis exposed and control dogs at 3 and 6 weeks following sensitization.

Significant differences in ELISA reactions in sera from M. bovis exposed and control dogs conducted with Triton X-100 and Protein A were detected at 3 weeks ($p \leq .02$). Significant differences in ELISA reactions were also detected at 6 ($p \leq .05$), 9 ($p \leq .05$), 12 ($p \leq .01$) and 15 ($p \leq .02$) weeks (Figure 7).

Significant differences in ELISA reactions with sera from M. bovis exposed and control dogs were detected at 9($p \leq .05$), 12 ($p \leq .02$) and 15 ($p \leq .05$) weeks using Triton X-100 and goat anti-dog conjugate (Figure 8). No significant difference in ELISA reactions was detected at 6 weeks post-exposure.

A comparison of ELISA results with sera from M. bovis exposed and control dogs receiving tuberculin skin tests at 6 and 12 weeks with M. bovis PPD and M. avium PPD are shown in Figures 5-8. A significant increase ($p \leq .05$) in ELISA

reactions at 9, 12 and 15 weeks was detected in sera of *M. bovis* exposed dogs receiving comparative tuberculin skin tests. Increased ELISA reactions were detected with *M. bovis* PPD and Protein A or goat anti-dog conjugates. Significant increases in ELISA reactions in sera of *M. bovis* exposed dogs receiving multiple skin tests were not detected with the Triton X-100 extract of *M. bovis*. No significant difference in ELISA reactions was detected in sera of *M. bovis* exposed dogs at 3 and 6 weeks before administration of comparative tuberculin skin tests.

No significant differences in ELISA reactions were detected in sera of control dogs which received comparative tuberculin skin tests at 6 and 12 weeks with *M. bovis* PPD or *M. avium* PPD.

DISCUSSION

A tuberculin skin test for detecting delayed-type hypersensitivity (DTH) in the dog was developed. Tuberculin was injected in the skin of the cervical region of the dog. Reporting skin test responses as increase in skin thickness at the injection site is preferred to cross-sectional diameter of the area of induration. Previous reports indicate that increases in skin thickness correlated with the immunologic status of the dog better than cross-sectional diameter of the area of induration (11,89).

The largest skin test responses (mean value) were observed at 48 hours; however, some dogs had higher responses at 24 or at 72 hours. Previous investigations in dogs have also reported peak DTH responses at 48 hours when measuring increase in skin thickness in the pinna of the ear (89). Responses observed in control dogs at 24 hours may be due to residual Arthus reactions to polysaccharide and protein antigens in commercial PPD preparations as previously reported (18).

Antigen-specific induction of DTH responses was evaluated using <u>M. bovis</u> PPD and <u>M. avium</u> PPD. Greater DTH responses were detected to <u>M. bovis</u> PPD than to <u>M. avium</u> PPD in dogs injected with killed <u>M. bovis</u>. Mean responses at 24, 48 and 72 hours to <u>M. bovis</u> PPD were greater than to <u>M. avium</u> PPD

during comparative tuberculin skin tests at 6 and 12 weeks. No detectable skin test responses were observed in control dogs to either antigen at 6 and 12 weeks.

Induction of skin test responsiveness to tuberculin antigens in dogs after tuberculin tests at 6 and 12 weeks was not observed in the present study. Previous studies by Thilsted and Shifrine reported that repeated skin tests induced a moderate degree of tuberculin sensitivity in 2 of 8 nonimmunized dogs after 2 skin tests with PPD tuberculin at 7 day intervals (89). Because responses to PPD tuberculin were observed on the initial test, exposure to mycobacteria in the environment or saprophytes is an important consideration of the previous study. Further studies are needed to evaluate time interval periods between repeat skin tests and determine the effects on skin test responsiveness.

Positive tuberculin responses observed in dogs injected with killed M. bovis provided evidence of successful sensitization to M. bovis. The specificity of the sensitization to M. bovis was evidenced by greater reactions to M. bovis PPD than to M. avium PPD. Additional investigations are required to obtain information concerning DTH responses in the cervical region of dogs exposed to viable M. bovis.

Dogs that were sensitized by subcutaneous injection of heat-killed M. bovis in mineral oil developed a granuloma at

the site of injection. The granuloma appeared 4 weeks after injection of sensitinogen at which time treatment was started. The dogs were treated with Benzapin injections for a short time and then administered Clavamox and Batril orally for the duration of the study or until the granuloma softened. These subcutaneous granulomas have been observed previously in cattle sensitized by heat-killed M. bovis in mineral oil (39).

Examination of skin biopsy sections from tuberculin injection sites showed infiltration of mononuclear cells and edema causing separation of the collagen fibers in the dermal connective tissue. These results are in agreement with previous reports describing the changes within the dermis at the skin test site (7,89,107).

Lymphocyte transformation assays have been used to monitor cell-mediated responses in animals experimentally or naturally exposed to M. bovis or other pathogenic mycobacteria (67,91,92). Previous investigations revealed a positive correlation between degree of lymphocyte transformation and DTH reactions in the dog. Therefore, the lymphocyte transformation assay may be used as a quantitative measure of cell-mediated responses in the dog (90).

The results of in vitro lymphocyte stimulation test reported herein were obtained using isolated peripheral blood lymphocytes from dogs injected with killed M. bovis cells in oil and controls. Lymphocyte responses from dogs exposed to

M. bovis and controls stimulated by M. bovis PPD or PHA were consistent with previous reports on antigen and mitogen induced transformation studies.

Higher stimulation indices (mean values) were observed to each of the 3 concentrations of M. bovis PPD for lymphocytes from M. bovis exposed dogs than from control dogs. The highest stimulation indices (mean values) to M. bovis PPD were observed at 9 weeks for lymphocytes from dogs injected with killed M. bovis. Significant increases in lymphocyte responses were detected at 3, 9 and 12 weeks with 10 μg M. bovis PPD.

Concentrations of PHA and PPD were varied to determine the optimal concentration to stimulate lymphocyte transformation that would show a recognizable distinction between M. bovis exposed and control dogs.

