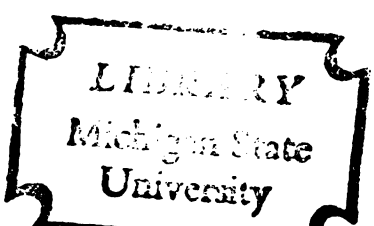

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Virgil Duane Troyer

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EVALUATION OF TRANSFER FACTOR ACTIVITY AND DELAYED-TYPE HYPERSENSITIVITY TO *BRUCELLA* WITH THE MOUSE FOOTPAD ASSAY

By

Virgil Duane Troyer

A THESIS

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ABSTRACT

EVALUATION OF TRANSFER FACTOR ACTIVITY AND DELAYED-TYPE HYPERSENSITIVITY TO BRUCELLA WITH THE MOUSE FOOTPAD ASSAY

By

Virgil Duane Troyer

A footpad assay was developed to study delayed-type hypersensitivity to Brucella in mice treated with bovine and murine dialyzable transfer factor preparations. A nucleoprotein antigen, Brucellergen, elicited cutaneous delayed-type hypersensitivity reactions in infected rabbits and cattle, and positive footpad responses evident at 24 and 48 hours in infected mice. Histological studies of the footpads showed mixed PMN-mononuclear infiltrates at 24 hours and predominantly mononuclear infiltrates at 48 hours. Footpad testing with the antigen dilution used in these studies was shown to sensitize a proportion of mice as demonstrated by swelling in subsequent footpad tests.

There was evidence of transfer activity in one transfer factor preparation from Brucella infected mice. Transfer activity was not consistently demonstrated in other murine transfer factor or whole cell preparations or in bovine transfer factor preparations. Possible explanations for these results are discussed.

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INTRODUCTION

Transfer factor is a dialyzable moiety of lymphoid cell extracts capable of passively transferring delayed-type hypersensitivity from a sensitized individual to a non-sensitized individual. There is also evidence that immunity or resistance to diseases involving the cell mediated immune system may be transferred by transfer factor (95). Originally, descriptions of transfer factor activity were limited to human subjects, but recently a number of animal models have been developed, including some involving cross-species transfer. Of particular interest in this thesis are those involving the bovine and the mouse (67, 87, 130).

The study outlined here was undertaken to develop an assay for the demonstration of delayed-type hypersensitivity to Brucella in mice and subsequently to use that assay in a mouse model to study transfer of delayed hypersensitivity from sensitized bovine and murine animals using dialyzable transfer factor. This model would not only serve as a research tool to study theoretical aspects of transfer factor, but also serve as a convenient in vivo assay of the biological activity of transfer factor preparations before their use in other species or man.

LITERATURE REVIEW

Delayed Type Hypersensitivity

Historical Background

The first description of delayed-type hypersensitivity (DTH) is usually credited to Koch, although the phenomenon may have been first described by Jenner nearly a century earlier in his "reaction of immunity." Koch observed, in 1890, that viable tubercle bacilli or culture filtrates (Old Tuberculin) injected subcutaneously evoked an inflammatory reaction in previously infected guinea pigs that was more intense than the reaction observed in uninfected animals. Mantoux described the reaction elicited by intradermal injection of tuberculin in 1910. Thereafter the tuberculin reaction became a standard test in the detection of tuberculosis. (149)

Several early studies indicated that DTH differed from other forms of hypersensitivity. Zinsser reported in 1925 (163) that DTH or anaphylactic reactivity could be selectively induced by different sensitization procedures, and that DTH was not passively transferred by serum antibodies. This work was supported by the histological studies of Dienes and Mallory (37) who demonstrated the slow development of a predominantly mononuclear infiltrate in the tuberculin reaction as opposed to the rapid development of a polymorphonuclear infiltrate in anaphylactic skin reactions. The role of sensitized lymphoid cells in DTH was established by Landsteiner and Chase

(24, 91) in 1942, in a set of investigations that finally set DTH apart from serum-mediated immunologic reactions. They demonstrated the passive transfer of contact sensitivity and tuberculin sensitivity in guinea pigs by peritoneal exudate cells, but not by serum.

