# Some immunologic responses in cattle experimentally exposed to Mycobacterium bovis



by

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# I. INTRODUCTION

Tuberculosis is one of the oldest recorded diseases (43). It is referred to in ancient Egyptian hieroglyphs (64) and Egyptian mummies contain tuberculous lesions (36).

In 1882 Robert Koch identified the organism that causes tuberculosis as an acid-fast bacillus, isolated it and reproduced the disease in laboratory animals by inoculation with the tubercle bacillus (39, 40). In 1896 Lehmann and Neumann proposed the genus name Mycobacterium to include the acid-fast organisms causing tuberculosis and leprosy (27).

In 1898 Theobald Smith isolated a tubercle bacillus from tuberculous cattle and differentiated it from the human tubercle bacillus by greater pathogenicity for rabbits (77-79). Different growth rates and biochemical reactions are additional criteria useful for differentiating the two tubercle bacilli that commonly cause progressive disease in mammals, Mycobacterium tuberculosis (human type) and Mycobacterium bovis (bovine type) (37). When bovine tuberculosis is brought under control in an area, the proportion of infections in cattle caused by Mycobacterium avium complex increases (15). Lesions of these infections are usually associated with the lymph nodes of the gastrointestinal tract.

Mycobacterium bovis is a microaerophilic, nonmotile, nonsporeforming rod (7, 36, 81, 88). The organism produces

no detectable toxins. It has a slow generation time, often requiring longer than 3 weeks for primary isolation. It is resistant to water soluble disinfectants and lysosomal action. It does not produce niacin or reduce nitrates. The organism forms short cords of parallel cells in liquid media (50). Mycobacterium bovis growth is inhibited by glycerol. It forms mammilate colonies that are smooth and dome-shaped with thin edges on certain egg containing media. The organism is gram-positive, but does not stain well with aniline dyes because of the high lipid content in the cell wall. It is routinely stained with hot carbolfuchsin, then resists decoloration by organic acids (acid-fast).

Bovine tuberculosis occurs worldwide (23, 36, 45, 65, 84, 90). It is characteristically a slow wasting disease of cattle, and on postmortem examination partial or complete destruction of organs, particularly the lungs and lymph nodes, is observed (64). Tuberculosis spreads within herds of cattle primarily by the airborne route or by ingestion of contaminated feed or water (24). Congenital infections have been reported (24, 72) as well as genital tract lesions (61, 85).

Transmission of bovine tuberculosis to man also occurs, but was brought under control in most developed countries early in this century when pasteurization of milk and milk products, together with the slaughter of tuberculin test-

positive cattle, became common practice (2, 6). Because cattle is the main domestic species in which the bovine tubercle bacillus is naturally maintained, in the absence of infected cattle the bovine type disappears from the human population (15, 30).

The objectives of this study were:

1. To assay the biologic activity of three purified protein derivative tuberculins of Mycobacterium bovis (European Economic Community standard, proposed international standard and USDA standard) in cattle experimentally exposed to M. bovis
2. To obtain information on the delayed-type hypersensitivity responses to the same tuberculins in cattle from a herd in which Mycobacterium paratuberculosis infection had been diagnosed
3. To compare the in vitro lymphocyte blastogenesis responses to the same tuberculins using cells from cattle experimentally exposed to M. bovis
4. To produce a purified protein derivative tuberculin of M. paratuberculosis and to evaluate its potency in guinea pigs sensitized with killed M. paratuberculosis and in cattle naturally infected with M. paratuberculosis.

## II. LITERATURE REVIEW

Live tubercle bacilli cause little specific antibody formation in animals and relatively little humoral antibody, which fails to provide protection against mycobacterial infections (12, 21, 22, 73). Mycobacterium bovis is not readily destroyed inside professional phagocytes, therefore protection against infection with M. bovis is associated with cell-mediated immunity (44, 51, 69), so named because the protection can be transferred with specifically sensitized cells (38). It was not recognized until 1945 that the immune mechanism involving specific recognition was related to a particular category of lymphocyte (11). The mechanism of acquired resistance to intracellular parasites was later linked to the development of resistant properties in host macrophages (55, 56).

The "Koch phenomenon" was observed in 1891 as a delayed local reaction to a subcutaneous injection of tubercle bacilli into a guinea pig with tuberculosis (42). This type of cellular hypersensitivity (delayed-type hypersensitivity) is associated with, and responsible for, the lesions of tuberculosis (13). Hypersensitivity develops approximately 2 to 4 weeks after exposure (23). Hypersensitivity may wane in cattle with advanced tuberculosis, but the disease can often be detected on clinical examination (23).

The tuberculous granuloma (tubercle) is mainly cellular (15, 76). Bacilli are engulfed by macrophages, but resist being killed and multiply, resulting in death of the macrophage and release of the bacilli and materials that sensitize attracted polymorphonuclear leukocytes (PMNs) and lymphocytes. Lymphocytes, in turn, release soluble lymphokines which attract and activate macrophages (58). Macrophages in the area of infection assume a distinctive appearance and are called epithelioid cells because of their large, vesicular nuclei and extensive pale cytoplasm with poorly defined borders. Several epithelioid cells may coalesce to form a multinucleated giant cell, and this mixture of epithelioid and giant cells forms the center of young tubercles. The lesion may develop into a classical tubercle with central necrosis, with or without calcification, and a periphery of lymphocytes, plasma cells, PMNs, unaltered macrophages and fibrous connective tissue.

Early in his study of tuberculosis, Koch obtained a "brownish transparent fluid" which he said protected against tuberculosis in guinea pigs and also cured established disease (41). His claims for the use of the liquid, tuberculin, as a therapeutic agent were later disproven, but it was shown to be a valuable diagnostic agent for the detection of tuberculosis (18). The methods of administration of tuberculin include a cutaneous test (von Pirquet), a percutaneous

patch test (Moro), an intradermal test (Mantoux) and an ophthalmic test (18, 23). The intradermal test is the most widely used in animals (87).

Tuberculin is a mixture of the soluble components of mycobacteria grown as a floating culture on liquid media (4). Many types of tuberculin have been prepared (47) differing in strain of tubercle bacillus, type of medium, incubation period and method of concentration of tuberculoprotein. Production involves growing the organisms on medium, killing the organisms, separating them from the culture filtrate (CF) and concentrating the CF by heat (Old Tuberculin) or precipitation (purified protein derivative) with ammonium sulfate (AS) or trichloracetic acid (TCA) (3, 48).

A more refined tuberculin was produced by the introduction of a protein-free medium to replace Koch's original glycerinated meat infusion broth (14, 54). The active principle of tuberculin was further purified from the CF by precipitation with AS or TCA (75). These tuberculin antigens detect infections with the homologous species of mycobacteria used in their production. They may also detect infections with heterologous species of mycobacteria, but the skin reactions are usually not as large (16, 17, 25, 33, 35, 49, 57, 70). In cattle, this sensitization may be due to infection with M. tuberculosis, M. paratuberculosis, M. avium complex, Nocardia or acid-fast bacteria from the soil or

water (35). These cross reactions limit the reliability of the tuberculin skin test (47).

Tuberculin purified protein derivative (PPD) is a heterogeneous mixture of tuberculoprotein and bacterial by-products such as nucleic acids, polysaccharides, lipids and ash (47). Large differences in potency are common when PPDs are tested in different species (20, 89). Therefore, to insure that different preparations of tuberculin have similar potency, it is important that they be compared to a well-defined standard (91). It is recommended that a representative sample of each tuberculin product be calibrated (standardized) by comparing its activity with that of a reference standard at the dose and under the conditions (in the biological system) in which it will be used in practice.

Because of the difficulties associated with standardization in humans or domestic animals, sensitized guinea pigs are often used to evaluate the potency and sensitivity of tuberculins (46, 47). Periodic comparisons of bovine PPDs with a reference preparation are carried out in cattle to assure the validity of assays in guinea pigs (28, 52, 53, 83). Guinea pig assays are more practical to conduct than cattle assays but still involve the maintenance of live animals for extended periods. It would be desirable to develop an in vitro method of standardization of tuberculins that

would be faster, less expensive, more sensitive and more specific than guinea pig assays.

Lymphocytes and macrophages collaborate in the recovery from, and acquired resistance to, intracellular pathogens (26). It was first reported in 1963 that lymphocytes from sensitized subjects responded to exposure to the sensitinogen in vitro by morphologic transformation into lymphoblasts in preparation for division (71, 74). A more objective method for assessing lymphocyte transformation than morphology is to measure the amount of radioactively labeled thymidine incorporated during incubation (10, 31).

Lymphocyte blastogenesis assays have been used as an in vitro correlate of delayed-type hypersensitivity reactions in tuberculin skin tests (8, 9, 59, 60, 62, 63, 66, 67). This comparison led to studies to determine the usefulness of the assay for evaluating the biologic activity of tuberculins in vitro (68) and for use in the diagnosis of tuberculosis (1, 5, 29, 34, 86). The in vitro assay has also been used to diagnose paratuberculosis (Johne's disease) in cattle (82).

# III. MATERIALS AND METHODS

## A. Experimental Exposure of Cattle to *Mycobacterium bovis*

### 1. Preparation of inoculum

A cell suspension of *M. bovis* (ATCC 19210) was prepared from a 21-day-old subculture. The organism was incubated at 37°C in Dubos broth with Tween 80 and Dubos Oleic Albumin Complex (DOAC) (Dubos albumin broth). On the morning of the experimental exposure, the cells were harvested by centrifugation at 1,000 X g for 30 minutes. The wet weight was determined and the cells were resuspended in sterile Butterfield's buffer to a final concentration of 0.025 mg per ml. The inoculum was tested for viability and purity by Ziehl-Neelsen stain and subculture in Dubos albumin broth. For composition of Dubos albumin broth and Butterfield's buffer see the Appendix.

### 2. Experimental exposure

Twenty-two neutered male Jersey calves from the same herd that were 6 to 7 months of age were inoculated intratracheally with 0.2 ml of *M. bovis* suspension containing 0.005 mg (wet weight) of cells.

## B. Evaluation of *M. bovis* Antigens

### 1. *Mycobacterium bovis* antigens

Three *M. bovis* antigens were tested:

1. European Economic Community standard for bovine tuberculin Purified Protein Derivative (PPD) (EEC-PPD)
2. Proposed international standard for bovine tuberculin PPD (PIS-PPD)
3. USDA standard PPD SR 318201 (USDA-PPD)

All three tuberculin PPDs were produced from *M. bovis* strain AN5 incubated for 10 weeks. The medium on which the bacilli were grown and the method of precipitation of the tuberculoprotein differed (3, 28):

| | Tuberculin PPD | |
|---|---|---|
| | EEC and PIS | USDA |
| Medium | Modified Dorset-Henley | Modified Reid's |
| Precipitation method | Trichloracetic acid | Ammonium sulfate |

The tuberculins each contained 1 mg tuberculoprotein per ml. Two dilutions of each tuberculin were prepared with isotonic phosphate buffered saline (pH 7.3) containing 0.0005% Tween 80:

| | Dilutions | |
|---|---|---|
| EEC | 1:2 | 1:10 |
| PIS | Undiluted | 1:5 |
| USDA | Undiluted | 1:5 |

2. Delayed-type hypersensitivity skin tests conducted in cattle experimentally exposed to M. bovis

Tuberculin tests were conducted 8 weeks after exposure to M. bovis. Nineteen animals received 0.1 ml of each tuberculin dilution intradermally. Three calves died before the tests. Six injections were randomly allocated to three sites on each side of the neck. The skin thickness was measured at the time of injection and at 72 hours with a dermal thickness gauge by two observers and recorded. The assay was repeated after an interval of 10 weeks. Each animal again received six injections, but the sites were rotated so that the previous injection sites were not used. The three injection sites on each side of the neck formed an equilateral triangle with sides of 15 cm that was centered in the neck (Figures 1 and 2). Four male Jersey calves were used as controls. They were from the same herd as the experimentally exposed animals and were of similar age but were housed separately. They each received the same six injections as the experimentally exposed animals at 8 and 18 weeks after infection.

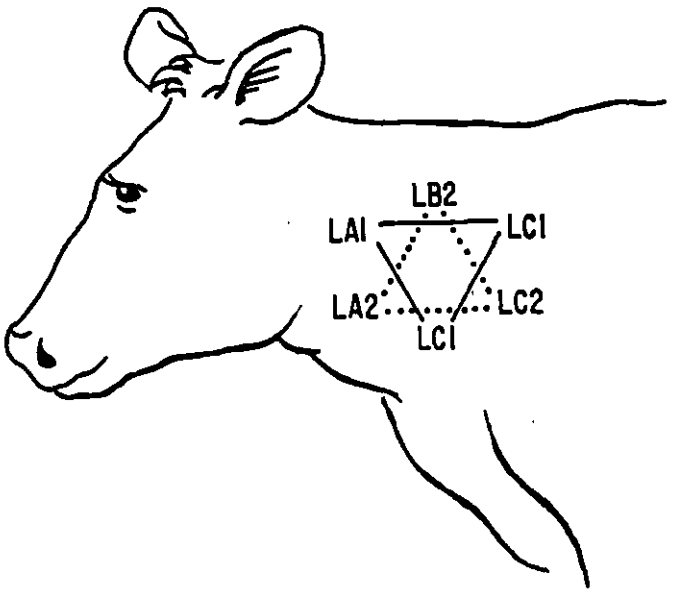


Figure 1. Injection sites of M. bovis PPDs on the left side of the neck at 8 and 18 weeks after exposure. Eight week sites are labeled LA1, LB1 and LC1; eighteen week sites are labeled LA2, LB2 and LC2

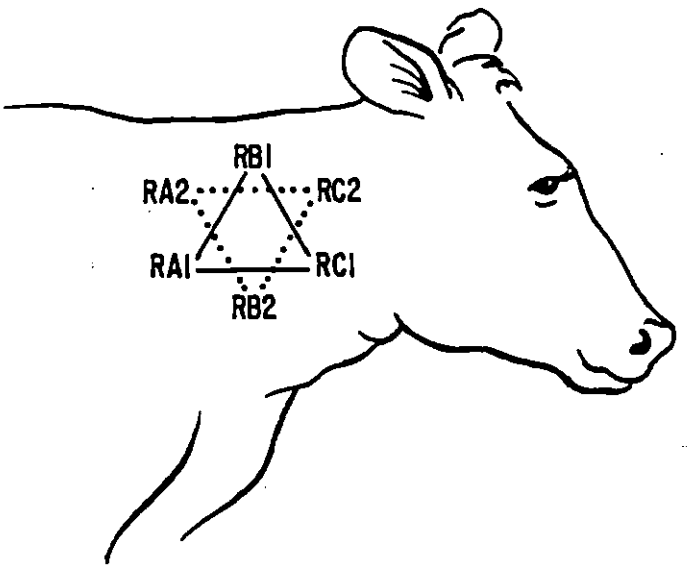


Figure 2. Injection sites of M. bovis PPDs on the right side of the neck at 8 and 18 weeks after exposure. Eight week sites are labeled RA1, RB1 and RC1; eighteen week sites are labeled RA2, RB2 and RC2

3. In vitro lymphocyte blastogenesis assay

Forty milliliters of whole blood were collected from each of three calves (calves 32, 42 and 51) experimentally exposed to M. bovis 11 weeks after exposure into 4.5 ml of 50% acid-citrate-dextrose (ACD) in siliconized 50 ml screw cap test tubes. Blood was also collected from one control calf (calf 90) at the same time. The blood was diluted with an equal volume of sterile PBS (pH 7.2). Twelve milliliters of dilute blood were layered over 10 ml of Histopaque-1077 (Sigma, St. Louis, MO). For composition see the Appendix. Four tubes were prepared for each animal.

The tubes were centrifuged at 400 X g for 40 minutes. The central milky phase of lymphocyte-rich suspension was transferred to a sterile siliconized tube containing 10 ml of Hanks' Balanced Salt Solution (HBSS) with 1.75 mg of sodium bicarbonate (Flow Laboratories, Subsidiary of Flow General, McLean, VA) and centrifuged at 200 X g for 15 minutes. The supernatant was poured off quickly and the pellet of cells resuspended in 2 ml of HBSS. Lysing agent for white blood cell counts (American Scientific, McGaw Park, IL) was added to a 150 ul sample of each cell suspension to lyse erythrocytes; then the mononuclear cell concentration was determined using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Each sample was divided into equal volumes and the concentration of cells was adjusted to 2.5

X $10^6$ cells per ml using:

1. Medium 199 with Earles' salts and glutamine (Flow Laboratories) plus fetal calf serum, penicillin, streptomycin, Hepes buffer (Flow Laboratories) (M199)
2. M199 plus indomethacin (M199I)

For composition of the media see the Appendix.

Three M. bovis antigens were tested using cells from each animal:

1. EEC standard for bovine tuberculin PPD (EEC-PPD)
2. Proposed international standard for bovine tuberculin PPD (PIS-PPD)
3. USDA standard PPD SR 318201 (USDA-PPD)

The antigens were dialyzed in phosphate buffered saline to remove the phenol used in their preparation. Phytohemagglutinin (PHA) (Difco, Detroit, MI) was added to cells from each animal in separate tubes as a nonspecific mitogen.

| Antigen | Quantity(ug) |
|---|---|
| EEC-PPD | 10, 1, 0.1 |
| PIS-PPD | 10, 1, 0.1 |
| USDA-PPD | 10, 1, 0.1 |
| PHA | 2.5, 1 |

Each quantity of each antigen was added to cells suspended in M199 and M199I from each animal. Two hundred microliters of the lymphocyte-rich suspension, containing

$5 \times 10^5$ cells, were added to each well of a 96-well tissue culture plate (Costar, Cambridge, MA) containing the various quantities of the different antigens. The cultures were prepared in triplicate and control cultures without antigen were included. The plates were incubated at 37°C in a 5% $CO_2$, 100% humidified atmosphere:

| | Antigen | Incubation period(days) |
|---|---|---|
| 1. | EEC-PPD | 5 |
| 2. | PIS-PPD | 5 |
| 3. | USDA-PPD | 5 |
| 4. | PHA | 3 |

Eighteen hours before harvest each culture was exposed to one microCurie tritiated thymidine (New England Nuclear Corporation, Boston, MA). Four hours before harvest, each culture was killed with one drop of 50% phenol. Cultures were harvested onto filter paper using a Titertek Cell Harvester (Flow Laboratories). The paper was allowed to dry, then the pads were removed and placed into vials containing toluene plus Metric-Pak 2a70 (Research Products International Corp., Elk Grove Village, IL). Radioactivity was measured in a Minaxi Tri-carb 4000 series liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

4. Necropsy

Three of the experimentally exposed calves (28, 38 and 47) died before application of a tuberculin test. These

were necropsied and tissues with gross lesions were collected for mycobacteriologic and histopathologic examination. The remaining 19 exposed calves and 4 control calves were necropsied after the second tuberculin test. The following tissues were examined for grossly visible lesions of tuberculosis:

1. Mandibular lymph nodes
2. Parotid lymph nodes
3. Medial retropharyngeal lymph nodes
4. Deep cervical lymph nodes
5. Tracheal injection site
6. Superficial cervical lymph nodes
7. Thoracic lymph nodes (including tracheobronchial)
8. Lung
9. Liver
10. Hepatic lymph nodes
11. Spleen
12. Mesenteric lymph nodes
13. Kidney
14. Popliteal lymph nodes

Tissues were collected from each calf for laboratory examination. Approximately half of each sample was placed in 10% buffered formalin for histopathologic examination. The other half was placed in a solution of saturated sodium borate for mycobacteriologic examination. The following

tissues were collected:

1. Mandibular and parotid lymph nodes
2. Medial retropharyngeal lymph nodes
3. Thoracic lymph nodes
4. Tracheal injection site lesion (if present)
5. Lung
6. Liver
7. Spleen
8. Pool of other tissues with gross lesions

5. Histopathology

The tissues collected in 10% buffered formalin were stored for a minimum of 1 week to allow complete fixation. Specimens of the lymph nodes of the thoracic cavity were sectioned to include a lesion, if possible. Sections selected for examination were placed in a plastic cassette (Lab-Tek Division, Miles Laboratories, Naperville, IL) and immersed in Decalcifying Solution (American Scientific Products, Division of American Hospital Supply Corporation, McGaw Park, IL) overnight.

The cassettes were washed in flowing tap water, then processed through a 13 hour cycle of an Autotechnicon-Duo automatic tissue processor (Technicon Corporation, Tarrytown, NY) to remove moisture and replace it with paraffin. Tissues in the processor passed through eight containers of dehydrant, two of clearing agent and two of melted paraffin.