Higher blastogenic responses were observed following stimulation by PHA as compared to stimulation by M. bovis PPD. The PHA stimulated lymphocyte cultures demonstrated a dose-related effect. Increased lymphocyte transformation responses were observed with 25 μg PHA as compared to 10 μg PHA. Higher lymphocyte responses (mean stimulation index) were observed with the higher concentration of PHA for both M. bovis exposed and control dogs.

Comparing lymphocyte responses to M. bovis PPD concentrations revealed that higher stimulation indices (mean

value) were detected for lymphocytes from dogs exposed to M. bovis than from controls. However, PPD stimulated cultures did not demonstrate a dose related response.

Variability in stimulation indices as determined by ^{3}H-thymidine incorporation is common to the lymphocyte stimulation test. Large variability in stimulation indices was observed which increased standard error for statistical analysis of results. To evaluate lymphocyte transformation assays, many variables that influence results need consideration. A few to include are cell cycle kinetics, temporal variations, age of donor, serum factors in cultures, duration of incubation period and cell viability (56).

Results of lymphocyte blastogenic assays with M. bovis PPD demonstrated the variability of lymphocyte responses. At 6 weeks, the stimulation indices to M. bovis PPD for most sensitized and control animals tended to markedly decrease compared to 3 week responses. Lymphocyte responses at 9 weeks increased to peak stimulation indices (mean value) observed and demonstrated a significant difference between M. bovis exposed and control lymphocytes. At 12 weeks, 3 of 6 (animals no. 8596,8794,8763) dogs exposed to M. bovis were observed to have decreased lymphocyte responses (mean stimulation indices).

In previous investigations, a decrease in lymphocyte transformation induced by PPD was observed 32 days following

immunization of dogs (88). The present study demonstrated a similar decline in lymphocyte responses with dogs exposed to M. bovis at 6 weeks following injection of sensitinogen. An explanation for this reduction in lymphocyte responses that occurs in both man and dogs to PPD is unknown (88).

In this study, the optimal concentration of M. bovis PPD for lymphocyte blastogenic tests conducted in dogs was observed to be 10 μg/well. At 9 and 12 weeks following injection of sensitinogen, significant differences in stimulation indices between M. bovis exposed and control dogs lymphocyte responses was detected with 10 μg M. bovis PPD. Stimulation index responses to 10 μg M. bovis PPD at 3, 6 and 12 weeks did not reveal detectable differences using 0.1 and 1.0 μg M. bovis PPD. A lower stimulation index mean was observed with 10 μg M. bovis PPD was observed at 9 weeks; however, the difference between lymphocyte responses of M. bovis exposed and control dogs was significant. Significantly greater responses were observed with lymphocytes from dogs injected with killed M. bovis at 3, 9 and 12 weeks using 10 μg M. bovis PPD.

In a previous report, repeated tuberculin skin tests appeared to increase in vitro bovine lymphocyte responses to M. bovis PPD (39). Results of this investigation demonstrated that repeated skin tests at 6 and 12 weeks did not increase in vitro lymphocyte responses to PPD in dogs.

Indomethacin is a potent inhibitor of prostaglandin synthesis and has been used to enhance immune responses by regulating prostaglandin production (30,34,35,69,70). Since prostaglandins inhibit many immune responses, blocking prostaglandin production with indomethacin should restore these immune functions and increase lymphocyte responsiveness.

The potential for indomethacin to enhance lymphocyte blastogenic responses was being further evaluated in the dog after experimental exposure to *M. bovis*. Other reports have cited the enhancing effects of indomethacin in the dog (30,69,70). The data show that indomethacin was not consistently effective in enhancing lymphocyte transformation. Previous studies have indicated that indomethacin can significantly enhance lymphocyte blastogenic responses to antigenic or mitogenic stimulation (70). The results at 3 weeks are in agreement with previous findings. Increased lymphocyte responses (stimulation index mean values) to each of 3 concentrations of *M. bovis* PPD with indomethacin were observed only at 3 weeks with lymphocytes from dogs injected with killed *M. bovis*. The addition of indomethacin to PHA stimulated lymphocyte cultures did not increase stimulation index for *M. bovis* exposed or control dogs.

Previous investigations with indomethacin have documented significantly enhanced *in vitro* lymphocyte blastogenic responses to PHA in dogs (30) to conA and PPD in cattle (69)

and to SRBC/PPD combined in swine (70). However, the data show that indomethacin did not significantly and consistently enhance in vitro lymphocyte blastogenic responses of dogs to either PPD or PHA. Isolated animal results each week detected increased lymphocyte responses in cultures stimulated with M. bovis PPD or PHA with indomethacin.

The discrepancy of these experimental results concerning indomethacin could be due to absence of optimal concentration of indomethacin in the cultures. This study was designed to evaluate a standard dilution of indomethacin and the potentiating effects of this concentration on cultures stimulated with various concentrations of antigen or mitogen. Maximal stimulation, as determined by previous studies, is observed with optimal indomethacin concentration of 5 µl/ml for SRBC/PPD induced effects with swine spleen cells (70) 1 µg/ml for PHA induced effects with dog lymphocytes (30) and 0.5 µg/well for PPD or 1.0 µg/well for conA induced effects with bovine lymphocytes (69). The concentration of indomethacin reveals major dose-related enhancing effect producing varied stimulation levels at various concentrations (70). Optimal concentration of indomethacin for a specific lymphocyte culture should result in maximal stimulation.

In cultures containing indomethacin, lymphocyte responses of dogs injected with killed M. bovis were significantly

higher than lymphocyte responses from control dogs following stimulation with M. bovis PPD or PHA. Maximal stimulation index (mean value) were observed at 9 weeks with lymphocytes from M. bovis exposed dogs. Dose-related results were observed with lymphocytes from both M. bovis exposed and control dogs to PHA concentrations. These results observed for cultures containing indomethacin were similar to those without indomethacin.

Low ELISA reactions were obtained using a potassium chloride (KCl) and Triton X-100 extracts prepared from autoclaved M. bovis ATCC 19210. The soluble protein concentration of the KCl extract was 0.020 mg/ml and of the Triton X-100 extract was 1.085 mg/ml.