Subsequent investigations demonstrated that DTH could be induced to a variety of bacterial and non-bacterial antigens. *In vivo* manifestations of DTH other than the classic cutaneous reaction have also been demonstrated, including contact sensitivity, allograft rejection, and systemic reactions; and *in vitro* assays that correlate to DTH have been developed (35, 43, 149).

Induction of DTH

DTH may be induced by active immunization or result from an infectious disease process. In either case DTH is induced by factors or processes that differ from those that normally induce the humoral immune response.

DTH is an immune response in a number of infectious diseases, often of a chronic nature involving obligate or facultative intracellular organisms (107). The classic example is tuberculosis, though DTH has been detected in other bacterial, fungal, viral, and parasitic diseases (36, 43, 140, 159). A representative sample of these diseases and the corresponding skin test antigens is listed below:

Disease	Skin Test Antigen
Tuberculosis	PPD
Brucellosis	Brucellergen
Lymphogranuloma Venereum (LGV)	Frei test antigen
Candidiasis	Oidiomycin
Coccidioidomycosis	Coccidioidin
Histoplasmosis	Histoplasmin

Disease	Skin Test Antigen
Blastomycosis	Blastomysin
Mumps	Mumps antigen
Leishmaniasis	Montenegro test antigen

The active induction of classical DTH is dependent on a number of factors, including the chemical nature of the antigen, vehicle for injection, route of injection, and the dose of antigen (43). Zinsser in his early work separating DTH from anaphylaxis (163) demonstrated that injection of viable tubercle bacilli but not of cell-free extracts would induce DTH. Others demonstrated that dead bacilli could also induce DTH, though the degree of sensitivity was less than with viable cells (149). Most antigens inducing DTH are proteins or contain protein, while antigens composed of polysaccharide are ineffectual (43). Antigens of low potency, that are slowly absorbed tend to induce DTH preferentially (132). The reasons why certain antigen-types evoke DTH responses while others evoke humoral responses is still not fully understood (132).

Though soluble proteins alone will not induce DTH, injection of these and other substances in certain adjuvants will evoke DTH reactivity. Dienes discovered in 1926 that systemic DTH to egg albumin and horse serum could be induced by injecting the protein directly into a tuberculous focus (37). This and subsequent observations that injection of antigens in liquid paraffin or vaseline would induce greater sensitivity, led to the development of complete Freund's adjuvant, a water-in-oil emulsion containing tubercle bacilli (149).

The route of injection and the dose of antigen have also been shown to affect induction of DTH. In general the intradermal injection

of antigen is more effective than injection by other routes (132, 43, 32). Smaller doses of antigen favor induction of DTH, while larger doses may stimulate antibody production or even induce tolerance (43).

Dvorak et al. (46), has repeatedly shown that cutaneous basophil hypersensitivity (CBH), formerly called "Jones-Mote" reactivity, may be induced by protein antigens injected with saline or mycobacterium-free adjuvants. Injection of these antigens in complete Freund's adjuvant induces classical DTH. Other differences in the induction and time course of CBH are discussed below.

Contact sensitivity to simple chemical compounds, is usually induced by painting the sensitizing agent on the bare skin of a subject. Contact sensitivity has also been induced however by intradermal injection without adjuvant, and by injection with Freund's adjuvant (149).

In Vivo Expressions of DTH

Cutaneous Reactivity

Macroscopic

Systemic DTH reactivity can be demonstrated locally by the intradermal injection of the specific antigen. Macroscopically the reaction is undetectable for several hours. Erythema and induration then appear at 6-12 hours, reaching a maximum at 24-48 hours (43, 47). Reactions in humans usually reach maximum intensity at 48 hours, whereas the reaction in some animals, such as guinea pigs (45, 46, 149) and mice (31, 52), have been maximal at 24 hours. The reactions subside slowly, depending on the intensity at the peak, and severe reactions may show a central area of necrosis (43, 132). The delayed

onset, late peak, and palpable induration are characteristics of the reaction that set DTH apart from antibody mediated hypersensitivity reactions.

