IN VIVO AND IN VITRO EVALUATION OF CELL-MEDIATED IMMUNITY IN THE DOG

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
ALFRED MAURICE LEGENDRE
1974

THESI

ABSTRACT

IN VIVO AND *IN VITRO* EVALUATION OF CELL-MEDIATED IMMUNITY IN THE DOG

By

Alfred Maurice Legendre

Methods to evaluate naturally and artificially induced cell-mediated immunity in the dog were tested. Twenty dogs of mixed age, sex and breed were injected intradermally with Monilia, Dermatophyton O, Trichophyton, Aspergillus and Streptokinase-Streptodornase to determine if a delayed skin reaction occurred regularly to ubiquitous antigens. On the basis of visible reactions and histological examination of biopsied injection sites, it was concluded that only Streptokinase-Streptodornase had promise.

To test artificially induced cell mediated immunity, 10 of the dogs were injected subcutaneously with viable BCG in incomplete Freund's adjuvant, and 10 dogs were sensitized with DNCB in DMSO applied to the plantar surface of the foot. Three weeks or more after sensitization the following tests were made: intradermal tuberculin testing, skin challenge with DNCB and *in vitro* migration inhibition of blood leukocytes by tuberculin.

Undiluted tuberculin was unsuitable for intradermal testing in the dog because of false positive reactions. Tuberculin diluted 1:50 was 90% accurate as an intradermal test in differentiating between BCG

and DNCB sensitized dogs. Tuberculin diluted 1:50 produced a reaction characterized by a predominantly polymorphonuclear leukocyte infiltration in BCG sensitized dogs. Half of the BCG sensitized dogs also had a distinct mononuclear cell infiltration. The response of sensitized dogs to tuberculin appears to be different than in other species.

All DNCB and 30% of the BCG sensitized dogs had microscopic but not gross responses to DNCB challenge. DNCB in DMSO is not a suitable agent for skin sensitization in dogs.

The direct radial migration inhibition of peripheral dog leukocytes by 1:100 tuberculin was 80% accurate in differentiating between BCG and DNCB sensitized dogs. The indirect method, which measures the migration inhibition effect of the supernates of dog leukocyte cultures on normal guinea pig peritoneal cells, produced an excessive number of false positive reactions. The indirect method was inferior to the direct method in evaluating cell mediated immunity in the dog. Tuberculin 1:100 was more effective than more purified tuberculoproteins in the migration inhibition test. The average of a number of migration inhibition tests is more accurate than individual tests. The direct radial migration inhibition test of peripheral leukocytes appears to be a good *in vitro* method to study cell mediated immunity in the dog.

IN VIVO AND *IN VITRO* EVALUATION OF CELL-MEDIATED IMMUNITY IN THE DOG

By

Alfred Maurice Legendre

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Small Animal Surgery and Medicine

1974

Dedicated to my parents and my wife

ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. Leonard Gideon and Dr. Robert Michel for their suggestions and help, and to Dr. Robert Schirmer, my major professor, for his counsel, guidance and support in this study and during the past three years.

A special thanks to Dr. Virginia Mallmann for the many hours she spent on the planning and development of this project.

I also want to thank Mrs. Jane Walsh for performing the *in vitro* studies, Ms. Roberta Milar for preparation of the tissue sections, and Ms. Janice Fuller for the skillful editing and typing of the manuscript.

Lastly, to the students of the immunology research group, my sincere appreciation for the many hours devoted to the project. Without their help, the successful completion of this study would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Cellular immunity or delayed type hypersensitivity plays an essential role in the immune defense system. Acquired immunity to many viral, fungal and intracellular bacterial infections, such as tuberculosis and brucellosis, is largely cell-mediated immunity, sometimes augmented by humoral or antibody-mediated immunity. Immune surveillance is a function of cellular immunity which recognizes and destroys neoplastic cells as they develop as well as foreign, transplanted cells. The congenital lack of this system, as in DiGeorge's syndrome, generally results in death at an early age.

The basic cell of the immune system is the lymphocyte, which probably originates in the bone marrow or fetal liver. The lymphocyte may then develop under the influence of the bursa of Fabricius or bursa equivalent and becomes a B-lymphocyte capable of giving rise to antibody producing plasma cells or, under the influence of the thymus, develops into a T-lymphocyte. The T-lymphocyte is the cell responsible for cell mediated or delayed type hypersensitivity responses. The responses and interactions of the B-lymphocyte (antibody system) and the T-lymphocyte (cell-mediated system) provide immunological competence and protection.

The T-lymphocyte is a long-lived, small lymphocyte which circulates in the lymph, blood and other tissues, throughout most of the body.

The majority of the lymphocytes in peripheral blood are T-lymphocytes. After T-lymphocytes are sensitized by an antigen, they can react immunologically specifically with the same antigen. The T-lymphocyte-antigen reaction releases effector molecules or lymphokines from the lymphocyte. The lymphokines such as migration inhibition factor, macrophage activating factor, lymphocyte transformation factor and chemotactic factor appear to attract cells into the area of the reaction, immobilizes the cells and causes cellular proliferation. This sequence of events results in a grossly visible response which appears at 24 to 48 hours at the site of a positive skin test. The reaction is characterized by a mononuclear cell infiltrate.

There are 2 methods currently used to detect and evaluate cellular immunity *in vivo*. The first method utilizes a ubiquitous antigen which naturally induces sensitization in a large percentage of the population. This method assumes that previous contact with the antigen has occurred. *Candida* is a suitable antigen in man because it produces a delayed type hypersensitivity reaction in most people tested intradermally. The second method involves sensitization with a chemical hapten, such as dinitrochlorobenzene (DNCB), not found in nature. The individual is then skin tested with the hapten after sufficient time for sensitization to develop.

In vitro methods which became available in the last decade have expanded the understanding of cellular immunity. The *in vitro* methods generally measure the effects of the lymphokines produced by T-lymphocyte-antigen reaction. Currently the most accepted tests are lymphocyte transformation and inhibition of macrophage migration. The results of

these tests correlate well with clinical evaluation and *in vivo* methods.

The importance of cellular immunity and methods of evaluation is reasonably well established in man. Little work has been done in the dog except in the area of transplant immunology. This study applies the methods currently used in man to evaluate cellular immunity in the dog. The response to 5 ubiquitous agents was studied, as was the response to DNCB sensitization. Dogs were infected with BCG (Bacillus Calmette Guerin) and their responses evaluated by intradermal testing and migration inhibition of leukocytes *in vitro*. Hopefully a system for evaluation of cellular immunity can be developed for the dog.

LITERATURE REVIEW

The role of cellular immunity in immune protection has been extensively investigated in the last decade. In a review of cell-mediated immunity,[61] a panel of experts of the World Health Organization stated that cellular immunity plays an important role in defense against intracellular bacterial, viral, fungal and protozoal infection. David[24] stated that cellular immunity is important in homograft rejection and protection against cancer cells. As the function of cellular immunity has become apparent, a variety of *in vivo* and *in vitro* methods were developed to evaluate this part of the immune system.

Intradermal skin testing was among the first methods used and is still the mainstay of demonstrating delayed hypersensitivity *in vivo*. The tuberculin reaction is considered the classical example of delayed type hypersensitivity or cellular immune response. Tuberculin was the first antigen shown to elicit a delayed type hypersensitivity reaction. Koch,[39] in 1891, first used tuberculin as a therapeutic agent because of the reaction it produced when injected into tuberculous patients. Intradermal tuberculin testing is the most widely accepted method for demonstrating sensitivity to the tuberculosis organism.

The role of delayed hypersensitivity was very controversial, largely because sensitivity could not be transferred by serum. The cellular nature of the delayed hypersensitivity response was demonstrated by

Landsteiner and Chase[41] in 1941. After inducing delayed hypersensitivity to a picryl chloride compound, peritoneal exudate cells transferred from sensitized guinea pigs to normal guinea pigs conferred the ability to respond to picryl chloride in a delayed manner. Transfer of serum or heat killed cells did not transfer the delayed type reaction. Chase[15] demonstrated transfer of delayed hypersensitivity to tuberculin with leukocytes or transfer factor from leukocytes.

Though the tuberculin reaction is considered the classical example of delayed hypersensitivity, many other bacterial and fungal antigens produce delayed hypersensitivity reactions. Hemolytic streptococci and their soluble extracellular products, Streptokinase-Streptodornase (SK-SD), can produce delayed type hypersensitivity reactions. Lawrence[42] passively transferred cellular immunity in man with cells from sensitized individuals. He also found that 69% of the population had delayed type sensitivity to SK-SD without a history of prior streptococcal infection, which suggests previous exposure that induced a cellular immune response. The use of ubiquitous antigens to which a large percentage of the population react in a delayed manner offers a good method of *in vivo* evaluation of cellular immunity. If an individual failed to respond to a battery of ubiquitous antigens, his cellular immune mechanism would be suspect. A group of antigens has evolved empirically that are used in man to evaluate immune responsiveness. Candida, Tricophyton, Streptokinase-Streptodornase and mumps antigen are commonly used.[40,56,12]

Intradermal tuberculin testing is presently the most frequently used method for demonstrating delayed hypersensitivity in the dog. The

occurrence of tuberculosis in the dog and the use of intradermal testing is well documented. Berg,[5] in a study of BCG infected dogs, or dogs with naturally occurring tuberculosis, reported that the use of tuberculin was not reliable. Tuberculin produced nonspecific reactions in control dogs which were equal in severity to the delayed hypersensitivity reactions of some infected dogs. Nonspecific reactions reached maximum intensity at 24 hours, while delayed hypersensitivity reactions were not maximum until 48 to 72 hours. The use of tuberculin had only a 50% reliability. Heat concentrated synthetic medium tuberculin produced a distinctly positive reaction in all infected dogs without false positive reactions in controls. Positive skin reactions were noted as early as 9 days postinfection. Snider[54] reviewed the published cases of tuberculosis in the dog and cat from 1890 to 1969 and reported that intradermal skin tests with tuberculin were unreliable. Awad[2] reported experimental and Paroti[46] clinical success with the use of BCG in intradermal skin testing in the dog. A study by Snider *et al.*[55] of clinical tuberculosis in dogs and cats in Pennsylvania showed that only 50% of infected animals tested were positive to intradermal testing with tuberculin or *M. bovis* antigen.

Delayed hypersensitivity reactions as seen in tuberculin sensitivity produce a characteristic type of inflammatory response. Gell and Hinde[28] described the skin reactions of sensitized rabbits to tuberculin testing. Perivascular cuffing of skin vessels with mononuclear and polymorphonuclear leukocytes characterized the reaction at 4 hours postinfection. Mononuclear cells were more prominent in the dermis while in the subcutaneous area polymorphonuclear leukocytes

predominated. By 22 hours postinjection the numbers of polymorphonuclear cells had decreased and histiocytic hyperplasia with perivascular cuffing of mononuclear cells was the prominent feature. Focal accumulations of polymorphonuclear cells in the dermis were felt to be due to injection trauma and tissue necrosis. Turk and Oort[58] demonstrated the histological features of delayed type tuberculin hypersensitivity in guinea pigs passively sensitized by cell transfer. Polymorphonuclear leukocytes were prominent at 4 hours, but decreased in numbers by 12 hours postinjection. At 12 hours mononuclear cells predominated in the dermis, but polymorphonuclear leukocytes persisted in the subcutaneous area. Up to 12 hours postinjection there was no significant difference between the inflammatory response of sensitized and normal guinea pigs. After 12 hours the sensitized animals showed greater cellular infiltration. Cell mediated reactions in the rabbit and guinea pig are predominantly a mononuclear reaction, but polymorphonuclear leukocytes are also found in significant numbers.

Skin sensitization with a chemical hapten such as DNCB is another *in vivo* method for evaluation of cell-mediated immunity. Skin test with hapten after adequate time for immune response produces a delayed hypersensitivity reaction. This method has been used in man in the clinical evaluation of an individual's cellular immunity.[31] DNCB sensitization evaluates the ability of the immune system at that time to respond to a new antigen not found in nature. The mechanism of DNCB sensitization was clarified by experiments on guinea pigs by Eisen.[25] The ability of the hapten to form covalent bonds with skin protein was necessary to the production of a delayed hypersensitivity

response. Eisen and Tabachnick[26] reported that DNCB combined with protein in the epidermis and not the corium. Only DNCB conjugates in the deeper parts of the epidermis produced delayed type hypersensitivity. DNCB conjugates in the cornified layers of the epidermis failed to elicit a response. A quantitative method of DNCB sensitization was developed by Catalona *et al.*[13] The skin is sensitized with 2000 µgm in .1 ml acetone applied to a 3 cm^2 area, and a challenge dose of 50 µgm is also applied at the time. After the 7 to 21 days needed for immune response, a spontaneous flare occurs at the test site where sensitized T-lymphocytes reacted with the DNCB bound in the epidermis. If no spontaneous flare occurs by 14 days, then a rechallenge dose of 50 µg DNCB is applied and the area is observed for 24 to 48 hours. Equivocal reactions are biopsied and evaluated by histopathologic examination. The reaction is graded as follows:

+4 - spontaneous flare at sensitization and challenge site

+3 - spontaneous flare at sensitization site

+2 - response at rechallenge site only

+1 - equivocal reaction--positive reaction noted on histopathologic examination

0 - no reaction on histopathologic examination

The microscopic reaction is characterized by marked lymphocytic and histiocytic infiltration at the dermal-epidermal junction and mononuclear perivascular cuffing in the dermis.

Catalona *et al.*[14] found that 96.5% of the individuals sensitized with DNCB responded to primary challenge by spontaneous flare. Joseph *et al.*[38] sensitized dogs with DNCB by applying 2000 µgm in .1 ml acetone to a 3 cm^2 area on the inner thigh area and challenged at 14 days

postsensitization with 50 and 100 μgm DNCB. All sensitized dogs reacted when challenged with 100 μgm DNCB. Others report greater difficulty in sensitizing dogs with DNCB.[3]

In the last decade, more *in vitro* methods of evaluating cell mediated immunity have been developed and are becoming available to the clinician. These methods measure the effects of the lymphokines produced when sensitized T-lymphocytes interact with specific antigen. The advantage of the *in vitro* system lies in the evaluation of each lymphokine individually. In contrast, the *in vivo* system evaluates the sum effect of all lymphokines produced. The *in vitro* system also eliminates many of the *in vivo* variables. *In vitro* methods have contributed to the understanding of the mechanism involved in cellular immune response.

A recent review by Bloom *et al.*,[9] in 1973, categorized the *in vitro* methods into lymphocyte-mediated cytotoxicity, lymphocyte transformation, and migration inhibition. Lymphocyte mediated cytotoxicity measures the cytotoxic effect of the cellular immune response. It is the *in vitro* correlate of immune surveillance and neoplastic cell destruction. Lymphocyte transformation methods evaluate the mitotic activity of a lymphocyte culture after stimulation. Sensitized T-lymphocytes undergo blastogenesis when they encounter specific antigen. This method quantitates the magnitude of the lymphocyte response. Migration inhibition methods measure the presence of migration inhibition factor (MIF). Migration inhibition factor is a soluble, nondialyzable substance released when sensitized T-lymphocytes encounter specific antigen. This factor inhibits the migration of normal leukocytes.

postsensitization with 50 and 100 μgm DNCB. All sensitized dogs reacted when challenged with 100 μgm DNCB. Others report greater difficulty in sensitizing dogs with DNCB.[3]

In the last decade, more *in vitro* methods of evaluating cell mediated immunity have been developed and are becoming available to the clinician. These methods measure the effects of the lymphokines produced when sensitized T-lymphocytes interact with specific antigen. The advantage of the *in vitro* system lies in the evaluation of each lymphokine individually. In contrast, the *in vivo* system evaluates the sum effect of all lymphokines produced. The *in vitro* system also eliminates many of the *in vivo* variables. *In vitro* methods have contributed to the understanding of the mechanism involved in cellular immune response.

A recent review by Bloom *et al.*,[9] in 1973, categorized the *in vitro* methods into lymphocyte-mediated cytotoxicity, lymphocyte transformation, and migration inhibition. Lymphocyte mediated cytotoxicity measures the cytotoxic effect of the cellular immune response. It is the *in vitro* correlate of immune surveillance and neoplastic cell destruction. Lymphocyte transformation methods evaluate the mitotic activity of a lymphocyte culture after stimulation. Sensitized T-lymphocytes undergo blastogenesis when they encounter specific antigen. This method quantitates the magnitude of the lymphocyte response. Migration inhibition methods measure the presence of migration inhibition factor (MIF). Migration inhibition factor is a soluble, nondialyzable substance released when sensitized T-lymphocytes encounter specific antigen. This factor inhibits the migration of normal leukocytes.