The tissues were then removed from the cassettes and hand embedded into liquid paraffin in metal molds using a Tissue Embedding Center (Lab-Tek Products, Division of Miles Laboratories, Inc., Westmont, IL). The molds were placed on a cold plate until the paraffin was firm enough for sectioning.

The paraffin tissue blocks were sectioned into ribbons of slices (6 um thickness) on a Microtome (Leitz, Germany). A representative section of each ribbon was selected and separated to be mounted on a 25 X 75 mm glass microscope slide. Mounting sections consisted of placing several drops of filtered distilled water containing Albumin Fixative (Harleco, Division of EM Industries, Inc., Gibbstown, NJ) on the slide, floating the section on the water, and smoothing out any wrinkles present while slowly heating the slide on a Slide Warmer (Chicago Surgical and Electrical Co., Melrose Park, IL). The slides remained on the warming board until most of the water had evaporated. They were placed on end to drain then placed in an Electric Laboratory Drier (Lipshaw, Detroit, MI) for approximately 15 minutes to dry completely.

Hematoxylin and eosin (H and E) staining was performed on an Autotechnicon-Duo adapted for tissue staining by automatic cycle. Coverslips were mounted with Permount (Fisher Scientific Co., Chemical Manufacturing Division, Fairlawn,

NJ). The slides were examined microscopically for typical tuberculous lesions.

6. Mycobacteriologic examination

Representative tissues were placed in saturated sodium borate solution and examined for the presence of M. bovis; an initial attempt to isolate was made on the following tissues from each animal:

1. Medial retropharyngeal lymph nodes
2. Thoracic lymph nodes
3. Liver

If no acid-fast colony was observed within 8 weeks, the pooled sample of other tissues with gross lesions was examined. The tissues were processed in a Blickman Biological Safety Cabinet (Blickman & Co., Weehawken, NJ).

The samples were first decontaminated in 200 ml of sodium hypochlorite (NaOCl) (1:1000). Excess fat was trimmed off and discarded. Tissues were then placed in blender jars containing approximately 50 ml of nutrient broth with phenol red. Each sample was macerated using a household blender to a smooth suspension. Twenty milliliters of suspension were placed in a 20 X 125 mm screw cap test tube and identified as untreated. Seven milliliters of suspension were placed in a test tube containing 5 ml of 0.5 N sodium hydroxide (NaOH) for 5 to 7 minutes. The sample was then neutralized with 6 N hydrochloric acid

(HCl) and centrifuged for 20 minutes at 1650 X g. Any pellicle floating near the top was discarded, and most of the fluid decanted. The sediment at the bottom of the tube was resuspended in the remaining fluid and identifed as treated.

Eight tubes of media were inoculated with each tissue sample. Four tubes were inoculated with the untreated suspension:

1. Modified Stonebrink medium
2. Herrold's egg yolk agar medium with glycerin, with malachite green, with mycobactin
3. Herrold's egg yolk agar medium without glycerin, without malachite green, without mycobactin
4. Lowenstein-Jensen medium without glycerin

Four tubes were inoculated with the treated suspension:

1. Modified Stonebrink medium
2. Middlebrook 7H-10 agar with Middlebrook OADC (oleic acid, bovine albumin fraction V, dextrose, beef catalase) enrichment
3. Herrold's egg yolk agar medium with glycerin, with malachite green, without mycobactin
4. Lowenstein-Jensen medium with glycerin

For composition of the media see the Appendix.

Inoculated media were slanted at 30 degrees and incubated overnight at 37°C. The next day, the tubes were placed upright and returned to the 37°C incubator. They

were examined weekly for 8 weeks for the presence of typical M. bovis colonies. If a suspect colony was identified, a smear was made, stained by the Ziehl-Neelsen technique and examined microscopically for the presence of acid-fast bacilli. Each colony of acid-fast bacilli was used to inoculate a tube of Dubos broth containing Tween 80 and DOAC.

The Dubos albumin broth was incubated at 37°C until the sedimented cells formed a button at least 3 mm in diameter. Then a smear was made from the broth, stained by the Ziehl-Neelsen technique and examined microscopically for acid-fast purity. A sensitivity assay was conducted to confirm the identity of the organism. The following liquid media were inoculated:

| | Medium | Concentration Antimicrobial Agent |
|---|---|---|
| 1. | Dubos medium serum broth (freezer tube for culture repository) | None |
| 2. | Dubos albumin broth (control) | None |
| 3. | Proskauer and Beck medium with 5% horse serum | None |
| 4. | Dubos albumin broth plus Isoniazid (INH) (Squibb and Sons, Princeton, NJ) | 10 ug/ml |
| 5. | Dubos albumin broth plus Thiophen-2-carboxylic acid hydrazide (TCH) (Aldrich Chemical Co., Inc., Milwaukee, WI) | 15 ug/ml |

The inoculated media were incubated for 2 weeks at 37°C then visually observed for turbidity. Turbidity less than that seen in the control tube was considered negative; all others were considered positive. The tube of Proskauer and Beck medium was examined for growth characteristics.

### C. Comparison of *M. bovis* Antigens and a *M. paratuberculosis* Antigen

#### 1. Production of *M. paratuberculosis* purified protein derivative

*Mycobacterium paratuberculosis* Strain 18 was inoculated into modified Dorset-Henley liquid medium (see the Appendix for composition) dispensed in 100 ml aliquots into Erlenmeyer culture flasks. One loopful of the seed culture was transferred to each Erlenmeyer flask using a clover leaf-shaped loop. Four hundred flasks were inoculated. Cultures were incubated for 11 weeks at 37°C. Then the incubator room was vented and the flasks autoclaved at 121°C for 30 minutes. The cells were separated from the culture filtrate (CF) using a fine mesh screen covered with two layers of 15 inch diameter Kendall Filter Disks (Kendall Co., Walpole, MA). The yield was 1579 grams of cells and 25.1 liters of CF.

Five percent phenol was added to the CF to give a final concentration of 0.5% and a volume of 27.9 liters. The CF was concentrated through an ultrafiltration membrane (UFM)

(Amicon Corp., Lexington, MA) with an exclusion point of 10,000 molecular weight, washed with phenolized phosphate buffer (PPB) No. 1 (see Table 1 for composition) until colorless, then precipitated with an equal volume of saturated ammonium sulfate (AS) $((NH_4)_2SO_4)$. The precipitate was dissolved in 3.5 liters of PPB No. 1, then clarified by centrifugation at 27,000 X g for 30 minutes at 4°C. The PPD was washed free of AS with PPB No. 3 (Table 1) using the UFM, then centrifuged at 27,000 X g for 30 minutes at 4°C. The micro-Kjehldahl test for nitrogen was done on the PPD and protein estimated. Total yield was 1.3 liters of PPD containing 5.15 mg tuberculoprotein per ml. Before use, the PPD was passed through a 0.22 micrometer pore size membrane filter.

Table 1. Composition of Phenolized Phosphate Buffers (PPB) in g per liter

| Chemical | No.1 | No.3 |
|---|---|---|
| $Na_2HPO_4.12\ H_2O$ | 11.46 | 4.77 |
| $NaH_2PO_4.H_2O$ | 0.66 | |
| $KH_2PO_4$ | 0.36 | |
| Phenol (100 ml of 5% aqueous solution) | 5.0 | 5.0 |

2. Evaluation of M. paratuberculosis PPD in sensitized guinea pigs

The biologic activity of the M. paratuberculosis PPD was evaluated in guinea pigs. Forty white female guinea pigs from one source weighing 500 to 700 g which had not been used in a previous test were sensitized by intramuscular injection of 1/4 ml of a suspension of one gram of heat-killed M. paratuberculosis per 10 ml mineral oil (containing 25 mg cells) into the medial surface of each hind leg. Thirty-six days later, they were divided into four groups, 10 guinea pigs per group. Guinea pigs in each group received three dilutions of one of four M. paratuberculosis antigens:

| | M. paratuberculosis antigen | mg/ml |
|---|---|---|
| 1. | M. paratuberculosis PPD--new product (USA) | 5.15 |
| 2. | M. paratuberculosis PPD--Canada (Canada) | 1 |
| 3. | M. paratuberculosis PPD--Netherlands (Netherlands) | 1 |
| 4. | Johnin Old Tuberculin (OT)--USDA (USDA-OT) | 1 |

Dilutions

1:50

1:100

1:200

The abdomen of each guinea pig was shaved and then a depilatory applied at least 4 hours before injection of antigens. Each guinea pig received 0.1 ml injections of the three dilutions of an antigen intradermally. Skin reactions were measured as the area of erythema produced 24 and 48 hours after injection of the product. One nonsensitized guinea pig was injected with the three dilutions of each product. There were no reactions in the nonsensitized guinea pigs.

3. Evaluation of *M. paratuberculosis* PPD in naturally infected cattle

The *M. paratuberculosis* PPD was evaluated in eight cattle from which *M. paratuberculosis* was isolated from feces collected from the rectum. Three products were injected simultaneously:

| | *M. paratuberculosis* antigen | mg/ml |
|---|---|---|
| 1. | *M. paratuberculosis* PPD--new product | 5.15 |
| 2. | *M. paratuberculosis* PPD--Canada | 1 |
| 3. | Johnin OT--USDA-OT | 1 |

The two PPDs were injected intradermally into two sites on one side of the neck and the OT was injected intradermally on the other side. The skin thickness was measured at the time of injection and at 72 hours with a dermal thickness gauge and recorded. Four nonsensitized cattle were

injected with the new product. Three had no reactions and one had a 3 mm increase in skin thickness at 72 hours.

4. Delayed-type hypersensitivity skin tests conducted in cattle from a herd in which M. paratuberculosis was diagnosed

The specificity of the three M. bovis PPDs was evaluated in 23 cattle in a herd in which M. paratuberculosis infection had been previously diagnosed. Simultaneously injected were:

1. EEC standard for bovine tuberculin PPD (EEC-PPD)
2. Proposed international standard for bovine tuberculin PPD (PIS-PPD)
3. USDA standard PPD SR 318201 (USDA-PPD)
4. M. paratuberculosis PPD--new product (USA-PPD)

All four products were injected into each animal. Two sites on each side of the neck received 0.1 ml tuberculin intradermally. Skin responses were measured at the time of injection and at 72 hours with a dermal thickness gauge and recorded.

## D. Statistics

Data were reported as means $\pm$ standard error. The number of observations used to calculate each value were reported as N. The significance of differences between mean values was tested using Student's two-tailed t test (80).

Confidence limits for means were based on the t distribution.

Responses to intradermal injections of tuberculins were compared by analyses of variance (80). They also provided F tests of the null hypothesis that the population means were identical.

Relative potencies for tuberculins were estimated using a statistical analysis of parallel lines bioassays (19, 32).

# IV. RESULTS

## A. Evaluation of *M. bovis* Antigens

### 1. Delayed-type hypersensitivity skin tests conducted at 8 weeks

The data from tuberculin skin tests conducted in 18 calves were used to construct three 6X6 Latin squares (Table 2).

Two readers observed the skin test responses. The mean observations were:

| | |
|---|---|
| Reader 1 | 21.54 (± 0.21) mm |
| Reader 2 | 22.76 (± 0.21) mm |

There were significant differences in the mean skin test responses to all six dilutions in the calves (P>F=0.0001) (Table 3).

The PPDs were injected into each of six sites on the neck of each calf (see Figures 1 and 2, page 12). There were significant differences in the mean skin test responses at different sites (P>F=0.0001). The mean responses were:

| Site | Response (mm) |
|---|---|
| LA | 22.7 (± 0.4) |
| LB | 23.2 (± 0.4) |
| LC | 20.2 (± 0.4) |
| RA | 24.3 (± 0.4) |
| RB | 20.4 (± 0.4) |
| RC | 22.2 (± 0.4) |

Table 2. Skin test responses (mm) to PPDs injected intradermally in the neck of 18 calves experimentally exposed to M. bovis 8 weeks post exposure

| Calf | PPD | | | | | |
|---|---|---|---|---|---|---|
| | EEC-PPD | | PIS-PPD | | USDA-PPD | |
| | 1:2 | 1:10 | No | 1:5 | No | 1:5 |
| 26 | 21.9[a] | 14.8 | 28.1 | 19.8 | 21.4 | 15.3 |
| | 21.8 | 14.5 | 30.5 | 19.5 | 18.5 | 15.5 |
| 29 | 37.7 | 17.8 | 34.9 | 19.2 | 24.6 | 16.0 |
| | 46.5 | 17.5 | 36.0 | 20.0 | 23.5 | 18.0 |
| 30 | 45.2 | 16.9 | 34.2 | 25.3 | 24.0 | 14.8 |
| | 45.5 | 18.0 | 36.0 | 26.0 | 27.5 | 18.0 |
| 31 | 19.5 | 8.3 | 29.4 | 15.0 | 15.9 | 15.7 |
| | 19.0 | 8.5 | 28.0 | 14.5 | 17.0 | 13.5 |
| 32 | 31.3 | 14.3 | 38.0 | 11.5 | 21.1 | 13.3 |
| | 43.0 | 17.0 | 43.0 | 12.5 | 25.0 | 15.5 |
| 33 | 31.3 | 17.0 | 51.4 | 25.9 | 39.5 | 32.0 |
| | 31.5 | 13.5 | 42.0 | 23.0 | 33.0 | 28.5 |
| 34 | 18.6 | 7.7 | 23.9 | 14.5 | 20.8 | 9.5 |
| | 17.5 | 9.5 | 24.0 | 16.5 | 13.5 | 11.0 |
| 35 | 22.3 | 9.4 | 20.9 | 13.0 | 12.8 | 10.6 |
| | 23.0 | 10.5 | 25.0 | 14.5 | 16.0 | 11.5 |
| 36 | 19.2 | 11.8 | 36.8 | 20.0 | 34.4 | 15.8 |
| | 21.0 | 13.5 | 36.8 | 21.0 | 37.0 | 19.5 |

[a]Value on top is from Reader 1; value on bottom is from Reader 2.

Table 2. Continued

| Calf | PPD | | | | | |
|---|---|---|---|---|---|---|
| | EEC-PPD | | PIS-PPD | | USDA-PPD | |
| | 1:2 | 1:10 | No | 1:5 | No | 1:5 |
| 40 | 27.3[a] | 18.4 | 36.3 | 14.9 | 18.2 | 15.1 |
| | 28.5 | 24.0 | 35.5 | 16.0 | 21.5 | 13.5 |
| 42 | 30.7 | 14.3 | 24.0 | 23.1 | 16.5 | 17.4 |
| | 30.0 | 14.5 | 27.5 | 22.0 | 21.0 | 17.0 |
| 43 | 20.9 | 10.6 | 17.5 | 16.4 | 17.7 | 9.5 |
| | 22.0 | 14.0 | 24.5 | 19.5 | 20.5 | 11.5 |
| 44 | 25.5 | 18.3 | 28.9 | 23.4 | 41.5 | 29.1 |
| | 27.0 | 18.0 | 31.5 | 24.5 | 49.0 | 34.0 |
| 45 | 26.5 | 12.4 | 43.9 | 16.5 | 23.9 | 12.7 |
| | 30.5 | 16.0 | 41.5 | 19.5 | 27.5 | 13.5 |
| 49 | 34.8 | 13.2 | 36.5 | 18.0 | 20.7 | 12.1 |
| | 34.0 | 15.0 | 40.0 | 17.0 | 22.0 | 14.0 |
| 50 | 35.2 | 9.3 | 47.6 | 14.6 | 18.1 | 10.8 |
| | 36.5 | 12.0 | 54.0 | 21.0 | 19.5 | 11.5 |
| 51 | 26.2 | 15.4 | 31.4 | 19.7 | 23.5 | 16.7 |
| | 19.5 | 18.0 | 30.5 | 23.0 | 22.5 | 17.0 |
| 52 | 15.7 | 7.1 | 25.2 | 10.4 | 19.0 | 9.1 |
| | 16.5 | 8.5 | 28.5 | 13.0 | 17.5 | 9.8 |

Table 3. Skin test response (mean value) in mm to two dilutions of three PPDs injected intradermally in the neck of 18 calves experimentally exposed to *M. bovis* 8 weeks post exposure

| Calf | Response[a] |
|---|---|
| 26 | 20.13 |
| 29 | 25.98 |
| 30 | 27.62 |
| 31 | 17.02 |
| 32 | 23.79 |
| 33 | 30.72 |
| 34 | 15.58 |
| 35 | 15.79 |
| 36 | 23.90 |
| 40 | 22.43 |
| 42 | 21.50 |
| 43 | 17.05 |
| 44 | 29.22 |
| 45 | 23.70 |
| 49 | 23.11 |
| 50 | 24.18 |
| 51 | 21.95 |
| 52 | 15.02 |

[a]N=12 (two readers per calf), standard deviation=0.63. See Table 2 for actual responses.

Comparisons were made of the mean responses at different sites at three levels of significance. Values of t were calculated and compared to $t_{table}$. If $t_{calculated} < t_{table}$ then the difference was not significant at that level.

At the 90% level:

Site LA = Site LB

Site LA = Site RC

Site LC = Site RB

At the 95% level:

Site LA = Site LB = Site RC

Site LC = Site RB

At the 99% level:

Site LA = Site LB = Site RC

Site LB = Site RA

Site LC = Site RB

Results are summarized in Tables 4 and 5.

There were significant differences in the mean skin test responses to the different dilutions of the three tuberculin PPDs (P>F=0.0001). The hypotheses (dilutions are equal vs. dilutions are not equal) were tested using the mean square for the interaction of calf*site as an error term ($MS_{calf*site}$=54.38). The mean responses were:

Table 4. Calculated t values for comparisons of PPD skin test responses (mean values) in mm at two sites on the neck of 18 cattle experimentally exposed to *M. bovis*[a] 8 weeks post exposure

| Site Comparison | t[b] | Alpha 0.10[c] | 0.05[d] | 0.01[e] |
|---|---|---|---|---|
| LA vs. LB | 0.9764 | | | |
| LA vs. LC | 4.8821 | *** | *** | *** |
| LA vs. RA | 3.1245 | *** | *** | *** |
| LA vs. RB | 4.4915 | *** | *** | *** |
| LA vs. RC | 0.9764 | | | |
| LB vs. LC | 5.8585 | *** | *** | *** |
| LB vs. RA | 2.1481 | *** | *** | |
| LB vs. RB | 5.4679 | *** | *** | *** |
| LB vs. RC | 1.9528 | *** | | |
| LC vs. RA | 8.0066 | *** | *** | *** |
| LC vs. RB | 0.3906 | | | |
| LC vs. RC | 3.9057 | *** | *** | *** |
| RA vs. RB | 7.6161 | *** | *** | *** |
| RA vs. RC | 4.1010 | *** | *** | *** |
| RB vs. RC | 3.5151 | *** | *** | *** |

[a]Significant differences are indicated by '***.'
[b]N=36, df=102, MSE=4.72.
[c]$t_{10\%}$=1.6599.
[d]$t_{5\%}$=1.9835.
[e]$t_{1\%}$=2.6429.

Table 5. Summary of comparisons of skin test responses (mean values) in mm to two dilutions of three PPDs at six sites on the neck of 18 calves at three levels of significance[a] 8 weeks post exposure

| | | Alpha | | |
|---|---|---|---|---|
| Site[b] | Mean[c] | 0.10 | 0.05 | 0.01 |
| RA | 24.3 | A | E | H |
| LB | 23.2 | B | F | H I |
| LA | 22.7 | B C | F | I |
| RC | 22.2 | C | F | I |
| RB | 20.4 | D | G | J |
| LC | 20.2 | D | G | J |

[a]Means identified by the same letter are not significantly different.
[b]Sites are listed in decreasing order of response.
[c]N=36.

| PPD | Response (mm) |
|---|---|
| EEC-PPD 1/2 | 27.9 (± 1.2) |
| EEC-PPD 1/10 | 13.9 (± 1.2) |
| PIS-PPD Undiluted | 33.4 (± 1.2) |
| PIS-PPD 1/5 | 18.4 (± 1.2) |
| USDA-PPD Undiluted | 23.5 (± 1.2) |
| USDA-PPD 1/5 | 15.8 (± 1.2) |

The responses to the six PPD injections were compared at three levels of significance: 90%, 95% and 99%. At all three levels, all responses were significantly different. The least significant differences (LSD) were calculated for the three levels of significance:

| Confidence level | LSD |
|---|---|
| 90% | 0.8501 |
| 95% | 1.0158 |
| 99% | 1.3443 |

Relative potencies were calculated for the three PPDs. To conduct a statistical analysis of a parallel lines bioassay, three assumptions are made:

1. The dilutions are regularly spaced (proportional). If a PPD is injected at dilutions a, b, c and d, then $a/b = b/c = c/d$.
2. The response to each PPD at the different dilutions is linear.