Microscopic

The microscopic picture of the DTH reaction is even more characteristic and usefull in distinguishing humoral from cell mediated DTH reactions. Dienes and Mallory (37) demonstrated early on that the DTH reaction was characterized by a predominantly mononuclear infiltrate, whereas the humoral (anaphylactic) reactions were composed primarily of PMN's. This basic definition has held to today, though there has been considerable controversy regarding the relative proportions of mononuclear cell types, the presence and role of non-mononuclear cell-types, and the factors involved in the evolution of the local cutaneous DTH reaction. Some of this confusion is due to conclusions drawn from biopsies taken at different times after antigen injection and experiments done on different species. The guinea pig and man, for example, display similar macroscopic DTH reactions, although the histology of the reactions differs markedly (149). Variations in preparation and staining of tissue samples has also yielded significantly different histological pictures (149), in one case revealing a cell type not previously evident (45, 46).

Two investigators, Turk and Dvorak, have conducted extensive studies of the histology of the DTH reactions in guinea pigs and humans. Their descriptions and conclusions will be primarily reviewed below. Some of the problems outlined above will be evident.

Prior to Turk's studies in 1965-1967 attempts were made to

distinguish lymphocyte and monocyte cell types on morphological characteristics. It was felt however, that these distinctions were artificial and influenced by variations in the tissue section preparation as well as variations in the tissue itself (149). Turk and Diengdoh (150) developed a staining method to distinguish macrophages from lymphocytes by the number of cytoplasmic granules staining for acid phosphatase. They stained frozen sections with an acid phosphatase-methyl green pyronin stain. Macrophages and PMN's both contained numerous stained granules, but could be distinguished from each other by nuclear shape. Lymphocytes showed few or no acid phosphatase staining granules.

Using this staining technique, Turk et al. (150) examined the histology of skin reactions in guinea pigs at 4, 8, 24, and 48 hour intervals after intradermal injection of a variety of antigens. They found that granulocytes predominated in the reaction at 4 hours, comprising 60-70% of the perivascular infiltrate. After this time they formed 25% or less of the cells. At the peak of the reaction, 24 hours, macrophages formed 50-60% of the mononuclear cells in the infiltrate, but by 48 hours they formed only 30% of the mononuclear cells, the remaining 70% being lymphocytes.

A similar study was conducted on humans by Turk's group (151). Antigen was injected at two skin sites, which were biopsied at 24 and 48 hours respectively. The dilution of antigen, 1:10,000 Old Tuberculin, was chosen to yield minimal non-specific reactions and epidermal damage. Granulocytes were not seen to any extent. Turk attributed this to differences between guinea pigs and humans, but the times of

biopsy and the use of a non-irritating dilution of antigen may have contributed to this observation. Acid-phosphatase staining cells, Turk's definition of macrophages, comprised only 10-13% of the mononuclear perivascular infiltrate at both 24 and 48 hours. It was observed, as reported by others, that capillaries and venules were filled with mononuclear cells (149).

Dvorak and his colleagues developed a tissue fixation method designed primarily to preserve basophilic granulocytes (45, 46). Tissues were post-fixed in osmium tetroxide and 1μm, Epon-embedded sections were stained with Giemsa stain for light microscopic evaluation. Using this technique, Dvorak was able to demonstrate the presence of basophils, not detectable by normal tissue preparation methods, in certain types of DTH reactions.

Dvorak's group used this tissue preparation method in extensive studies of classic DTH in guinea pigs and human subjects, partially to evaluate the presence of basophils in these reaction-types. Distinctions between macrophage and lymphocyte mononuclear cells were made on a morphological basis, though frequently no distinction was made.

In the guinea pig (45, 46), they found a perivascular infiltrate of mononuclear cells beginning at 6 hours and continuing in intensity to 24 hours. PMN's comprised only a minority of cells during any time in the reaction, though this was found to be dependent on a low dose of skin-test antigen. Basophils were found in significant numbers in these classic DTH reactions, particularly if the interval between immunization and skin test was relatively short. In one study (46), skin tests at 1 week post-immunization

contained 13-25% basophils, whereas skin tests at 6 weeks contained less than 7%.