Migration inhibition and lymphocyte transformation are the two techniques most frequently used in evaluation of cellular immunity. The two *in vitro* methods should be correlated with each other and with *in vivo* responses when cellular immunity is evaluated. The methodology of performing the *in vitro* techniques is described by Bloom and Glade.[8]

The migration inhibition method of evaluating cellular immune response has contributed greatly to the understanding of cellular immunology. Rich and Lewis,[47] in 1928, were the first to report a decrease in migration of cells from tuberculous animals when the tissue culture medium contained tuberculin. An increase in cytotoxic effects of tuberculin on the cells of tuberculous animals was also observed. The use of cells in tissue culture was prompted by the inability to demonstrate an antibody involved in the tuberculin response. The observation of cytotoxicity has been confirmed in many reports, but the development of an *in vitro*, quantitative method of measuring migration inhibition was not developed until 1957. Hall and Scherago,[32] using small pieces of a buffy coat preparation of human peripheral leukocytes, showed inhibition of the leukocytes of tuberculous individuals with tuberculin. Later, Hall and Scherago[33] demonstrated in guinea pigs experimentally infected with *M. tuberculosis* that the leukocytes from infected animals were markedly inhibited in their migration by tuberculin. The migration inhibition of leukocytes developed prior to the development of delayed type hypersensitivity to intradermal injection of tuberculin. Treatment of the infected guinea pigs with streptomycin and isoniazid produced an increase in the migration of leukocytes as the disease became inactive, though the skin test remained

positive.[34] A similar response was noted with human leukocytes when the disease was in remission.[30] Johnson and Scherago[37] demonstrated a direct correlation between migration of leukocytes exposed to histoplasmin and skin sensitivity to histoplasmin in guinea pigs experimentally infected with *Histoplasma capsulatum*. This experiment demonstrated the use of migration inhibition to evaluate cellular immunity in a disease other than tuberculosis.

George and Vaughn,[29] in 1962, developed the capillary tube method of measuring migration inhibition. Leukocytes from sensitized animals were packed in capillary tubes and incubated in culture medium with and without antigen. The leukocytes migrate out of the capillary tubes onto a coverslip in a fan-shaped pattern. Migration is measured by projection of the culture chamber on drawing paper and calculation of the area of migration with a planimeter. Percent inhibition is calculated by this formula:

$$\frac{\text{area of migration with antigen}}{\text{area of migration without antigen}} \times 100 = \%\ \text{migration}$$

$$100 - \%\ \text{migration} = \%\ \text{migration inhibition}$$

The capillary tube method has gained wide acceptance in evaluation of migration inhibition. Moore and Scherago[45] demonstrated migration inhibition of leukocytes with histoplasmin from dogs experimentally infected with *Histoplasma capsulatum*. David *et al.*,[17] using guinea pigs sensitive to tuberculin, ovalbumin and diphtheria toxoid, demonstrated that migration inhibition was antigen specific. Only the cells from animals showing delayed hypersensitivity reactions to skin testing showed migration inhibition. Antigens eliciting only antibody production were incapable of producing migration inhibition. Cells from

unsensitized animals could not be sensitized by incubation with sera from sensitized animals. David *et al.*[18] showed that as few as 2.5% sensitized cells in a population of normal cells would produce migration inhibition when the culture was incubated with specific antigen. Sensitized cells killed by freezing failed to inhibit migration. David *et al.*[19] demonstrated carrier specificity with migration inhibition in animals sensitized with hapten protein conjugates. This carrier specificity differs from antibody-antigen reactions in that antibodies react with the hapten regardless of the protein carrier. David *et al.*[20] demonstrated the loss of ability to produce migration inhibition in sensitized cells incubated with trypsin. The cells recovered the ability to inhibit migration suggesting the resynthesization of the material removed by trypsin.

David[21] showed that puromycin could prevent the migration inhibition of sensitized cells. Puromycin inhibits protein synthesis; therefore, it was felt that production of migration inhibition factor requires active protein synthesis.

Bloom and Bennett[6] and David[22] independently demonstrated in guinea pigs that the lymphocyte was the cell responsible for reaction to the antigen and that the macrophage was only an indicator cell. The inhibition of macrophage migration was due to a soluble substance(s) elaborated from sensitized lymphocytes exposed to specific antigen. This substance was appropriately called migration inhibition factor (MIF). Søborg[51] demonstrated inhibition of migration of human leukocytes with Brucella antigen in subjects reacting to Brucella skin test or experimentally sensitized with killed organisms. He found the

migration inhibition method was the best *in vitro* correlate of cellular hypersensitivity. Thor[57] transferred the ability to produce MIF to nonsensitized lymphocytes with an RNA extract of sensitized lymphocytes. Bloom and Bennett[7] detected MIF in lymphocyte cultures as early as 6 hours after addition of specific antigen. Lymphocyte transformation was not necessary for MIF production, but supernates rich in MIF increase blast transformation in normal lymphocyte cultures. MIF enriched supernates injected intradermally in guinea pigs produced inflammatory reactions with a predominantly mononuclear infiltration. The specificity of the migration inhibition test was confirmed by David and Schlossman[23] using various DNP-oligolysines. This study suggests a highly specific binding site acting as a cellular receptor. Danish workers detected inhibition and enhanced migration of human leukocytes from patients with Sjogren's syndrome,[52] glomerulonephritis,[4] and Hashimoto's thyroiditis[53] when their cells were incubated with respective tissue antigens. Anderson *et al.*[1] reported inhibition of migration of human cells from patients with mammary tumors when the cells were incubated with mammary tumor extracts. Extracts of normal mammary tissue failed to produce inhibition. Hardt *et al.*[35] demonstrated migration inhibition of leukocytes from patients with biliary cirrhosis and chronic active hepatitis with liver mitochondrial antigens. Borstaff *et al.*[11] confirmed Hardt's findings. Using tolerant animals, Borel and David[10] demonstrated that unresponsiveness to skin testing correlated well with a lack of migration inhibition response. Rocklin *et al.*,[49] using an indirect migration inhibition system, demonstrated production of MIF in cell cultures of individuals reacting intradermally to PPD,

SK-SD and Candida antigens. They incubated human leukocytes with the antigens for 3 days recovering a cell-free supernate. Assay for the presence of MIF in the supernate was done using normal guinea pig peritoneal exudate cells and evaluating the degree of inhibition. No MIF activity was demonstrated in mixed leukocyte cultures incubated for 3 days, unless the patient had received a previous transfusion. Rocklin *et al.*[50] found that patients with thymic aplasia were unable to manifest delayed hypersensitivity reactions *in vivo* or migration inhibition *in vitro*.

Mallmann *et al.*,[44] using a tuberculoprotein found in *M. bovis*, were able to differentiate guinea pigs infected with *M. bovis* from those infected with *M. tuberculosis*. The migration inhibition test used was a radial migration of a spot of cells attached to a glass surface.

Winkelstein[59] found that, if 2.5% of a leukocyte population were from the lymph node of a sensitized animal, migration inhibition occurred in the presence of specific antigen. No inhibition of migration could be produced with up to a 20% population of thymic or bone marrow cells from a sensitized animal.

Leu *et al.*[43] demonstrated that MIF inhibited the migration of peritoneal macrophages but not alveolar macrophages. Killed or live peritoneal macrophages adsorbed MIF, but alveolar macrophages did not. Incubation of an MIF-rich supernate with peritoneal macrophages removed 1/2 the MIF activity in 15 minutes. MIF adsorption shows a direct dose response at low concentrations with saturation characteristics. Adsorption is time and temperature dependent and can be abolished by

pretreatment of macrophages with proteolytic enzymes. These characteristics suggest a receptor site on the macrophage.

Remold[48] reported that MIF is heterogeneous and different antigens produce MIF of slightly different molecular weights. Human MIF has a lower molecular weight than guinea pig MIF. Inactivation of MIF by chymotrypsin suggests that it is a protein. MIF was shown to be different from lymphotoxin and chemotactic factor.

Churchill *et al.*[16] demonstrated migration inhibition with specific tumor antigens in guinea pigs with experimentally produced hepatomas. Radiation, trypsinization and freeze storage did not inhibit the ability of the tumor cells to inhibit migration. Only specific tumors produced migration inhibition, and inhibition was not noted with unrelated tumors and normal spleen cells. Hilborg[36] demonstrated tumor immunity by migration inhibition in patients with the tumor and in contact individuals. Histoincompatible tissue homogenates did not produce MIF. Waxman and Lockskin[60] reported migration inhibition to tuberculin in leukocytes of patients with miliary tuberculosis and anergy to intradermal tuberculin testing. This would suggest normal production of immune mediators, but deficiency of nonimmunological inflammatory response.

Fauser *et al.*[27] used the radial migration technique to detect production of MIF by lymphocytes of chickens. Migration inhibition occurred when chickens were experimentally infected with BCG and Marek's disease virus and the leukocytes incubated with specific antigen.

Migration inhibition has been shown to be a good *in vitro* correlate for delayed hypersensitivity reactions. It has been used in evaluating

pretreatment of macrophages with proteolytic enzymes. These characteristics suggest a receptor site on the macrophage.

Remold[48] reported that MIF is heterogeneous and different antigens produce MIF of slightly different molecular weights. Human MIF has a lower molecular weight than guinea pig MIF. Inactivation of MIF by chymotrypsin suggests that it is a protein. MIF was shown to be different from lymphotoxin and chemotactic factor.

Churchill *et al.*[16] demonstrated migration inhibition with specific tumor antigens in guinea pigs with experimentally produced hepatomas. Radiation, trypsinization and freeze storage did not inhibit the ability of the tumor cells to inhibit migration. Only specific tumors produced migration inhibition, and inhibition was not noted with unrelated tumors and normal spleen cells. Hilborg[36] demonstrated tumor immunity by migration inhibition in patients with the tumor and in contact individuals. Histoincompatible tissue homogenates did not produce MIF. Waxman and Lockskin[60] reported migration inhibition to tuberculin in leukocytes of patients with miliary tuberculosis and anergy to intradermal tuberculin testing. This would suggest normal production of immune mediators, but deficiency of nonimmunological inflammatory response.

Fauser *et al.*[27] used the radial migration technique to detect production of MIF by lymphocytes of chickens. Migration inhibition occurred when chickens were experimentally infected with BCG and Marek's disease virus and the leukocytes incubated with specific antigen.

Migration inhibition has been shown to be a good *in vitro* correlate for delayed hypersensitivity reactions. It has been used in evaluating

pretreatment of macrophages with proteolytic enzymes. These characteristics suggest a receptor site on the macrophage.

Remold[48] reported that MIF is heterogeneous and different antigens produce MIF of slightly different molecular weights. Human MIF has a lower molecular weight than guinea pig MIF. Inactivation of MIF by chymotrypsin suggests that it is a protein. MIF was shown to be different from lymphotoxin and chemotactic factor.

Churchill *et al.*[16] demonstrated migration inhibition with specific tumor antigens in guinea pigs with experimentally produced hepatomas. Radiation, trypsinization and freeze storage did not inhibit the ability of the tumor cells to inhibit migration. Only specific tumors produced migration inhibition, and inhibition was not noted with unrelated tumors and normal spleen cells. Hilborg[36] demonstrated tumor immunity by migration inhibition in patients with the tumor and in contact individuals. Histoincompatible tissue homogenates did not produce MIF. Waxman and Lockskin[60] reported migration inhibition to tuberculin in leukocytes of patients with miliary tuberculosis and anergy to intradermal tuberculin testing. This would suggest normal production of immune mediators, but deficiency of nonimmunological inflammatory response.

Fauser *et al.*[27] used the radial migration technique to detect production of MIF by lymphocytes of chickens. Migration inhibition occurred when chickens were experimentally infected with BCG and Marek's disease virus and the leukocytes incubated with specific antigen.

Migration inhibition has been shown to be a good *in vitro* correlate for delayed hypersensitivity reactions. It has been used in evaluating

tumor immunity, autoimmune conditions as well as identification of cellular hypersensitivity states to a variety of bacterial and viral agents.

MATERIALS AND METHODS

Animals

Twenty dogs of both sexes, various breeds, between 5 months and 4 years of age were used in this study. The dogs were housed in concrete cages and exercised daily in accordance with Public Law 89-544 (The Animal Welfare Act). Cages were cleaned twice daily and dogs fed[a] once daily. Fresh water was provided *ad libitum*. All animals were vaccinated against canine distemper and infectious canine hepatitis at least 3 months prior to the start of the study.

Experimental Groups

Two experimental groups of 10 dogs each were formed. All dogs were skin tested and biopsied prior to sensitization with BCG or DNCB. The first group was tested with undiluted tuberculin intradermally prior to sensitization, while the second group was tested with a 1:50 dilution of tuberculin. Five dogs of each group received BCG, while the other 5 dogs were sensitized with DNCB. Variations in the *in vitro* testing existed between the 2 groups.

---

[a]Ken-L-Meal, Quaker Oats Company, Chicago, Illinois.

Intradermal Testing

The hair of both flanks was clipped closely with a #40 Oster blade immediately prior to injection. No cleansing of the skin was done. The skin was marked with vertical lines to identify the injection sites. Intradermal injection of .1 ml of each concentration of antigen was made using a tuberculin syringe with a 25-gauge needle. The accuracy of the intradermal injection was evaluated by the presence of a small skin bleb. Injections were made in duplicate at the ends of the lines on the right and left side. The following antigens were injected:

Antigen	Concentration
Streptokinase-Streptodornase[b] (SK-SD)	50 u/.1 ml
Monilia Mix[c]	1:10 and 1:100
Dermatophyton O[c] (*Candida albicans*)	1:10 and 1:100
Aspergillus Mix[c]	1:10 and 1:100
Trichophyton Mix[c]	1:10 and 1:100
Diluent[c] (control)	

The injection sites were evaluated at 1, 4, 24 and 48 hours. The reactions were evaluated visually and by digital palpation for signs of skin thickening, induration and erythema. The following scale was used in evaluating the reactions:

0 - No evidence of inflammatory changes

+1 - Slight thickening noted only on palpation

+2 - Skin thickening with an area of induration less than 1.0 cm in diameter

+3 - Skin thickening with induration greater than 1.0 cm in diameter usually accompanied by erythema and exudation.

---

[b]Varizyme, American Cyanamid Co., Princeton, New Jersey.

[c]Hollister Stier, Downer's Grove, Illinois.

In addition to visual and digital evaluation, the injection sites of the second group were evaluated by measuring skin fold thickness with calipers prior to and 24 hours after intradermal injection.

At 24 hours postinjection the sites on the right side were biopsied using an 8 mm Keyes cutaneous punch[d] after the animal had been anesthetized with intravenous sodium thiamylal.[e] The biopsies were fixed in 10% buffered formalin and tissue sections were made and stained with hematoxylin-eosin. The histological sections were examined for the degree of inflammatory changes and cellular infiltration. The reactions were graded according to the following criteria:

Grade 0 - No increase in numbers of inflammatory cells in dermis or subcutaneous area.

Grade I - Very slight increase in numbers of inflammatory cells. Cellular infiltrate is diffuse with no aggregates of inflammatory cells.

Grade II - Slight infiltration of dermis and/or subcutaneous area with inflammatory cells. Occasional small focal accumulations of inflammatory cells (less than 20 per high power field).

Grade III - Slight to moderate infiltration of dermis and subcutaneous area with inflammatory cells. Occasional focal accumulation of moderate numbers of inflammatory cells (less than 50 cells per high power field).

---

[d]American Hospital Supply, Detroit, Michigan.

[e]Surital Sodium, Parke, Davis & Company, Detroit, Michigan.

Grade IV - Moderate infiltration of the dermis and subcutaneous area with inflammatory cells. Moderate numbers of foci containing large numbers of inflammatory cells (100 to 200 per high power field).

Grade V - Moderate to severe infiltration of large areas of the dermis and subcutaneous area with inflammatory cells. The involvement is diffuse with many areas having hundreds of cells per high power field. Edema and hemorrhage may be evident.

Grade VI - Severe infiltration of the dermis and subcutaneous area with inflammatory cells. There is replacement of many of the normal skin structures with inflammatory elements. Edema, hemorrhage, and necrosis are prominent features.

DTH - Indicates that the reaction shows a mononuclear infiltration especially in the perivascular and adnexal areas suggesting a delayed hypersensitivity response.

In grading the reactions, injection trauma was not considered.

Bacillus of Calmette-Guerin[f] (BCG) Sensitization

Ten of the dogs were randomly selected for sensitization with BCG. Hair was clipped from the plantar surface of the right hind foot just above the pad and the area was cleansed with alcohol. Five milligrams, wet weight, of viable BCG in .5 ml of saline and .5 ml of Freund's incomplete adjuvant[g] were injected subcutaneously. The injection site was observed every other day for 21 days. At 21 days postinjection the

---

[f]Viable cultures prepared by Dr. Virginia Mallmann, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan.

[g]Difco Company, Detroit, Michigan.

right and left popliteal lymph nodes in the first group of dogs (5 BCG sensitized) were surgically removed, measured, and fixed in 10% buffered formalin. Tissue sections were made and stained with H & E and Ziehl-Neelsen acid-fast stain. The nodes were examined for histological changes and presence of acid-fast organisms.

Dinitrochlorobenzene (DNCB)[h] Sensitization

The remaining 10 dogs were sensitized with DNCB by skin application of the chemical by the method of Catalona.[19] A modification was made using DNCB in DMSO[i] in lieu of DNCB in acetone for sensitization. The plantar surface of the right hind leg was clipped and cleansed with acetone. A sensitization dose of 2000 µgm DNCB in .1 ml DMSO was applied to a 3 cm^2 area just above the pad. A challenge dose of 100 µgm DNCB in .1 ml of acetone was applied to a 3 cm^2 area approximately 4 cm proximal to the sensitization dose. The sites were observed every other day for spontaneous flare. At 21 days postsensitization, the animals were rechallenged with 100 µgm DNCB in .1 ml acetone. The site was evaluated as previously described and biopsied at 24 hours. Histological evaluation was made using the criteria previously described. The popliteal lymph nodes were surgically removed, measured, and fixed in 10% buffered formalin. The tissue sections were stained with H & E and Ziehl-Neelsen stains and evaluated for histological changes.