Previous reports have observed a 3-fold or more increase in soluble protein content with Triton X-100 extracts compared to KCl extracts of M. bovis from equal volumes of packed cells. (40) Triton X-100, a nonionic detergent, has been used to solubilize viral, transplantation and tumor-associated antigens as well as cell wall and cell-membrane components of Escherichia coli (40). When EDTA, a chelating agent, was used with Triton X-100 an increased amount of cell wall-associated protein was obtained (40). Generally, only a small percentage of total protein of cells is solubilized by KCl (40).

Previous investigations suggest that autoclaved extracts of mycobacteria lack specificity and reactivity (41,43). Loss

of biologic activity and decreased potency have been noted with an autoclaved lysozyme extract of M. bovis (41). Autoclaving may have denatured or altered reactive components of the lysozyme extract. Moreover, antigenicity as measured by crossed immunoelectrophoresis (CIE) was substantially reduced when culture filtrates and sonic cell extracts of M. bovis were autoclaved (43).

Since detectable differences in ELISA reactions between positive and negative controls were not observed using either the KCl or Triton X-100 extract, a prepared Triton X-100 extract of M. bovis An-5 was obtained and used for the ELISA tests.

Results of ELISA reported herein evaluating sera of dogs exposed to M. bovis appears to be of practical value. Positive ELISA reactions were detected at 3, 6, 9, 12 and 15 weeks in sera of dogs following exposure to M. bovis. Significant increases in ELISA reactions were observed in sera of dogs experimentally exposed to M. bovis as compared to ELISA reactions in sera of control dogs. These positive increases in ELISA reactions in sera from experimentally exposed animals were detected with all antigen and conjugate assays. Significant differences between ELISA reactions of M. bovis exposed and control dogs were detected by 3 weeks following injection of sensitinogen.

Information presented herein suggests M. bovis PPD

provided for greater differentiation between positive and negative ELISA reactions with sera from dogs. A detectable margin between positive and negative responses was observed with mean PPD responses not observed with mean Triton X-100 responses each week tested. Results observed in this investigation with sera from dogs indicated Protein A provided greater differences between positive and negative ELISA reactions.

Results of ELISA indicated that M. bovis exposed dogs that received multiple skin tests had higher ELISA reactions than M. bovis exposed dogs that were not skin tested. Statistically significant ($p \leq .05$) increases in ELISA reactions were detected at 9, 12 and 15 weeks in sera of dogs exposed to M. bovis and skin tested at 6 and 12 weeks. These significant increases were detected by M. bovis PPD and not by Triton X-100 extract of M. bovis. No increase in ELISA reactions were observed in sera of control dogs that were repeatedly skin tested.

Repeated skin testing of an animal previously exposed to mycobacteria appeared to have a "booster-type" effect on serum antibody levels. Previous investigations have reported increases in antibody levels to mycobacterial antigens in the sera of cattle after tuberculin skin tests (76,94).

Further studies are necessary with dogs to explore the practical importance and validate the significance of this

observation. Further investigation with dogs experimentally exposed to viable *M. bovis* or dogs naturally exposed to *M. bovis* are important.

The results of the present study showed positive tuberculin reactions in *M. bovis* exposed dogs, increased lymphocyte responses from *M. bovis* exposed dogs to *M. bovis* PPD *in vitro* and detection of increased levels of specific antibody to *M. bovis* in sera from *M. bovis* exposed dogs; all results provide evidence of successful sensitization of dogs to *M. bovis*.

SUMMARY

The cell-mediated and humoral immune responses of 6 adult dogs inoculated with heat-killed *Mycobacterium bovis* in oil were evaluated. Tuberculin skin tests were conducted to measure delayed-type hypersensitivity (DTH) responses *in vivo*. The cervical region provided a suitable skin test site for detecting DTH in dogs injected with killed *M. bovis*. Measurements of DTH to tuberculin purified protein derivative were recorded at 24, 48 and 72 hours. Skin test reactions were quantitated by measuring increase in skin thickness at the injection site and cross-sectional diameter of the indurated and erythematous area. Maximal DTH responses were generally observed at 48 hours in dogs exposed to heat-killed *M. bovis* in oil. No detectable skin test responses were observed at 48 hours in nonexposed control dogs. Skin test responses to *M. bovis* PPD were greater than responses to *M. avium* PPD in dogs exposed to killed *M. bovis*. Multiple tuberculin skin tests did not induce tuberculin sensitivity in control dogs.

Lymphocyte blastogenic assays were used to evaluate *in vitro* lymphocyte responses following *M. bovis* PPD or PHA stimulation of peripheral blood lymphocytes isolated from the dogs. Significant differences in lymphocyte blastogenic responses between *M. bovis* exposed and control dogs were

detected at 9 weeks post-exposure with each of 3 concentrations of *M. bovis* PPD. Higher blastogenic responses (mean stimulation index) to *M. bovis* PPD were observed with lymphocytes from dogs injected with *M. bovis* sensitinogen than from control dogs. Lymphocyte responses (mean stimulation index) to PHA were greater than responses to *M. bovis* PPD for both *M. bovis* exposed dogs and control dogs.

A modified enzyme-linked immunosorbent assay (ELISA) was evaluated to detect mycobacterial antibodies in the sera of dogs experimentally exposed to killed *M. bovis* cells in mineral oil. The tests were conducted using *M. bovis* PPD and a Triton X-100 extract of *M. bovis*. Horseradish peroxidase labeled Protein A or affinity purified goat anti-dog (H+L) were used as conjugates. Significant ($p \leq .01$) increases in ELISA reactions were observed with sera from dogs following injection of heat-killed *M. bovis* in oil. No significant increases in ELISA reactions were detected in sera of nonexposed (control) dogs. Results also indicated higher ELISA reactions were observed with sera from *M. bovis* exposed dogs that received skin tests at 6 and 12 weeks. No difference in ELISA reactions could be detected in sera of control dogs receiving repeated tuberculin skin tests at 6 and 12 weeks.