Dvorak et al. (47) conducted an extensive study of CBH, contact sensitivity, and classic DTH reactions in human subjects, using light and electron microscopy and immunoflourescent techniques. In the studies of classic DTH to several microbial antigens, biopsies were taken at 48 hours after antigen injection. As before, an intense perivascular infiltrate of mononuclear cells was observed, composed mostly of small or activated lymphocytes with a smaller proportion of macrophages. Basophils were observed in 61% of the reactions, but in relatively low numbers in all but 19% of the reactions. Neutrophils and eosinophils were noted, but in low numbers in most reactions. Mast cells at the reaction site exhibited variably reduced numbers of cytoplasmic granules. This "degranulation" was much less extensive than that seen in antibody-mediated allergic skin reactions, but was a constant feature of the test reactions.

Significantly new findings were also reported from this study (47) regarding the presence and consequences of enhanced vascular permeability in DTH reactions. These findings were described in detail in a serial biopsy study of contact dermatitis, though similar phenomena were seen in the classic DTH reactions biopsied at 48 hours. The microvasculature of the reaction sites exhibited compaction and congestion with tight intravascular packing of erythrocytes. Electron microscopy often revealed large gaps between endothelial cells of the affected venules, through which plasma and less frequently erythrocytes were extravasated into the perivascular space. Intervascular deposition of fibrin, detected by flourescent antibody,

was evident in nearly all reactions at 24 hours. It was suggested that vascular leakage of fibrinogen and other clotting factors led to this deposition of fibrin. It should be noted that immunoglobulin and complement (C_3) deposits were not detected by immunoflourescent techniques in these reactions.

From the evidence presented in these studies, Dvorak has proposed that mast cells and/or basophils are stimulated by lymphokines released at the test site from antigen-stimulated lymphocytes to release a variety of vasoactive agents which mediate the observed vascular permeability changes (47). The possible role of mast cell products in DTH skin reactions in the mouse has also been suggested by Gershon et al. (50).

Few histological studies have been done on DTH reactions after 48 hours. Several studies of the tuberculin reaction in animals have shown typical tuberculous foci containing epithelioid cells, giant cells, and small lymphocytes. Some of these cells may arise as a consequence of tissue damaged during the immunological reaction (149).

Effector Cells

In addition to the histological studies outlined above, which demonstrate the cell-types present at the reaction site, considerable research has been done to determine the origin and role of the "effector" cells in the cutaneous DTH reaction. In a series of classic investigations by McCluskey et al.(27, 111), it was demonstrated that the vast majority of infiltrating cells in the skin reaction site lacked specificity for the antigen used to elicit the reaction. When lymph node cells from specifically immunized donors, labeled with

^{3}H-thymidine, were used to passively transfer DTH to non-reactive guinea pigs, less than 8% of the mononuclear cells at the test site were labeled. In other experiments, separate donors were immunized to non-cross reacting antigens and the donor cells of only one specificity were labeled. Non-sensitized animals received ^{3}H-thymidine labeled cells for one specificity and unlabeled cells for the other specificity. Subsequent skin tests by both antigens at separate sites revealed equivalent accumulation of the labeled cells at both test sites. When unsensitized recipients were repeatedly injected with ^{3}H-thymidine before receiving unlabeled donor cells, the great majority of cells at the test site were labeled. These studies suggest that the DTH reaction is initiated by the arrival of a small number of specifically sensitized cells at the antigen injection site, but that these antigen specific cells are not in themselves the cells responsible for the macroscopic induration or the microscopic cellular infiltration.

Using a modification of McCluskey's model, Lubaroff and Waksman demonstrated that a large percentage of the non-specific infiltrating cells were blood-borne bone-marrow derived cells (105, 106). They passively transferred DTH to unsensitized, thymectomized, heavily irradiated rats. Maximal reactions were only achieved when bone marrow cells, from normal or sensitized rats, were administered to the recipients prior to the lymph node cells from the sensitized donor (105). Using an immunoflourescent technique to identify bone marrow and lymph node cells from genetically different donors, approximately 75% of the cells at the test site were shown to be from the bone marrow donor and approximately 25% from the sensitized

lymph node cell donor (106). A similar participation by non-sensitized bone marrow cells in the DTH response was reported by Youdim et al. (160) in immune irradiated mice that responded to antigen injection only after administration of normal bone marrow cells. Irradiation had been shown to abrogate DTH reactivity, but not destroy the specific antigen-reactive cells.