3. The dose-response lines for the different PPDs analyzed are parallel.

To evaluate these criteria, the data were evaluated by an analysis of variance, then F statistics constructed to compare sources of variation (Tables 6 and 7).

Important comparisons are:

1. $Dilution_{linear}$-indicates fit of data to line
2. $PPD*Dilution_{linear}$-indicates parallelism of dose-response lines.

Parallelism was significant; therefore, there was a 99% probability that the lines were not parallel. To determine which of the dose-response lines were not parallel, regression lines were calculated and the slopes compared (Figure 3, Table 8).

If a comparison was significant, the lines were not parallel. Comparisons involving USDA-PPD were significant, therefore no comparison with the other PPDs could be included in this analysis. The analysis of variance was repeated excluding USDA-PPD data (Tables 9 and 10).

The data, excluding USDA-PPD, met the assumptions of linearity and parallelism. Data were expressed as an average of the observations by the two readers. Averages were calculated for the data for a particular dilution of a PPD and for PPDs. Table 11 lists the average skin test responses to EEC-PPD and PIS-PPD.

Table 6. Analysis of variance of skin test responses to three PPDs in cattle experimentally exposed to *M. bovis* 8 weeks post exposure

| Source | df | SS | MS |
|---|---|---|---|
| PPD | 2 | 1608.46 | 804.23 |
| $Dilution_{linear}$ | 1 | 8071.56 | 8071.56 |
| $PPD*Dil._{linear}$ | 2 | 561.34 | 280.67 |
| Animal | 17 | 4468.06 | 262.82 |
| Animal*PPD | 34 | 2434.40 | 71.60 |
| $Animal*Dil._{linear}$ | 17 | 1152.85 | 67.81 |
| $An.(PPD)*Dil._{linear}$ | 34 | 1234.59 | 36.31 |
| Error | 108 | 564.53 | 5.23 |
| Total | 215 | | |

Table 7. Results of F tests for sources of variation from Table 6[a]

| | | F | |
|---|---|---|---|
| PPD | :Animal*PPD | 11.23 | *** |
| $Dilution_{linear}$ | :Animal*$Dilution_{linear}$ | 119.03 | *** |
| PPD*$Dil._{linear}$ | :An.(PPD)*$Dil._{linear}$ | 7.73 | *** |
| Animal | :Error | 50.22 | *** |
| An.*PPD | :Error | 13.69 | *** |
| An.*$Dil._{linear}$ | :Error | 12.97 | *** |

[a]Alpha=0.01, comparisons that are significant are indicated by '***.'

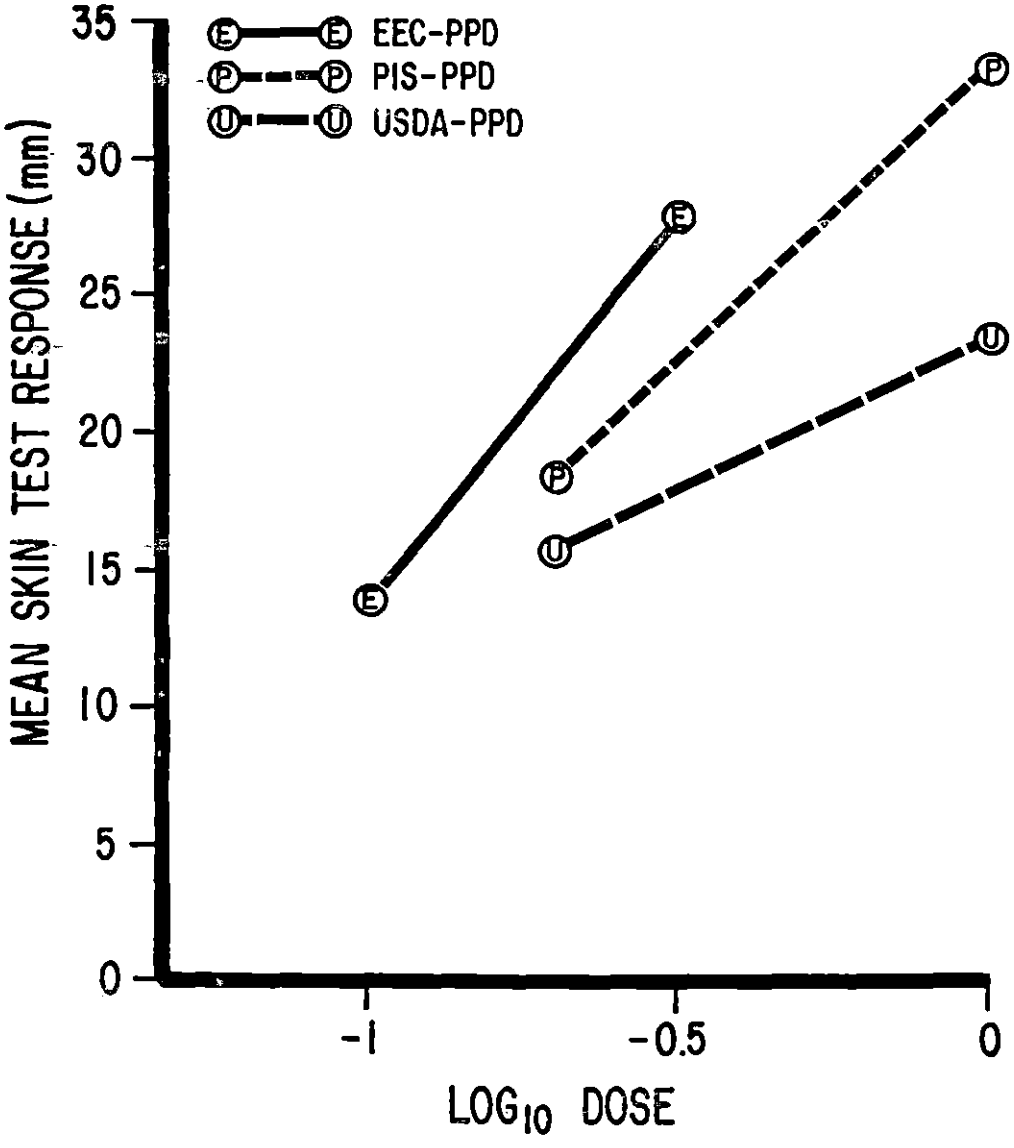


Figure 3. Dose-response lines for three PPDs injected intradermally in the neck of 18 calves experimentally exposed to M. bovis. $Slope_{EEC-PPD}$= 20.01, $slope_{PIS-PPD}$= 21.44, $slope_{USDA-PPD}$= 11.02

Table 8. Comparison of slopes of all possible pairs of regression lines ($log_{10}$dose of PPD vs. skin test response) 8 weeks post exposure of cattle[a]

| Comparison | t[b] | |
|---|---|---|
| EEC-PIS | 0.45 | |
| EEC-USDA | 2.79 | *** |
| PIS-USDA | 3.16 | *** |

[a]Alpha=0.05, comparisons that are significant are indicated by '***.'

[b]$t_{70df}$=1.994.

Table 9. Analysis of variance of skin test responses to EEC-PPD and PIS-PPD in 18 cattle experimentally exposed to M. bovis 8 weeks post exposure

| Source | df | SS | MS |
|---|---|---|---|
| PPD | 1 | 926.69 | 926.69 |
| $Dilution_{linear}$ | 1 | 7555.96 | 7555.96 |
| $PPD*Dil._{linear}$ | 1 | 8.95 | 8.95 |
| Animal | 17 | 3077.31 | 181.02 |
| Animal*PPD | 17 | 671.58 | 39.50 |
| $Animal*Dil._{linear}$ | 17 | 1497.36 | 88.08 |
| $An.(PPD)*Dil._{linear}$ | 17 | 605.07 | 35.59 |
| Error | 72 | 387.98 | 5.39 |
| Total | 143 | | |

Table 10. Results of F tests for sources of variation from Table 9[a]

| | | F | |
|---|---|---|---|
| PPD | :Animal*Dilution$_{linear}$ | 23.47 | *** |
| Dilution$_{linear}$ | :Animal*Dilution$_{linear}$ | 285.79 | *** |
| PPD*Dil.$_{linear}$ | :An.(PPD)*Dil.$_{linear}$ | 0.25 | |
| Animal | :Error | 33.67 | *** |
| An.*PPD | :Error | 7.35 | *** |
| An.*Dil.$_{linear}$ | :Error | 16.38 | *** |

[a]Alpha=0.01, significant comparisons are indicated by '***.'

Table 11. Skin test responses (mean values) in mm to two dilutions of EEC-PPD and PIS-PPD in cattle experimentally exposed to M. bovis 8 weeks post exposure

| | EEC-PPD | | PIS-PPD | |
|---|---|---|---|---|
| | Dilution | | Dilution | |
| | 1:2 | 1:10 | Undiluted | 1:5 |
| | 27.86 | 13.87 | 33.44 | 18.45 |
| Mean | 20.87 | | 25.94 | |

The slopes of the regression lines (Figure 3) for each of the two PPDs were:

| PPD | Slope |
|---|---|
| EEC-PPD | 20.01 |
| PIS-PPD | 21.44 |

The slopes were not significantly different at the 5% level; therefore, a common regression coefficient was calculated by fitting one line to the data from both PPDs. Slope= 20.73. To calculate the relative potency (R) of PPD 1 to PPD 2, the following formula was used:

$\log_{10}R = (y_1 - y_2)/b_1 - (x_1 - x_2)$

y = average skin test response to PPD

x = average dilution of PPD

$b_1$= common regression coefficient

The relative potency of EEC-PPD vs. PIS-PPD was calculated to be 0.88. Therefore, the skin test response elicited by 1 unit of PIS-PPD was equivalent to the response elicited by 0.88 units of EEC-PPD. The 95% confidence limits were (0.69,1.14).

2. Delayed-type hypersensitivity skin tests conducted at 18 weeks

The PPDs were injected intradermally into the neck of each of the same experimentally exposed cattle 18 weeks after exposure (Table 12).

Table 12. Skin test responses (mm) to PPDs injected intradermally in the neck of 18 calves experimentally exposed to M. bovis 18 weeks post exposure

| | PPD | | | | | |
|---|---|---|---|---|---|---|
| | EEC-PPD | | PIS-PPD | | USDA-PPD | |
| Calf | 1:2 | 1:10 | No | 1:5 | No | 1:5 |
| 26 | 24.8[a] | 11.2 | 21.5 | 21.5 | 17.3 | 7.7 |
| | 26.4 | 11.5 | 22.3 | 24.7 | 25.0 | 9.0 |
| 29 | 11.0 | 5.1 | 13.2 | 8.9 | 12.5 | 6.3 |
| | 11.5 | 4.6 | 15.5 | 8.1 | 11.4 | 6.1 |
| 30 | 21.5 | 13.3 | 32.0 | 19.4 | 19.3 | 13.2 |
| | 24.5 | 13.5 | 33.4 | 23.3 | 19.9 | 11.3 |
| 31 | 11.0 | 5.9 | 17.8 | 14.4 | 15.1 | 9.3 |
| | 12.5 | 7.5 | 18.5 | 18.0 | 17.0 | 11.5 |
| 32 | 16.6 | 7.1 | 15.8 | 7.7 | 8.5 | 7.8 |
| | 16.9 | 7.5 | 16.6 | 9.5 | 10.8 | 7.5 |
| 33 | 34.1 | 13.8 | 36.7 | 17.5 | 27.5 | 21.4 |
| | 32.5 | 13.0 | 39.7 | 18.3 | 27.2 | 17.2 |
| 34 | 3.3 | 3.5 | 8.2 | 6.2 | 7.4 | 9.2 |
| | 5.5 | 4.1 | 8.7 | 8.8 | 9.0 | 5.9 |
| 35 | 14.2 | 3.9 | 14.0 | 9.1 | 11.0 | 3.3 |
| | 14.0 | 4.5 | 42.4 | 9.0 | 11.4 | 4.1 |
| 36 | 10.0 | 14.3 | 15.6 | 7.6 | 11.9 | 7.0 |
| | 9.0 | 14.5 | 14.0 | 9.2 | 12.0 | 7.0 |

[a]Value on top is from Reader 1; value on bottom is from Reader 2.

Table 12. Continued

| Calf | PPD | | | | | |
|---|---|---|---|---|---|---|
| | EEC-PPD | | PIS-PPD | | USDA-PPD | |
| | 1:2 | 1:10 | No | 1:5 | No | 1:5 |
| 37 | 15.3[a] | 5.8 | 20.5 | 11.6 | 14.9 | 10.6 |
| | 14.0 | 8.0 | 19.6 | 11.9 | 14.8 | 12.0 |
| 40 | 12.9 | 9.4 | 26.5 | 12.6 | 13.4 | 9.2 |
| | 12.7 | 9.4 | 22.2 | 12.6 | 13.5 | 10.1 |
| 42 | 19.4 | 6.8 | 23.5 | 14.2 | 13.2 | 9.1 |
| | 21.3 | 8.0 | 27.4 | 14.3 | 14.1 | 11.9 |
| 43 | 7.3 | 5.1 | 8.1 | 5.6 | 5.1 | 7.2 |
| | 6.3 | 5.4 | 7.9 | 7.5 | 6.0 | 6.8 |
| 44 | 17.8 | 8.7 | 25.9 | 14.7 | 22.1 | 12.0 |
| | 19.3 | 10.6 | 27.1 | 17.3 | 23.0 | 13.6 |
| 45 | 15.6 | 7.7 | 14.5 | 10.2 | 11.8 | 5.2 |
| | 16.2 | 7.1 | 13.0 | 10.9 | 13.2 | 5.5 |
| 49 | 15.4 | 12.1 | 21.1 | 17.3 | 12.4 | 12.5 |
| | 16.7 | 11.5 | 20.8 | 18.3 | 14.0 | 13.2 |
| 51 | 8.3 | 8.0 | 11.7 | 9.0 | 9.2 | 6.2 |
| | 13.5 | 10.0 | 11.4 | 9.7 | 10.2 | 7.4 |
| 52 | 7.7 | 5.6 | 13.3 | 11.6 | 10.0 | 7.7 |
| | 7.8 | 5.7 | 10.1 | 10.5 | 10.3 | 8.4 |

Two readers observed the skin test responses. The mean observations were:

Reader 1 12.78 (± 0.22) mm

Reader 2 13.71 (± 0.22) mm

There were significant differences in the mean responses to all six dilutions in the calves (P>F=0.0001). The mean responses are listed in Table 13.

The PPDs were injected into each of six sites on the neck of each calf (see Figures 1 and 2, page 12). There were significant differences in the mean skin test responses at different sites (P>F=0.0001). The mean responses were:

| Site | Response (mm) |
|---|---|
| LA | 14.2 (± 0.4) |
| LB | 12.0 (± 0.4) |
| LC | 12.8 (± 0.4) |
| RA | 12.4 (± 0.4) |
| RB | 13.8 (± 0.4) |
| RC | 14.3 (± 0.4) |

Comparisons were made of the mean responses at different sites at three levels of significance.

At the 90% level:

Site LA = Site RB = Site RC

Site LB = Site LC = Site RA

Table 13. Skin test response (mean value) in mm to two dilutions of three PPDs injected intradermally in the neck of 18 calves experimentally exposed to *M. bovis* 18 weeks post exposure

| Calf | Response[a] |
|---|---|
| 26 | 18.58 |
| 29 | 9.52 |
| 30 | 20.38 |
| 31 | 13.21 |
| 32 | 11.02 |
| 33 | 24.91 |
| 34 | 6.65 |
| 35 | 11.74 |
| 36 | 11.01 |
| 37 | 13.25 |
| 40 | 13.71 |
| 42 | 15.27 |
| 43 | 6.52 |
| 44 | 17.68 |
| 45 | 10.91 |
| 49 | 15.44 |
| 51 | 9.55 |
| 52 | 9.06 |

[a]N=12 (two readers per calf), standard deviation=0.65. See Table 12 for actual responses.

At the 95% level:

Site LA = Site RB = Site RC

Site LB = Site LC = Site RA

Site LC = Site RB

At the 99% level:

Site LA = Site RB = Site RC

Site LB = Site LC = Site RA

Site LC = Site RA = Site RB

Results are summarized in Tables 14 and 15.

There were significant differences in the mean skin test responses to the different dilutions of the three tuberculin PPDs ($P>F=0.0001$). The hypotheses (dilutions are equal vs. dilutions are not equal) were tested using the mean square for the interaction of calf*site as an error term ($MS_{calf*site}=23.21$). The mean responses were:

| PPD | Response (mm) |
|---|---|
| EEC-PPD 1/2 | 15.2 (± 0.8) |
| EEC-PPD 1/10 | 8.4 (± 0.8) |
| PIS-PPD Undiluted | 19.7 (± 0.8) |
| PIS-PPD 1/5 | 12.8 (± 0.8) |
| USDA-PPD Undiluted | 14.0 (± 0.8) |
| USDA-PPD 1/5 | 9.3 (± 0.8) |

The responses to the six PPD injections were compared at three levels of significance: 90%, 95% and 99%.

Table 14. Calculated t values for comparisons of PPD skin test responses (mean values) in mm at two sites on the neck of 18 cattle experimentally exposed to *M. bovis*[a] 18 weeks post exposure

| Site Comparison | t[b] | Alpha 0.10[c] | 0.05[d] | 0.01[e] |
|---|---|---|---|---|
| LA vs. LB | 4.2399 | *** | *** | *** |
| LA vs. LC | 2.6924 | *** | *** | *** |
| LA vs. RA | 3.4400 | *** | *** | *** |
| LA vs. RB | 0.8992 | | | |
| LA vs. RC | 0.1673 | | | |
| LB vs. LC | 1.5475 | | | |
| LB vs. RA | 0.7999 | | | |
| LB vs. RB | 3.3407 | *** | *** | *** |
| LB vs. RC | 4.4072 | *** | *** | *** |
| LC vs. RA | 0.7476 | | | |
| LC vs. RB | 1.7932 | *** | | |
| LC vs. RC | 2.8597 | *** | *** | *** |
| RA vs. RB | 2.5408 | *** | *** | |
| RA vs. RC | 3.6073 | *** | *** | *** |
| RB vs. RC | 1.0665 | | | |

[a]Significant differences are indicated by '***.'
[b]N=36, df=102, MSE=5.08.
[c]$t_{10\%}$=1.6599.
[d]$t_{5\%}$=1.9835.
[e]$t_{1\%}$=2.6429.

Table 15. Summary of comparisons of skin test responses (mean values) in mm to two dilutions of three PPDs at six sites on the neck of 18 calves at three levels of significance[a] 18 weeks post exposure

| | | Alpha | | |
|---|---|---|---|---|
| Site[b] | Mean[c] | 0.10 | 0.05 | 0.01 |
| RC | 14.3 | A | C | F |
| LA | 14.2 | A | C | F |
| RB | 13.8 | A | C D | F G |
| LC | 12.8 | B | D E | G H |
| RA | 12.4 | B | E | G H |
| LB | 12.0 | B | E | H |

[a]Means identified by the same letter are not significantly different.
[b]Sites are listed in decreasing order of response.
[c]N=36, standard error=0.4.

At all three levels, all responses were significantly different. The least significant differences (LSD) were calculated for the three levels of significance:

| Confidence level | LSD |
|---|---|
| 90% | 0.8820 |
| 95% | 1.0539 |
| 99% | 1.3947 |

Relative potencies were calculated for the three PPDs. Results of an analysis of variance and F tests are summarized in Tables 16 and 17.

The data met the assumptions of linearity and parallelism (Figure 4). Data were expressed as an average of the observations by the two readers. Averages were calculated for the data for a particular dilution of a PPD and for PPDs. Table 18 lists the average skin test responses to the three PPDs.