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APPENDIX A

Lymphocyte Blastogenic Assays (counts per minute data)

Animal No.	Week	Not stimulated	0.1 µg/well	1.0 µg/well	10 µg/well
			Concentrations of *Mycobacterium bovis* PPD		
8770	-4	1397	----	----	491
		1197	----	----	535
		----	----	----	462
	-2	707	----	----	446
		364	----	----	483
		620	----	----	277
	3	1210	4342	3563	3705
		880	4303	3662	5497
		766	6559	3170	4689
	6	192	564	349	338
		182	472	194	187
		301	----	225	----
	9	1035	7458	6904	6533
		644	7248	7745	7084
		----	9499	7654	7710
	12	60	647	396	650
		56	401	250	693
		61	----	365	519
8596	-4	571	----	----	2494
		429	----	----	1687
		319	----	----	----
	-2	147	186	311	305
		312	97	250	202
		198	----	458	----
	3	164	516	477	317
		115	415	490	573
		172	----	422	437
	6	142	205	206	146
		159	327	107	159
		----	190	253	147
	9	106	1703	2709	2729
		174	2297	2632	3099
		98	2007	2178	3256
	12	67	128	84	64
		96	52	56	92
		57	70	52	61

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD 0.1 µg/well	1.0 µg/well	10 µg/well
8782	-4	803	----	----	2434
		976	----	----	2044
		----	----	----	1428
	-2	243	129	300	383
		129	150	196	260
		247	----	324	207
	3	723	7704	5156	6071
		999	6861	5349	7191
		819	7152	5682	4949
	6	208	717	1158	1358
		149	540	847	1571
		188	841	557	1174
	9	75	489	453	405
		76	497	739	266
		77	610	738	425
	12	66	1148	697	355
		84	725	412	502
		49	----	826	503
8794	-4	595	----	----	669
		581	----	----	827
		615	----	----	862
	-2	69	128	150	58
		93	203	181	66
		----	----	260	72
	3	603	1962	1750	2851
		815	1899	2035	1578
		696	2342	3910	2109
	6	1320	1494	1692	716
		793	1362	1061	559
		502	----	1493	----
	9	281	12585	9356	3421
		357	12912	13073	3171
		178	14480	11447	2926
	12	86	60	61	89
		70	55	60	81
		102	----	----	99

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD 0.1 μg/well	1.0 μg/well	10 μg/well
8763	-4	207	----	----	269
		215	----	----	158
		147	----	----	278
	-2	465	153	404	621
		565	89	400	419
		----	259	690	382
	3	460	4091	3127	3017
		522	4776	4545	1729
		512	3675	4974	3300
	6	291	240	156	380
		419	94	215	341
		262	127	170	280
	9	108	629	551	471
		70	379	1036	326
		90	573	773	253
	12	72	51	54	62
		65	45	45	50
		68	56	79	68
8745	-4	499	----	----	103
		534	----	----	104
		372	----	----	----
	-2	241	575	608	517
		335	493	803	505
		----	----	----	----
	3	407	1613	1196	1237
		327	1363	1054	815
		609	1150	1334	750
	6	175	287	560	377
		119	250	405	578
		184	----	562	475
	9	1042	6724	5109	2551
		835	8464	5318	2025
		----	7686	6102	2177
	12	195	946	963	693
		233	875	1009	1292
		----	----	1312	741

Animal No.	Week	Not stimulated	Concentrations of *Mycobacterium bovis* PPD 0.1 µg/well	1.0 µg/well	10 µg/well
8721	-4	16729	----	----	8166
		21434	----	----	4312
		9405	----	----	12611
	-2	303	235	612	713
		192	166	316	731
		399	----	510	754
	3	118	394	126	109
		204	153	175	111
		----	----	----	----
	6	302	261	256	308
		201	176	124	529
		138	196	215	363
	9	118	154	184	411
		86	243	164	160
		139	235	----	224
	12	108	129	105	126
		96	129	103	74
		167	83	116	67
8174	-4	551	----	----	860
		379	----	----	1170
		----	----	----	----
	-2	1872	2563	1764	2781
		1401	1390	2284	1082
		2352	----	1769	2085
	3	383	2191	1143	1401
		247	1132	1407	1268
		243	1166	797	1593
	6	19701	9969	21058	12573
		22150	4024	10883	20902
		25095	----	----	8442
	9	143	358	299	302
		105	453	424	358
		124	400	290	247
	12	65	68	83	88
		98	68	58	119
		119	90	87	132

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD 0.1 μg/well	1.0 μg/well	10 μg/well
8238	-4	200	----	----	628
		189	----	----	820
		109	----	----	----
	-2	----	----	----	----
		----	----	----	----
		----	----	----	----
	3	87	84	97	93
		88	168	199	95
		161	245	134	55
	6	4721	2751	3232	4271
		3865	3559	3899	4495
		2499	----	----	----
	9	118	387	373	541
		142	299	446	448
		107	509	245	406
	12	60	78	59	102
		83	59	72	73
		81	65	114	55
8563	-4	560	----	----	649
		490	----	----	542
		----	----	----	----
	-2	698	505	564	317
		449	384	538	502
		298	359	821	296
	3	589	1798	1463	1818
		349	1655	1525	1997
		391	2036	2086	2207
	6	31102	4447	14650	13995
		20203	7631	15486	21605
		----	----	----	----
	9	238	616	433	324
		338	777	549	469
		296	515	553	364
	12	82	133	79	104
		88	126	86	101
		91	----	106	92

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD 0.1 μg/well	1.0 μg/well	10 μg/well
8718	-4	3406	----	----	2138
		2302	----	----	1704
		4263	----	----	----
	-2	285	152	293	251
		138	148	207	162
		156	210	232	179
	3	1993	7395	6242	3947
		2196	5962	6558	4649
		2030	5806	7387	6441
	6	527	1085	1931	719
		287	892	1076	583
		----	892	----	739
	9	131	720	379	739
		207	489	363	504
		132	498	657	598
	12	853	1083	991	526
		420	1279	1245	281
		----	----	----	351
7946	-4	356	----	----	320
		184	----	----	327
		228	----	----	----
	-2	366	335	420	197
		456	209	225	235
		----	314	375	234
	3	----	----	----	----
		----	----	----	----
		----	----	----	----
	6	149	223	68	171
		131	106	109	169
		165	188	94	----
	9	95	95	188	120
		74	108	145	134
		81	103	135	155
	12	58	82	131	105
		77	89	147	147
		78	----	----	----