---

[h] 2-4 Dinitrochlorobenzene, K and K Laboratories, Inc., Plainview, New York.

[i] Dimethylsulfoxide, J. T. Baker Chemical Company, Phillipsburg, New Jersey.

In vivo and *In vitro* Evaluation of BCG Sensitized Dogs

Prior to sensitization blood was drawn on all dogs for complete blood counts and for *in vitro* migration inhibition by tuberculin. One-tenth milliliter of mammalian tuberculin[j] (1:50 dilution) was injected intradermally in each flank. The first group received undiluted tuberculin which produced a nonspecific inflammatory response; thereafter, 1:50 dilution of tuberculin was substituted. The areas were observed at 1, 4, 24 and 48 hours and were evaluated by the criteria previously described. The injection site on the right side was biopsied at 24 hours. Tissue sections were made and evaluated microscopically by the criteria previously described. Twenty-one days postsensitization, blood was drawn on all dogs for *in vitro* evaluation of migration inhibition with tuberculin. Blood was also drawn on the second group for a complete blood count. The same tuberculin testing procedure with biopsy was repeated on all dogs at this time using 1:50 dilution of mammalian tuberculin. The second group (10 dogs) were also given intradermal injections of .1 ml of the PPD of *M. bovis*[k] (3.21 mg protein per ml) and *M. avium*[l] (1.42 mg protein per ml) and the reactions were evaluated and biopsied at 24 hours as previously described.

---

[j]Mammalian Tuberculin, United States Department of Agriculture, Lansing, Michigan.

[k]PPD-9 - *M. bovis* Strain AN5, U.S.D.A., A.P.H.I.S. - U.S.D.L., Ames, Iowa.

[l]PPD-10 - *M. avium* Strain D_4, U.S.D.A., A.P.H.I.S. - U.S.D.L., Ames, Iowa.

In vitro Test of Migration Inhibition of Dog Leukocytes with Tuberculin by the Radial Migration Method

Nine milliliters of blood were collected from the jugular vein into a syringe containing 1 ml of 0.1 M citrated saline. The blood was centrifuged in a capped tube for 20 minutes at 860 x g. The plasma was removed with a pipette and discarded. The buffy coat was drawn by capillary action into micro blood tubes.[m] The tubes were sealed with warm, sterile paraffin. The filled micro blood tubes were placed in a test tube and centrifuged for 15 minutes at 700 x g. After centrifugation, the micro blood tubes were scored with a diamond pen at or slightly below the RBC-WBC interface and broken carefully. The leukocytes were aspirated into a syringe with a 25-gauge needle containing 0.3 ml of culture medium.[n] The cells were mixed gently with medium and the cell concentration adjusted using a hemacytometer to 10^7 leukocytes per ml. Four separate drops of a cell suspension were placed in each plastic tissue culture dish[o] and allowed to attach for 5 minutes. The spots of cells were flushed gently with Hank's balanced salt solution[p] to remove any red blood cells and nonadherent leukocytes. Two milliliters of culture medium was added to the petri dishes.

---

[m]Micro blood collecting tubes, Scientific Products, Evanston, Illinois.

[n]Medium 199, Microbiological Associates, Inc., Bethesda, Maryland. 1% L-Glutamine Solution, Grand Island Biological, Grand Island, New York; 1% Amino Acid Solution, Grand Island Biological, Grand Island, New York; 1% Vitamin Mix, Grand Island Biological, Grand Island, New York; Sodium Bicarbonate to buffer to 7.0, Microbiological Associates, Bethesda, Maryland.

[o]Tissue Culture Dish, Falcon Plastics, Los Angeles, California.

[p]Hanks' Balanced Salt Solution, Grand Island Biological, Grand Island, New York.

The medium for the control cultures contained no antigen. The medium for test cultures contained 1 test antigen. In the direct test, the diameter of each spot was measured prior to incubation and after 24 hours of incubation at 37 C. The spots were measured with an eyepiece micrometer in a light microscope at 100X magnification. The following formulas were used to calculate migration inhibition:

spot diameter postincubation - spot diameter preincubation

= units of migration

$$\frac{\text{total units of migration of 4 spots in antigen medium}}{\text{total units of migration of 4 spots in control medium}} \times 100 = \% \text{ migration}$$

100 - % migration = migration inhibition

In the indirect migration inhibition test, supernate fluids from direct test plates after 24-hour incubation were added to spots of guinea pig peritoneal exudate cells collected as described. Peritoneal cells were collected by injecting 3 ml of sterile mineral oil intraperitoneally into normal nonsensitized guinea pigs. Three days after mineral oil stimulation 50 cc of Hank's buffered salt solution was injected intraperitoneally into live, nonanesthetized animals. The abdomen was massaged and as much fluid as possible aspirated with a syringe. The peritoneal fluid was centrifuged in a conical tube at 700 x g for 10 minutes. The cell pellet was resuspended and rinsed 3 times with BSS to remove the remaining mineral oil. The rinsed cells were mixed with medium and the concentration adjusted to 10^7 cells per ml.

The spots were measured, the cultures incubated for 24 hours, and the spot remeasured. Percent migration inhibition is calculated as previously described.

In addition to mammalian tuberculin, PPD of *M. bovis*, PPD of *M. tuberculosis*,[q] Band 24[r] and Brucella antigens[s] were also used as the antigens in some of the migration inhibition methods.

---

[q]PPD, Parke, Davis & Company, Detroit, Michigan.

[r]Band 24 - purified tuberculo-protein, Dr. Virginia Mallmann, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan.

[s]Brucellergen, Merck Sharp and Dohme, West Point, Pennsylvania.

RESULTS

Intradermal Testing

The results of the gross and microscopic evaluation of the intradermal injection sites with various ubiquitous antigens are listed (Table 1). Most of the antigens produced an immediate type sensitivity response characterized by a polymorphonuclear infiltrate. Streptokinase-Streptodornase 50 u produced a mononuclear reaction in 10 dogs (50%), but only 4 dogs (20% had gross reactions of the delayed type. Dermatophyton 0 1:10 produced a mononuclear response in 4 dogs (20%), but a grossly detectable delayed type reaction in only 1 of the dogs.

BCG Sensitization

The subcutaneous injection site of 5 mg BCG in 0.5 ml of incomplete Freund's adjuvant was swollen in all dogs by 10 days postinjection and had ulcers of varying size 3 weeks to a month postinjection. Dog 12 had a relatively mild response to infection, and dogs 5 and 14 had severe responses. The right popliteal nodes of all BCG sensitized dogs were grossly enlarged. Microscopic examination of the right popliteal nodes of dogs 1 through 10 demonstrated the presence of acid-fast organisms in the 5 BCG sensitized dogs (Table 2). No lymphadenopathy or acid-fast organisms were found in the lymph nodes of DNCB sensitized dogs.

Table 1. Gross and microscopic evaluation of the reaction to intradermal injection of various antigens (intradermal testing was done prior to DNCB or BCG sensitization)

	Antigens									
	SK-SD 50 u	Monilia 1:100	Monilia 1:10	Derm O 1:10	Derm O 1:100	Tricho 1:10	Tricho 1:100	Asper 1:10	Asper 1:100	Diluent
Dog 1, adult, male, mixed breed										
1 hour	-	-	2	-	-	3	1	3	1	-
4 hours	-	2	2	1	-	2	1	3	2	-
24 hours	1	1	1	-	-	2	-	3	1	-
48 hours	1	-	1	-	-	2	-	3	1	-
Histopath.	IV(DTH)	I	III	II(DTH)	I	IV	II	V	IV(DTH)	I
Dog 2, adult, female, Beagle										
1 hour	-	-	3	-	-	2	-	3	-	-
4 hours	-	-	1	-	-	1	-	2	-	-
24 hours	-	-	1	-	-	1	-	2	1	-
48 hours	-	-	-	-	-	1	-	2	-	-
Histopath.	IV(DTH)	II	III	-	I	I	I	V	III	I
Dog 3, adult, female, Beagle										
1 hour	-	1	2	-	-	1	-	1	-	-
4 hours	-	-	1	-	-	-	-	-	-	-
24 hours	-	-	1	-	-	-	-	1	-	-
48 hours	-	-	1	-	-	-	-	1	-	-
Histopath.	III(DTH)	II(DTH)	III	-	-	II	I	IV	II	-
Dog 4, adult, male, Beagle										
1 hour	-	-	2	-	-	1	-	2	-	-
4 hours	-	-	1	-	-	1	-	1	-	-
24 hours	-	-	1	-	-	1	-	2	-	-
48 hours	-	-	1	-	-	1	-	1	-	-
Histopath.	II(DTH)	II(DTH)	II	II(DTH)	I	-	I	IV	III	I

Table 1 (continued)

	Antigens									
	SK-SD 50 u	Monilia 1:100	Monilia 1:10	Derm O 1:10	Derm O 1:100	Tricho 1:10	Tricho 1:100	Asper 1:10	Asper 1:100	Diluent
Dog 5, adult, male, Beagle										
1 hour	-	1	2	-	-	3	1	3	1	-
4 hours	-	1	1	-	-	3	-	3	1	-
24 hours	-	1	1	-	-	3	-	3	1	-
48 hours	-	-	-	-	-	3	-	2	1	-
Histopath.	II(DTH)	II(DTH)	II	II(DTH)	II	III	I	II	III(DTH)	I
Dog 6, adult, female, Poodle										
1 hour	-	1	2	-	1	3	-	3	1	-
4 hours	-	1	2	-	-	3	1	3	1	-
24 hours	1	-	1	-	-	2	-	2	1	-
48 hours	1	-	1	-	-	2	-	2	1	-
Histopath.	II(DTH)	II	III	-	II	II	II	IV	III	III
Dog 7, adult, female, Poodle										
1 hour	-	-	1	-	-	1	-	2	1	-
4 hours	-	-	1	-	-	1	-	1	1	-
24 hours	-	-	-	-	-	-	-	2	1	-
48 hours	-	-	-	-	-	1	-	2	1	-
Histopath.	I	II	I	I	I	II	-	III	II	II
Dog 8, adult, female, mixed breed										
1 hour	-	1	3	-	-	2	1	3	1	-
4 hours	-	-	1	-	-	1	-	2	1	-
24 hours	-	-	1	-	-	2	-	2	1	-
48 hours	-	-	1	-	-	1	-	2	1	-
Histopath.	II	II	III	III	I	II	II	III	II	III

Table 1 (continued)

	Antigens									
	SK-SD 50 u	Monilia 1:100	Monilia 1:10	Derm O 1:10	Derm O 1:100	Tricho 1:10	Tricho 1:100	Asper 1:10	Asper 1:100	Diluent
Dog 9, adult, male, Beagle										
1 hour	-	1	2	-	-	2	-	3	1	-
4 hours	-	1	2	-	-	2	-	2	1	-
24 hours	1	-	2	-	-	1	-	2	1	-
48 hours	-	-	2	-	-	1	-	2	1	-
Histopath.	III(DTH)	II	II	III	II	II	II(DTH)	III	II	I
Dog 10, adult, male, Dalmatian										
1 hour	-	1	2	1	1	2	1	2	1	-
4 hours	-	1	2	1	-	3	1	2	2	-
24 hours	-	1	2	-	-	2	-	2	1	-
48 hours	-	1	1	-	-	2	-	2	2	-
Histopath.	IV(DTH)	II	III	II	II	II	I	III	IV	III
Dog 11, 6 months, male, mixed breed										
1 hour	-	1	2	-	-	2	1	3	-	-
4 hours	-	-	1	-	-	2	-	2	-	-
24 hours	-	-	1	-	-	2	-	2	-	-
48 hours	-	-	2	-	-	1	-	2	-	-
Histopath.	II(DTH)	IV	II	II	II	V	III	V	IV	I
Dog 12, 6 months, female, mixed breed										
1 hour	-	1	3	-	-	2	1	2	1	-
4 hours	-	-	2	-	-	2	-	2	-	-
24 hours	-	-	2	-	-	1	-	2	1	-
48 hours	-	-	-	-	-	1	-	2	-	-
Histopath.	-	I	III	I	I	V	IV	IV	IV	I

Table 1 (continued)

	Antigens									
	SK-SD 50 u	Monilia 1:100	Monilia 1:10	Derm O 1:10	Derm O 1:100	Tricho 1:10	Tricho 1:100	Asper 1:10	Asper 1:100	Diluent
Dog 13, adult, male, Beagle										
1 hour	-	1	2	-	-	2	1	3	1	-
4 hours	-	-	3	-	-	2	-	2	-	-
24 hours	1	-	2	-	-	2	-	3	-	-
48 hours	-	-	1	-	-	2	-	2	-	-
Histopath.	II	I	III	I	-	V	IV	V	IV	-
Dog 14, adult, male, mixed breed										
1 hour	-	1	3	1	1	2	1	3	1	1
4 hours	1	1	2	-	-	2	1	2	1	-
24 hours	1	1	2	-	-	2	-	3	1	-
48 hours	-	-	1	-	-	2	-	3	-	-
Histopath.	III	II	III	III	III(DTH)	V	III(DTH)	V	V	II
Dog 15, adult, male, Beagle										
1 hour	-	1	2	-	-	2	1	2	-	-
4 hours	-	-	1	-	-	2	-	1	-	-
24 hours	-	-	1	1	1	2	-	2	-	-
48 hours	-	-	1	-	-	1	-	1	-	-
Histopath.	III	III	III	III(DTH)	III	V	II	V	IV	II
Dog 16, 8 months, female, mixed breed										
1 hour	-	-	2	-	-	2	-	2	-	-
4 hours	-	-	-	-	-	1	-	2	-	-
24 hours	-	-	1	-	-	1	-	2	-	-
48 hours	-	-	1	-	-	1	-	2	-	-
Histopath.	I	II	I	III	II(DTH)	IV	II	IV	III	-

Table 1 (continued)

	Antigens									
	SK-SD 50 u	Monilia 1:100	Monilia 1:10	Derm O 1:10	Derm O 1:100	Tricho 1:10	Tricho 1:100	Asper 1:10	Asper 1;100	Diluent
Dog 17, 8 months, female, mixed breed										
1 hour	-	-	2	-	-	1	-	1	-	-
4 hours	-	-	1	-	-	-	-	2	-	-
24 hours	-	-	1	-	-	1	-	2	1	-
48 hours	-	-	1	-	-	-	-	2	-	-
Histopath.	II	III	IV	III	IV	V	II	V	III	I
Dog 18, 8 months, male, mixed breed										
1 hour	-	-	2	-	-	2	1	2	-	-
4 hours	-	-	1	-	-	1	-	2	-	-
24 hours	-	-	1	-	-	2	-	2	-	-
48 hours	-	-	-	-	-	1	-	2	-	-
Histopath.	II	II	IV	I	II	II	II	V	II(DTH)	II
Dog 19, 10 months, female, mixed breed										
1 hour	-	1	2	-	-	1	1	3	-	-
4 hours	-	1	2	-	-	1	1	3	-	-
24 hours	-	-	2	-	-	1	-	3	-	-
48 hours	-	-	2	-	-	1	-	3	-	-
Histopath.	III(DTH)	III	IV	I(DTH)	-	IV	II	V	II	-

Table 1 (continued)

	Antigens									
	SK-SD 50 u	Monilia 1:100	Monilia 1:10	Derm O 1:10	Derm O 1:100	Tricho 1:10	Tricho 1:100	Asper 1:10	Asper 1:100	Diluent
Dog 20, 8 months, male, mixed breed										
1 hour	-	-	3	-	-	3	-	3	-	-
4 hours	-	-	2	-	-	2	-	3	-	-
24 hours	-	-	2	-	-	2	1	3	-	-
48 hours	-	-	2	-	-	2	-	2	-	-
Histopath.	I	I	IV	II	I	V	I	VI	IV	I

DTH - Changes were characterized by a mononuclear cell infiltration suggestive of a delayed type hypersensitivity reaction.

Gross evaluation - 1 to 3 in order of increasing severity
Microscopic evaluation - I to VI in order of increasing severity

Table 2. Size of popliteal nodes of dogs 3 weeks postinjection of BCG or DNCB in the right hind foot and presence of acid-fast organisms in right popliteal node

Dog No. - Sensitizing antigen	Left popliteal node (cm x cm x cm)	Right popliteal node (cm x cm x cm)	Acid-fast organisms observed in right popliteal lymph node
1 - DNCB	3.5 x .5 x .3	3.5 x 1.0 x .3	Negative
2 - BCG	.5 x .5 x .5	4 x 2 x 1	Positive
3 - BCG	1.3 x .6 x .6	4.8 x 2.6 x 1.3	Positive
4 - DNCB	1 x .5 x .2	1.8 x .5 x .3	Negative
5 - BCG	1.3 x .5 x .5	3.8 x 2.0 x 1.5	Positive
6 - DNCB	.75 x .5 x .38	.75 x .5 x .5	Negative
7 - BCG	1 x .3 x .3	1.4 x 1 x 1.3	Positive
8 - DNCB	1.4 x 1.3 x .3	1.8 x 1.0 x .4	Negative
9 - BCG	2 x .5 x .1	5 x 3 x 2	Positive
10 - DNCB	2 x 1 x .5	2.5 x .5 x .1	Negative

Peripheral Blood Values Pre- and Postsensitization

The total leukocyte, lymphocyte and monocyte counts of peripheral venous blood presensitization and 3 weeks postsensitization are reported (Table 3). No significant differences were noted pre- and postsensitization.