The slopes of the regression lines (Figure 4) for each of the three PPDs were:

| PPD | Slope |
|---|---|
| EEC-PPD | 9.66 |
| PIS-PPD | 9.92 |
| USDA-PPD | 6.84 |

The slopes were not significantly different at the 5% level; therefore, a common regression coefficient was

Table 16. Analysis of variance of skin test responses to three PPDs in cattle experimentally exposed to *M. bovis* 18 weeks post exposure

| Source | df | SS | MS |
|---|---|---|---|
| PPD | 2 | 990.12 | 495.06 |
| $Dilution_{linear}$ | 1 | 2044.88 | 2044.88 |
| $PPD*Dil._{linear}$ | 2 | 51.40 | 25.70 |
| Animal | 17 | 4746.44 | 279.20 |
| Animal*PPD | 34 | 592.86 | 17.44 |
| $Animal*Dil._{linear}$ | 17 | 906.80 | 53.34 |
| $An.(PPD)*Dil._{linear}$ | 34 | 533.48 | 15.69 |
| Error | 108 | 578.69 | 5.36 |
| Total | 215 | | |

Table 17. Results of F tests for sources of variation from Table 16[a]

| | | F | |
|---|---|---|---|
| PPD | :Animal *PPD | 28.39 | *** |
| $Dilution_{linear}$ | :Animal*$Dilution_{linear}$ | 38.34 | *** |
| PPD*$Dil._{linear}$ | :An.(PPD)*$Dil._{linear}$ | 1.64 | |
| Animal | :Error | 52.09 | *** |
| An.*PPD | :Error | 3.25 | *** |
| An.*$Dil._{linear}$ | :Error | 9.95 | *** |

[a]Alpha=0.01, significant comparisons are indicated by '***.'

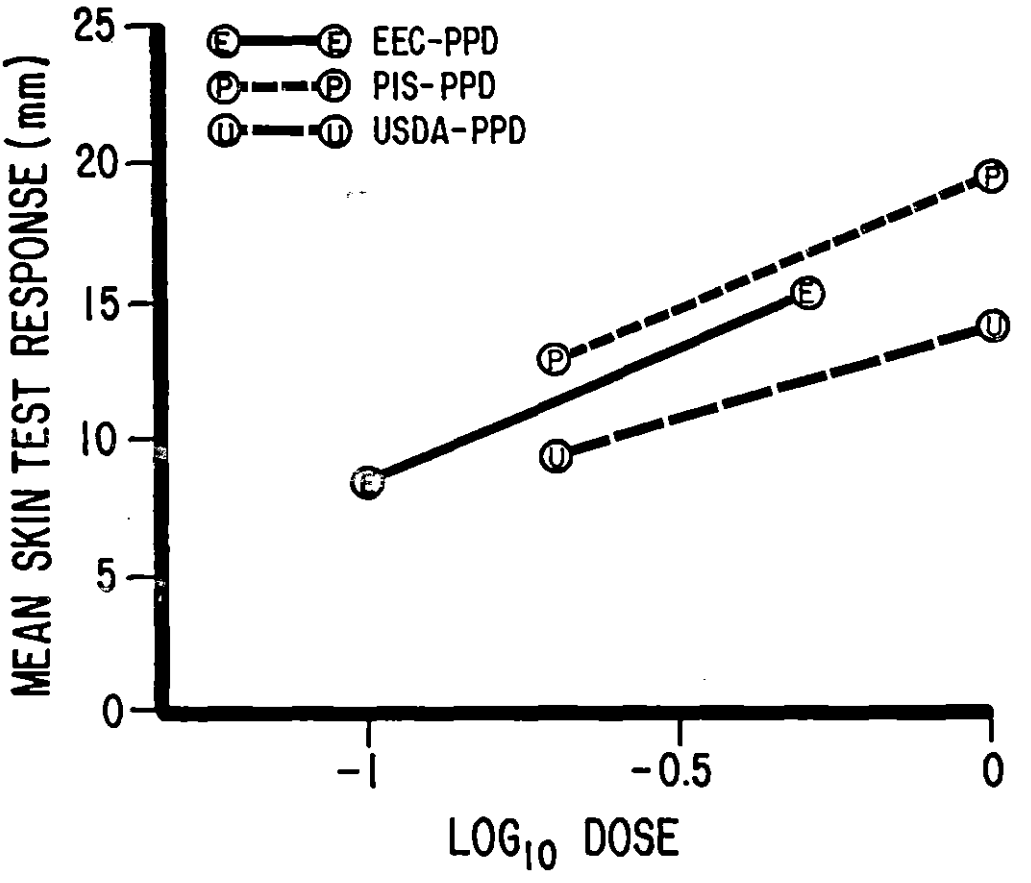


Figure 4. Dose-response lines for three PPDs injected intradermally in the neck of 18 calves experimentally exposed to M. bovis. $Slope_{EEC-PPD}$= 9.66, $slope_{PIS-PPD}$= 9.92, $slope_{USDA-PPD}$= 6.84

Table 18. Skin test responses (mean values) in mm to two dilutions of three PPDs in 18 cattle experimentally exposed to *M. bovis* 18 weeks post exposure

| EEC-PPD | | PIS-PPD | | USDA-PPD | |
|---|---|---|---|---|---|
| Dilution | | Dilution | | Dilution | |
| 1:2 | 1:10 | No | 1:5 | No | 1:5 |
| 15.19 | 8.44 | 19.74 | 12.81 | 14.04 | 9.26 |
| Mean | 11.81 | | 16.27 | | 11.65 |

calculated by fitting one line to the data from all three PPDs. Slope= 8.80.

Relative potencies were calculated and are listed in Table 19. The skin test response elicited by 1 unit of PIS-PPD was equivalent to the response elicited by 1.61 units of EEC-PPD and 3.35 units of USDA-PPD.

3. In vitro lymphocyte blastogenesis assay

Three wells in each row of a culture plate contained cells but no antigen or mitogen. The counts per minute (CPM) of radioactivity in each of those three wells were averaged and served as a background count ($CPM_{background}$). The CPM in the wells containing cells plus antigen or mitogen were averaged ($CPM_{test}$). A stimulation index (SI) was calculated by dividing $CPM_{test}$ by $CPM_{background}$ (Tables 20, 21, 22 and 23).

The response to indomethacin was different for the different antigens (P>F=0.0011). The SI was the same whether or not indomethacin was added to cells plus EEC-PPD or PIS-PPD. The SI was larger when indomethacin was added to cells plus USDA-PPD (Table 24).

No significant differences in responses to M. bovis antigens were observed following the addition of indomethacin (P>F=0.3862). The mean responses to the three M. bovis antigens were:

Table 19. Relative potencies (R) of three PPDs in cattle experimentally exposed to *M. bovis* 18 weeks post exposure

| PPD comparison | R | 95% confidence limits |
|---|---|---|
| EEC-PPD vs. PIS-PPD | 1.61 | (1.10,2.53) |
| USDA-PPD vs. PIS-PPD | 3.35 | (2.29,5.30) |

Table 20. Results of lymphocyte blastogenesis assay with cells with indomethacin and without indomethacin from three cattle experimentally exposed to M. bovis (Mitogen = PHA)[a]

| Calf #[b] | Mitogen Quantity (ug) | CPM Average Background | CPM Average Test | SI |
|---|---|---|---|---|
| 32 | 2.5 | 664 | 2203 | 3.32 |
| | 1 | | 9826 | 14.80 |
| 32I | 2.5 | 1929 | 5282 | 2.74 |
| | 1 | | 4924 | 2.55 |
| 42 | 2.5 | 350 | 9850 | 13.86 |
| | 1 | | 5502 | 15.72 |
| 42I | 2.5 | 1476 | 9421 | 6.38 |
| | 1 | | 4715 | 3.19 |
| 51 | 2.5 | 669 | 14240 | 21.28 |
| | 1 | | 12898 | 19.28 |
| 51I | 2.5 | 841 | 6813 | 8.10 |
| | 1 | | 5551 | 6.60 |
| 90 | 2.5 | 336 | 9188 | 25.10 |
| | 1 | | 14043 | 38.37 |
| 90I | 2.5 | 331 | 2567 | 7.76 |
| | 1 | | 5718 | 17.27 |

[a]Calf 90 was a control.

[b]Calf numbers followed by I indicate suspensions were prepared in M199I.

Table 21. Results of lymphocyte blastogenesis assay with cells with indomethacin and without indomethacin from three cattle experimentally exposed to *M. bovis* (Antigen = EEC-PPD)[a]

| Calf #[b] | Antigen Quantity (ug) | CPM Average Background | CPM Average Test | SI |
|---|---|---|---|---|
| 32 | 10 | 11022 | 10257 | 0.93 |
| | 1 | | 8014 | 0.73 |
| | 0.1 | | 12634 | 1.15 |
| 32I | 10 | 11855 | 9828 | 0.83 |
| | 1 | | 11838 | 1.00 |
| | 0.1 | | 10362 | 0.87 |
| 42 | 10 | 8573 | 8853 | 1.03 |
| | 1 | | 7284 | 0.85 |
| | 0.1 | | 7518 | 0.88 |
| 42I | 10 | 4211 | 6193 | 1.47 |
| | 1 | | 10398 | 2.47 |
| | 0.1 | | 16028 | 3.81 |
| 51 | 10 | 5113 | 3297 | 0.64 |
| | 1 | | 3741 | 0.73 |
| | 0.1 | | 5703 | 1.12 |
| 51I | 10 | 16191 | 10446 | 0.64 |
| | 1 | | 14891 | 0.92 |
| | 0.1 | | 18379 | 1.14 |
| 90 | 10 | 9056 | 6868 | 0.76 |
| | 1 | | 6369 | 0.70 |
| | 0.1 | | 6336 | 0.70 |
| 90I | 10 | 20560 | 7104 | 0.34 |
| | 1 | | 9972 | 0.48 |
| | 0.1 | | 18227 | 0.89 |

[a]Calf 90 was a control.
[b]Calf numbers followed by I indicate suspensions were prepared in M199I.

Table 22. Results of lymphocyte blastogenesis assay with cells with indomethacin and without indomethacin from three cattle experimentally exposed to *M. bovis* (Antigen = PIS-PPD)[a]

| Calf #[b] | Antigen Quantity (ug) | CPM Average Background | CPM Average Test | SI |
|---|---|---|---|---|
| 32 | 10 | 3493 | 3632 | 1.04 |
| | 1 | | 4148 | 1.19 |
| | 0.1 | | 11854 | 3.39 |
| 32I | 10 | 4966 | 3871 | 0.78 |
| | 1 | | 3859 | 0.78 |
| | 0.1 | | 15283 | 3.08 |
| 42 | 10 | 4454 | 5116 | 1.15 |
| | 1 | | 2817 | 0.63 |
| | 0.1 | | 22788 | 5.12 |
| 42I | 10 | 4354 | 6309 | 1.45 |
| | 1 | | 2909 | 0.67 |
| | 0.1 | | 15431 | 3.54 |
| 51 | 10 | 4383 | 4639 | 1.06 |
| | 1 | | 3931 | 0.90 |
| | 0.1 | | 15379 | 3.51 |
| 51I | 10 | 6703 | 8661 | 1.29 |
| | 1 | | 3276 | 0.49 |
| | 0.1 | | 21616 | 3.22 |
| 90 | 10 | 6757 | 9138 | 1.35 |
| | 1 | | 4162 | 0.62 |
| | 0.1 | | 8205 | 1.21 |
| 90I | 10 | 7256 | 6943 | 0.96 |
| | 1 | | 6805 | 0.94 |
| | 0.1 | | 19165 | 2.64 |

[a]Calf 90 was a control.

[b]Calf numbers followed by I indicate suspensions were prepared in M199I.

Table 23. Results of lymphocyte blastogenesis assay with cells with indomethacin and without indomethacin from three cattle experimentally exposed to *M. bovis* (Antigen = USDA-PPD)[a]

| Calf #[b] | Antigen Quantity (ug) | CPM Average Background | CPM Average Test | SI |
|---|---|---|---|---|
| 32 | 10 | 467 | 522 | 1.12 |
| | 1 | | 570 | 1.22 |
| | 0.1 | | 413 | 0.88 |
| 32I | 10 | 7627 | 8733 | 1.14 |
| | 1 | | 5022 | 0.66 |
| | 0.1 | | 3679 | 0.49 |
| 42 | 10 | 600 | 1531 | 2.55 |
| | 1 | | 688 | 1.15 |
| | 0.1 | | 497 | 0.83 |
| 42I | 10 | 5980 | 5845 | 0.98 |
| | 1 | | 5275 | 0.88 |
| | 0.1 | | 5407 | 0.90 |
| 51 | 10 | 665 | 7290 | 10.96 |
| | 1 | | 6776 | 10.19 |
| | 0.1 | | 5443 | 8.18 |
| 51I | 10 | 7811 | 6934 | 0.89 |
| | 1 | | 5503 | 0.70 |
| | 0.1 | | 8533 | 1.09 |
| 90 | 10 | 9696 | 14623 | 1.51 |
| | 1 | | 5494 | 0.57 |
| | 0.1 | | 16771 | 1.73 |
| 90I | 10 | 11589 | 10686 | 0.92 |
| | 1 | | 8353 | 0.72 |
| | 0.1 | | 11103 | 0.96 |

[a]Calf 90 was a control.
[b]Calf numbers followed by I indicate suspensions were prepared in M199I.

Table 24. Results of lymphocyte blastogenesis assay with cells with indomethacin and without indomethacin from three cattle experimentally exposed to *M. bovis*[a]

| Antigen | Antigen Quantity (ug) | Stimulation index | |
|---|---|---|---|
| | | Indomethacin | No indomethacin |
| EEC | 10 | 1 | 1 |
| | 1 | 1 | 1 |
| | 0.1 | 2 | 1 |
| PIS | 10 | 1 | 1 |
| | 1 | 1 | 1 |
| | 0.1 | 3 | 4 |
| USDA | 10 | 1 | 5 |
| | 1 | 1 | 4 |
| | 0.1 | 1 | 3 |

[a]N=3, standard error=3.

| | SI |
|---|---|
| Indomethacin | 1.2 (± 0.5) |
| No indomethacin | 2.0 (± 0.5) |

However, the response by cells from calf 42 to EEC-PPD plus indomethacin was larger than the response to EEC-PPD alone. Similarly, the response by cells from calf 51 to USDA-PPD plus indomethacin was smaller than the response to USDA-PPD alone. These differences in response were seen at all three quantities of antigen.

| | Quantity of EEC-PPD (ug) | | |
|---|---|---|---|
| Calf # | 10 | 1 | 0.1 |
| 42 | 1.03 | 0.85 | 0.88 |
| 42I | 1.47 | 2.47 | 3.81 |
| | Quantity of USDA-PPD (ug) | | |
| Calf # | 10 | 1 | 0.1 |
| 51 | 10.96 | 10.19 | 8.18 |
| 51I | 0.89 | 0.70 | 1.09 |

The smaller response to the M. bovis antigens plus indomethacin was not consistent for cells from all of the calves (P>F=0.0145). The changes in response observed when indomethacin and the M. bovis antigens were added to the cells of each of the three calves were different for different calves. Cells from calves 32 and 42 responded no differently to the M. bovis antigens plus indomethacin than to the M. bovis antigens alone. Cells from calf 51

responded with a higher SI to the M. bovis antigens plus indomethacin than they did to the M. bovis antigens alone (Table 25).

4. Necropsy findings

The experimentally exposed calves that survived and controls were necropsied after the second PPD test. Tissues were examined for gross lesions. A summary of necropsy findings follows as Table 26.

5. Histopathologic examination

Slides were prepared from thoracic lymph nodes and examined for tuberculous lesions. Control calves were numbers 88-91 (Table 27).

Lesions varied in stage of development. Less mature lesions were composed of a mixture of epithelioid cells and inflammatory cells, primarily lymphocytes and a few polymorphonuclear leukocytes. Several multinucleated giant cells formed by the fusion of epithelioid cells were present in lesions (Figures 5 and 6).

Mature lesions consisted of a center of caseous necrosis, with or without calcification, surrounded by an area of epithelioid cells and multinucleated giant cells, with an outer boundary of inflammatory cells and fibrous connective tissue (Figures 7 and 8). Careful examination of some appropriately stained lesions at high magnification revealed tubercle bacilli (Figure 9).

Table 25. Results of lymphocyte blastogenesis assay with cells from three cattle experimentally exposed to *M. bovis* with indomethacin and without indomethacin[a]

| | Stimulation index | |
|---|---|---|
| Calf # | Indomethacin | No indomethacin |
| 32 | 1.1 | 1.3 |
| 42 | 1.8 | 1.6 |
| 51 | 1.2 | 4.1 |

[a]N=9, standard error=0.5.

Table 26. Necropsy summary from 19 calves experimentally exposed to *M. bovis* and 4 control calves

| Calf # | 34 | 29 | 37 | 27 | 31 | 33 |
|---|---|---|---|---|---|---|
| Date | 10/18 | 10/18 | 10/18 | 10/18 | 10/21 | 10/21 |
| Mandibular ln | N | N | N | N | N | N |
| Parotid ln | N | N | N | N | N | N |
| M. retropharyngeal ln | N | N | N | N | N | N |
| Deep cervical ln | C | C | C | C | C | C |
| Tracheal lesion (cm) | 10 | 2 | 1.5 | 0.5 | N | 4 |
| Superficial cervical ln | N | E | N | N | E | N |
| Thoracic ln | C | C | N | C | C | C |
| Lung | P | P | N | C | C | C |
| Liver | C | N | N | N | C | C |
| Hepatic ln | N | N | N | N | N | N |
| Spleen | N | N | N | N | N | N |
| Mesenteric ln | N | N | N | E | N | N |
| Kidney | N | I | N | N | N | N |
| Popliteal ln | N | N | N | N | N | N |

ln= Lymph nodes
N = No gross lesions observed
E = Enlarged
C = Calcified granuloma
P = Pneumonia
I = Infarct

Table 26. Continued

| Calf # | 26 | 52 | 44 | 40 | 35 | 45 |
|---|---|---|---|---|---|---|
| Date | 10/22 | 10/23 | 10/24 | 10/28 | 11/1 | 11/1 |
| Mandibular ln | N | N | N | N | N | N |
| Parotid ln | N | E | N | N | N | N |
| M. retropharyngeal ln | N | N | N | N | N | N |
| Deep cervical ln | C | N | C | C | C | C |
| Tracheal lesion (cm) | 2 | N | 2 | 3 | 1.5 | 1.5 |
| Superficial cervical ln | C | N | N | C | C | C |
| Thoracic ln | C | N | N | N | C | N |
| Lung | N | N | N | N | N | N |
| Liver | N | N | N | N | N | N |
| Hepatic ln | N | N | N | N | N | N |
| Spleen | N | N | N | N | N | N |
| Mesenteric ln | N | N | N | N | N | N |
| Kidney | N | N | N | N | N | N |
| Popliteal ln | N | N | N | N | N | N |

ln= Lymph nodes
N = No gross lesions observed
E = Enlarged
C = Calcified granuloma

Table 26. Continued

| Calf # | 36/46 | 49 | 30 | 43 | 51 | 32 |
|---|---|---|---|---|---|---|
| Date | 11/1 | 11/1 | 11/1 | 11/4 | 11/4 | 11/4 |
| Mandibular ln | N | N | N | N | N | N |
| Parotid ln | N | N | N | N | N | N |
| M. retropharyngeal ln | N | N | N | N | N | N |
| Deep cervical ln | C | C | C | C | C | C |
| Tracheal lesion (cm) | 2 | 1 | 3 | N | N | 1 |
| Superficial cervical ln | C | C | C | C | C | C |
| Thoracic ln | N | C | C | C | C | C |
| Lung | N | P | N | C | N | C |
| Liver | N | A | C | N | N | N |
| Hepatic ln | N | N | N | C | N | N |
| Spleen | N | N | N | N | N | N |
| Mesenteric ln | N | N | N | N | N | N |
| Kidney | N | N | N | N | N | N |
| Popliteal ln | N | N | N | N | N | N |

ln= Lymph nodes
N = No gross lesions observed
C = Calcified granuloma
P = Pneumonia
A = Abscess

Table 26. Continued

| Calf # | 42 | 91 | 89 | 90 | 88 |
|---|---|---|---|---|---|
| Date | 11/4 | 11/6 | 11/6 | 11/6 | 11/6 |
| Mandibular ln | N | N | N | N | N |
| Parotid ln | N | N | N | N | N |
| M. retropharyngeal ln | N | N | N | N | N |
| Deep cervical ln | C | N | N | N | N |
| Tracheal lesion (cm) | N | N | N | N | N |
| Superficial cervical ln | C | N | N | N | N |
| Thoracic ln | C | N | N | N | N |
| Lung | N | N | N | N | N |
| Liver | N | N | N | N | N |
| Hepatic ln | N | N | N | N | N |
| Spleen | N | N | N | N | N |
| Mesenteric ln | N | N | N | N | N |
| Kidney | N | N | N | N | N |
| Popliteal ln | N | N | N | N | N |

ln= Lymph nodes
N = No gross lesions observed
C = Calcified granuloma

Table 27. Histopathologic findings in thoracic lymph nodes

| Calf # | Lesions present |
|---|---|
| 26 | -[a] |
| 29 | +[b] |
| 30 | + |
| 31 | + |
| 32 | + |
| 33 | + |
| 34 | + |
| 35 | - |
| 36 | + |
| 37 | + |
| 40 | - |
| 42 | + |
| 43 | + |
| 44 | + |
| 45 | - |
| 49 | - |
| 50 | + |
| 51 | + |
| 52 | + |
| 88 | - |
| 89 | - |
| 90 | - |
| 91 | - |

[a]- = no lesions observed.
[b]+ = tuberculous lesions observed.