		Concentrations of phytohemagglutinin (PHA)		
Animal No.	Week	Not stimulated	25 μg/well	10 μg/well
8770	-4	150	1066	966
		196	1240	867
		----	----	967
	-2	2873	13998	13697
		5810	11645	11621
		4008	12537	15366
	3	216	3275	1836
		594	7229	2646
		----	6072	3078
	6	2368	5174	6292
		5194	5265	9307
		3516	3774	9128
	9	201	12656	5815
		176	11377	5062
		195	12806	5712
	12	187	2711	1837
		187	2624	1805
		----	2599	----
8596	-4	112	72	170
		104	152	98
		74	81	75
	-2	1658	21455	39544
		1201	23829	26054
		----	13847	35203
	3	143	24495	27522
		90	27522	24930
		228	11445	26227
	6	278	1094	797
		157	1759	683
		158	962	708
	9	144	55643	37701
		105	49577	35505
		114	56483	34810
	12	81	121	105
		97	112	90
		74	70	142

Animal No.	Week	Not stimulated	25 µg/well	10 µg/well
		Concentrations of phytohemagglutinin (PHA)		
8782	-4	449	1683	2439
		445	1564	2120
		----	1324	1959
	-2	193	4510	8830
		227	4385	7562
		252	4361	9053
	3	763	9177	8878
		672	10741	10564
		926	5392	6499
	6	1364	48808	31803
		1307	50090	27225
		----	53716	26012
	9	240	47381	25952
		359	47711	25153
		340	53411	25419
	12	674	24142	13427
		711	16684	11875
		----	20513	11492
8794	-4	2090	404	1708
		1147	635	1620
		1558	916	----
	-2	209	12438	12695
		235	10962	13888
		228	11442	12526
	3	143	29910	22765
		105	26396	19609
		115	29862	23859
	6	5516	34035	29801
		6116	38622	25155
		----	41288	18965
	9	2413	17112	11236
		2148	18439	17303
		2468	16040	11899
	12	73	86	104
		60	96	87
		53	80	78

		Concentrations of phytohemagglutinin (PHA)		
Animal No.	Week	Not stimulated	25 µg/well	10 µg/well
8763	-4	714	450	872
		856	712	895
		1058	882	1006
	-2	392	15660	20927
		352	19796	18568
		675	22679	18732
	3	389	24431	23855
		247	31166	18593
		299	14341	18100
	6	488	13495	13012
		614	6796	10469
		509	8764	10651
	9	200	34476	19127
		158	35226	18529
		171	32663	18961
	12	58	121	147
		101	159	124
		65	251	118
8745	-4	389	5301	3947
		266	5733	3111
		----	3544	3599
	-2	237	3238	2951
		242	5551	2291
		168	3135	3154
	3	606	5971	2724
		337	6281	2168
		385	5632	2114
	6	1853	58481	33255
		2049	55583	27498
		3139	56795	28794
	9	563	23333	14118
		513	22497	9635
		469	25199	8119
	12	167	25315	15157
		193	23535	15630
		204	22515	22163

Animal No.	Week	Not stimulated	25 µg/well	10 µg/well
		Concentrations of phytohemagglutinin (PHA)		
8721	-4	338	30959	21406
		224	26031	23096
		262	27103	26530
	-2	1000	18368	23423
		698	16703	26330
		1510	13222	24631
	3	----	----	----
		----	----	----
		----	----	----
	6	1478	29194	12500
		1197	36740	8990
		1807	28576	14191
	9	239	41057	18459
		203	40311	23529
		194	42621	17577
	12	128	5651	1322
		109	4955	1630
		123	5612	1368
8174	-4	145	155	187
		166	304	167
		----	----	----
	-2	213	25374	27788
		170	26059	29994
		213	25616	31077
	3	848	29627	18700
		613	24005	15997
		----	18635	17121
	6	1691	19556	6666
		1169	----	8732
		----	----	----
	9	824	33900	24617
		1074	30837	22111
		789	31737	14698
	12	458	4273	2598
		419	3996	2269
		----	4636	2367

Animal No.	Week	Concentrations of phytohemagglutinin (PHA)		
		Not stimulated	25 μg/well	10 μg/well
8238	-4	120	149	229
		151	179	164
		156	146	200
	-2	249	890	432
		154	893	741
		167	1022	587
	3	232	652	205
		473	393	272
		----	813	301
	6	779	3110	703
		563	2084	427
		----	----	424
	9	364	14793	6617
		384	15258	10391
		304	15593	6711
	12	99	2239	748
		100	2231	582
		105	2081	535
8563	-4	1356	843	2696
		1000	667	3770
		----	1299	----
	-2	636	4257	5834
		228	4827	4238
		----	3504	5332
	3	234	8682	4089
		136	9557	5171
		151	10786	5694
	6	1145	26467	24714
		1382	25575	15508
		1895	18255	16466
	9	5716	12850	8080
		6976	14787	9818
		7175	11945	8289
	12	----	----	----
		----	----	----
		----	----	----

Animal No.	Week	Not stimulated	25 μg/well	10 μg/well
		Concentrations of phytohemagglutinin (PHA)		
8718	-4	144	1383	503
		220	1447	550
		212	1253	790
	-2	93	1879	755
		159	1453	801
		171	1622	649
	3	774	7975	5722
		937	10068	4210
		1505	4932	3928
	6	395	12236	4736
		435	9635	4785
		495	7813	5491
	9	157	3516	1621
		140	3951	1667
		98	3662	1460
	12	----	----	----
		----	----	----
		----	----	----
7946	-4	296	592	267
		237	383	258
		289	604	244
	-2	333	8076	6657
		408	8905	6526
		438	6311	6858
	3	----	----	----
		----	----	----
		----	----	----
	6	280	178	197
		328	149	205
		184	203	187
	9	182	34059	16118
		162	30580	16314
		140	35629	16810
	12	96	462	508
		96	492	396
		74	416	574