DNCB Sensitization

A mild scurf of the sensitization site was found 1 to 2 days after application of DNCB-DMSO. No significant increase in size of the right popliteal nodes was noted in DNCB sensitized dogs (Table 2).

Challenge of Sensitized Dogs with DNCB

No gross reaction was detected at any of the sites 24 hours after challenge. Microscopically, the sites had a slight response in all 10 of the DNCB sensitized dogs (100%) and 3 of the BCG sensitized dogs (30%) (Table 4).

Tuberculin Testing Pre- and Postsensitization

Recording of gross and microscopic reactions to undiluted tuberculin is given in Table 4 and a compilation given in Table 5. Undiluted tuberculin caused severe microscopic inflammatory reactions characterized by edema, necrosis and polymorphonuclear leukocyte infiltration in sensitized and nonsensitized dogs. Two dogs (20%) presensitization, 3 dogs (60%) postsensitization with DNCB, and all BCG sensitized dogs had gross reactions to undiluted tuberculin.

Table 3. Leukocyte, lymphocyte and monocyte counts per cubic millimeter of peripheral blood of dogs prior to and after BCG or DNCB sensitization

Dog No.	Leukocytes/mm^3	Lymphocytes/mm^3	Monocytes/mm^3
11 - Pre	10,000	2,300	400
- Post	14,700	3,675	441
12* - Pre	18,800	3,000	2,444
- Post	14,900	4,172	745
13 - Pre	6,900	1,794	414
- Post	7,700	2,464	77
14* - Pre	9,800	1,666	588
- Post	9,800	1,568	392
15* - Pre	9,460	2,914	376
- Post	10,700	3,317	107
16* - Pre	10,300	2,578	824
- Post	12,600	2,520	630
17* - Pre	9,100	3,003	728
- Post	9,800	3,332	1,274
18 - Pre	15,600	3,588	312
- Post	10,800	2,160	1,080
19 - Pre	10,000	2,800	1,000
- Post	9,200	3,404	920
20 - Pre	12,700	3,048	635
- Post	9,600	2,784	288

*BCG sensitized dogs.

Table 4. Gross and microscopic evaluation of sites of intradermal injection of undiluted tuberculin and DNCB challenge in dogs prior to and 3 weeks after BCG or DNCB sensitization

Dog No. Sensitizing Antigen	Tuberculin				DNCB 100 µgm	
	Gross		Microscopic			
	Presens.	Postsens.	Presens.	Postsens.	Postsens. Gross	Postsens. Micros.
1-DNCB	1*	1	V*	V	0	I
2-BCG	0	3	V	VI	0	0
3-BCG	0	3	V	VI	0	0
4-DNCB	0	2	V	IV	0	I
5-BCG	1	3	IV	VI	0	I
6-DNCB	0	1	II	II	0	I
7-BCG	0	1	IV	V	0	0
8-DNCB	0	2	III	III	0	I
9-BCG	0	1	V	VI	0	I
10-DNCB	0	3	IV	V	0	II

	Gross Postsensitization	Microscopic Postsensitization
11-DNCB	0 (1 mm)**	I
12-BCG	0 (0 mm)	0
13-DNCB	0 (0 mm)	I
14-BCG	0 (.5 mm)	0

Table 4 (continued)

Dog No. Sensitizing Antigen	Gross Postsensitization	Microscopic Postsensitization
15-BCG	0 (0 mm)	0
16-BCG	0 (0 mm)	I
17-BCG	0 (0 mm)	0
18-DNCB	0 (0 mm)	I
19-DNCB	0 (0 mm)	III
20-DNCB	0 (0 mm)	I

*Gross evaluations are graded 1 to 3 and microscopic evaluation I to VI in increasing degree of inflammatory reaction.

**Increase in skin fold thickness 24 hours after DNCB challenge.

Table 5. Compilation of gross and microscopic reactions of dogs 1-10 to undiluted tuberculin pre- and postsensitization

	Presensitization		Postsensitization	
	Positive	Negative	Positive	Negative
Gross Reaction				
BCG Sens.	1	4	5	0
DNCB Sens.	1	4	5	0
	Grade			
	>III	III or less	>III	III or less
Microscopic Reaction				
BCG Sens.	5	0	5	0
DNCB Sens.	3	2	3	2

Intradermal Testing with Tuberculin Diluted 1:50 Pre- and Postsensitization

Recording of gross and microscopic reactions to 1:50 tuberculin injected intradermally pre- and postsensitization is reported in Table 6 and a compilation given in Table 7. Gross reactions were 90% accurate in differentiating between BCG and DNCB sensitized dogs. Microscopically, 1:50 tuberculin produced less severe responses in unsensitized dogs and differentiation of BCG from DNCB sensitized dogs was 70% accurate.

The tuberculin reaction in BCG sensitized dogs was interpreted as a delayed type hypersensitivity reaction in only half the dogs tested. These dogs had clear-cut mononuclear cell infiltration though the predominant cell was the polymorphonuclear leukocyte. The other BCG sensitized dogs had minimal mononuclear cell infiltration.

Direct Migration-Inhibition Pre- and Postsensitization

The results of direct inhibition test of the radial migration of peripheral leukocytes by 1:100 tuberculin are reported in Tables 8 and 9 and Figures 1 through 8. After preliminary examination of the results, 35% or greater inhibition was considered positive. Only cells from 1 dog (dog 3) had migration inhibition prior to sensitization with BCG or DNCB. Compilation of migration inhibition in BCG and DNCB sensitized dogs was made in Table 10.

Migration Inhibition in Sensitized Dogs with Various Antigens

The results of direct inhibition test of radial migration of peripheral leukocytes by tuberculin 1:100, PPD, Band 24 and Brucellergen 6 weeks postsensitization are given in Table 9 and Figures 5 through 8.

Table 6. Gross and microscopic evaluation of intradermal tuberculin (1:50) injection sites prior to and 3 weeks after BCG or DNCB sensitization and direct migration inhibition of peripheral leukocytes by 1:100 tuberculin

Dog No. - Sensitizing Antigen	Gross		Microscopic		Direct Migration Inhibition	
	Pre-sens.	Post-sens.	Pre-sens.	Post-sens.	Pre-sens. %	Post-sens. %
1-DNCB	-	0**	-	II**	0	63
2-BCG	-	2	-	V	0	34
3-BCG	-	1	-	IV	59	48
4-DNCB	-	0	-	IV	0	10
5-BCG	-	2	-	II(DTH)***	19	0
6-DNCB	-	0	-	II	0	50
7-BCG	-	0	-	III	27	34
8-DNCB	-	0	-	I	10	0
9-BCG	-	1	-	II	16	80
10-DNCB	-	1	-	IV	0	13
11-DNCB	0	0(0 mm)*	III	III(DTH)	8	18

Table 6 (continued)

Dog No. - Sensitizing Antigen	Gross		Microscopic		Direct Migration Inhibition	
	Pre-sens.	Post-sens.	Pre-sens.	Post-sens.	Pre-sens. %	Post-sens. %
12-BCG	0	2(2 mm)	III	V	23	15
13-DNCB	0	0(0 mm)	II	II	0	0
14-BCG	1	3(2.5 mm)	III	V(DTH)	17	53
15-BCG	1	2(1.5 mm)	III(DTH)	V(DTH)	28	45
16-BCG	0	1(1 mm)	I	III(DTH)	23	41
17-BCG	0	2(1.5 mm)	III	V(DTH)	19	45
18-DNCB	0	0(0 mm)	II	III	0	0
19-DNCB	0	0(0 mm)	III	II	27	10
20-DNCB	0	0(0 mm)	V	II	18	50

*Increase in skin fold thickness prior to and 24 hours after intradermal tuberculin injection.

**Gross evaluations are graded 1 to 3 and microscopic evaluation I to VI in increasing degree of inflammatory reaction.

***DTH - changes were characterized by a mononuclear cell infiltration suggestive of delayed type hypersensitivity.

Table 7. Compilation of gross and microscopic evaluation of intradermal injection sites of 1:50 tuberculin pre- and postsensitization

	Presensitization*		Postsensitization**	
	Positive	Negative	Positive	Negative
Gross Reaction				
BCG Sens.	2	8	9	1
DNCB Sens.	0	10	1	9
	Grade			
	>III	III or less	>III	III or less
Microscopic Reaction				
BCG Sens.	0	5	6	4
DNCB Sens.	1	4	2	8

*Ten dogs.

**Twenty dogs.

Table 8. Direct and indirect inhibition of leukocyte migration by 1:100 tuberculin prior to and after sensitization with BCG and DNCB

Dog No. - Sensitizing Antigen	Direct					Indirect
	Pre-sens. %	3 wks Post. %	8 wks Post. %	11 wks Post. %	12 wks Post. %	12 wks Post. %
1-DNCB	0	63	42	31	0	9
2-BCG	0	34	45	34	25	69
3-BCG	59	48	40	44	12	58
4-DNCB	0	10	15	40	0	40
5-BCG	19	0	34	34	27	53
6-DNCB	0	50	50	25	0	38
7-BCG	27	34	38	80	53	48
8-DNCB	10	0	0	0	0	18
9-BCG	16	80	60	37	32	34
10-DNCB	0	13	19	0	23	24

Table 9. Direct inhibition of leukocyte migration by 1:100 tuberculin, PPD, Band 24 and Brucellergen prior to and after sensitization with BCG and DNCB

Dog No. Sensitizing Antigen	Tuberculin				PPD	Band 24	Brucellergen
	Pre-sens.	Pre-sens.	3 wks Post.	6 wks Post.	6 wks Post.	6 wks Post.	6 wks Post.
11-DNCB	15	8	18	23	34	34	45
12-BCG	0	23	15	0	43	29	29
13-DNCB	10	0	0	30	30	20	20
14-BCG	0	17	53	60	40	60	20
15-BCG	23	28	45	58	-	43	29
16-BCG	20	23	41	58	58	0	58
17-BCG	0	19	45	50	38	50	50
18-DNCB	12	0	0	25	25	38	25
19-DNCB	0	27	10	13	50	38	50
20-DNCB	13	18	50	63	50	0	50

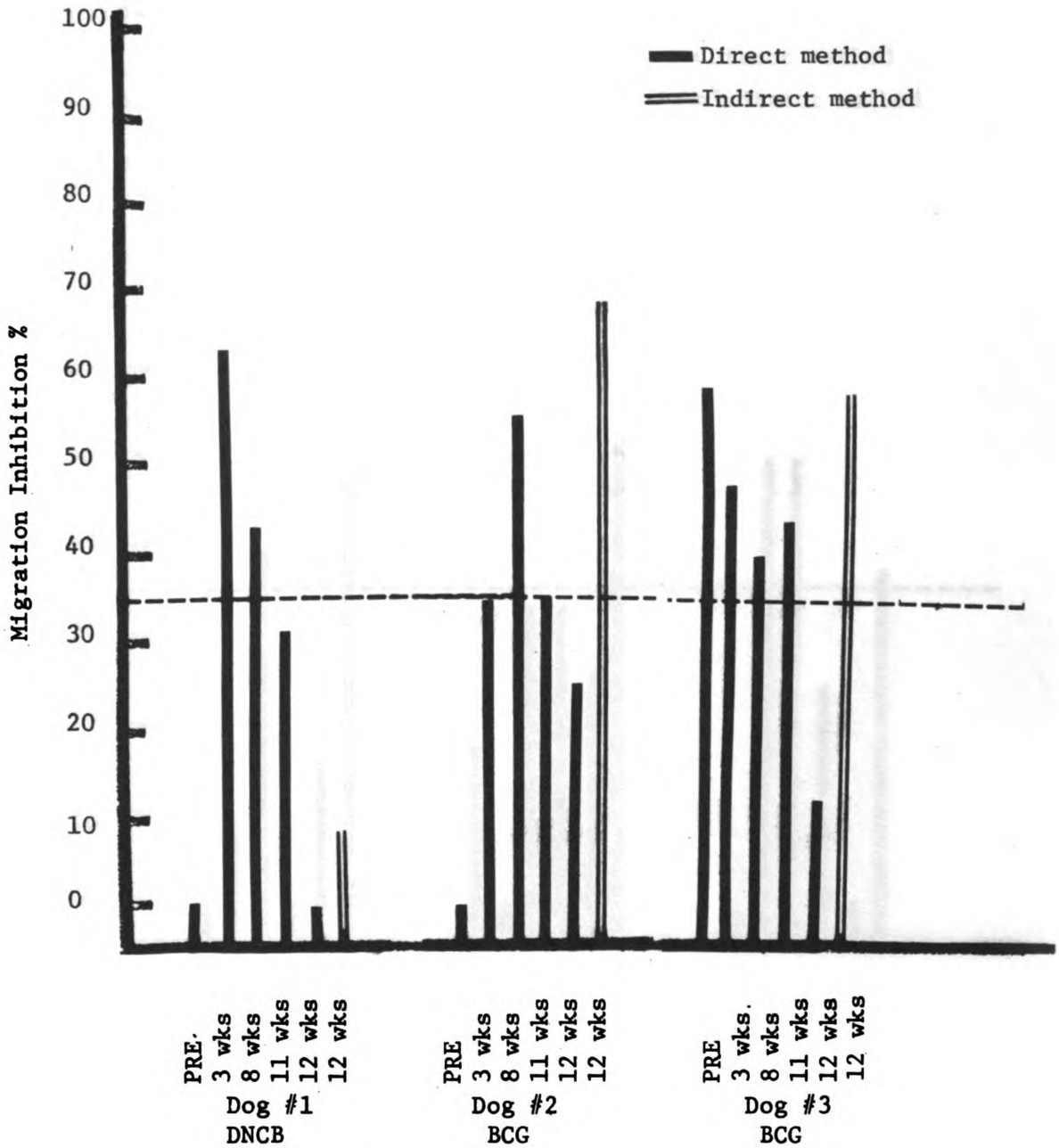

Figure 1. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin in culture media. Migration inhibition before sensitization (PRE) with BCG (Bacillus Calmette Guerin) or DNCB (Dinitrochlorobenzene) and at intervals postsensitization. Indirect migration inhibition using normal guinea pig peritoneal cells cultured with cell free supernate of the dog leukocyte cultures with 1:100 mammalian tuberculin. Wks = weeks; PRE = presensitization. Dogs 1, 2 and 3. Inhibition $\geq$ 35% chosen empirically as positive test.

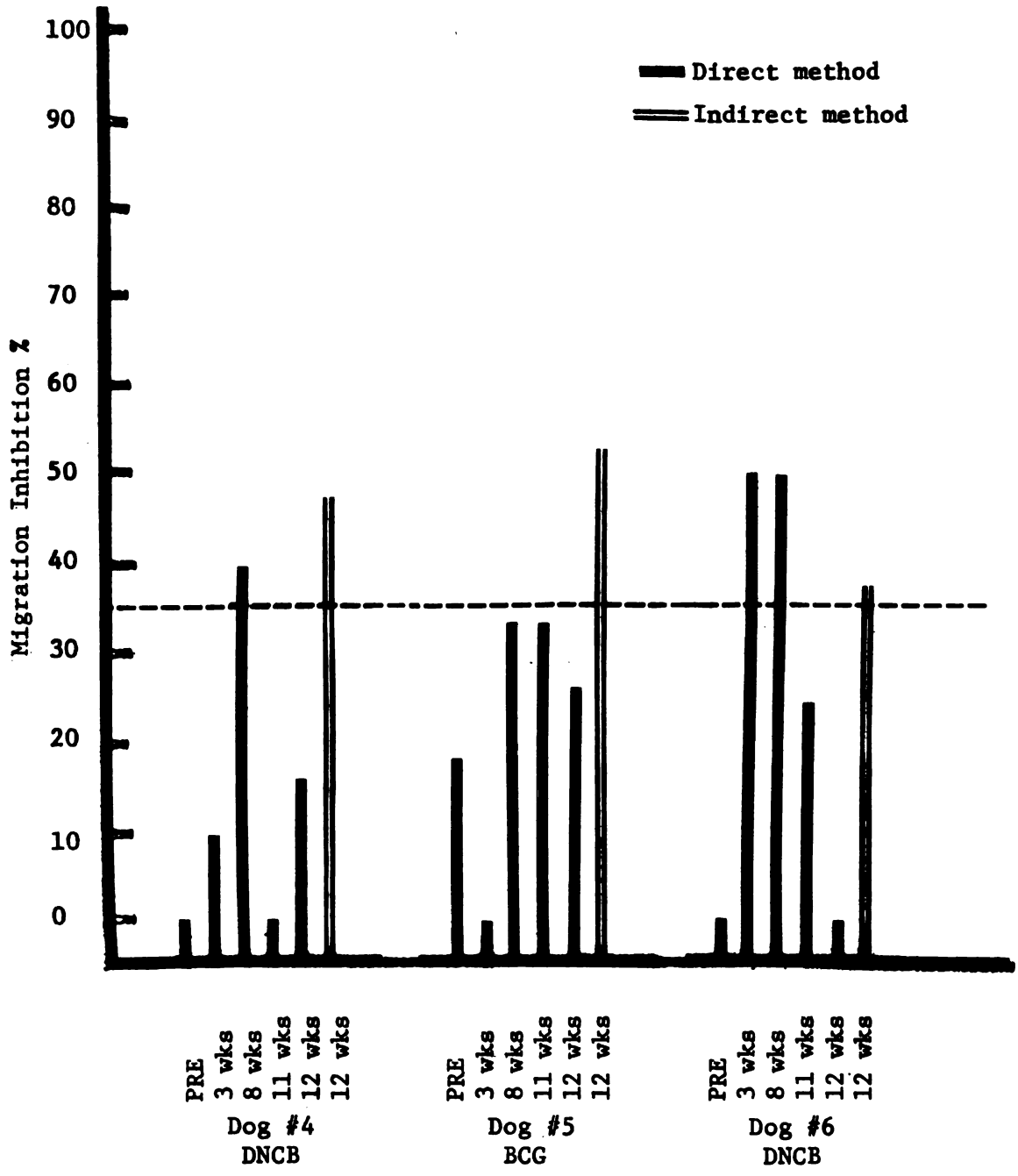

Figure 2. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin in culture media. Migration inhibition before sensitization (PRE) with BCG (Bacillus Calmette Guerin) or DNCB (Dinitrochlorobenzene) and at intervals postsensitization. Indirect migration inhibition using normal guinea pig peritoneal cells cultured with cell free supernate of the dog leukocyte cultures with 1:100 mammalian tuberculin. Wks = weeks; PRE = presensitization. Dogs 4, 5 and 6. Inhibition $\geq$ 35% chosen empirically as positive test.