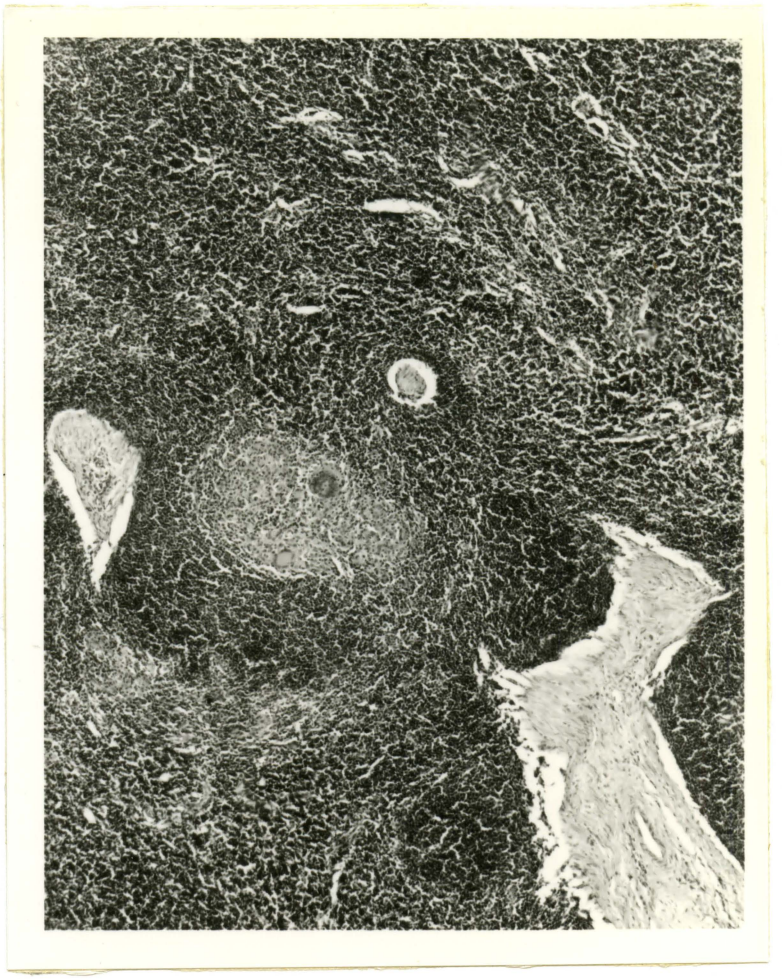

Figure 5. Tracheobronchial lymph node with developing tubercle. H and E stain. x63

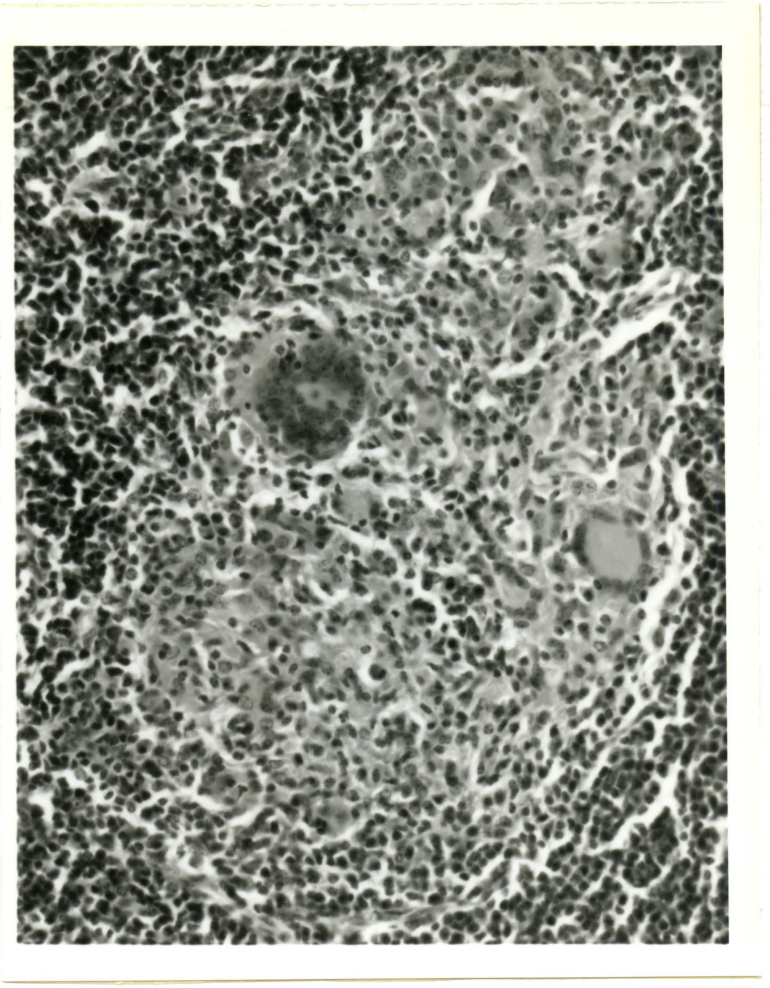

Figure 6. Same as Figure 5 showing multinucleated giant cell. H and E stain. x250

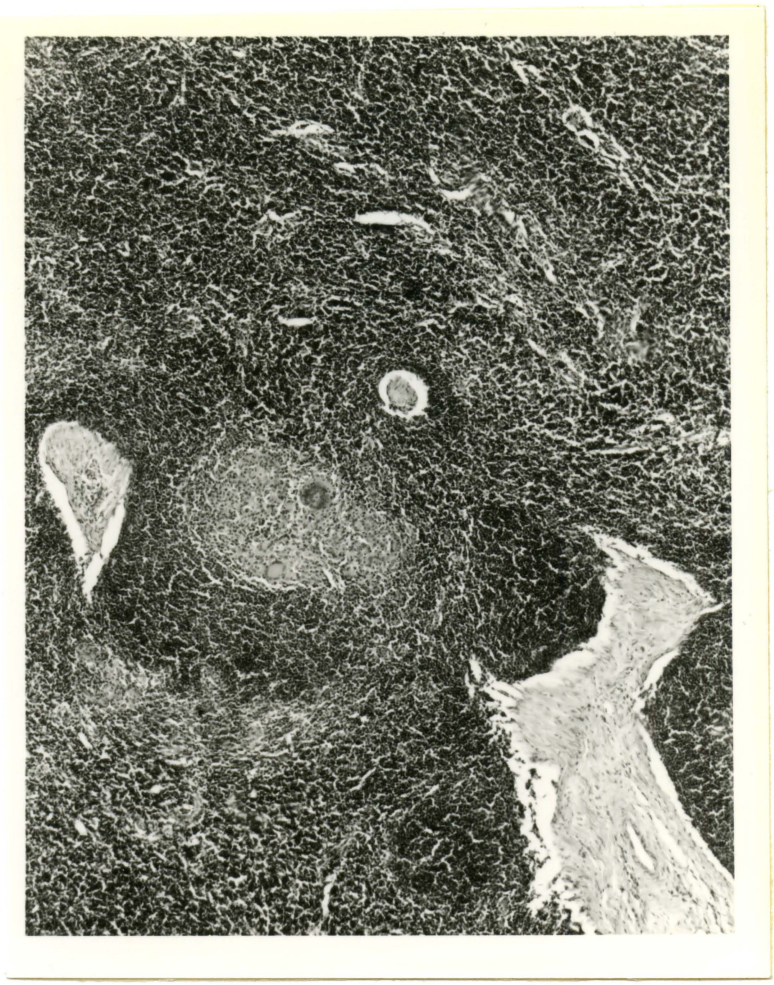

Figure 7. Tracheobronchial lymph node with mature tubercle. H and E stain. x63

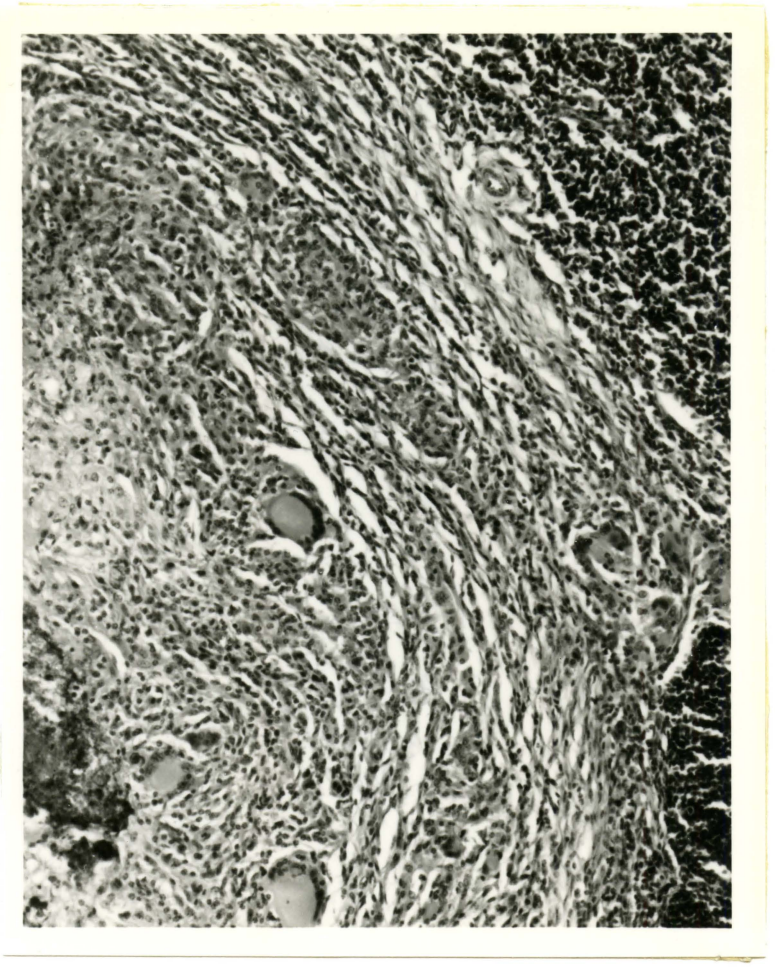

Figure 8. Same as Figure 7 showing an area of caseous necrosis and calcification bordered by epithelioid cells mixed with other inflammatory cells. Several multinucleated giant cells are present. Fibrous connective tissue has formed at the periphery of the lesion. H and E stain. x150

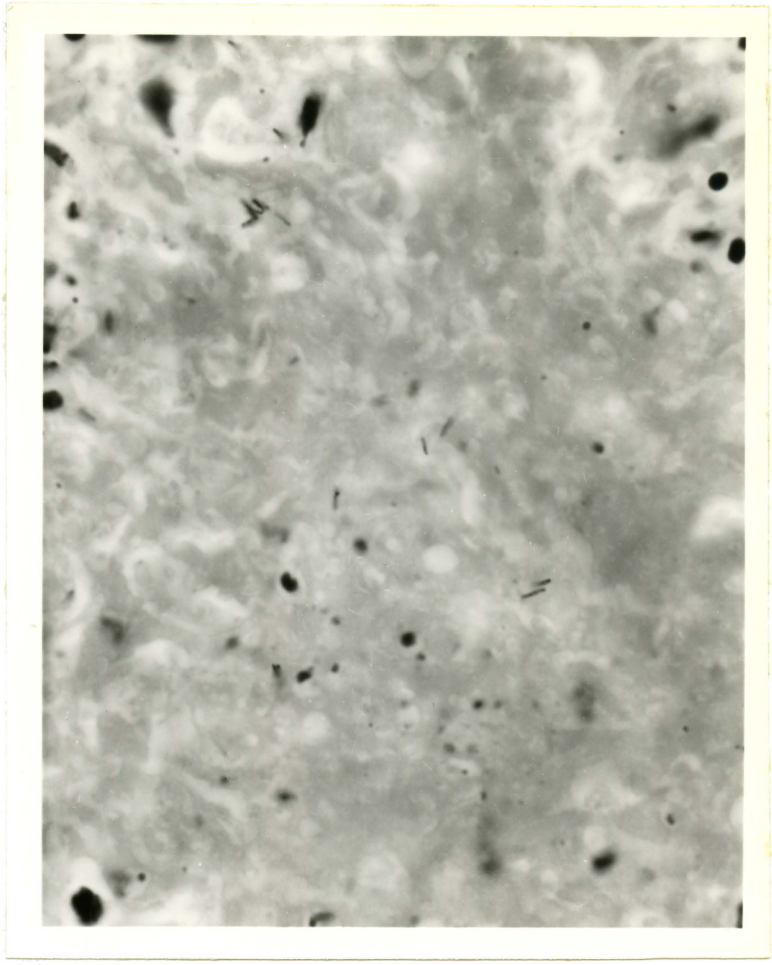

Figure 9. Area of necrosis in tracheobronchial lymph node showing several tubercle bacilli. New fuchsin stain. x1000

6. Mycobacteriologic examination

Lesions from each calf were collected for mycobacterial isolation. Colonies of non-photochromogenic, slowly growing, acid-fast bacteria suspected of being M. bovis were inoculated into Proskauer and Beck medium with 5% horse serum to determine growth characteristics. Flocculent growth with many clumped cells was suggestive of M. bovis or M. tuberculosis, while smooth suspensions of uniform growth were suggestive of Mycobacterium avium strains.

Colonies were tested for susceptibility to isoniazid (INH) and thiophen-2-carboxylic acid hydrazide (TCH). Susceptible strains showed no apparent growth. Table 28 summarizes the susceptibility of M. bovis, M. tuberculosis and M. avium complex to INH and TCH. Mycobacterium bovis was isolated from each of the 19 experimentally exposed calves used in the PPD evaluation.

## B. Comparison of M. bovis Antigens and a M. paratuberculosis Antigen

1. Evaluation of M. paratuberculosis PPD in sensitized guinea pigs at 24 hours

Mycobacterium paratuberculosis tuberculins were injected into each of 40 guinea pigs (see Appendix, Tables 67-70). One of the guinea pigs injected with three dilutions of the Netherlands PPD died before results were obtained.

Table 28. Susceptibility of three mycobacteria to isoniazid (INH) and thiophen-2-carboxylic acid hydrazide (TCH) (growth indicates resistance)

| Organism | Growth in: INH | TCH |
|---|---|---|
| *M. bovis* | - | - |
| *M. tuberculosis* | - | + |
| *M. avium* complex | + | + |

Two readers observed the skin test responses after 24 hours. The mean observations were:

Reader 1 242 (± 5) $mm^2$

Reader 2 266 (± 5) $mm^2$

There were significant differences in skin test responses to each of the four tuberculins tested (P>F=0.0019). The average skin test responses were:

| Tuberculin | Number of observations | Area ($mm^2$) |
|---|---|---|
| USA | 60 | 287 (± 19) |
| Canada | 60 | 300 (± 19) |
| Netherlands | 54 | 225 (± 20) |
| USDA-OT | 60 | 200 (± 19) |

The skin test responses (mean values) were compared at three levels of significance. No significant differences in skin test responses were detected between USA and Canada. There were also no differences between Netherlands and USDA-OT (P<0.1). At the 95% level, the same differences were seen; USA = Canada, Netherlands = USDA-OT. At the 99% level, there were no differences between Canada, USA and Netherlands. Also, there was no difference between Netherlands and USDA-OT; Canada = USA = Netherlands, Netherlands = USDA-OT (Tables 29, 30, 31 and 32).

Three dilutions of each tuberculin were injected. There was a significant difference in skin test responses

Table 29. Comparison of skin test responses (mean values) for each of four tuberculins in guinea pigs sensitized with killed M. paratuberculosis at 24 hours (Alpha=0.10)[a]

| Tuberculin comparison | Lower confidence limit | Difference between means | Upper confidence limit | |
|---|---|---|---|---|
| USA-Canada | -33.61 | 12.80 | 59.21 | |
| USA-Netherlands | 14.48 | 62.16 | 109.85 | *** |
| USA-USDA-OT | 40.65 | 87.07 | 133.48 | *** |
| Canada-Netherlands | 27.28 | 74.96 | 122.65 | *** |
| Canada-USDA-OT | 53.45 | 99.87 | 146.28 | *** |
| Netherlands-USDA-OT | -22.78 | 24.90 | 72.59 | |

[a]df=35, MSE=22637, critical value of t=1.68957. Comparisons that are significant are indicated by '***.'

Table 30. Comparison of skin test responses (mean values) for each of four tuberculins in guinea pigs sensitized with killed M. paratuberculosis at 24 hours (Alpha=0.05)[a]

| Tuberculin comparison | Lower confidence limit | Difference between means | Upper confidence limit | |
|---|---|---|---|---|
| USA-Canada | 42.97 | 12.80 | 68.57 | |
| USA-Netherlands | -4.86 | 62.16 | 119.46 | *** |
| USA-USDA-OT | -31.31 | 87.07 | 142.83 | *** |
| Canada-Netherlands | -17.66 | 74.96 | 132.26 | *** |
| Canada-USDA-OT | -44.11 | 99.87 | 155.63 | *** |
| Netherlands-USDA-OT | 32.40 | 24.90 | 82.20 | |

[a]df=35, MSE=22637, critical value of t=2.03011. Comparisons that are significant are indicated by '***.'

Table 31. Comparison of skin test responses (mean values) for each of four tuberculins in guinea pigs sensitized with killed M. paratuberculosis at 24 hours (Alpha=0.01)[a]

| Tuberculin comparison | Lower confidence limit | Difference between means | Upper confidence limit | |
|---|---|---|---|---|
| USA-Canada | -62.02 | 12.80 | 87.62 | |
| USA-Netherlands | -14.71 | 62.16 | 139.04 | |
| USA-USDA-OT | 12.24 | 87.07 | 161.89 | *** |
| Canada-Netherlands | -1.91 | 74.96 | 151.84 | |
| Canada-USDA-OT | 25.04 | 99.87 | 174.69 | *** |
| Netherlands-USDA-OT | -51.97 | 24.90 | 101.77 | |

[a]df=35, MSE=22637, critical value of t=2.72381. Comparisons that are significant are indicated by '***.'

Table 32. Summary of skin test responses (mean values) in $mm^2$ for each of four tuberculins at three levels of significance in guinea pigs at 24 hours[a]

| | | | Alpha | | |
|---|---|---|---|---|---|
| Tuberculin | Mean | N | 0.10 | 0.05 | 0.01 |
| Canada | 300 | 60 | A | C | E |
| USA | 287 | 60 | A | C | E |
| Netherlands | 225 | 54 | B | D | E F |
| USDA-OT | 200 | 60 | B | D | F |

[a]Means with the same letter are not significantly different.

(mean values) to them (P>F=0.0001). The mean responses were:

| Dilution | N | Area ($mm^2$) |
|---|---|---|
| 1:50 | 78 | 382 (± 6) |
| 1:100 | 78 | 241 (± 6) |
| 1:200 | 78 | 139 (± 6) |

The skin test responses to the dilutions were compared at three levels of significance. The confidence intervals for the three means were:

| Confidence level | Dilution | | |
|---|---|---|---|
| | 1:50 | 1:100 | 1:200 |
| 90% | (373,391) | (232,250) | (130,148) |
| 95% | (371,393) | (230,252) | (128,150) |
| 99% | (367,397) | (226,256) | (124,154) |

The effect of reader on the mean skin test response to the four tuberculins was examined. There was no significant interaction (P>F=0.1665). Therefore, the differences in response to each of the tuberculins were the same for each of the two readers.

The effect of dilution on the mean skin test response to the four tuberculins was significant (P>F=0.0001). Therefore, the differences in response observed at different dilutions were not the same for the four tuberculins (Table

33). The product-dilution interaction was compared at three levels of significance (Tables 34, 35, 36 and 37).