The role of a relatively few antigen-reactive lymphocytes in the DTH response is also indicated by results reported by David (35) and Bloom and Bennett (12) using the in vitro macrophage migration inhibition assay. David initially demonstrated that as few as 2.5% peritoneal exudate cells from sensitized guinea pigs mediated the migration inhibition. Bloom and Bennett subsequently separated lymphocytes and macrophages from PEC suspensions and showed that as few as 0.6% purified sensitive lymphocytes would cause significant antigen-dependent macrophage migration inhibition, but that purified macrophages from sensitive guinea pigs exhibited no inhibition in the presence of antigen.

The role of thymus-derived lymphocytes (T-cells) as effector cells in DTH responses is indicated by the failure of athymic mice to develop or display DTH reactivity (122, 161) and by the observed cellular proliferation in T-cell rich paracortical areas of lymph nodes following sensitization (149). In studies using local passive transfer of DTH with PEC in guinea pigs, Turk and Polak demonstrated initially that lymphocytes, but not macrophages were responsible for the transfer (152). Jaffer et al. (61) used the same model to demonstrate that T-cells and not B-cells were primarily responsible for the DTH transfer. They used a lymphocyte population containing

less than 5% B-cells, prepared by passing PEC through an anti-immunoglobulin column. Successful transfer was achieved by these T-cells as evaluated both macro- and microscopically. Attempts to transfer DTH with the column-retained B-cells, released by dextranase digestion of the support, could not be evaluated due to non-specific inflammation induced by residues in the effluent.

Youdim et al. demonstrated in a series of experiments in mice that treatment of sensitized lymphoid cells with anti-theta serum and complement, a treatment that lyses thymus-derived cells, abolished their capacity to transfer DTH to non-immune mice (160, 161). Immune thymocytes alone did not transfer DTH in these studies.

Cutaneous Basophil Hypersensitivity

Raffel and Newel (125) conducted investigations in 1958 on a delayed-type reaction induced by injecting antigen-antibody complexes in saline or mycobacterium-free adjuvants. They suggested that the sensitivity induced was not only different than Arthus and immediate-type hypersensitivity, but significantly different than classic DTH because of its early appearance (5-7 days) after immunization and disappearance at a time (16-20 days) when DTH reactions are reaching their maximum reactivity and antibody production is beginning. They suggested the term "Jones-Mote" reaction, in reference to a report 20 years earlier by Jones and Mote (70) of a similar sensitivity in humans induced by repeated intradermal injection of rabbit serum proteins without adjuvant. Controversy continued regarding this distinction (148) until Dvorak et al. demonstrated that these reactions could indeed be distinguished by extensive infiltration of

basophilic granulocytes in addition to mononuclear cells at the test site (45, 46). As noted above, Dvorak's detection of basophils was accomplished by a fixation technique that preserved the previously undetected basophil. He suggested the term "cutaneous basophil hypersensitivity" (CBH) to denote the reaction. CBH was also found to be induced by injection of soluble proteins without mycobacterial adjuvants, to display relatively non-indurated reactions with significantly less change in vascular permeability and fibrin deposition then seen with classic DTH, and to be resistant to carrageenan injection, a treatment that markedly diminishes classic tuberculin DTH (44, 45, 46, 128).

CBH resembles DTH in that the cutaneous reaction is delayed, reaching maximum intensity at 24-48 hours. CBH is also lymphocyte mediated, being sensitive to treatment by anti-lymphocyte serum (128) and being passively transferred by viable sensitized lymphocytes (46).

The role of the basophil in the CBH reaction has not been established. Basophils do not bind or ingest specific antigen at the test site (46). Overt basophil degranulation by explosive release of granules has not been detected, though careful light- and electron-microscopic studies have shown evidence for a slow "piecemeal" release of granules from basophils at the reaction site over a course of several days (42).