Concentrations of *Mycobacterium bovis* PPD with indomethacin added to cultures

Animal No.	Week	Not stimulated	0.1 μg/well	1.0 μg/well	10 μg/well
8770	-4	316	----	----	881
		243	----	----	531
		----	----	----	362
	-2	343	708	658	412
		725	428	716	467
		531	734	983	346
	3	479	6857	7791	7843
		696	8111	6586	11149
		681	5505	6288	8448
	6	898	292	568	2008
		747	439	429	1711
		1198	----	515	----
	9	69	3640	4272	2881
		79	3750	3984	4506
		80	3877	4825	4323
	12	187	4403	3215	4317
		193	4137	3189	4510
		291	----	4122	3893
8596	-4	389	----	----	5670
		432	----	----	3747
		----	----	----	----
	-2	138	311	146	242
		148	177	236	427
		168	277	258	314
	3	362	2775	2530	2069
		334	3002	1711	2291
		----	1938	1587	2256
	6	172	130	281	122
		105	143	147	146
		139	166	205	146
	9	1840	1789	2712	1712
		1459	1765	2176	1690
		----	1371	1952	1387
	12	68	47	64	75
		83	39	63	76
		57	42	84	45

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD with indomethacin added to cultures 0.1 µg/well	1.0 µg/well	10 µg/well
8782	-4	774	----	----	806
		875	----	----	900
		----	----	----	----
	-2	182	304	183	527
		108	229	235	521
		134	184	338	----
	3	1894	17576	17047	14717
		1642	15674	12786	17501
		1809	13999	18291	18787
	6	274	1180	1085	712
		288	637	896	620
		215	483	632	623
	9	198	1020	1143	769
		166	1001	1263	844
		136	1105	1512	974
	12	131	199	230	285
		65	225	289	195
		70	109	228	139
8794	-4	390	----	----	404
		301	----	----	374
		378	----	----	466
	-2	204	196	146	142
		135	109	178	106
		142	155	----	134
	3	236	851	865	696
		159	925	1187	672
		----	1001	1088	718
	6	697	208	319	336
		1181	209	606	546
		----	----	----	501
	9	1375	22082	17528	7678
		1093	19436	20794	7264
		1223	22436	20121	7007
	12	97	153	218	244
		77	88	164	185
		86	86	218	188

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD with indomethacin added to cultures 0.1 µg/well	1.0 µg/well	10 µg/well
8763	-4	322	----	----	528
		255	----	----	528
		----	----	----	404
	-2	193	124	131	430
		86	79	116	245
		----	118	----	----
	3	2723	13753	21277	20783
		2150	20547	23836	13946
		1798	18799	18230	13644
	6	243	218	460	185
		278	439	292	278
		425	177	----	277
	9	260	1199	1036	648
		266	1727	2525	684
		----	1080	2759	1138
	12	75	88	61	62
		63	62	74	80
		83	66	86	53
8745	-4	229	----	----	269
		338	----	----	125
		168	----	----	183
	-2	205	151	182	1021
		235	132	150	591
		272	188	----	725
	3	237	2123	2037	1965
		180	1902	1761	1407
		224	3002	2393	1272
	6	437	8455	7082	3914
		347	10703	12057	3561
		355	9744	9122	3497
	9	161	713	682	417
		156	951	1248	453
		180	1044	905	436
	12	78	637	451	572
		80	311	525	396
		72	----	603	398

Animal No.	Week	Not stimulated	Concentrations of *Mycobacterium bovis* PPD with indomethacin added to cultures 0.1 μg/well	1.0 μg/well	10 μg/well
8721	-4	394	----	----	322
		253	----	----	----
		----	----	----	----
	-2	1400	735	831	3782
		2734	953	514	3155
		----	----	----	----
	3	----	----	----	----
		----	----	----	----
		----	----	----	----
	6	208	676	808	482
		197	554	1036	707
		----	493	----	743
	9	90	86	119	97
		77	84	114	117
		86	107	138	104
	12	126	317	214	405
		115	185	238	324
		----	----	----	305
8174	-4	324	----	----	568
		321	----	----	591
		----	----	----	----
	-2	404	383	521	522
		254	410	408	666
		531	564	339	788
	3	267	267	209	277
		77	210	298	257
		149	253	264	----
	6	2373	4372	13748	13638
		2369	2262	13314	16630
		----	----	----	----
	9	143	1313	1338	857
		149	1509	868	816
		----	1679	782	621
	12	104	323	211	427
		78	266	210	341
		59	198	179	329

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD with indomethacin added to cultures: 0.1 μg/well	1.0 μg/well	10 μg/well
8238	-4	----	----	----	----
		----	----	----	----
		----	----	----	----
	-2	----	----	----	----
		----	----	----	----
		----	----	----	----
	3	81	66	131	105
		84	75	102	100
		----	----	109	63
	6	1277	3268	1919	1150
		1838	3442	1263	682
		----	----	----	----
	9	1518	2557	3788	1431
		1363	2203	2539	2236
		1320	3015	1947	2405
	12	68	123	121	123
		62	78	146	98
		----	88	----	----
8563	-4	611	----	----	1172
		539	----	----	1180
		----	----	----	----
	-2	625	1305	616	1721
		510	938	751	1406
		----	1334	----	858
	3	810	1829	1672	1804
		520	2305	1792	1967
		929	1833	2202	2326
	6	6291	14462	16200	8188
		7167	28519	14657	6070
		----	----	----	8649
	9	182	672	371	1125
		145	402	382	696
		260	328	657	934
	12	93	61	64	82
		75	53	80	90
		83	89	85	60