The differences between mean responses to tuberculins were examined for significance. $t^*$ was calculated as $(Mean_1 - Mean_2) / (SEM_1 + SEM_2)^{1/2}$. $t^*$ was compared to $t_{table}$ at three levels of significance. If $t^* > t_{table}$ the difference was significant.

| Confidence level | $t_{table}$ |
|---|---|
| 90% | 1.64 |
| 95% | 1.96 |
| 99% | 2.57 |

Values of $t^*$ for various comparisons are listed in Table 38.

The interaction between tuberculin, dilution and reader was examined. There was no significant interaction ($P>F=0.6237$). Therefore, the effect of different dilutions on tuberculin was the same for each of the two readers.

Relative potencies were calculated for the four tuberculins. The data were evaluated by an analysis of variance, then F statistics constructed to compare sources of variation (Tables 39 and 40).

Important comparisons are:

1. Lack of Fit-indicates lack of fit of data to line
2. $Tuberculin*Dilution_{linear}$-indicates parallelism of dose-response lines.

Table 33. Average area of response ($mm^2$) per guinea pig at 24 hours using three dilutions of four *M. paratuberculosis* tuberculins[a]

| | Dilution | | | | |
|---|---|---|---|---|---|
| Antigen | 1:50 | 1:100 | 1:200 | Total | N |
| USA | 413.5 | 289.0 | 159.6 | 862.1 | 20 |
| Canada | 457.6 | 280.2 | 162.6 | 900.4 | 20 |
| Netherlands | 314.7 | 220.1 | 140.8 | 675.6 | 18 |
| USDA-OT | 333.8 | 173.8 | 93.3 | 600.9 | 20 |

[a]$MSE_{Netherlands}$=12, all others=11.

Table 34. Confidence intervals for product-dilution interaction from Table 33 (USA)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (396,432) | (271,307) | (142,178) |
| 95% | (392,436) | (267,311) | (138,182) |
| 99% | (386,442) | (261,317) | (132,188) |

Table 35. Confidence intervals for product-dilution interaction from Table 33 (Canada)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (440,476) | (262,298) | (145,181) |
| 95% | (436,480) | (258,302) | (141,185) |
| 99% | (430,486) | (252,308) | (135,191) |

Table 36. Confidence intervals for product-dilution interaction from Table 33 (Netherlands)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (296,334) | (201,239) | (122,160) |
| 95% | (292,338) | (197,243) | (118,164) |
| 99% | (285,345) | (190,250) | (111,171) |

Table 37. Confidence intervals for product-dilution interaction from Table 33 (USDA-OT)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (316,352) | (156,192) | (75,111) |
| 95% | (312,356) | (152,196) | (71,115) |
| 99% | (306,362) | (146,202) | (65,121) |

Table 38. Calculated t values for comparisons of skin test responses (mean values) to four tuberculins at different dilutions and different levels of significance in guinea pigs sensitized with *M. paratuberculosis* at 24 hours[a]

| Comparison | t* | Confidence level | | |
|---|---|---|---|---|
| | | 90% | 95% | 99% |
| $USA_{50}$-$Canada_{50}$ | 2.82 | *** | *** | *** |
| $USA_{100}$-$Canada_{100}$ | 0.58 | NS | NS | NS |
| $USA_{200}$-$Canada_{200}$ | 0.19 | NS | NS | NS |
| $USA_{50}$-$Netherlands_{50}$ | 6.18 | *** | *** | *** |
| $USA_{100}$-$Netherlands_{100}$ | 4.31 | *** | *** | *** |
| $USA_{200}$-$Netherlands_{200}$ | 1.19 | NS | NS | NS |
| $USA_{50}$-$USDA\text{-}OT_{50}$ | 5.13 | *** | *** | *** |
| $USA_{100}$-$USDA\text{-}OT_{100}$ | 7.38 | *** | *** | *** |
| $USA_{200}$-$USDA\text{-}OT_{200}$ | 2.12 | *** | *** | NS |
| $Canada_{50}$-$Netherlands_{50}$ | 8.93 | *** | *** | *** |
| $Canada_{100}$-$Netherlands_{100}$ | 3.75 | *** | *** | *** |
| $Canada_{200}$-$Netherlands_{200}$ | 1.37 | NS | NS | NS |
| $Canada_{50}$-$USDA\text{-}OT_{50}$ | 7.96 | *** | *** | *** |
| $Canada_{100}$-$USDA\text{-}OT_{100}$ | 6.80 | *** | *** | *** |
| $Canada_{200}$-$USDA\text{-}OT_{200}$ | 1.92 | *** | NS | NS |
| $Netherlands_{50}$-$USDA\text{-}OT_{50}$ | 1.19 | NS | NS | NS |
| $Netherlands_{100}$-$USDA\text{-}OT_{100}$ | 2.87 | *** | *** | *** |
| $Netherlands_{200}$-$USDA\text{-}OT_{200}$ | 3.25 | *** | *** | *** |

[a]Significant differences are indicated by '***.' Differences that are not significant are indicated by 'NS.'

Table 39. Analysis of variance of skin test responses to four tuberculins in sensitized guinea pigs at 24 hours

| Source | df | SS | MS |
|---|---|---|---|
| Tuberculin | 3 | 412487.02 | 137495.67 |
| Animal(Tuberculin) | 35 | 792309.79 | 22637.42 |
| Dilution | 2 | 2279227.53 | 1139613.76 |
| $Dilution_{linear}$ | 1 | 2260877.07 | 2260877.07 |
| Lack of Fit | 1 | 18350.46 | 18350.46 |
| Tuberculin*Dilution | 6 | 86359.69 | 14393.28 |
| $T*D_{linear}$ | 3 | 71338.07 | 23779.36 |
| Residual | 3 | 15021.62 | 5007.21 |
| Dil.*Animal(Tuberculin) | 70 | 302902.69 | 4327.18 |
| $Dil._{linear}$*An.(Tub.) | 35 | 212819.90 | 6080.57 |
| Residual | 35 | 90082.79 | 2573.79 |
| Error | 117 | 183772.50 | 1570.71 |
| Total | 233 | | |

Table 40. Results of F tests for sources of variation from Table 39[a]

| | | F | |
|---|---|---|---|
| Tuberculin | :Animal(Tuberculin) | 6.07 | *** |
| Dilution | :Dilution*Animal(Tub.) | 263.36 | *** |
| Dil.$_{linear}$ | :Dilution$_{linear}$*Animal(Tub.) | 371.82 | *** |
| Lack of Fit | :Residual | 7.13 | ** |
| Tuberculin*Dil. | :Dilution*Animal(Tub.) | 3.33 | *** |
| T*D$_{linear}$ | :Dilution$_{linear}$*Animal(Tub.) | 3.91 | ** |

[a]Significant comparisons are indicated by '**' when significant at 5% and '***' when significant at 1%.

Parallelism was significant; therefore, there was a 95% probability that the lines were not parallel. To determine which of the dose-response lines were not parallel, regression lines were calculated and the slopes compared (Figure 10, Table 41).

If a comparison was significant, the lines were not parallel. All of the comparisons involving the Netherlands PPD were significant, therefore it was not comparable to the other tuberculins. The analysis of variance was repeated excluding Netherlands data (Tables 42 and 43).

The data met the assumptions of linearity and parallelism. Data are expressed as an average of the observations by two readers. Tables 44, 45 and 46 list the observations for each tuberculin.

Mean values were calculated for a particular dilution of a tuberculin and for tuberculins (Table 47).

The slopes of the regression lines (Figure 10) for each of the three tuberculins were:

| Tuberculin | Slope |
|---|---|
| USA | 421.72 |
| Canada | 489.98 |
| USDA-OT | 399.46 |

The slopes were not significantly different at the 5% level; therefore, a common regression coefficient was calculated by fitting one line to all of the data from all three

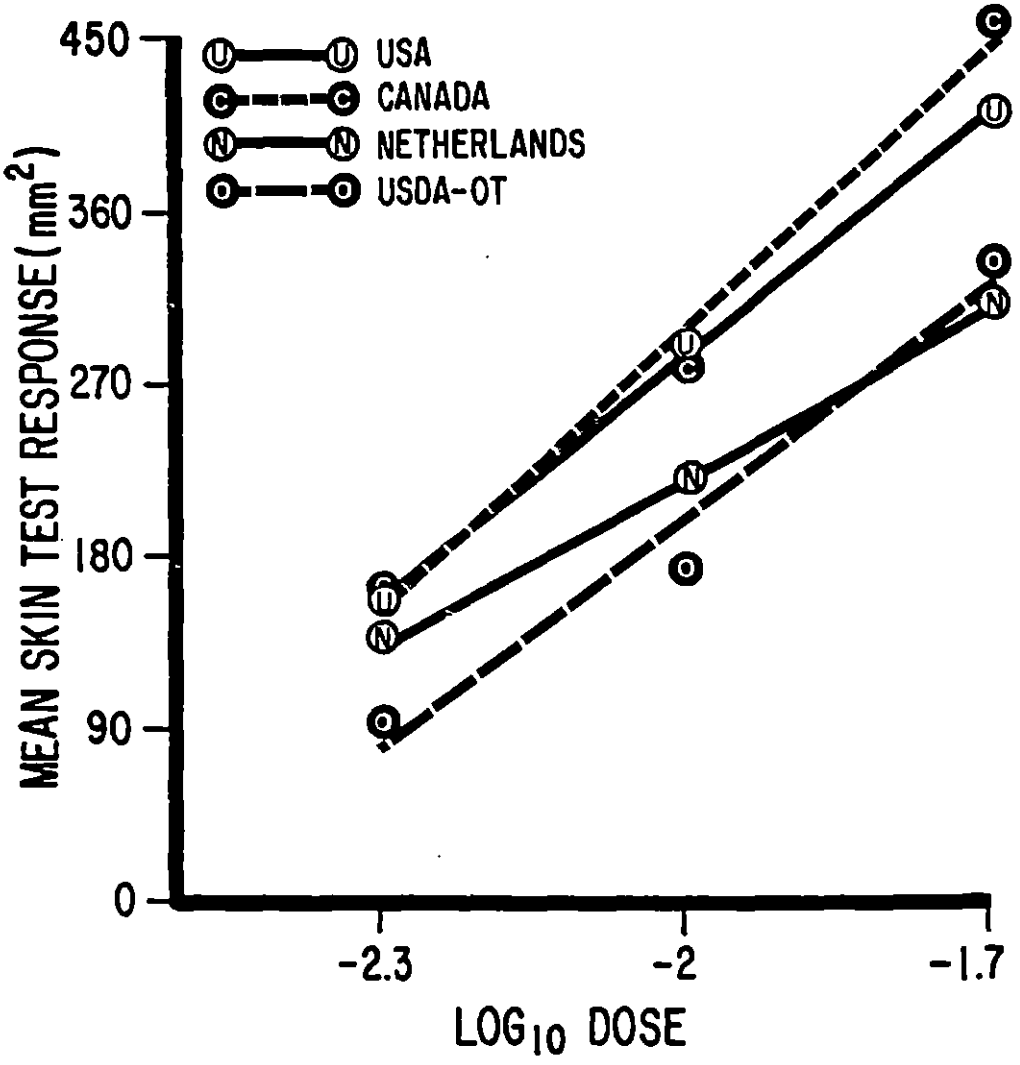


Figure 10. Dose-response lines for four tuberculins injected intradermally in the abdomen of guinea pigs sensitized with M. paratuberculosis. $Slope_{USA}$= 421.72, $slope_{Canada}$= 489.98, $slope_{Netherlands}$= 288.73, $slope_{USDA-PPD}$= 399.46

Table 41. Comparison of slopes of all possible pairs of regression lines ($log_{10}$dose of tuberculin vs. skin test response) at 24 hours[a]

| Comparison | t[b] | |
|---|---|---|
| USA-Canada | 1.04 | |
| USA-Netherlands | 2.45 | *** |
| USA-USDA-OT | 0.39 | |
| Canada-Netherlands | 3.56 | *** |
| Canada-USDA-OT | 1.53 | |
| Netherlands-USDA-OT | 2.40 | *** |

[a]Alpha=0.05, comparisons that are significant are indicated by '***.'

[b]$t_{52df}$=2.007.

Table 42. Analysis of variance of skin test responses to three tuberculins in sensitized guinea pigs at 24 hours

| Source | df | SS | MS |
|---|---|---|---|
| Tuberculin | 2 | 354355.91 | 177177.96 |
| Animal(Tuberculin) | 27 | 732987.98 | 27147.70 |
| Dilution | 2 | 2097364.58 | 1048682.29 |
| $Dilution_{linear}$ | 1 | 2077174.53 | 2077174.53 |
| Lack of Fit | 1 | 20190.05 | 20190.05 |
| Tuberculin*Dilution | 4 | 29137.35 | 7284.34 |
| $T*D_{linear}$ | 2 | 16130.07 | 8065.04 |
| Residual | 2 | 13007.28 | 6503.64 |
| Dil.*Animal(Tub.) | 54 | 241570.07 | 4473.52 |
| $Dil._{linear}$*An.(Tub.) | 27 | 163873.40 | 6069.39 |
| Residual | 27 | 77696.67 | 2877.65 |
| Error | 90 | 147431.50 | 1638.13 |
| Total | 179 | | |

Table 43. Results of F tests for sources of variation from Table 42[a]

| | | F | |
|---|---|---|---|
| Tuberculin | :Animal(Tuberculin) | 6.53 | *** |
| Dilution | :Dilution*Animal(Tub.) | 234.42 | *** |
| Dil.$_{linear}$ | :Dilution$_{linear}$*Animal(Tub.) | 342.24 | *** |
| Lack of Fit | :Residual | 7.02 | ** |
| Tuberculin*Dil. | :Dilution*Animal(Tub.) | 1.63 | |
| T*D$_{linear}$ | :Dilution$_{linear}$*Animal(Tub.) | 1.33 | |

[a]Significant comparisons are indicated by '**' when significant at 5% and '***' when significant at 1%.

Table 44. Skin test responses (mean values) in $mm^2$ to three dilutions of USA tuberculin in 10 guinea pigs sensitized with M. paratuberculosis at 24 hours[a]

| Dilution | | |
|---|---|---|
| 1:50 | 1:100 | 1:200 |
| 641 | 386 | 201 |
| 313 | 179 | 96 |
| 349 | 265 | 128 |
| 392 | 252 | 208 |
| 418 | 450 | 187 |
| 385 | 165 | 112 |
| 366 | 246 | 119 |
| 416 | 356 | 220 |
| 358 | 205 | 103 |
| 498 | 385 | 221 |

[a]Each observation is an average of observations by two readers.

Table 45. Skin test responses (mean values) in $mm^2$ to three dilutions of Canada tuberculin in 10 guinea pigs sensitized with M. paratuberculoisis at 24 hours[a]

| Dilution | | |
|---|---|---|
| 1:50 | 1:100 | 1:200 |
| 412 | 242 | 142 |
| 491 | 355 | 225 |
| 474 | 294 | 194 |
| 340 | 246 | 162 |
| 333 | 213 | 110 |
| 732 | 433 | 296 |
| 542 | 284 | 131 |
| 358 | 244 | 142 |
| 405 | 258 | 122 |
| 490 | 232 | 100 |

[a]Each observation is an average of observations by two readers.

Table 46. Skin test responses (mean values) in $mm^2$ to three dilutions of USDA-OT tuberculin in 10 guinea pigs sensitized with M. paratuberculosis at 24 hours[a]

| Dilution | | |
|---|---|---|
| 1:50 | 1:100 | 1:200 |
| 322 | 138 | 84 |
| 198 | 155 | 106 |
| 406 | 217 | 109 |
| 326 | 166 | 92 |
| 232 | 127 | 68 |
| 292 | 113 | 66 |
| 432 | 138 | 96 |
| 471 | 270 | 148 |
| 319 | 178 | 75 |
| 340 | 234 | 89 |

[a]Each observation is an average of observations by two readers.

Table 47. Skin test responses (mean values) in $mm^2$ to three dilutions of three tuberculins in 10 sensitized guinea pigs at 24 hours

| | Tuberculin | | |
|---|---|---|---|
| Dilution | USA | Canada | USDA-OT |
| 1:50 | 414 | 458 | 334 |
| 1:100 | 289 | 280 | 174 |
| 1:200 | 159 | 163 | 93 |
| Mean | 287 | 300 | 200 |

tuberculins. Slope= 437.05. Relative potencies were calculated:

| Tuberculin comparison | Relative potency | Confidence interval |
|---|---|---|
| USA vs. USDA-OT | 0.63 | (0.55,0.72) |
| Canada vs. USDA-OT | 0.59 | (0.52,0.68) |

Therefore, 1 unit of USDA-0T produced the same response as 0.63 units of USA and 0.59 units of Canada. Confidence intervals for those estimates were calculated at the 95% level of significance.

2. Evaluation of M. paratuberculosis PPD in sensitized guinea pigs at 48 hours

Four M. paratuberculosis tuberculins were injected intradermally on the abdomens of guinea pigs (see Appendix, Tables 71-74). Skin test responses were measured 24 hours after injection (see previous section) and 48 hours after injection. One of the guinea pigs injected with three dilutions of the Netherlands PPD died before results were obtained.

Three readers observed the skin test responses. The mean observations were:

| | N | Mean observations |
|---|---|---|
| Reader 1 | 27 | 139 (± 8) $mm^2$ |
| Reader 2 | 90 | 147 (± 4) $mm^2$ |
| Reader 3 | 117 | 158 (± 4) $mm^2$ |

Four tuberculins were injected. There were no significant differences in responses to them (P>F=0.1795).

Three dilutions of each tuberculin were injected. There was a significant difference in skin test responses (mean values) to them (P>F=0.0001). The mean responses were:

| Dilution | N | Area ($mm^2$) |
|---|---|---|
| 1:50 | 78 | 234 (± 5) |
| 1:100 | 78 | 147 (± 5) |
| 1:200 | 78 | 74 (± 5) |

The skin test responses to the dilutions were compared at three levels of significance. The confidence intervals for the three dilution means were:

| Confidence level | Dilution | | |
|---|---|---|---|
| | 1:50 | 1:100 | 1:200 |
| 90% | (226,242) | (139,155) | (66,82) |
| 95% | (225,243) | (138,156) | (65,83) |
| 99% | (222,246) | (135,159) | (62,86) |

The effect of reader on the mean skin test response to the four tuberculins was examined. There was no significant interaction (P>F=0.4139). Therefore, the differences in response to the different tuberculins was consistently observed by the three readers.

The effect of dilution on the mean skin test response to the four tuberculins was significant (P>F=0.0033). Therefore, the differences in response observed at different dilutions was not the same for the four tuberculins (Table 48). The product-dilution interaction was compared at three levels of significance (Tables 49, 50, 51 and 52).

The differences between mean responses were examined for significance. $t^*$ was calculated and was compared to $t^{table}$ at three levels of significance. It $t^* > t^{table}$ the difference was significant.

| Confidence level | $t_{table}$ |
|---|---|
| 90% | 1.64 |
| 95% | 1.96 |
| 99% | 2.57 |

Values of $t^*$ for various comparisons are listed in Table 53.

The interaction between tuberculin, dilution and reader was examined. There was no significant interaction (P>F=0.7965). Therefore, the effect of different dilutions on tuberculins was the same for each of the two readers.

Relative potencies were calculated for the four tuberculins. The data were evaluated by an analysis of variance, then F statistics constructed (Tables 54 and 55).

Important comparisons are:

1. Lack of Fit-indicates lack of fit of data to line

Table 48. Average area of response ($mm^2$) per guinea pig at 48 hours using three dilutions of four *M. paratuberculosis* antigens[a]

| Antigen | Dilution 1:50 | 1:100 | 1:200 | Total | N |
|---|---|---|---|---|---|
| USA | 246.8 | 163.5 | 76.6 | 486.9 | 20 |
| Canada | 266.8 | 173.5 | 97.5 | 537.8 | 20 |
| Netherlands | 189.6 | 133.6 | 81.1 | 404.3 | 18 |
| USDA-OT | 228.8 | 117.2 | 40.4 | 386.4 | 20 |

[a] $MSE_{Netherlands}=10$, all others=9.