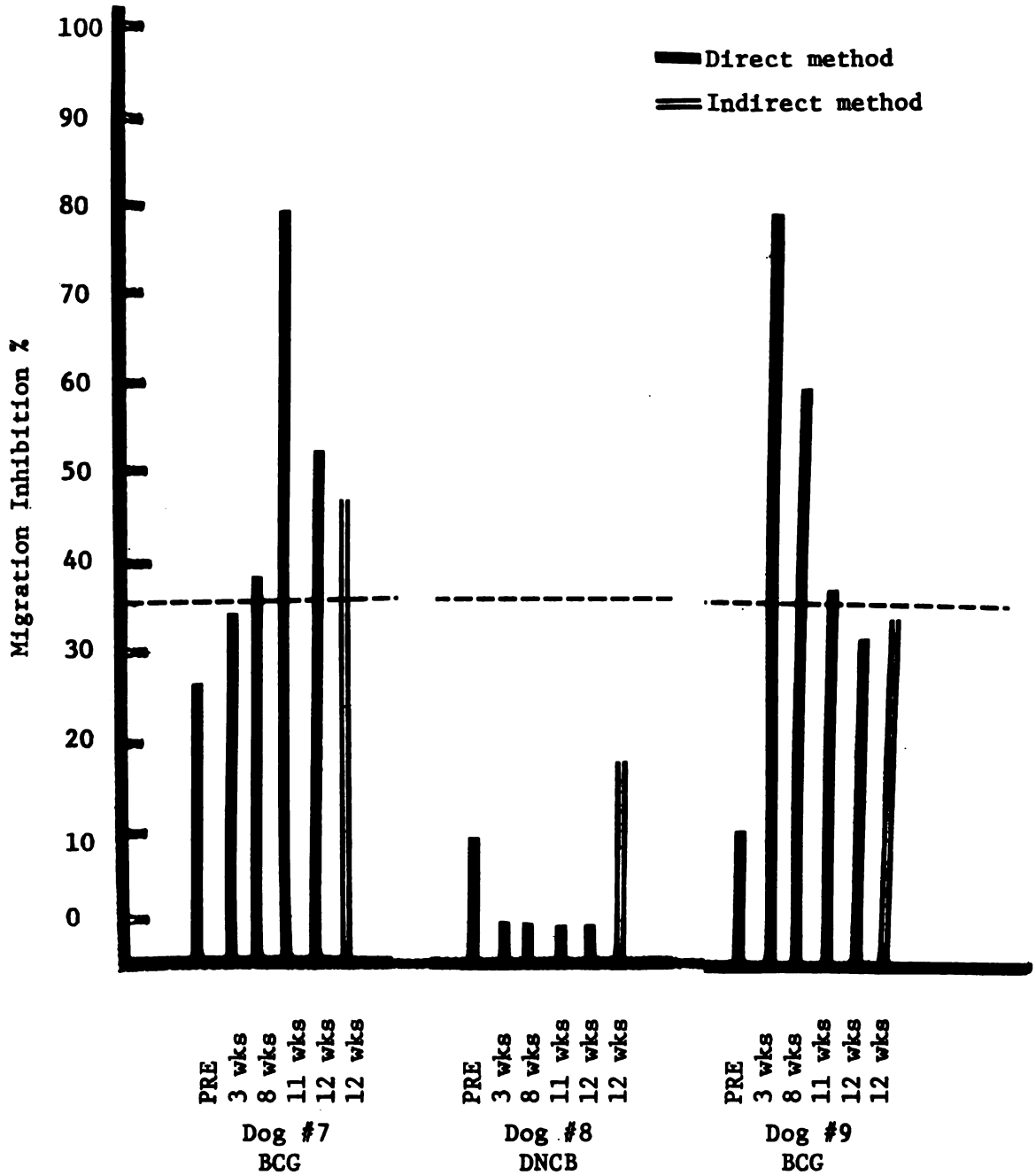

Figure 3. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin in culture media. Migration inhibition before sensitization (PRE) with BCG (Bacillus Calmette Guerin) or DNCB (Dinitrochlorobenzene) and at intervals postsensitization. Indirect migration inhibition using normal guinea pig peritoneal cells cultured with cell free supernate of the dog leukocyte cultures with 1:100 mammalian tuberculin. Wks = weeks; PRE = presensitization. Dogs 7, 8 and 9. Inhibition $\geq$ 35% chosen empirically as positive test.

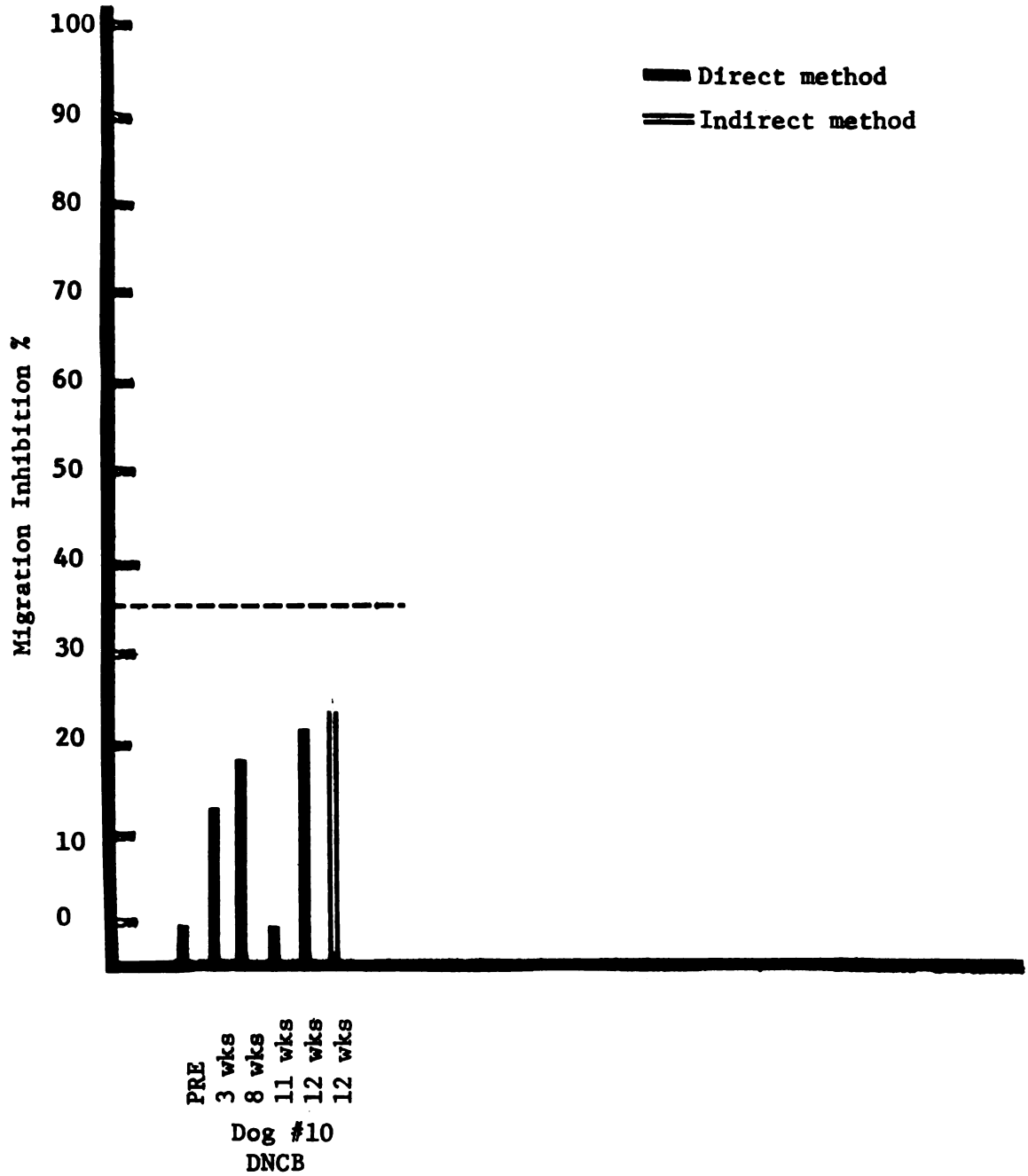

Figure 4. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin in culture media. Migration inhibition before sensitization (PRE) with BCG (Bacillus Calmette Guerin) or DNCB (Dinitrochlorobenzene) and at intervals postsensitization. Indirect migration inhibition using normal guinea pig peritoneal cells cultured with cell free supernate of the dog leukocyte cultures with 1:100 mammalian tuberculin. Wks = weeks; PRE = presensitization. Dog 10. Inhibition $\geq$ 35% chosen empirically as positive test.

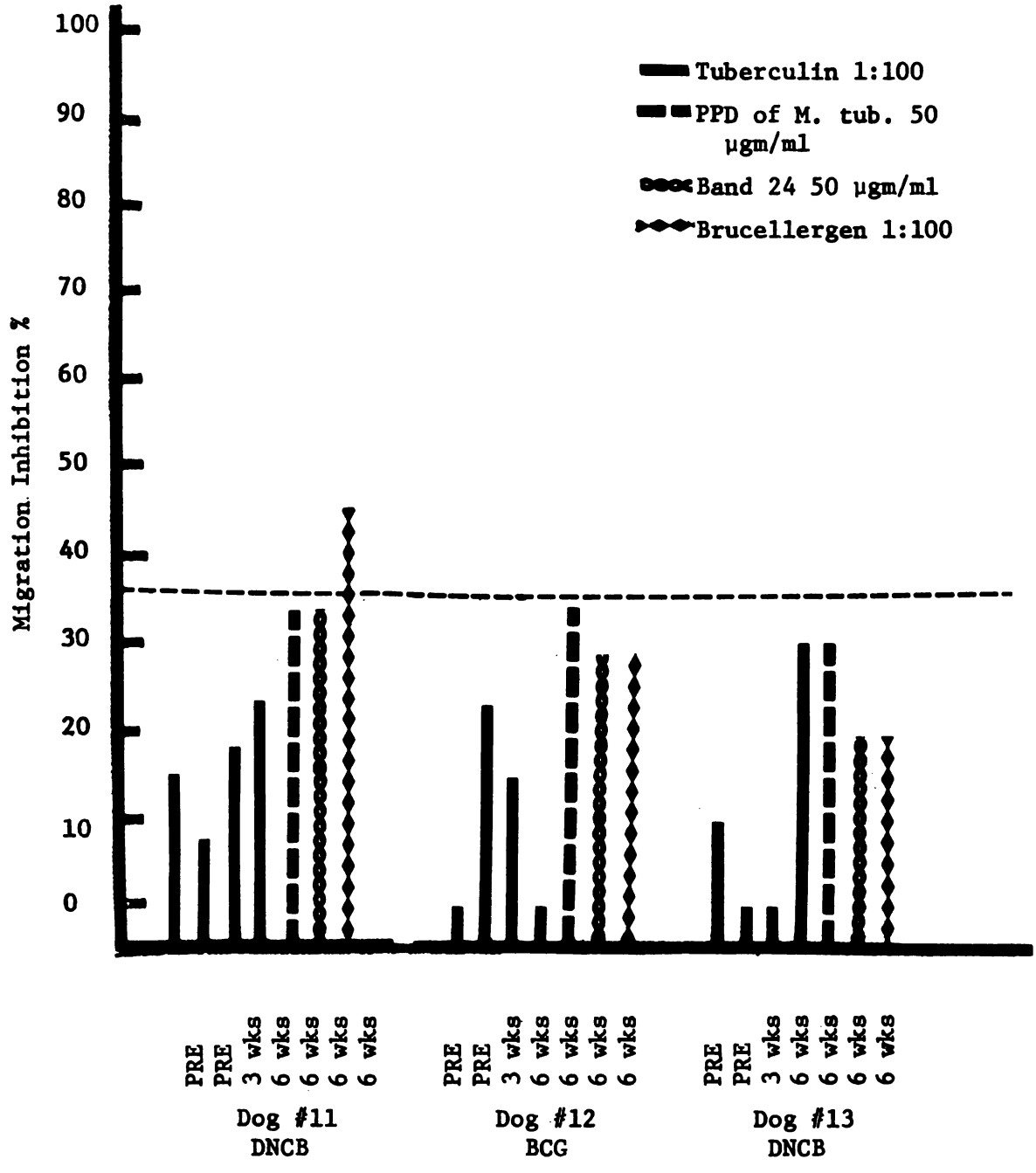

Figure 5. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin, PPD of *M. tuberculosis* (50 µgm/ml), Band 24 (50 µgm/ml) and Brucellergen 1:100. PRE = presensitization; wks = weeks. Dogs 11, 12 and 13. Inhibition ≥ 35% chosen empirically as positive test.

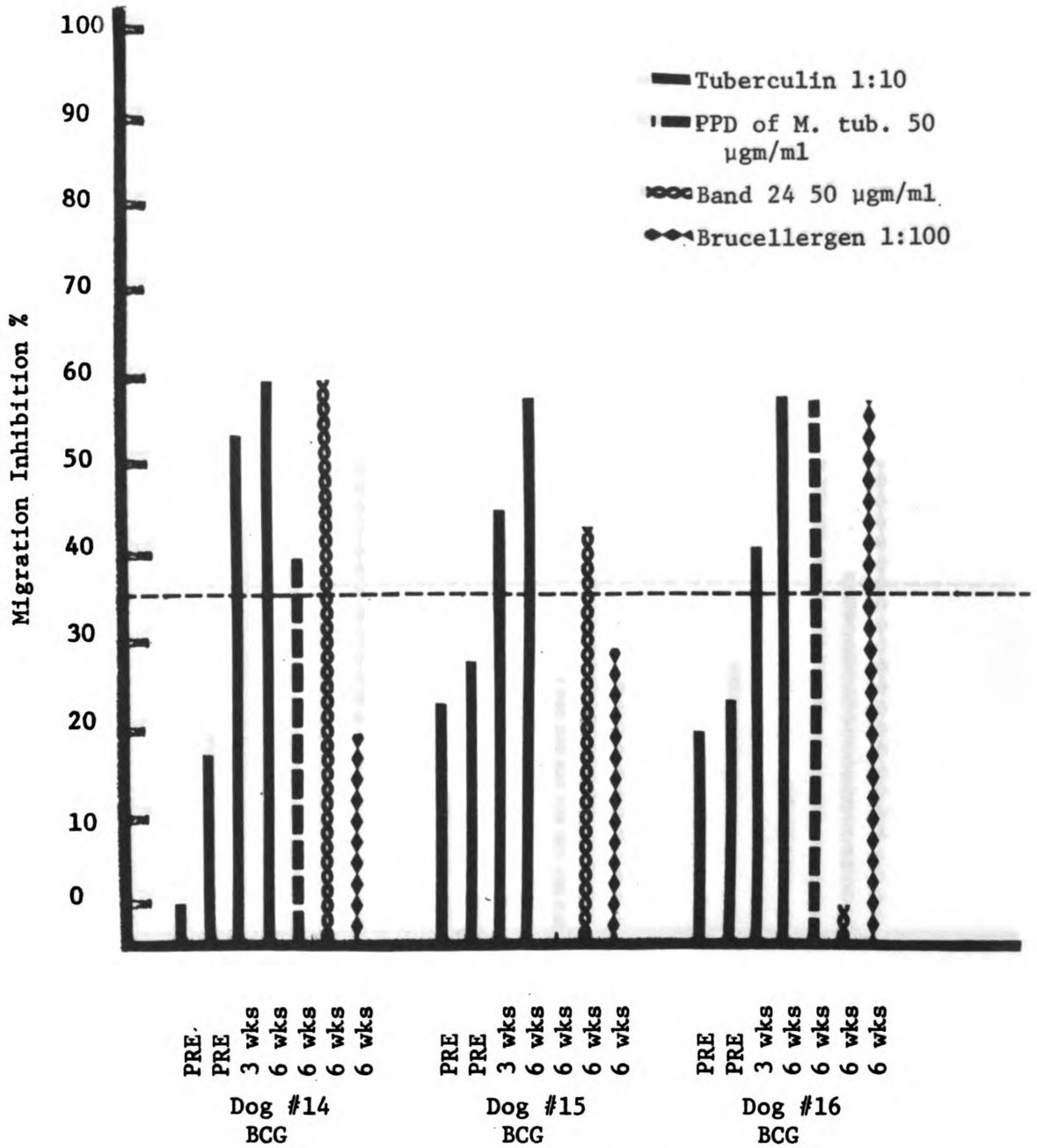

Figure 6. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin, PPD of *M. tuberculosis* (50 µgm/ml), Band 24 (50 µgm/ml) and Brucellergen 1:100. PRE = presensitization; wks = weeks. Dogs 14, 15 and 16. Inhibition ≥ 35% chosen empirically as positive test.