Animal No.	Week	Not Stimulated	Concentrations of *Mycobacterium bovis* PPD with indomethacin added to cultures		
			0.1 μg/well	1.0 μg/well	10 μg/well
8718	-4	399	----	----	733
		351	----	----	1219
		----	----	----	----
	-2	335	326	474	2073
		224	391	346	1756
		208	344	349	1444
	3	1492	6132	7503	6591
		1694	7418	6918	8704
		1616	4832	7181	5956
	6	364	552	524	480
		312	757	793	547
		350	557	564	413
	9	463	3042	3109	2778
		501	3230	2922	3309
		----	2911	2899	2412
	12	202	292	310	735
		138	272	264	700
		327	240	243	533
7946	-4	221	----	----	215
		309	----	----	373
		208	----	----	169
	-2	318	943	759	373
		230	539	490	283
		----	869	722	----
	3	284	570	301	468
		665	163	578	149
		----	----	----	----
	6	1428	3623	3312	1427
		1029	2759	2222	1766
		1090	3108	2974	1441
	9	126	179	176	135
		117	156	226	209
		91	135	276	154
	12	76	63	66	93
		69	72	70	118
		----	117	135	78

Concentrations of phytohemagglutinin (PHA) with indomethacin added to cultures

Animal No.	Week	Not stimulated	25 μg/well	10 μg/well
8770	-4	387	141	861
		376	264	561
		----	----	480
	-2	382	3920	4939
		333	4167	5127
		484	4485	4824
	3	295	9892	5301
		322	12117	8016
		445	9015	6120
	6	473	5590	8572
		619	9127	9705
		----	6854	----
	9	155	20547	11455
		200	21641	9118
		212	22278	12060
	12	128	7228	4889
		138	8625	3684
		150	9226	4138
8596	-4	101	74	170
		169	71	135
		128	----	242
	-2	1182	33321	52202
		1017	41919	54668
		----	28814	49202
	3	375	24242	34574
		198	22152	33880
		146	26408	29399
	6	584	17725	16626
		568	10870	22483
		829	7616	9530
	9	216	58421	41883
		213	69162	39368
		295	54151	40698
	12	57	26056	17269
		120	26847	15493
		95	26689	17711

Animal No.	Week	Not stimulated	25 µg/well	10 µg/well
		Concentrations of phytohemagglutinin (PHA) with indomethacin added to cultures		
8782	-4	238	1713	1922
		123	1697	2224
		199	2269	2787
	-2	312	8562	10983
		305	6478	14086
		280	6419	13463
	3	685	17527	21473
		638	17439	18251
		578	11257	15665
	6	1527	51949	60155
		1438	66365	38799
		----	40052	46068
	9	450	41364	25756
		293	39097	31348
		224	45753	19644
	12	84	25421	10813
		98	23780	11593
		91	23896	7965
8794	-4	696	513	1364
		822	558	1050
		620	663	----
	-2	422	6900	5532
		423	5275	5693
		392	6847	6977
	3	116	32867	26628
		125	34175	19067
		----	22635	36636
	6	527	21057	12099
		537	19737	13452
		----	10666	14402
	9	1595	28459	21739
		1948	22764	19237
		----	26303	20489
	12	58	57854	30558
		71	54267	32332
		57	56035	----

		Concentrations of phytohemagglutinin (PHA) with indomethacin added to cultures		
Animal No.	Week	Not stimulated	25 μg/well	10 μg/well
8763	-4	878	681	4662
		1312	438	2235
		----	659	3762
	-2	213	8883	10413
		150	9074	13393
		198	7757	10739
	3	354	21533	19911
		269	20232	14686
		344	16141	23873
	6	480	8236	6127
		416	8725	5872
		468	9670	6652
	9	204	37012	29675
		140	38000	22880
		182	38576	24448
	12	76	135	107
		62	66	109
		80	65	110
8745	-4	158	3315	6587
		131	7603	6503
		----	5206	8546
	-2	227	2921	1864
		223	2885	4662
		----	----	----
	3	362	5371	3651
		246	5224	3953
		355	5448	3791
	6	807	44052	22425
		1200	55532	22588
		1370	50148	23006
	9	496	42789	24682
		319	39701	23053
		300	38866	22160
	12	127	14456	7576
		201	10677	6506
		118	19429	5099

Animal No.	Week	Concentrations of phytohemagglutinin (PHA) with indomethacin added to cultures		
		Not stimulated	25 µg/well	10 µg/well
8721	-4	221	4807	16566
		161	6495	18452
		282	3843	17258
	-2	510	8362	14543
		869	18837	14456
		515	12133	13429
	3	----	----	----
		----	----	----
		----	----	----
	6	757	44898	19831
		1109	43308	24207
		1704	42566	----
	9	139	184	243
		129	163	228
		125	199	172
	12	383	33522	15780
		328	28012	17754
		----	28715	17721
8174	-4	298	315	209
		210	245	194
		202	220	203
	-2	604	34286	36500
		684	30101	37723
		833	25619	38069
	3	----	----	----
		----	----	----
		----	----	----
	6	596	2613	7122
		592	2714	13120
		----	----	----
	9	393	52179	35337
		555	40522	34293
		482	46587	28148
	12	179	11519	6752
		205	11330	5677
		162	11963	7131

Animal No.	Week	Concentrations of phytohemagglutinin (PHA) with indomethacin added to cultures		
		Not stimulated	25 μg/well	10 μg/well
8238	-4	----	----	----
		----	----	----
		----	----	----
	-2	84	1116	1476
		124	1354	2171
		81	839	1933
	3	101	215	332
		172	92	236
		----	----	406
	6	153	188	201
		151	158	202
		102	139	129
	9	221	10901	8107
		197	12804	8986
		238	10638	7888
	12	88	1215	614
		82	1285	470
		100	1159	881
8563	-4	1197	3216	607
		847	2836	604
		----	1998	----
	-2	274	5891	7443
		392	7850	6837
		267	8448	6167
	3	350	6768	3474
		243	8515	3917
		257	6382	3330
	6	1182	9976	9240
		981	11696	10978
		1401	10291	10317
	9	636	51669	24604
		641	51611	21258
		726	45143	24903
	12	----	----	----
		----	----	----
		----	----	----