Table 49. Confidence intervals for product-dilution interaction from Table 48 (USA)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (231,263) | (148,180) | (61,93) |
| 95% | (228,266) | (145,183) | (58,96) |
| 99% | (223,271) | (140,188) | (53,101) |

Table 50. Confidence intervals for product-dilution interaction from Table 48 (Canada)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (251,283) | (158,190) | (82,114) |
| 95% | (248,286) | (155,193) | (79,117) |
| 99% | (243,291) | (150,198) | (74,122) |

Table 51. Confidence intervals for product-dilution interaction from Table 48 (Netherlands)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (174,206) | (118,150) | (65,97) |
| 95% | (170,210) | (114,154) | (61,101) |
| 99% | (164,216) | (108,160) | (55,107) |

Table 52. Confidence intervals for product-dilution interaction from Table 48 (USDA-OT)

| Confidence level | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 90% | (213,245) | (101,133) | (24,56) |
| 95% | (210,248) | (98,136) | (21,59) |
| 99% | (205,253) | (93,141) | (16,64) |

Table 53. Calculated t values for comparisons of skin test responses (mean values) in mm to four tuberculins at different dilutions and different levels of significance in guinea pigs sensitized with *M. paratuberculosis* at 48 hours[a]

| Comparison | t* | Confidence level 90% | 95% | 99% |
|---|---|---|---|---|
| $USA_{50}$-$Canada_{50}$ | 1.54 | NS | NS | NS |
| $USA_{100}$-$Canada_{100}$ | 0.77 | NS | NS | NS |
| $USA_{200}$-$Canada_{200}$ | 1.61 | NS | NS | NS |
| $USA_{50}$-$Netherlands_{50}$ | 4.09 | *** | *** | *** |
| $USA_{100}$-$Netherlands_{100}$ | 2.14 | *** | *** | NS |
| $USA_{200}$-$Netherlands_{200}$ | 0.32 | NS | NS | NS |
| $USA_{50}$-$USDA\text{-}OT_{50}$ | 1.38 | NS | NS | NS |
| $USA_{100}$-$USDA\text{-}OT_{100}$ | 3.56 | *** | *** | *** |
| $USA_{200}$-$USDA\text{-}OT_{200}$ | 2.78 | *** | *** | *** |
| $Canada_{50}$-$Netherlands_{50}$ | 5.51 | *** | *** | *** |
| $Canada_{100}$-$Netherlands_{100}$ | 2.85 | *** | *** | *** |
| $Canada_{200}$-$Netherlands_{200}$ | 1.17 | NS | NS | NS |
| $Canada_{50}$-$USDA\text{-}OT_{50}$ | 2.92 | *** | *** | *** |
| $Canada_{100}$-$USDA\text{-}OT_{100}$ | 4.33 | *** | *** | *** |
| $Canada_{200}$-$USDA\text{-}OT_{200}$ | 4.39 | *** | *** | *** |
| $Netherlands_{50}$-$USDA\text{-}OT_{50}$ | 2.85 | *** | *** | *** |
| $Netherlands_{100}$-$USDA\text{-}OT_{100}$ | 1.17 | NS | NS | NS |
| $Netherlands_{200}$-$USDA\text{-}OT_{200}$ | 2.91 | *** | *** | *** |

[a]Significant differences are indicated by '***.' Differences that are not significant are indicated by 'NS.'

Table 54. Analysis of variance of skin test responses to four tuberculins in sensitized guinea pigs at 48 hours

| Source | df | SS | MS |
|---|---|---|---|
| Tuberculin | 3 | 99207.28 | 33069.09 |
| Animal(Tuberculin) | 35 | 699098.04 | 19974.23 |
| Dilution | 2 | 989150.94 | 494575.47 |
| $Dilution_{linear}$ | 1 | 986944.53 | 986944.53 |
| Lack of Fit | 1 | 2206.41 | 2206.41 |
| Tuberculin*Dilution | 6 | 36627.65 | 6104.61 |
| $T*D_{linear}$ | 3 | 33807.01 | 11269.00 |
| Residual | 3 | 2820.64 | 940.21 |
| Dil.*Animal(Tub.) | 70 | 236148.06 | 3373.54 |
| $Dil._{linear}$*An.(Tub.) | 35 | 162896.72 | 4654.19 |
| Residual | 35 | 73251.34 | 2092.90 |
| Error | 117 | 105460.50 | 901.37 |
| Total | 233 | | |

Table 55. Results of F tests for sources of variation from Table 54[a]

| | | F | |
|---|---|---|---|
| Tuberculin | :Animal(Tuberculin) | 1.66 | |
| Dilution | :Dilution*Animal(Tub.) | 146.60 | *** |
| Dil.linear | :$Dilution_{linear}$*Animal(Tub.) | 212.05 | *** |
| Lack of Fit | :Residual | 1.05 | |
| Tuberculin*Dil. | :Dilution*Animal(Tub.) | 1.81 | |
| T*$D_{linear}$ | :$Dilution_{linear}$*Animal(Tub.) | 2.42 | |

[a]Alpha=0.01. Significant comparisons are indicated by '***.'

2. Tuberculin*Dilution$_{linear}$-indicates parallelism of dose-response lines.

The value for parallelism was close to the significant value. To determine which of the dose-response lines were not parallel, regression lines were calculated and the slopes compared (Figure 11, Table 56).

If a comparison was significant, the lines were not parallel. Two of the three comparisons involving the Netherlands PPD were significant, therefore it was not comparable to the other PPDs. The analysis of variance was repeated excluding Netherlands data (Tables 57 and 58).

The data met the assumptions of linearity and parallelism. Data are expressed as an average of the observations by three readers (Tables 59, 60 and 61).

Mean values were calculated for a particular dilution of a tuberculin and for tuberculins (Table 62).

The slopes of the regression lines (Figure 11) for each of the three tuberculins were:

| Tuberculin | Slope |
|---|---|
| USA | 282.70 |
| Canada | 281.20 |
| USDA-0T | 312.84 |

The slopes were not significantly different at the 5% level; therefore, a common regression coefficient was

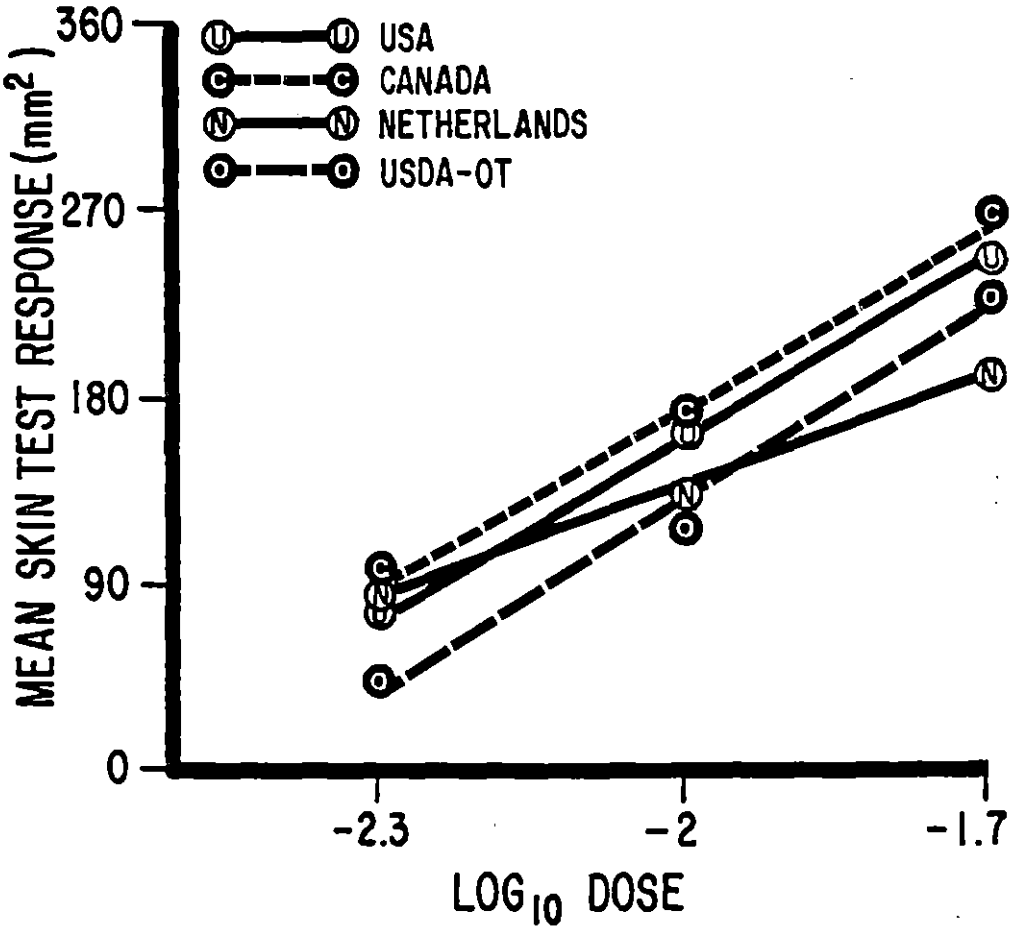


Figure 11. Dose-response lines for four tuberculins injected intradermally in the abdomen of guinea pigs sensitized with M. paratuberculosis. $Slope_{USA}$= 282.70, $slope_{Canada}$= 281.20, $slope_{Netherlands}$= 180.21, $slope_{USDA-PPD}$= 312.84

Table 56. Comparison of slopes of all possible pairs of regression lines ($log_{10}$ dose of tuberculin vs. skin test response) at 48 hours[a]

| Comparison | t[b] | |
|---|---|---|
| USA-Canada | 0.02 | |
| USA-Netherlands | 2.24 | *** |
| USA-USDA-OT | 0.63 | |
| Canada-Netherlands | 1.89 | |
| Canada-USDA-OT | 0.57 | |
| Netherlands-USDA-OT | 3.24 | *** |

[a]Alpha=0.05, comparisons that are significant are indicated by '***.'

[b]$t_{52df}$=2.007.

Table 57. Analysis of variance of skin test responses to four tuberculins in sensitized guinea pigs at 48 hours

| Source | df | SS | MS |
|---|---|---|---|
| Tuberculin | 2 | 79028.13 | 39514.06 |
| Animal(Tuberculin) | 27 | 638288.00 | 23640.30 |
| Dilution | 2 | 931360.30 | 465680.15 |
| $Dilution_{linear}$ | 1 | 928752.07 | 928752.07 |
| Lack of Fit | 1 | 2608.23 | 2608.23 |
| Tuberculin*Dilution | 4 | 4768.27 | 1192.07 |
| $T*D_{linear}$ | 2 | 2310.45 | 1155.22 |
| Residual | 2 | 2457.82 | 1228.91 |
| Dil.*Animal(Tuberculin) | 54 | 178148.10 | 3299.04 |
| $Dil.*An.(Tub.)_{linear}$ | 27 | 109752.72 | 4064.92 |
| Residual | 27 | 68395.38 | 2533.16 |
| Error | 90 | 96696.00 | 1074.40 |
| Total | 179 | | |

Table 58. Results of F tests for sources of variation from Table 57[a]

| | | F | |
|---|---|---|---|
| Tuberculin | :Animal(Tub.) | 1.67 | |
| Dilution | :Dilution*Animal(Tub.) | 141.16 | *** |
| Dil.$_{linear}$ | :Dilution$_{linear}$*Animal(Tub.) | 228.48 | *** |
| Lack of Fit | :Residual*Animal(Tub.) | 1.03 | |
| Tuberculin*Dil. | :Dilution*Animal(Tub.) | 0.36 | |
| T*D$_{linear}$ | :Dilution$_{linear}$*Animal(Tub.) | 0.28 | |

[a]Alpha=0.01. Significant comparisons are indicated by '***.'

Table 59. Skin test responses (mean values) in $mm^2$ to three dilutions of USA tuberculin in guinea pigs sensitized with M. paratuberculoisis at 48 hours[a]

| Dilution | | |
|---|---|---|
| 1:50 | 1:100 | 1:200 |
| 212 | 162 | 60 |
| 256 | 214 | 75 |
| 448 | 302 | 158 |
| 215 | 188 | 104 |
| 170 | 88 | 94 |
| 212 | 174 | 90 |
| 328 | 160 | 80 |
| 256 | 168 | 28 |
| 188 | 102 | 38 |
| 182 | 76 | 39 |

[a]Each observation is an average of observations by two readers.

Table 60. Skin test responses (mean values) in $mm^2$ to three dilutions of Canada tuberculin in guinea pigs sensitized with M. paratuberculosis at 48 hours[a]

| Dilution | | |
|---|---|---|
| 1:50 | 1:100 | 1:200 |
| 174 | 124 | 75 |
| 330 | 218 | 150 |
| 319 | 162 | 148 |
| 252 | 174 | 56 |
| 224 | 170 | 84 |
| 260 | 149 | 71 |
| 555 | 302 | 275 |
| 180 | 114 | 12 |
| 130 | 180 | 35 |
| 244 | 144 | 70 |

[a]Each observation is an average of observations by two readers.

Table 61. Skin test responses (mean values) in $mm^2$ to three dilutions of USDA-OT tuberculin in guinea pigs sensitized with M. paratuberculosis at 48 hours[a]

| Dilution | | |
|---|---|---|
| 1:50 | 1:100 | 1:200 |
| 154 | 126 | 48 |
| 284 | 146 | 38 |
| 186 | 108 | 69 |
| 236 | 94 | 43 |
| 220 | 102 | 30 |
| 155 | 52 | 28 |
| 305 | 248 | 52 |
| 380 | 178 | 57 |
| 168 | 63 | 39 |
| 201 | 56 | 0 |

[a]Each observation is an average of observations by two readers.

Table 62. Skin test responses (mean values) in $mm^2$ to three dilutions of four tuberculins in 10 sensitized guinea pigs at 48 hours

| | Tuberculin | | |
|---|---|---|---|
| Dilution | USA | Canada | USDA-OT |
| 1:50 | 247 | 267 | 229 |
| 1:100 | 164 | 174 | 117 |
| 1:200 | 77 | 98 | 40 |
| Mean | 163 | 180 | 129 |

calculated by fitting one line to all of the data from all three tuberculins. Slope= 292.25. Relative potencies were calculated:

| Tuberculin comparison | Relative potency | Confidence interval |
|---|---|---|
| USA vs. USDA-OT | 0.77 | (0.65,0.91) |
| Canada vs. USDA-OT | 0.67 | (0.56,0.79) |

Therefore, 1 unit of USDA-OT produced the same response as 0.77 units of USA and 0.67 units of Canada. Confidence intervals for those estimates were calculated at the 95% level of significance.

3. Evaluation of M. paratuberculosis PPD in naturally infected cattle

Three M. paratuberculosis tuberculins were injected intradermally into the neck of each of eight naturally infected cattle simultaneously (Table 63). A significant response of greater than 1 mm increase in skin thickness was observed in six of the eight cattle. The responses varied from diffuse swellings to well-difined lumps. The average skin test response for each cow was calculated as the sum of the increase in skin thickness for each tuberculin in a cow divided by 3. There were significant differences in responses between cows (P>F=0.0001). The average responses varied for 0.4 to 7.7 ($\pm$ 0.4) mm.

Table 63. Skin test responses to three tuberculins measured as increases in skin thickness (mm) in eight cattle naturally infected with *M. paratuberculosis*

| Cow # | Tuberculin | | |
|---|---|---|---|
| | USA | Canada | USDA-OT |
| 301 | 8.1 | 9.0 | 6.0 |
| 302 | 1.0 | 0.5 | 0.0 |
| 303 | 2.0 | 1.0 | 1.4 |
| 304 | 6.0 | 6.0 | 5.5 |
| 305 | 4.1 | 2.0 | 1.5 |
| 306 | 0.8 | 0.0 | 0.5 |
| 307 | 1.0 | 0.0 | 0.5 |
| 308 | 1.3 | 0.0 | 1.0 |

The skin test response (mean value) to each tuberculin was calculated as the sum of the increase in skin thickness for a tuberculin in each of eight cows divided by 8. There were significant differences in responses to the three tuberculins (P>F=0.00417) (Table 64).

The level of significance of the differences among tuberculins varied:

$t_{USA-Canada} = 2.84$

$t_{USA-USDA-OT} = 3.87$

$t_{Canada-USDA-OT} = 1.03$

For 14 degrees of freedom:

$t_{0.05} = 2.145$

$t_{0.01} = 2.977$

Therefore, the following statements can be made:

1. There were differences between USA and Canada at the 95% level.
2. There were differences between USA and USDA-OT at the 99% level.
3. There were no differences between Canada and USDA-OT.

4. Delayed-type hypersensitivity skin tests conducted in cattle from a herd in which M. paratuberculosis was diagnosed

The specificity of three M. bovis PPDs was evaluated in 23 cattle in a herd in which M. paratuberculosis infection

Table 64. Skin test responses (mean values) to three tuberculins measured as increases in skin thickness (mm) in eight cattle naturally infected with *M. paratuberculosis*

| | |
|---|---|
| USA | 3.9 |
| Canada | 3.0 |
| USDA-OT | 2.7 |

had been previously diagnosed. Four PPDs were injected into each animal (Table 65).

There were significant differences in the mean response to all four PPDs in each cow (P>F=0.0001). The responses ranged from 0.4 to 10.6 (± 1.1) mm increase.

There were significant differences in the mean skin test responses to the different PPDs (P>F=0.0001). The mean responses were:

| PPD | Mean response (mm) |
|---|---|
| USA | 5.7 (± 0.5) |
| PIS-PPD | 2.5 (± 0.5) |
| EEC-PPD | 1.6 (± 0.5) |
| USDA-PPD | 1.4 (± 0.5) |

The differences between the mean responses were evaluated with t tests. There were no significant differences between the responses to each of the M. bovis PPDs, however it should be noted that the sample size was small. The response to the M. paratuberculosis PPD was significantly different than responses to the M. bovis PPDs (Table 66).

Table 65. Skin test responses to three *M. bovis* PPDs and one *M. paratuberculosis* PPD measured as increases in skin thickness (mm) in 23 cattle from a herd in which *M. paratuberculosis* had been diagnosed

| Cow # | Tuberculin | | | |
|---|---|---|---|---|
| | USA | EEC-PPD | PIS-PPD | USDA-PPD |
| 1 | 10.5 | 0.5 | 3.5 | 4.5 |
| 2 | 0.7 | 0.8 | 0.0 | 0.0 |
| 3 | 0.0 | 0.9 | 3.5 | 0.0 |
| 4 | 11.7 | 8.5 | 5.8 | 8.5 |
| 5 | 3.8 | 1.0 | 2.5 | 0.5 |
| 6 | 6.8 | 1.3 | 0.2 | 0.0 |
| 7 | 15.7 | 3.3 | 6.5 | 0.0 |
| 8 | 5.0 | 0.0 | 0.0 | 0.3 |
| 9 | 11.5 | 1.5 | 7.0 | 0.0 |
| 10 | 10.0 | 1.2 | 6.0 | 1.5 |
| 11 | 8.5 | 3.0 | 4.1 | 2.5 |
| 12 | 3.5 | 0.0 | 0.0 | 0.5 |
| 13 | 0.5 | 0.0 | 1.0 | 0.5 |
| 14 | 17.6 | 8.7 | 8.2 | 8.0 |
| 15 | 0.5 | 1.5 | 1.5 | 1.1 |
| 16 | 0.3 | 0.0 | 1.3 | 0.0 |
| 17 | 0.6 | 1.2 | 2.7 | 0.0 |
| 18 | 5.0 | 0.0 | 2.5 | 0.0 |
| 19 | 7.5 | 0.0 | 0.0 | 0.5 |
| 20 | 3.5 | 0.5 | 0.6 | 0.4 |
| 21 | 5.2 | 0.5 | 0.5 | 0.0 |
| 22 | 3.0 | 1.3 | 0.0 | 0.0 |
| 23 | 0.0 | 0.1 | 0.6 | 2.5 |

Table 66. Calculated t values for comparisons between skin test responses (mean values) to three M. bovis PPDs and one M. paratuberculosis PPD in cattle from a herd in which M. paratuberculosis infection had been diagnosed[a]

| Comparison | t[b] | |
|---|---|---|
| USA-PIS-PPD | 4.89660 | *** |
| USA-EEC-PPD | 6.37018 | *** |
| USA-USDA-PPD | 6.67718 | *** |
| PIS-PPD-EEC-PPD | 1.47358 | |
| PIS-PPD-USDA-PPD | 1.78058 | |
| EEC-PPD-USDA-PPD | 0.30700 | |

[a]Alpha=0.05, comparisons that are significant are indicated by '***.'

[b]$t_{23}$ df=1.99656.