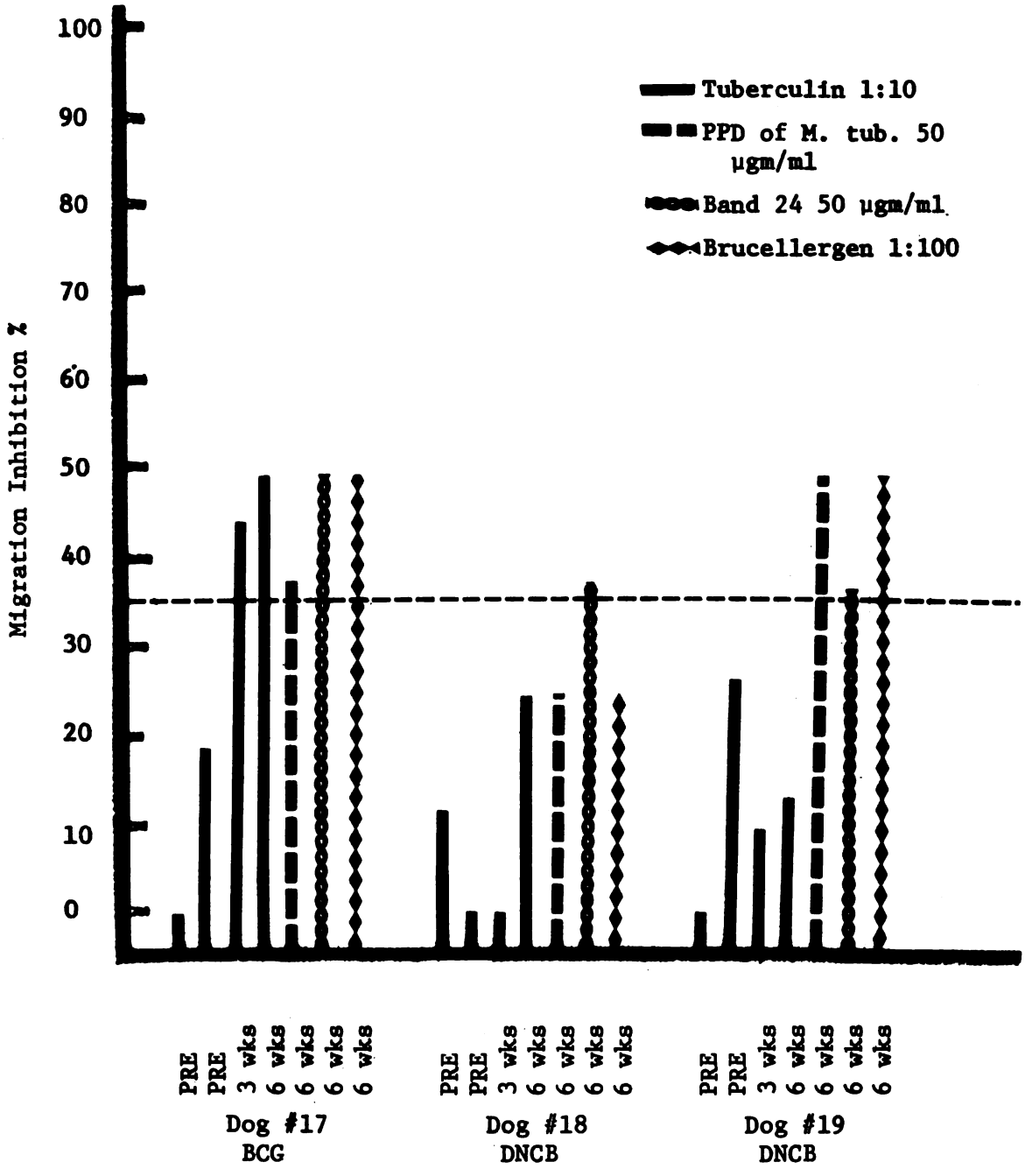

Figure 7. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin, PPD of *M. tuberculosis* (50 µgm/ml), Band 24 (50 µgm/ml) and Brucellergen 1:100. PRE = presensitization; wks = weeks. Dogs 17, 18 and 19. Inhibition ≥ 35% chosen empirically as positive test.

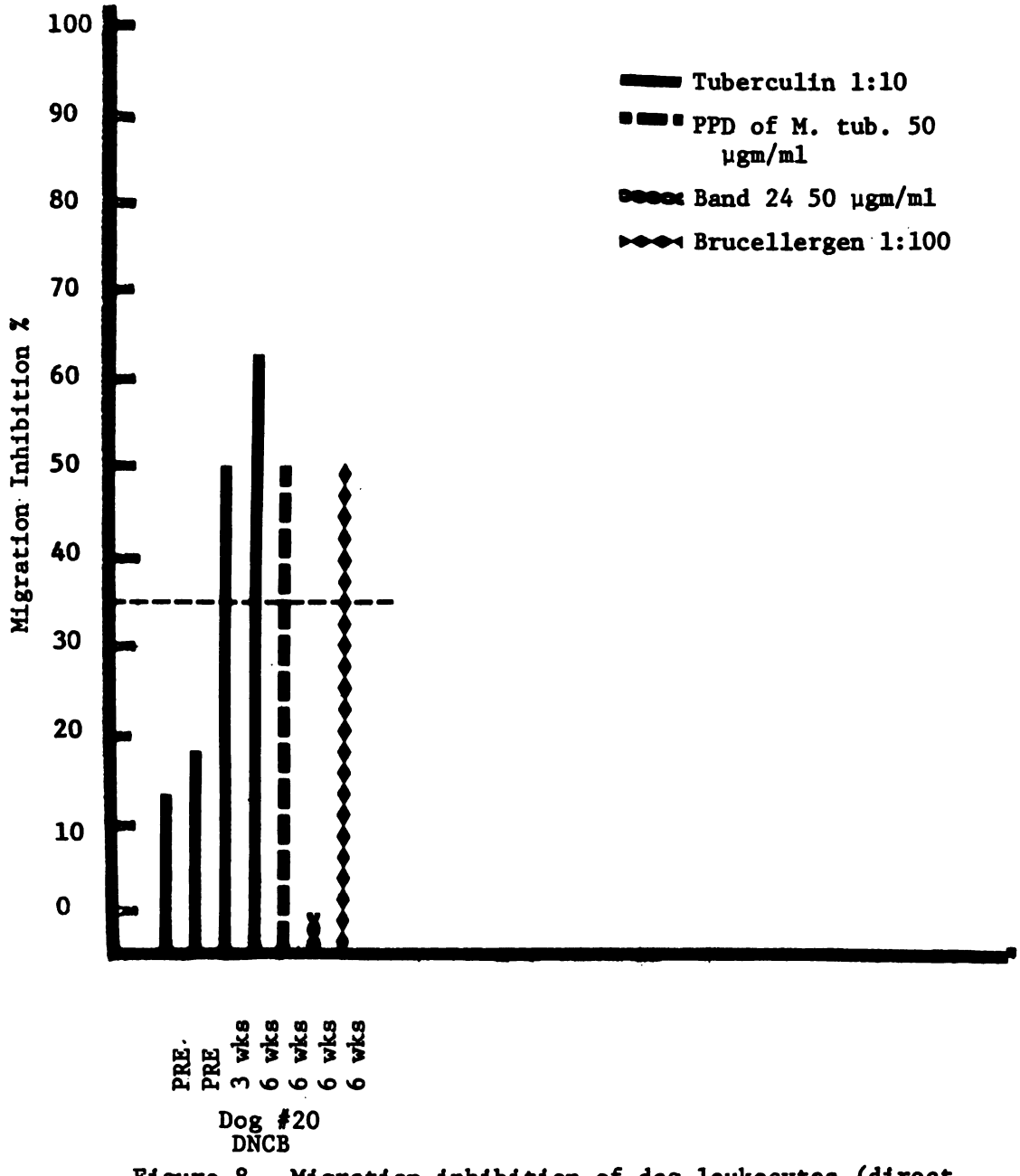

Figure 8. Migration inhibition of dog leukocytes (direct method) by 1:100 mammalian tuberculin, PPD of *M. tuberculosis* (50 µgm/ml), Band 24 (50 µgm/ml) and Brucellergen 1:100. PRE = presensitization; wks = weeks. Dog 20. Inhibition ≥ 35% chosen empirically as positive test.

Table 10. Compilation of migration inhibition by direct method with 1:100 tuberculin in dogs sensitized with BCG and DNCB 3, 6 and 8 weeks postsensitization

Dog No.	Weeks Postsens.	Positive*	Negative
1-10			
BCG (5)**	3	2	3
DNCB (5)	3	3	2
11-20			
BCG (5)	3	4	1
DNCB (5)	3	1	4
1-10			
BCG (5)	8	4	1
DNCB (5)	8	3	2
11-20			
BCG (5)	6	4	1
DNCB (5)	6	1	4
1-10			
BCG (5)	11	3	2
DNCB (5)	11	0	5

*Inhibition greater than 35% inhibition.

**Number in parentheses indicates number of dogs studied.

Compilation of their effectiveness in differentiating between BCG and DNCB sensitized dogs is given in Table 11. Tuberculin was most effective in differentiating between BCG and DNCB sensitized dogs. There was no correlation between tuberculin reactions and Brucellergen.

Direct and Indirect Migration Inhibition

The results of tests to compare ability of the direct and indirect migration to differentiate between BCG and DNCB sensitized dogs (dogs 11-20) are given in Table 12 and Figures 9 and 10, and a compilation is given in Table 13. The direct method was slightly better in differentiating between BCG and DNCB sensitized dogs. Fifty percent of the dogs were positive presensitization by the indirect test, while only 5% of the dogs were positive by the direct test.

Average of All Pre- and Postsensitization Direct Test Migration Inhibition

The average of all pre- and postsensitization direct migration inhibition tests by 1:100 tuberculin are given in Table 14 and Figures 11 and 12, and a compilation is given in Table 15. This method was 80% accurate in differentiating BCG and DNCB sensitized dogs.

Table 11. Comparison of the migration inhibition effects of mammalian tuberculin, PPD, Band 24 and Brucellergen 6 weeks post-sensitization with BCG and DNCB

	Tub.		PPD		Band 24		Brucellergen	
	Pos.*	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
BCG Sensitized dogs	4	1	3	1**	3	2	2	3
DNCB Sensitized dogs	1	4	2	3	2	3	3	2

*Inhibition $\geq$ 35% chosen empirically as positive test.

**One dog's test contaminated.

Table 12. Direct and indirect migration inhibition pre- and post-sensitization with BCG and DNCB

Dog No. - Sensitizing Antigen	Presensitization		Postsensitization	
	Direct %	Indirect %	Direct %	Indirect %
11-DNCB	8	21	18	21
12-BCG	23	37	15	24
13-DNCB	0	10	0	9
14-BCG	17	50	53	48
15-BCG	28	59	45	42
16-BCG	23	47	41	35
17-BCG	19	44	45	38
18-DNCB	0	33	0	12
19-DNCB	27	17	10	0
20-DNCB	18	18	50	37

Inhibition $\geq$ 35% chosen empirically as positive test.

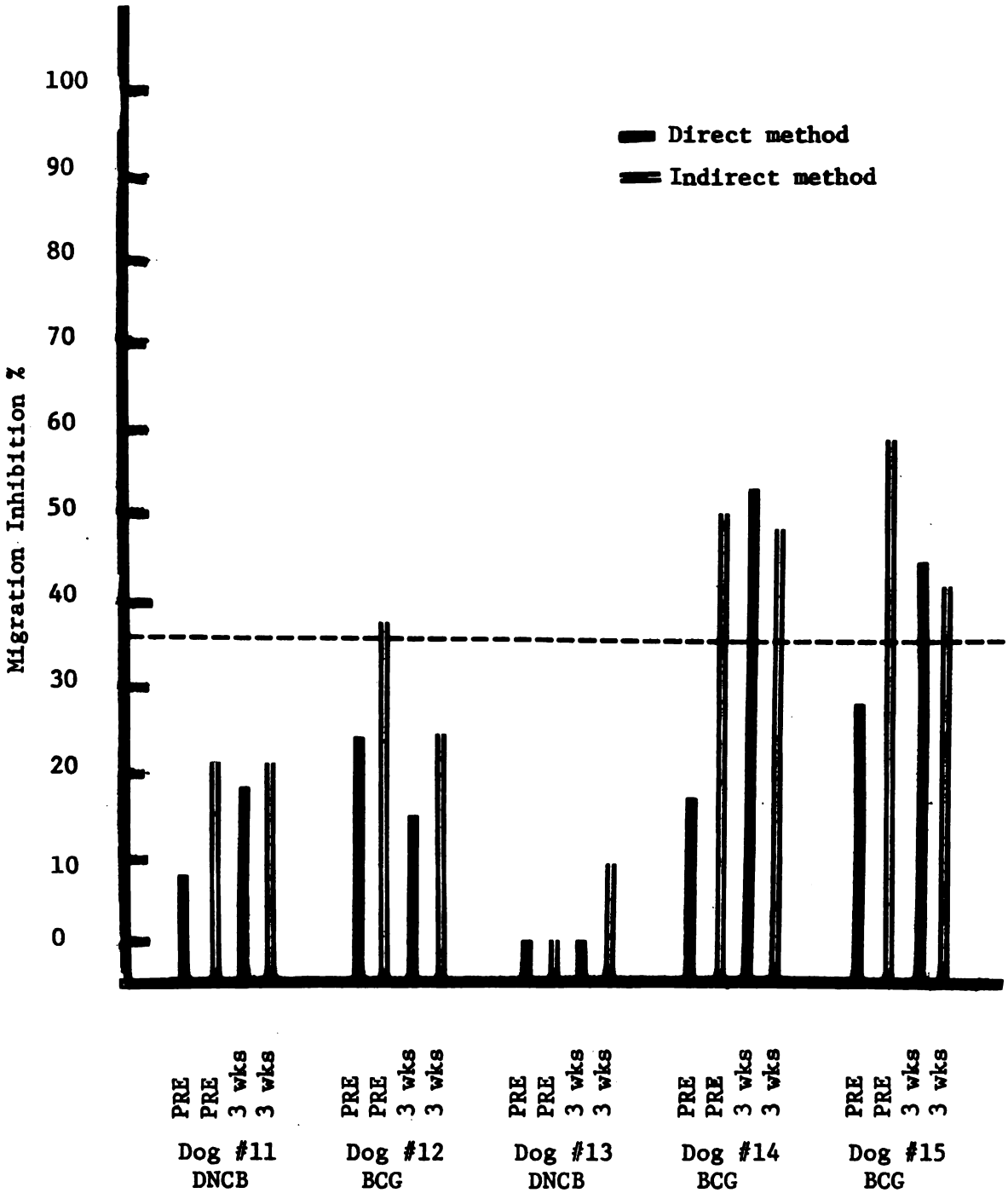

Figure 9. Comparison of the migration inhibition effect of 1:100 tuberculin by the direct and indirect method in dogs sensitized with BCG (Bacillus Calmette Guerin) and DNCB (Dinitrochlorobenzene) presensitization and 3 weeks postsensitization. The direct method measures the inhibition of migration of sensitized dog leukocytes incubated with 1:100 mammalian tuberculin. The indirect method measures the inhibition of migration of normal guinea pig peritoneal exudate cells incubated with the cell free supernate from the dog leukocyte cultures. Wks = weeks; PRE = presensitization. Dogs 11, 12, 13, 14 and 15. Inhibition $\geq$ 35% chosen empirically as positive test.