Animal No.	Week	Concentrations of phytohemagglutinin (PHA) with indomethacin added to cultures		
		Not stimulated	25 µg/well	10 µg/well
8718	-4	170	2758	3298
		179	3075	2092
		212	----	1985
	-2	277	3711	1925
		359	3038	1463
		----	2679	1789
	3	460	24276	14823
		558	26031	18336
		705	24091	17582
	6	720	11444	8570
		800	12649	5612
		760	10763	7341
	9	223	5475	6037
		276	8869	3567
		214	9025	4543
	12	139	6770	3703
		121	6675	3715
		----	5741	2847
7946	-4	176	1332	540
		157	1223	757
		185	1111	394
	-2	322	13251	7294
		269	13162	5477
		216	11655	----
	3	----	----	----
		----	----	----
		----	----	----
	6	969	25318	22877
		1146	21567	19502
		1258	32265	17978
	9	113	12492	8406
		166	13191	----
		----	----	----
	12	77	354	203
		88	386	313
		78	450	----

APPENDIX B

Absorbance data from ELISA tests

Animal No.	Test week	*Mycobacterium bovis* PPD antigen: Protein A	*Mycobacterium bovis* PPD antigen: Goat anti-dog
8770	-4	0.152	0.058
	-2	0.093	0.053
	3	0.421	0.146
	6	0.625	0.333
	9	1.422	0.858
	12	1.258	0.916
	15	1.570	1.304
8596	-4	0.053	0.008
	-2	0.088	0.011
	3	0.656	0.141
	6	0.282	0.168
	9	1.456	0.791
	12	1.376	1.080
	15	1.586	1.227
8782	-4	0.081	0.052
	-2	0.064	0.017
	3	0.436	0.316
	6	0.242	0.125
	9	0.782	0.618
	12	0.660	0.427
	15	1.305	1.100
8794	-4	0.215	0.063
	-2	0.231	0.072
	3	1.143	0.665
	6	1.239	0.624
	9	1.217	0.797
	12	1.313	0.911
	15	-----	-----
8763	-4	0.107	0.014
	-2	0.099	0.026
	3	0.161	0.065
	6	0.384	0.147
	9	0.171	0.055
	12	0.324	0.168
	15	0.465	0.210

Animal No.	Test week	*Mycobacterium bovis* PPD antigen: Protein A	*Mycobacterium bovis* PPD antigen: Goat anti-dog
8745	-4	0.104	0.006
	-2	0.080	0.031
	3	0.258	0.163
	6	0.145	0.192
	9	0.117	0.034
	12	0.129	0.108
	15	0.095	0.019
8721	-2	0.116	0.043
	-4	0.069	0.026
	3	0.081	0.045
	6	0.069	0.035
	9	0.073	0.044
	12	0.102	0.054
	15	0.058	0.047
8174	-4	0.234	0.202
	-2	0.261	0.404
	3	0.231	0.215
	6	0.189	0.322
	9	0.274	0.267
	12	0.201	0.271
	15	0.253	0.221
8238	-4	0.049	0.055
	-2	0.038	0.092
	3	0.032	0.196
	6	0.040	0.183
	9	0.097	0.399
	12	0.047	0.284
	15	0.054	0.199
8563	-4	0.198	0.054
	-2	0.116	0.073
	3	0.141	0.052
	6	0.135	0.070
	9	0.115	0.036
	12	0.120	0.045
	15	0.106	0.049
8718	-4	0.137	0.044
	-2	0.166	0.120
	3	0.138	0.080
	6	0.309	0.175
	9	0.263	0.070
	12	0.261	0.183
	15	0.210	0.122

Animal No.	Test week	*Mycobacterium bovis* PPD antigen: Protein A	Goat anti-dog
7946	-4	0.069	0.035
	-2	0.076	0.086
	3	0.100	0.090
	6	0.083	0.130
	9	0.068	0.081
	12	0.097	0.107
	15	0.109	0.089

Triton X-100 extract of M. bovis

Animal No.	Test week	Protein A	Goat anti-dog
8770	-4	0.112	0.071
	-2	0.104	0.066
	3	0.479	0.149
	6	0.397	0.153
	9	1.263	0.506
	12	1.200	0.536
	15	1.281	0.757
8596	-4	0.075	0.028
	-2	0.063	0.033
	3	0.156	0.058
	6	0.255	0.092
	9	0.705	0.352
	12	0.811	0.321
	15	1.390	0.810
8782	-4	0.096	0.061
	-2	0.112	0.072
	3	0.264	0.161
	6	0.200	0.093
	9	0.340	0.253
	12	0.380	0.176
	15	0.292	0.266
8794	-4	0.177	0.059
	-2	0.263	0.077
	3	0.718	0.401
	6	1.087	0.400
	9	1.031	0.437
	12	1.276	0.639
	15	-----	-----
8763	-4	0.131	0.039
	-2	0.134	0.043
	3	0.314	0.066
	6	0.490	0.088
	9	0.318	0.064
	12	0.666	0.186
	15	0.797	0.166
8745	-4	0.133	0.036
	-2	0.082	0.033
	3	0.247	0.126
	6	0.181	0.086
	9	0.150	0.049
	12	0.128	0.075
	15	0.102	0.055

Animal No.	Test week	Triton X-100 extract of *M. bovis*: Protein A	Triton X-100 extract of *M. bovis*: Goat anti-dog
8721	-4	0.147	0.040
	-2	0.080	0.032
	3	0.078	0.051
	6	0.104	0.047
	9	0.141	0.099
	12	0.086	0.036
	15	0.101	0.052
8174	-4	0.198	0.102
	-2	0.170	0.061
	3	0.193	0.106
	6	0.161	0.081
	9	0.205	0.103
	12	0.138	0.072
	15	0.183	0.095
8238	-4	0.046	0.056
	-2	0.041	0.077
	3	0.058	0.074
	6	0.050	0.107
	9	0.085	0.200
	12	0.068	0.137
	15	0.067	0.133
8563	-4	0.275	0.042
	-2	0.072	0.044
	3	0.102	0.049
	6	0.114	0.051
	9	0.091	0.037
	12	0.084	0.047
	15	0.088	0.061
8718	-4	0.211	0.072
	-2	0.205	0.066
	3	0.179	0.055
	6	0.180	0.083
	9	0.196	0.045
	12	0.201	0.066
	15	0.120	0.098
7946	-4	0.071	0.040
	-2	0.078	0.039
	3	0.095	0.038
	6	0.099	0.043
	9	0.155	0.063
	12	0.093	0.042
	15	0.134	0.049