# V. DISCUSSION

Delayed-type hypersensitivity tests were conducted in cattle experimentally exposed to M. bovis and in cattle in a herd in which M. paratuberculosis infection had been diagnosed. This potency assay was done to compare responses to tuberculins; results should not be directly applied to interpretation of routine field test for tuberculosis. Tuberculins are used routinely worldwide to detect tuberculosis, with or without disease (46). The only other surveillance tool in cattle in the United States is postmortem examinations to find grossly visible lesions, then follow-up by histopathologic and mycobacteriologic examination.

Bovine tuberculosis occurs worldwide (22, 34, 44, 64, 83, 89) and tuberculins are produced in several different countries for use in their eradication or control programs (3, 27). In order to interpret results of tuberculin tests using different products it is necessary to compare each product to a reference standard.

The process of comparing tuberculins involves determining their potency and specificity (46). In this study, three M. bovis PPD tuberculins produced in different countries were evaluated (standardized) simultaneously. The potency assay was conducted in cattle experimentally exposed to M. bovis. The organism was recovered from all of the test cattle at the end of the assay.

The potencies of the three tuberculins were determined to be:

| | Relative potency | |
|---|---|---|
| Tuberculin | 8 weeks | 18 weeks |
| European Economic Community | 0.88 | 1.61 |
| Proposed international standard | 1.00 | 1.00 |
| USDA standard | ---- | 3.35 |

A relative potency (R) could not be calculated for the USDA standard from the data collected from the tuberculin test injected 8 weeks after experimental exposure. The statistical analysis used to calculate R assumes the dose-response lines ($\log_{10}$ dose of tuberculin vs. skin test response) for the tuberculins being compared are parallel; this was not true for the USDA data. A preliminary study would have been useful to assure that this requirement would be satisfied. Such a study would also help determine the appropriate dilutions to be used in the tuberculin testing trial.

Skin test responses were observed by two readers. Although some reader differences were observed, each reader ranked the tuberculins in the same way.

| | Skin test response (mm) | |
|---|---|---|
| | 8 weeks | 18 weeks |
| Reader 1 | 21.54 ($\pm$ 0.21) | 12.78 ($\pm$ 0.22) |
| Reader 2 | 22.76 ($\pm$ 0.21) | 13.71 ($\pm$ 0.22) |

Significant differences among cattle were seen in responses to tuberculin injection. This phenomenon, biologic variation, is the reason several replications of each treatment were necessary to estimate a mean population response. Two animals rarely have the same response to identical treatments. Another factor that might have influenced the different responses was the stage of disease. Although the same dose of organisms was injected into each calf, some were probably not as well sensitized as others at the time of testing. This lower level of sensitization was seen as a smaller response, but the response was still easily observed, so that infection in these animals was detected by tuberculin skin testing.

Skin test responses were significantly different (P>F=0.0001) at each of the six sites used for injection. These differences were seen in both the 8 and 18 week assays.

| | Skin test response (mm) | |
|---|---|---|
| Site | 8 weeks | 18 weeks |
| LA | 22.7 | 14.2 |
| LB | 23.2 | 12.0 |
| LC | 20.2 | 12.8 |
| RA | 24.3 | 12.4 |
| RB | 20.4 | 13.8 |
| RC | 22.2 | 14.3 |

The standard error was 0.4. The site codes for 8 weeks refer to different areas on the neck than at 18 weeks (see Figures 1 and 2, page 12).

Skin test responses were significantly smaller at 18 weeks post infection than at 8 weeks. This was true for individual calves as well as for tuberculins. It is recommended that animals be used in tuberculin trials only once (86, 89) for this reason. It is also recommended that a skin test injection site be used only once (47). Otherwise, a local alteration in response may be observed.

Mean responses to each of the three tuberculins were lower in the 18 week test than in the 8 week test; moreover, the biologic activity of the EEC-PPD decreased more than that of the PIS-PPD, as measured by relative potency.

| | Skin test response (mm) | |
|---|---|---|
| Tuberculin | 8 weeks | 18 weeks |
| EEC-PPD | 20.9 | 11.8 |
| PIS-PPD | 25.9 | 16.2 |
| USDA-PPD | 19.6 | 11.6 |

While these differences in skin test response were obvious in a tuberculin testing trial, it is important to remember that all three tuberculins successfully identified each of the 19 tuberculous cattle as infected.

A preliminary study was done to evaluate the usefulness of the three M. bovis PPDs as antigens for in vitro lymphocyte blastogenesis assays. Blastogenesis assays have been used to diagnose tuberculosis (1, 5, 28, 32, 85). In this study, none of the M. bovis antigens elicited responses in the infected animals that were different from those in the control. However, the number of replications was too few and the responses too variable for this sample to be considered representative.

The specificity assay was conducted in cattle from a herd in which M. paratuberculosis infection had been diagnosed. Specificity testing is necessary because tuberculins detect infections with heterologous species of mycobacteria (16, 17, 24, 47, 69). In order to establish sensitivity to M. paratuberculosis antigens, a M. paratuberculosis PPD tuberculin was simultaneously injected. The product was produced for this study and standardized in guinea pigs and cattle in comparison to M. paratuberculosis PPDs from two other countries and a Johnin OT. Results of the specificity study showed that the response to each of the three M. bovis PPDs in cattle exposed to M. paratuberculosis was significantly smaller ($P<0.05$) than that to the M. paratuberculosis PPD.

The results of the preliminary assays testing M. paratuberculosis PPDs in guinea pigs and cattle support the

recommendation that tuberculins be standardized in the biologic system in which they will be used in practice (WHO). In guinea pigs, the following skin test responses were seen:

Canada > USA > USDA

In cattle, a different pattern was seen:

USA > Canada > USDA.

# VI. SUMMARY

The biologic activities of three reference Purified Protein Derivative tuberculins (PPDs) were compared by delayed-type hypersensitivity tests in cattle experimentally exposed to Mycobacterium bovis at 8 and 18 weeks after exposure. The PPDs compared were: European Economic Community standard (EEC-PPD), proposed international standard (PIS-PPD) and USDA standard (USDA-PPD). Positive responses were observed following the injection of each of the three PPDs into each of the exposed animals; no positive responses were observed in controls.

The biologic activity of the three PPDs was compared by calculating relative potencies using a parallel lines bioassay. The relative potency of EEC-PPD vs. PIS-PPD was 0.88 at 8 weeks after experimental exposure. It was not possible to calculate a relative potency for USDA-PPD at 8 weeks after exposure because the dose-response line was not parallel to the dose-response lines of EEC-PPD and PIS-PPD. Relative potencies were calculated for all three PPDs at 18 weeks after exposure: EEC-PPD vs. PIS-PPD was 1.61, USDA-PPD vs. PIS-PPD was 3.35.

An in vitro lymphocyte blastogenesis assay was conducted with cells from three of the cattle experimentally exposed to M. bovis and one control calf. Important

differences in biologic activity of the three PPDs were not detected.

Gross and/or microscopic lesions were observed on necropsy of each of the cattle exposed to M. bovis; M. bovis was isolated from tissues from each of the calves. Lesions were not observed in tissues from the control calves, nor was M. bovis isolated.

A M. paratuberculosis PPD was produced by precipitation with ammonium sulfate and standardized in sensitized guinea pigs and cattle naturally infected with M. paratuberculosis. The M. paratuberculosis PPD and three M. bovis PPDs (EEC-PPD, PIS-PPD and USDA-PPD) were simultaneously injected into cattle from a herd in which M. paratuberculosis infection had been diagnosed. The response to the M. paratuberculosis PPD was larger than the response to each of the M. bovis PPDs.

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# VIII. APPENDIX

## BUTTERFIELD'S BUFFER

Stock solution

| | |
|---|---|
| Potassium phosphate monobasic | 34.0 g |
| Distilled water | 500.0 ml |
| 1 N Sodium hydroxide to obtain pH 7.2 | 180 ml (approx.) |

Distilled water QS 1000 ml

Working solution

Add 1.25 ml of above to 998.75 ml distilled water for each 1000 ml buffer needed.

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## DUBOS ALBUMIN BROTH WITH TWEEN 80 AND DOAC

| Ingredients | For one liter Medium |
|---|---|
| Dubos broth base with Tween 80 (Difco 0385) | 6.5 g |
| Distilled water (double glass distilled) | 900.0 ml |
| Dubos oleic albumin complex (Difco 0375) | 100.0 ml |

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## HERROLD'S EGG YOLK MEDIUM[1]

| Ingredients | For 1020 ml Medium |
|---|---|
| Peptone (Difco 0118) | 9.0 g |
| Sodium chloride | 4.5 g |
| Agar (Special Noble-Difco 0142) | 15.3 g |
| Beef extract (Difco 0126) | 2.7 g |
| Glycerin | 27.0 ml |
| Distilled water | 870.0 ml |
| Egg yolks | 6 |
| 2% Malachite green dye solution (aqueous) | 5.0 ml |
| 1 N Sodium hydroxide solution to obtain pH 7.5 | 4.1 ml (approx.) |

[1]Medium is also prepared without glycerin or malachite green.

## HERROLD'S EGG YOLK MEDIUM WITH MYCOBACTIN

Prepare Herrold's egg yolk medium. Add contents of one 4-dram wide mouthed vial (2 mg) mycobactin dissolved in 4 ml ethyl alcohol.

## HISTOPAQUE-1077

| Ingredients | For 100 ml Medium |
|---|---|
| Ficoll (Type 400) | 5.7 g |
| Sodium diatrizoate | 9.0 g |

## LOWENSTEIN-JENSEN MEDIUM[1]

| Ingredients | For 1612 ml Medium |
|---|---|
| Lowenstein Medium Base (Difco 0444) | 37.2 g |
| Glycerin | 12.0 ml |
| Distilled water | 600.0 ml |
| Whole eggs[2] - aseptically prepared | 1000.0 ml (24 large) |

[1]Medium is also made without glycerin when specified.
[2]Eggs should be strictly fresh and must be from hens that have had no antibiotics.

## M199(I)

| Ingredients | For 121 ml Medium |
|---|---|
| Medium 199 (Flow Laboratories) | 100.0 ml |
| Fetal calf serum | 15.0 ml |
| Penicillin | 2 X $10^4$ units |
| Streptomycin | 20.0 mg |
| Hepes buffer | 2.1 ml |
| (Indomethacin) | (0.5 mg) |

## MIDDLEBROOK 7H-10 AGAR WITH MIDDLEBROOK OADC ENRICHMENT

| Ingredients | For one liter Medium |
|---|---|
| Middlebrook 7H-10 agar base (Difco 0627) | 20.0 g |
| Distilled water with 5 ml glycerin | 900.0 ml |
| Middlebrook OADC enrichment | 100.0 ml |

## MODIFIED DORSET-HENLEY MEDIUM

| Ingredients | For 50 liters Medium |
|---|---|
| L-asparagine | 700.0 g |
| Dipotassium phosphate | 74.6 g |
| Sodium citrate | 37.1 g |
| Magnesium sulfate | 75.0 g |
| Ferric citrate | 15.0 g |
| Glucose | 500.0 g |
| Glycerol | 4.0 l |
| Distilled water | 46.0 l |
| Zinc sulfate | 4.0 g |
| Manganese chloride | 0.4 g |
| Cobalt chloride | 0.069 g |

Verify pH is betweeen 6.7 and 6.9 but do not adjust

## MODIFIED P&B MEDIUM WITH 5% HORSE SERUM

| Ingredients | For one liter Medium |
|---|---|
| L-asparagine | 5.0 g |
| Potassium phosphate monobasic | 5.0 g |
| Potassium sulfate | 5.0 g |
| Glycerin | 20.0 ml |
| Distilled water (double glass distilled) | 930.0 ml |
| Magnesium citrate | 1.5 g |
| Horse serum (sterile) | 50.0 ml |

## MODIFIED STONEBRINK MEDIUM

| Ingredients | For 1200 ml Medium |
|---|---|
| Salt mixture | |
| Sodium pyruvate | 5.0 g |
| Potassium phosphate monobasic | 2.0 g |
| Distilled water | 300.0 ml |
| Sodium phosphate dibasic to obtain pH 6.5 | 1.4 g |
| Dye mixture | (approx.) |
| Crystal violet | 100.0 mg |
| Malachite green (oxalate form) | 800.0 mg |
| Distilled water | 100.0 ml |
| Whole eggs[1] - aseptically prepared | 800.0 ml |
| | (20 large) |

[1]Eggs should be strictly fresh and must be from hens that have had no antibiotics.

Table 67. Skin test responses ($mm^2$) to M. paratuberculosis tuberculin in sensitized guinea pigs at 24 hours (Tuberculin = USA)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 682[a] | 442 | 221 |
| | 600 | 330 | 180 |
| 2 | 351 | 198 | 104 |
| | 275 | 160 | 88 |
| 3 | 308 | 216 | 126 |
| | 390 | 315 | 130 |
| 4 | 420 | 238 | 209 |
| | 364 | 266 | 208 |
| 5 | 420 | 400 | 176 |
| | 416 | 500 | 198 |
| 6 | 345 | 154 | 117 |
| | 425 | 176 | 108 |
| 7 | 364 | 221 | 117 |
| | 368 | 270 | 121 |
| 8 | 406 | 368 | 220 |
| | 425 | 345 | 221 |
| 9 | 375 | 228 | 96 |
| | 340 | 182 | 110 |
| 10 | 510 | 345 | 204 |
| | 486 | 425 | 238 |

[a]Value on top is from Reader 1; value on bottom is from Reader 2.

Table 68. Skin test responses ($mm^2$) to M. paratuberculosis tuberculin in sensitized guinea pigs at 24 hours (Tuberculin = Canada)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 375[a] | 204 | 135 |
| | 450 | 280 | 150 |
| 2 | 442 | 374 | 225 |
| | 540 | 336 | 225 |
| 3 | 416 | 264 | 165 |
| | 532 | 325 | 224 |
| 4 | 336 | 204 | 143 |
| | 345 | 288 | 182 |
| 5 | 330 | 176 | 99 |
| | 336 | 247 | 121 |
| 6 | 798 | 416 | 308 |
| | 665 | 450 | 285 |
| 7 | 513 | 299 | 130 |
| | 570 | 270 | 132 |
| 8 | 375 | 209 | 130 |
| | 340 | 280 | 154 |
| 9 | 360 | 216 | 135 |
| | 450 | 300 | 110 |
| 10 | 476 | 165 | 110 |
| | 504 | 300 | 90 |

[a]Value on top is from Reader 1; value on bottom is from Reader 2.

Table 69. Skin test responses ($mm^2$) to *M. paratuberculosis* tuberculin in sensitized guinea pigs at 24 hours (Tuberculin = Netherlands)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 416[a] | 264 | 126 |
| | 442 | 260 | 150 |
| 2 | 273 | 180 | 99 |
| | 352 | 234 | 99 |
| 3 | 384 | 176 | 170 |
| | 352 | 234 | 234 |
| 4 | 390 | 240 | 132 |
| | 425 | 260 | 165 |
| 5 | 240 | 200 | 99 |
| | 286 | 273 | 170 |
| 6 | 299 | 176 | 117 |
| | 352 | 252 | 140 |
| 7 | 231 | 150 | 96 |
| | 280 | 240 | 143 |
| 8 | 182 | 187 | 130 |
| | 182 | 195 | 180 |
| 9 | 253 | 180 | 117 |
| | 325 | 260 | 168 |

[a]Value on top is from Reader 1; value on bottom is from Reader 2.

Table 70. Skin test responses ($mm^2$) to M. paratuberculosis tuberculin in sensitized guinea pigs at 24 hours (Tuberculin = USDA-OT)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 315[a] | 112 | 96 |
| | 330 | 165 | 72 |
| 2 | 187 | 130 | 96 |
| | 208 | 180 | 117 |
| 3 | 416 | 187 | 99 |
| | 396 | 247 | 121 |
| 4 | 308 | 112 | 88 |
| | 345 | 220 | 96 |
| 5 | 192 | 104 | 63 |
| | 273 | 150 | 72 |
| 6 | 345 | 96 | 70 |
| | 238 | 130 | 63 |
| 7 | 416 | 160 | 135 |
| | 448 | 117 | 56 |
| 8 | 420 | 247 | 144 |
| | 522 | 294 | 150 |
| 9 | 286 | 117 | 80 |
| | 352 | 240 | 70 |
| 10 | 364 | 220 | 88 |
| | 315 | 247 | 90 |

[a]Value on top is from Reader 1; value on bottom is from Reader 2.

Table 71. Skin test responses ($mm^2$) to M. paratuberculosis tuberculin in sensitized guinea pigs at 48 hours (Tuberculin = USA)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 204[a] | 156 | 50 |
| | 221 | 169 | 70 |
| 2 | 240 | 187 | 60 |
| | 272 | 240 | 90 |
| 3 | 320 | 304 | 150 |
| | 575 | 300 | 165 |
| 4 | 192 | 195 | 77 |
| | 238 | 182 | 132 |
| 5 | 165 | 77 | 88 |
| | 176 | 100 | 99 |
| 6 | 216 | 182 | 70 |
| | 208 | 165 | 110 |
| 7 | 280 | 165 | 90 |
| | 375 | 154 | 70 |
| 8 | 247 | 126 | 21 |
| | 266 | 210 | 35 |
| 9 | 182 | 117 | 35 |
| | 195 | 88 | 42 |
| 10 | 195 | 63 | 36 |
| | 169 | 90 | 42 |

[a]Value on top is from Reader 2; value on bottom is from Reader 3.

Table 72. Skin test responses ($mm^2$) to *M. paratuberculosis* tuberculin in sensitized guinea pigs at 48 hours (Tuberculin = Canada)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 165[a] | 117 | 72 |
| | 182 | 130 | 77 |
| 2 | 300 | 195 | 168 |
| | 360 | 240 | 132 |
| 3 | 308 | 102 | 154 |
| | 330 | 221 | 143 |
| 4 | 266 | 165 | 48 |
| | 238 | 182 | 64 |
| 5 | 187 | 132 | 77 |
| | 260 | 208 | 90 |
| 6 | 216 | 130 | 72 |
| | 304 | 168 | 70 |
| 7 | 560 | 300 | 280 |
| | 551 | 304 | 270 |
| 8 | 165 | 117 | 24 |
| | 195 | 110 | 0 |
| 9 | 117 | 165 | 35 |
| | 143 | 195 | 35 |
| 10 | 204 | 120 | 40 |
| | 285 | 169 | 99 |

[a]Value on top is from Reader 2; value on bottom is from Reader 3.

Table 73. Skin test responses ($mm^2$) to *M. paratuberculosis* tuberculin in sensitized guinea pigs at 48 hours (Tuberculin = Netherlands)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 140[a] | 99 | 45 |
| | 195 | 108 | 42 |
| 2 | 308 | 160 | 42 |
| | 350 | 150 | 56 |
| 3 | 209 | 180 | 88 |
| | 195 | 160 | 90 |
| 4 | 280 | 195 | 110 |
| | 294 | 165 | 108 |
| 5 | 143 | 110 | 35 |
| | 108 | 96 | 40 |
| 6 | 180 | 126 | 99 |
| | 176 | 88 | 88 |
| 7 | 132 | 121 | 64 |
| | 132 | 72 | 56 |
| 8 | 168 | 143 | 143 |
| | 117 | 165 | 120 |
| 9 | 156 | 140 | 130 |
| | 130 | 126 | 104 |

[a]Value on top is from Reader 1; value on bottom is from Reader 3.

Table 74. Skin test responses ($mm^2$) to M. paratuberculosis tuberculin in sensitized guinea pigs at 48 hours (Tuberculin = USDA-OT)

| Guinea pig | Dilution 1:50 | 1:100 | 1:200 |
|---|---|---|---|
| 1 | 143[a] | 120 | 54 |
| | 165 | 132 | 42 |
| 2 | 252 | 160 | 35 |
| | 315 | 132 | 42 |
| 3 | 228 | 108 | 66 |
| | 143 | 108 | 72 |
| 4 | 221 | 88 | 32 |
| | 252 | 99 | 54 |
| 5 | 187 | 50 | 32 |
| | 252 | 154 | 28 |
| 6 | 160 | 63 | 32 |
| | 150 | 42 | 24 |
| 7 | 288 | 256 | 54 |
| | 322 | 240 | 50 |
| 8 | 360 | 176 | 60 |
| | 400 | 180 | 54 |
| 9 | 168 | 63 | 30 |
| | 168 | 63 | 48 |
| 10 | 220 | 48 | 0 |
| | 182 | 63 | 0 |

[a]Value on top is from Reader 2; value on bottom is from Reader 3.