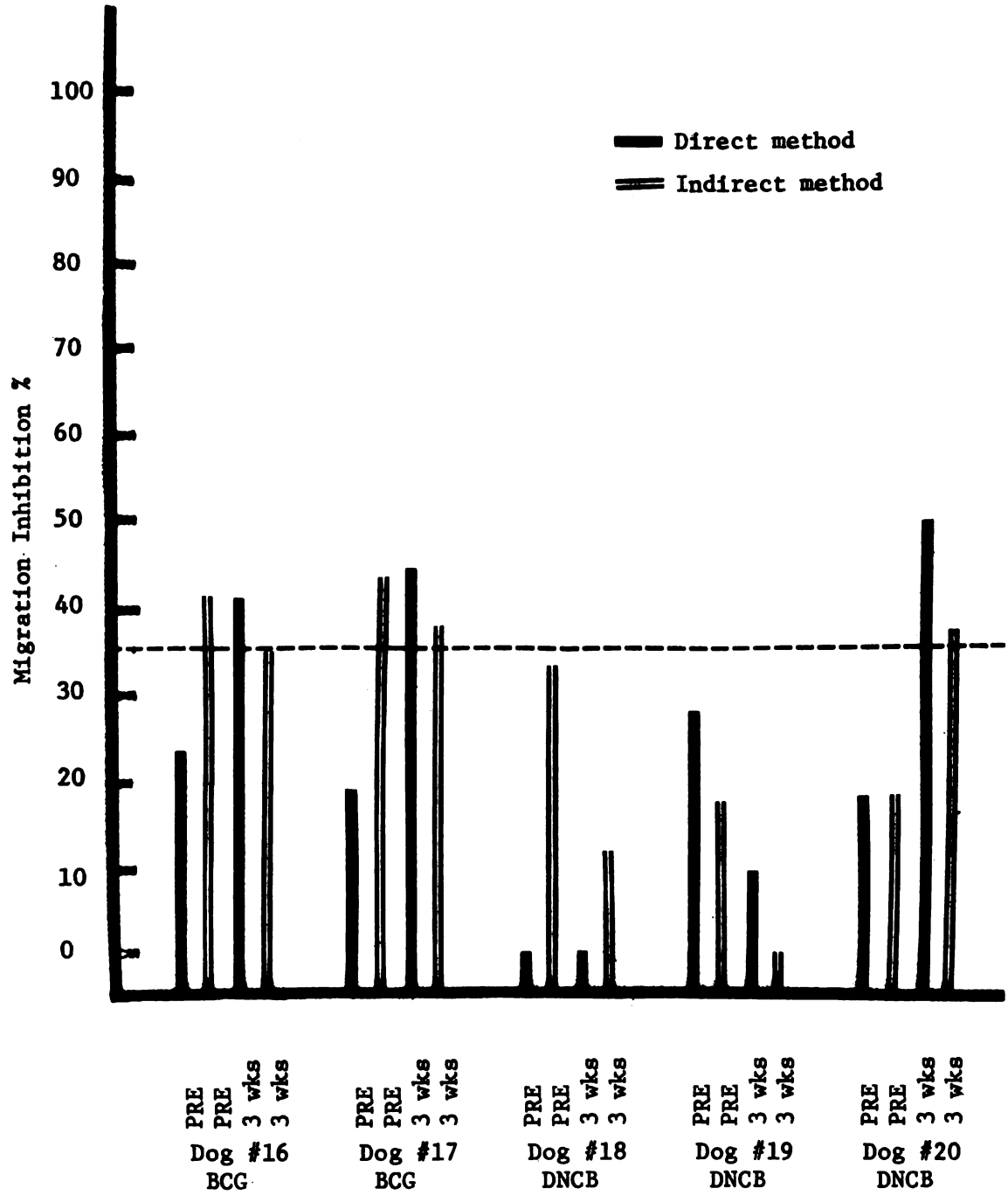

Figure 10. Comparison of the migration inhibition effect of 1:100 tuberculin by the direct and indirect method in dogs sensitized with BCG (Bacillus Calmette Guerin) and DNCB (Dinitrochlorobenzene) presensitization and 3 weeks postsensitization. The direct method measures the inhibition of migration of sensitized dog leukocytes incubated with 1:100 mammalian tuberculin. The indirect method measures the inhibition of migration of normal guinea pig peritoneal exudate cells incubated with the cell free supernate from the dog leukocyte cultures. Wks = weeks; PRE = presensitization. Dogs 16, 17, 18, 19 and 20. Inhibition $\geq$ 35% chosen empirically as positive test.

Table 13. Compilation of direct and indirect migration inhibition presensitization and 3 weeks postsensitization in dogs sensitized with BCG or DNCB

	Presensitization		Postsensitization			
			BCG		DNCB	
	Positive	Negative	Positive	Negative	Positive	Negative
Direct (10 dogs)	1	9	4	1	1	4
Indirect (10 dogs)	5	5	3	2	1	4

*Three weeks postsensitization.

Table 14. Average of all tests on each dog of direct migration inhibition pre- and postsensitization with BCG or DNCB

Dog No.	Presensitization Average of Direct MI %	Postsensitization Average of Direct MI %
1-DNCB	0	34
2-BCG	0	34.5
3-BCG	59	36
4-DNCB	0	16.25
5-BCG	19	23.75
6-DNCB	-	31.25
7-BCG	27	51.25
8-DNCB	10	0
9-BCG	16	52.25
10-DNCB	0	13.75
11-DNCB	11.5	20.5
12-BCG	11.5	7.5
13-DNCB	5	15
14-BCG	8.5	56.5
15-BCG	25.5	51.5
16-BCG	21.5	49.5
17-BCG	9.5	47.5
18-DNCB	6	12.5
19-DNCB	13.5	11.5
20-DNCB	15.5	56.5

Inhibition $\geq$ 35% chosen empirically as positive test.

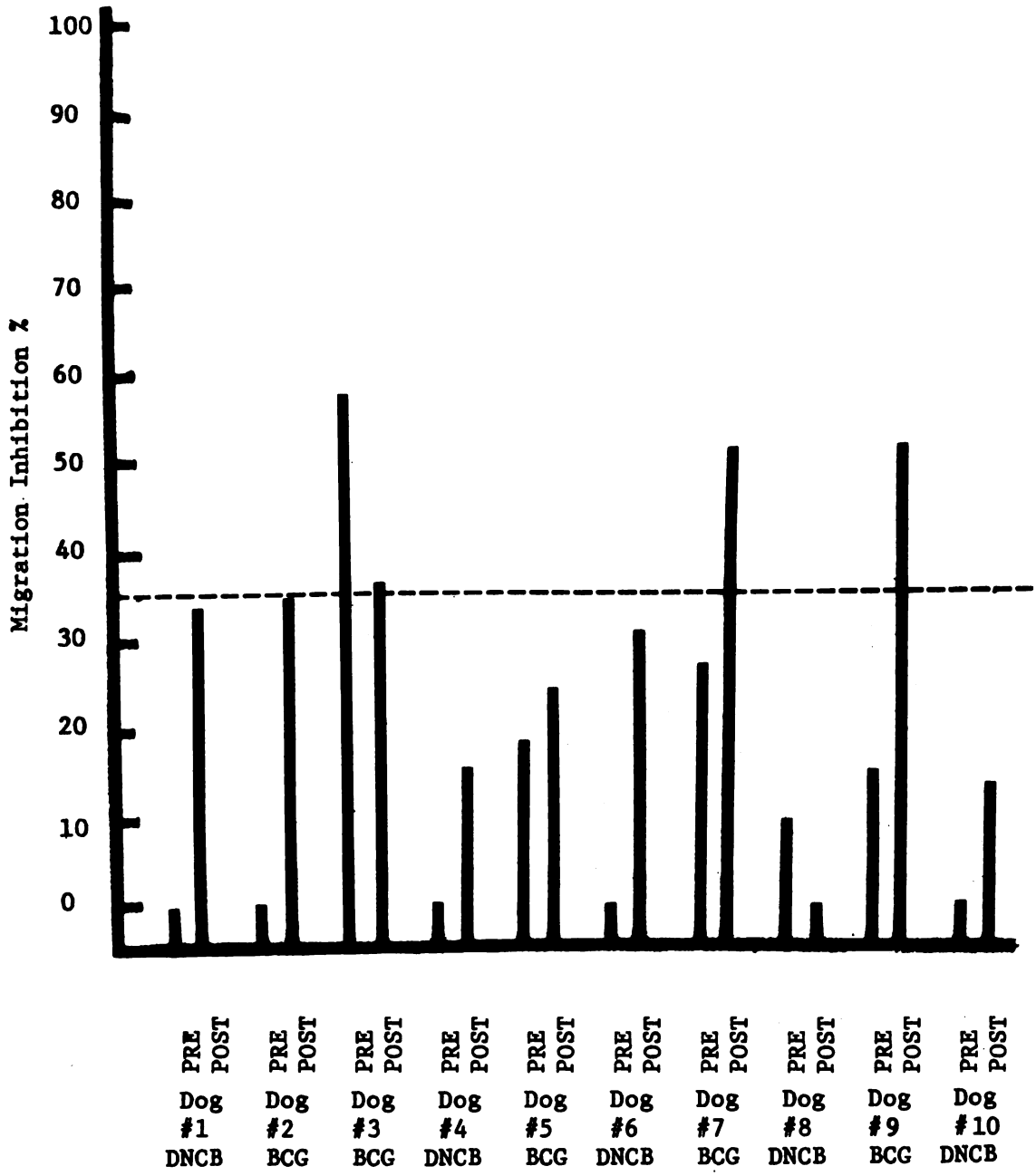

Figure 11. Averages of all direct migration inhibition studies using 1:100 mammalian tuberculin reported in Figures 1 through 8. PRE indicates an average of all migration inhibition studies pre-BCG (Bacillus Calmette Guerin) or pre-DNCB (Dinitrochlorobenzene) sensitization. POST indicates an average of all studies postsensitization. Dogs 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. Inhibition $\geq$ 35% chosen empirically as positive test.

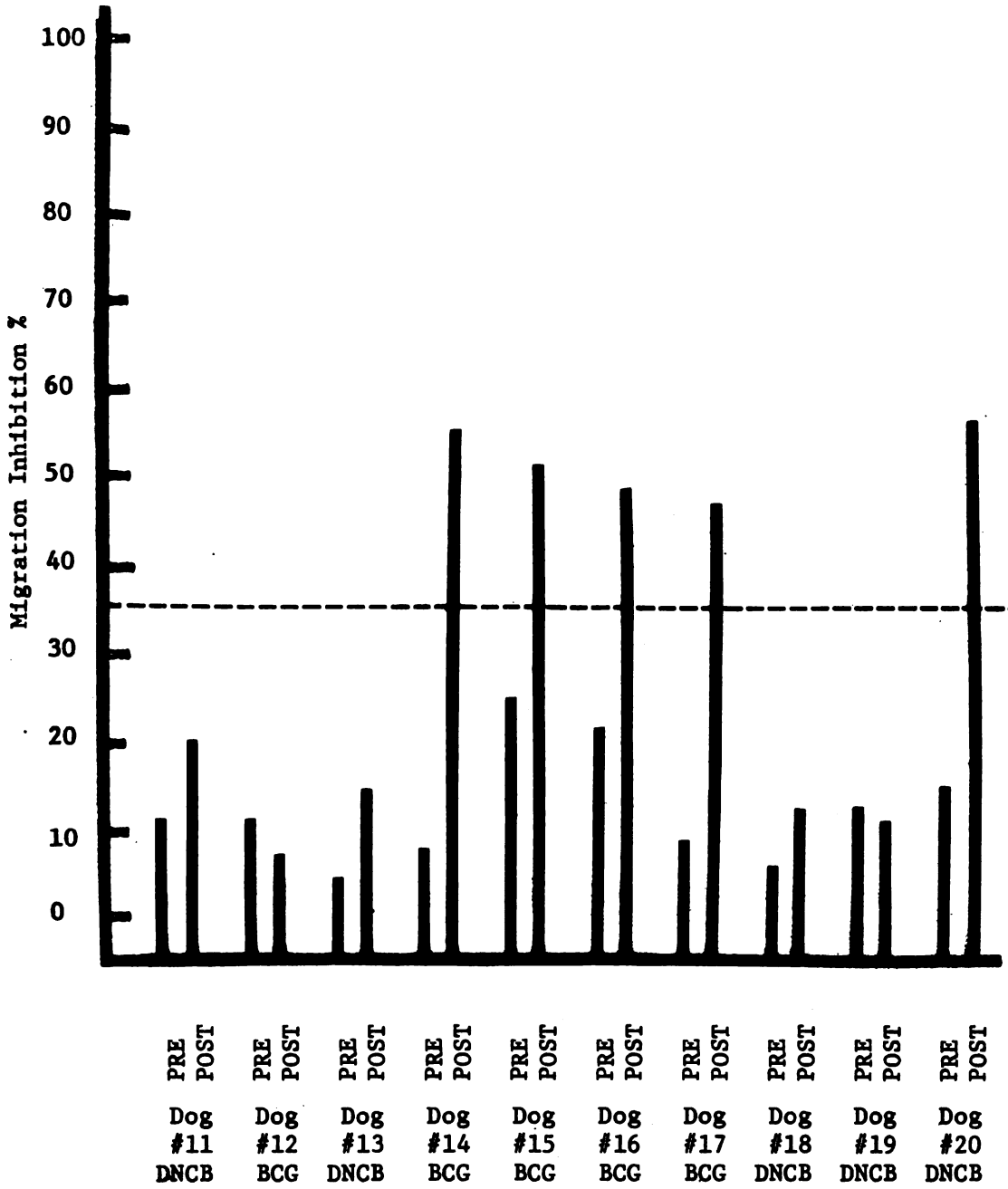

Figure 12. Averages of all direct migration inhibition studies using 1:100 mammalian tuberculin reported in Figures 1 through 8. PRE indicates an average of all migration inhibition studies pre-BCG (Bacillus Calmette Guerin) or pre-DNCB (Dinitrochlorobenzene) sensitization. POST indicates an average of all studies postsensitization. Dogs 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20. Inhibition $\geq$ 35% chosen empirically as positive test.

Table 15. Compilation of the average of all migration inhibition results by direct methods in dogs pre- and postsensitization with BCG and DNCB

	Presensitization		Postsensitization	
	Positive*	Negative	Positive	Negative
BCG	1	9	7	3
DNCB	0	10	1	9

*Inhibition ≥ 35% chosen empirically as positive test.

DISCUSSION AND CONCLUSIONS

This study was performed to evaluate the applicability of some of the current methods to the study of cell mediated immunity in the dog. The methods evaluated are currently employed in the clinical evaluation of the immune system in man.

Ubiquitous antigens, which regularly produce delayed type sensitivity reactions in most individuals, are used extensively in the evaluation of a given individual's cell-mediated competence. The antigens commonly used in intradermal testing in man are: *Candida albicans*, *Trichophyton*, PPD, Streptokinase-Streptodornase, and mumps.[12] Five antigen preparations were selected (Monilia, Dermatophyton O, Trichophyton, Aspergillus and Streptokinase-Streptodornase) which would be expected to produce delayed hypersensitivity reactions in the dog. Monilia, Aspergillus and Trichophyton produced immediate responses which were fully developed at 4 hours and were characterized by extensive polymorphonuclear leukocyte infiltration. These antigens were found to be unsuitable for demonstrating delayed hypersensitivity in the dog. Dermatophyton O (*Candida albicans*) 1:10 produced some mild mononuclear cell reactions of the delayed hypersensitivity type, but only 1 dog (5%) had a gross delayed hypersensitivity reaction. Streptokinase-Streptodornase 50 u produced delayed gross reactions in 4 dogs (20%) and a mononuclear cell infiltrate in 10 dogs (50%). Of the antigens

tested, only Streptokinase-Streptodornase, possibly at an increased concentration, offers a potentially useful antigen for demonstrating delayed hypersensitivity in the dog. Sensitization with DNCB is another standard method for evaluating cell mediated immune response to a new antigen. Ninety-six percent of individuals exposed experimentally to DNCB developed spontaneous flare reactions.[14] DNCB in DMSO instead of acetone was used in the initial sensitization to achieve good penetration of the DNCB. The possibility that the anti-inflammatory effects of DMSO interfered with sensitization was considered. When challenged with a 100 μgm dose of DNCB in acetone 21 days postsensitization, no gross reactions were noted, but all DNCB sensitized dogs and 3 BCG sensitized dogs (30%) had a mild microscopic response. The microscopic response indicates a slight sensitization, and a different method of sensitization could possibly produce gross reactions. Joseph[38] reported good results by sensitizing dogs with DNCB in acetone applied near the inguinal lymph nodes. Sensitization with DNCB in DMSO is not a practical method for clinical use.

Infection with BCG was accomplished by subcutaneous injection of viable BCG organisms. Lymphadenopathy and observation of acid-fast organisms in the right popliteal lymph node of dogs biopsied confirmed the establishment of a BCG infection.

Undiluted tuberculin was unsuitable for intradermal skin testing in the dog. Undiluted tuberculin produced a strong nonspecific inflammatory reaction in unsensitized dogs. All BCG and DNCB sensitized dogs responded with a grossly detectable reaction.

Tuberculin diluted 1:50 differentiated between BCG and DNCB sensitized dogs with 90% accuracy on evaluation of gross reactions, and 70% accuracy by microscopic evaluation. Tuberculin diluted 1:50 appears to be a superior product to undiluted tuberculin for diagnostic use in the dog.

The injection sites of 1:50 tuberculin were characterized by a predominantly polymorphonuclear leukocyte infiltration in BCG and DNCB sensitized dogs, but the inflammatory changes were more severe in the BCG sensitized dogs. Only half of the BCG sensitized dogs had a clear-cut mononuclear cell response, and this was overshadowed by a predominance of polymorphonuclear leukocytes. The polymorphonuclear leukocyte appears to play a greater role in the tuberculin reaction of sensitized dogs than it does in sensitized rabbits[28] or guinea pigs.[58]

In vitro techniques have been used extensively in man in the study of cell-mediated immunity. The migration inhibition method has been shown to be a versatile method for use in a variety of immunological conditions. The direct radial migration inhibition method using 1:100 tuberculin was 80% accurate in differentiating between BCG and DNCB sensitized dogs. It offers a promising method for use in the study of cellular immunity in the dog.

The direct method was superior to the indirect method of measuring migration inhibition. The indirect method had an excessive number of false positive reactions in dogs presensitization and in DNCB sensitized dogs. Only 1 dog (5%) had a false positive response presensitization by the direct method. The possibility of prior contact with

a *Mycobacterium* must be considered, but the dog was negative on intradermal skin testing.

Mammalian tuberculin 1:100 was as effective an antigen *in vitro* as the more purified tuberculoproteins (PPD of *M. tuberculosis* or Band 24). Brucellergen was used to confirm that all reactions were not nonspecific. Brucellergen produced inhibition patterns unrelated to BCG or DNCB sensitization. Five dogs (50%) had migration inhibition to Brucellergen suggesting the possibility of previous exposure to the *Brucella* organism.

Migration inhibition occurred in 1 or more tests of cells from 3 dogs of the first group (dogs 1 through 10) sensitized with DNCB. One dog in the second group (dogs 11 through 20) sensitized with DNCB had *in vitro* migration inhibition to 1:100 tuberculin. The possibility of infection of DNCB sensitized dogs with BCG in the first group must be considered as the dogs were exercised in the same run. The sites of inoculation ulcerated and possible transmission cannot be excluded. The second group of dogs were carefully segregated.

Maximum *in vitro* response to BCG infection did not occur until 6 to 8 weeks postsensitization in some dogs, and began to diminish by 11 weeks. Two dogs (dogs 2 and 7) were negative at 3 weeks but positive at 8 weeks postsensitization with BCG.

Two dogs (dogs 5 and 12) sensitized with BCG were never positive by migration inhibition test. Dog 5 had 2 migration inhibition values of 34% (35% or greater was selected as positive). It had a good gross response to intradermal test of 1:50 tuberculin, but microscopically poor cellular infiltration (grade II). In addition, dog 5 had the most

severe focal reaction to BCG infection of all the dogs sensitized. It is interesting to speculate that the dog was incapable of mounting a good immune cellular response to the organism and there was an additional compensatory nonspecific inflammatory response to contain the BCG infection. However, dog 12 had a mild reaction at the site of BCG infection and did not have even borderline positive migration inhibition values. It did have a strong gross response with good cellular infiltrate to intradermal injection of 1:50 tuberculin. The possibility exists that dog 12 had a good cell mediated response, not detected *in vitro*, which prevented a larger reaction at the site of BCG infection. Much additional information is needed before the mechanism of normal cell mediated responses can be effectively analyzed and evaluated and deviations determined and explained.

Averaging of the results of all the migration inhibition tests was most effective in differentiating BCG from DNCB sensitized dogs. This eliminated the variables seen on individual tests and demonstrated that the use of more than one test is preferable. Migration inhibition of leukocytes in the dog differs from other species in that the test measures mainly granulocyte migration. The macrophage is usually considered the cell influenced by migration inhibition factor. Migration of normal dog leukocytes is considerably less than the migration of human and bovine leukocytes obtained in this laboratory. Poor migration makes interpretation of migration inhibition more difficult.

The direct radial migration inhibition test appears to be a useful tool in the study of cell-mediated immunity in the dog. More work is

needed to compare findings obtained by migration inhibition with other *in vitro* methods such as lymphocyte transformation.

SUMMARY

Methods to evaluate naturally and artificially induced cell mediated immunity in the dog were tested. Twenty dogs of mixed age, sex and breed were injected intradermally with Monilia, Dermatophyton O, Trichophyton, Aspergillus and Streptokinase-Streptodornase to determine if a delayed skin reaction occurred regularly to ubiquitous antigens. On the basis of visible reactions and histological examination of biopsied injection sites, it was concluded that only Streptokinase-Streptodornase had promise.

To test artificially induced cell mediated immunity, 10 of the dogs were injected subcutaneously with viable BCG in incomplete Freund's adjuvant, and 10 dogs were sensitized with DNCB in DMSO applied to the plantar surface of the foot. Three weeks or more after sensitization the following tests were made: intradermal tuberculin testing, skin challenge with DNCB and *in vitro* migration inhibition of blood leukocytes by tuberculin.

Undiluted tuberculin was unsuitable for intradermal testing in the dog because of false positive reactions. Tuberculin diluted 1:50 was 90% accurate as an intradermal test in differentiating between BCG and DNCB sensitized dogs. Tuberculin diluted 1:50 produced a reaction characterized by a predominantly polymorphonuclear leukocyte infiltration in BCG sensitized dogs. Half of the BCG sensitized dogs also had

a distinct mononuclear cell infiltration. The response of sensitized dogs to tuberculin appears to be different than in other species.

All DNCB and 30% of the BCG sensitized dogs had microscopic but not gross responses to DNCB challenge. DNCB in DMSO is not a suitable agent for skin sensitization in dogs.

The direct radial migration inhibition of peripheral dog leukocytes by 1:100 tuberculin was 80% accurate in differentiating between BCG and DNCB sensitized dogs. The indirect method, which measures the migration inhibition effect of the supernates of dog leukocyte cultures on normal guinea pig peritoneal cells, produced an excessive number of false positive reactions. The indirect method was inferior to the direct method in evaluating cell mediated immunity in the dog. Tuberculin 1:100 was more effective than more purified tuberculoproteins in the migration inhibition test. The average of a number of migration inhibition tests is more accurate than individual tests. The direct radial migration inhibition test of peripheral leukocytes appears to be a good *in vitro* method to study cell mediated immunity in the dog.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Anderson, V., Benedixen, G., and Schiodt, T.: An *in vitro* demonstration of cellular immunity against autologous mammary carcinoma in man. Acta Med. Scand., 186, (1969): 101-103.

2. Awad, F. I.: Intradermal BCG test for the diagnosis of tuberculosis in dogs. Deutsch. Tieraertzl. Wschr., 69, (1962): 623-625.

3. Baker, E.: Allergy skin testing in the dog. J.A.V.M.A., 148, (1966): 1160-1162.

4. Benedixen, G.: Organ-specific inhibition of the *in vitro* migration of leukocytes in human glomerulonephritis. Acta Med. Scand., 184, (1968): 99-103.

5. Berg, O. A.: Tuberculin reactions in dogs. Acta Tuberc. Scand., 43(Suppl.), (1958): 9-46.

6. Bloom, B., and Bennett, B.: Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. Science, 153, (1966): 80-82.

7. Bloom, B., and Bennett, B.: Migration inhibition factor associated with delayed-type hypersensitivity. Fed. Proc., 27, (1968): 13-15.

8. Bloom, B., and Glade, P.: In vitro *Methods in Cell-Mediated Immunity*. Academic Press, New York, 1971.

9. Bloom, B., Ceppellini, R., Cerottini, J., David, J., Kunkel, H., Landy, M., Lawrence, H., Maini, R., Nussenzweig, V., Perlmann, P., Spitler, L., Rosen, F., and Zabriskie, J.: *In vitro* methods in cell-mediated immunity. Cellular Immunol., 6, (1973): 331-347.

10. Borel, Y., and David, J.: *In vitro* studies of the suppression of delayed hypersensitivity by the induction of partial tolerance. J. Exp. Med., 131, (1970): 603-610.

11. Brastoff, J., Roitt, I., and Doniach, D.: Leucocyte migration inhibition in autoimmune disease. Lancet, i, (1969): 1212-1213.

12. Buckley, C. E.: Immunologic evaluation of older patients. Postgraduate Medicine, 51, (1972): 235-242.

13. Catalona, W. O., Taylor, P. O., Robson, A., and Chretien, P.: A method for dinitrochlorobenzene contact sensitization. New England J. Med., 286, (1972): 399-402.

14. Catalona, W., Taylor, P., and Chretien, P.: Quantitative dinitrochlorobenzene contact sensitization in a normal population. Clin. Exp. Immunol., 12, (1972): 325-333.

15. Chase, M. W.: The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exp. Biol. Med., 59, (1945): 134-135.

16. Churchill, W., Zbar, B., Belli, J., and David, J.: Detection of cellular immunity to tumor antigens of a guinea pig hepatoma by inhibition of macrophage migration. J. Nat. Canc. Inst., 48, (1972): 541-549.

17. David, J., Al-Askari, S., Lawrence, H., and Thomas, L.: Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens. J. Immunol., 93, (1964): 264-273.

18. David, J., Lawrence, H., and Thomas, L.: Delayed hypersensitivity *in vitro*. J. Immunol., 93, (1964): 264-278.

19. David, J., Lawrence, H., and Thomas, L.: Delayed hypersensitivity *in vitro*. J. Immunol., 93, (1964): 279-282.

20. David, J., Lawrence, H., and Thomas, L.: The *in vitro* desensitization of sensitive cells by trypsin. J. Exp. Med., 120, (1964): 1189-1200.

21. David, J.: Suppression of delayed hypersensitivity *in vitro* by inhibition of protein synthesis. J. Exp. Med., 122, (1965): 1125-1134.

22. David, J.: Delayed hypersensitivity *in vitro*: Its mediation by cell free substances formed by lymphoid cell-antigen interaction. Proc. Nat. Acad. Sci., 56, (1966): 72-77.

23. David, J., and Schlossman, S.: Immunochemical studies on the specificity of cellular hypersensitivity. J. Exp. Med., 128, (1968): 1451-1459.

24. David, J. R.: Lymphocytic factors in cellular hypersensitivity. In *Immunobiology*, ed. by Good, R. A., and Fisher, D. W. Sinauer Associates, Inc., Stamford, Connecticut 06905, 1971.

25. Eisen, H., Orris, L., and Belman, S.: Elicitation of delayed allergic skin reactions with haptens: The dependence of elicitation on hapten combination with protein. J. Exp. Med., 95, (1952): 473-487.

26. Eisen, H., and Tabachnick, M.: Elicitation of allergic contact dermatitis in the guinea pig. J. Exp. Med., 108, (1958): 773-795.

27. Fauser, I., Purchase, H., Long, P., Velicer, L., Mallmann, V., Fauser, H., and Winegar, G.: Delayed hypersensitivity and leucocyte migration inhibition in chickens with BCG or Marek's disease infection. Avian Path., 2, (1973): 55-61.

28. Gell, P., and Hinde, I.: The histology of the tuberculin reaction and its modification by cortisone. Brit. J. Exp. Path., 32, (1951): 516-529.

29. George, M., and Vaughan, J.: *In vitro* cell migration as a model for delayed hypersensitivity. Proc. Soc. Exp. Biol. Med., 111, (1962): 514-521.

30. Gerloch, E., Scherago, M., and Hall, H.: The effect of chemotherapy on the tuberculin sensitivity of leukocytes from tuberculous patients. Am. Rev. Resp. Dis., 81, (1960): 100-101.

31. Gross, L.: Immunological defect in aged population and its relationship to cancer. Cancer, 18, (1965): 201-204.

32. Hall, H., and Scherago, M.: The sensitivity of human leukocytes to old tuberculin. Am. Rev. Tuberc., 75, (1957): 807-822.

33. Hall, H., and Scherago, M.: The development of leukocytic sensitivity to tuberculin in guinea pigs experimentally infected with *Mycobacterium tuberculosis* H37RV. Am. Rev. Tuberc., 76, (1957): 888-891.

34. Hall, H., and Scherago, M.: The effect of chemotherapy on the leukocytic sensitivity to tuberculin in guinea pigs experimentally infected with *Mycobacterium tuberculosis* H37RV. Am. Rev. Tuberc., 77, (1958): 815-822.

35. Hardt, F., Nerup, J., and Bendixen, G.: Antihepatic cellular hypersensitivity in hepatic cirrhosis. Lancet 1, (1969): 730.

36. Hilberg, R., Balcerzak, S., and LoBuglio, A.: A migration inhibition-factor assay for tumor immunity in man. Cellular Immunology, 7, (1973): 152-158.

37. Johnson, R., and Scherago, M.: The effect of histoplasmin *in vitro* on the migration of leukocytes from guinea pigs experimentally infected with *Histoplasma capsulatum*. Am. Rev. Resp. Dis., 81, (1960): 96-99.

38. Joseph, W., Melewicz, F., and Morton, D.: Delayed cutaneous hypersensitivity to dinitrochlorobenzene as a method of quantitating immunosuppressive therapy for organ transplant. Surgery, 69, (1971): 847-853.

39. Koch, R.: Fortsetzung der Mittheilungen über ein Heilmittel gegen Tuberculose. Deutsche Medicinishe Wochenschrift, 3, (1891): 101-102.

40. Lamb, D., Pilney, F., Kelly, W., and Good, R.: A comparative study of the incidence of anergy in patients with carcinoma, Hodgkin's disease and other lymphomas. J. Immunol., 89, (1962): 555-558.

41. Landsteiner, K., and Chase, M. W.: Experiments on transfer of cutaneous sensitivity to simple compounds. Proc. Soc. Exp. Biol. Med., 49, (1942): 688-690.

42. Lawrence, H. S.: The cellular transfer in humans of delayed cutaneous reactivity to hemolytic *Streptococcus*. J. Immunol., 68, (1952): 159-178.

43. Leu, R., Eddleston, A., Hodden, J., and Good, R.: Mechanism of action of migration inhibition factor. J. Exp. Med., 136, (1972): 589-603.

44. Mallmann, V., Carter, J., Dardas, T., Dardas, H., Fauser, H., and Mallmann, W.: Specific inhibition of radial migration *in vitro* of guinea pig leucocytes by tuberculo-protein. Bact. Proc. Abst. 971, page 86.

45. Moore, B., and Scherago, M.: The effect of histoplasmin on the *in vitro* migration of leukocytes from dogs injected with *Histoplasma capsulatum*. Am. Rev. Resp. Dis., 90, (1964): 806-808.

46. Parodi, A., Fontaine, M., Brion, A., Tisseur, H., and Goret, P.: Mycobacterial infections of domestic carnivores. Rec. Med. Vet., 141, (1965): 421-432.

47. Rich, A., and Lewis, M.: Mechanism of allergy in tuberculosis. Proc. Soc. Exp. Biol. Med., 25, (1928): 596-598.

48. Rewold, H.: Purification and characterization of lymphocyte mediators in cellular immunity. Transplant. Rev., 10, (1972): 152-176.

49. Rocklin, R., Meyers, O., and David, J.: An *in vitro* assay for cellular hypersensitivity in man. J. Immunol., 104, (1970): 95-102.

50. Rocklin, R., Rosen, F., and David, J.: *In vitro* lymphocyte response of patients with immunologic deficiency diseases. New England J. Med., 282, (1970): 1340-1343.

51. Søborg, M.: *In vitro* detection of cellular hypersensitivity in man. Acta Med. Scand., 182, (1967): 167-174.

52. Søborg, M., and Bertram, U.: Cellular hypersensitivity in Sjögren's syndrome. Acta Med. Scand., 184, (1968): 319-322.

53. Søborg, M., and Halberg, P.: Cellular hypersensitivity in Hashimoto's thyroiditis. Acta Med. Scand., 183, (1968): 101-105.

54. Snider, W. R.: Tuberculosis in canine and feline populations. Am. Rev. Resp. Dis., 104, (1971): 877-887.

55. Snider, W. R., Cohen, D., Reif, J. S., Stein, S. C., and Prior, J. E.: Tuberculosis in canine and feline populations. Am. Rev. Resp. Dis., 104, (1971): 866-876.

56. Speer, F., and Dockhorn, R.: *Allergy and Immunology in Children.* Charles C. Thomas, Springfield, Illinois, 1971: 220.

57. Thor, D.: Human delayed hypersensitivity: An *in vitro* correlate and transfer by an RNA extract. Fed. Proc., 27, (1968): 16-20.

58. Turk, J. L., and Oort, J.: A histological study of the early stages of the development of the tuberculin reaction after passive transfer of cells labelled with [^{3}H] thymidine. Immunol., 6, (1963): 140-147.

59. Winkelstein, A.: Cellular immunity *in vitro*: Elaboration of MIF by thymic and marrow lymphoid cells. Proc. Soc. Exp. Biol. Med., 140, (1972): 93-98.

60. Waxman, J., and Lockshin, M.: *In vitro* and *in vivo* cellular immunity in anergic miliary tuberculosis. Am. Rev. Resp. Dis., 107, (1973): 661-664.

61. World Health Organization: *Cell Mediated Immunity and Resistance to Infection.* Technical Series No. 519, Geneva, 1973: 1-64.