Contact Skin Sensitivity

Contact skin sensitivity has been induced in guinea pigs and man to a variety of simple chemical compounds, the most commonly

used being picryl chloride, dinitrochlorobenzene (DNCB) and dinitrofluorobenzene (DNFB) (149). Contact skin sensitivity is responsible for a variety of skin allergies in man, such as to poison ivy, cosmetics, drugs, and synthetic chemicals (36). There is evidence that these simple compounds may act as haptens and attach to skin proteins, which act as carriers (149). Sensitization can be accomplished by percutaneous application, intradermal injection, or injection by other routes if complete Freund's adjuvant is employed.

The reaction, elicited by application of the sensitizing compound at a skin test site distant from that used for sensitization, is characterized by a delayed onset of erythema, induration, and occasional vesiculation. Maximum intensity is displayed at 24 hours in the guinea pig and 48-72 hours or longer in man. Microscopically, the cellular infiltrate is much like that seen with intradermally injected antigen, with the exception that the mononuclear cell infiltrate extends into the epidermis (47, 149).

Systemic Reactions

Cutaneous DTH reactions, though elicited locally, represent a systemic hypersensitive state. When specific antigen is administered systemically, generalized hypersensitive reactions can be observed. Koch first observed this in 1890, when he saw a rise in temperature in tuberculous human subjects after subcutaneous injection of Old Tuberculin (149). This febrile reaction has been demonstrated in humans and animals by other investigators (31, 43, 149) and may be accompanied by other symptoms such as, prostration, malaise, irregular breathing, and lethargy. It has been

demonstrated that these reactions are neither examples of endotoxin fever nor anaphylactic shock (149). In highly sensitive subjects, inhalation of aerosols of the antigen may cause systemic reactions, often resembling an acute attack by the disease agent itself (113).

Biological Significance of DTH

DTH reactivity correlates closely to a number of cell-mediated immune phenomena. However, the role of the DTH reaction in these phenomena or the distinction between DTH and the phenomena has been the subject of much debate.

For many years the allergic reactivity of DTH was thought to be responsible for cell mediated immunity (CMI) to infection. The role of DTH in CMI is supported by the emergence of DTH reactivity at a time when infecting microorganisms are being eliminated (10, 107, 160). Transfer of CMI and DTH are both achieved using lymphocytes, and immunization techniques that induce antibody formation but not DTH confer no CMI protection (43, 149).

Accumulating evidence however, indicates that DTH and CMI do not always correlate. Sensitive animals may be desensitized by repeated injection of antigen and found to retain CMI to infection, though DTH can no longer be demonstrated. Immunization techniques that induce selectively DTH or CMI but not both, have offered strong evidence that the two mechanisms are indeed separate (59, 149, 162).

It has been demonstrated that allograft rejection is mediated by an immunologically specific reaction to surface antigens on cells of the grafted tissue. That DTH is involved in allograft rejection

is indicated by the extensive mononuclear infiltration into the grafted tissue (43, 149). Accelerated graft rejection can be passively transferred by viable lymphoid cells (43, 149) or transfer factor (95), but not serum. Typical cutaneous DTH reactions can also be elicited by the intradermal injection of cells to an animal that previously received a graft from the cell donor (149).

Antibody is also induced by grafted tissue and may cause complement-dependent cytotoxicity for suspensions of target cells, but the role of antibody in direct graft rejection is thought to be minor. In fact specific antibody may in some instances enhance graft survival (43).

The evidence for participation of DTH in auto-immune diseases is like that for allograft rejection: prominant mononuclear infiltrates and passive transfer, in some diseases, with living lymphoid cells. The pathology and mechanisms of these diseases are however not well understood and the relative roles of cellular and humoral factors are not to date readily separated (135, 149).

In Vitro Assays of DTH

In vitro assays of DTH were developed when it was observed that sensitized lymphocytes release a number of factors (lymphokines) following stimulation with specific antigen in vitro. These lymphokines mediated in vitro effects on other cell-types including migration inhibition of macrophages and PMN's; aggregation of macrophages; chemotactic attraction of PMN's, monocytes, and lymphocytes; stimulation of blastogenesis; cytotoxic changes; and activation of macrophages (35, 43, 133). Some of these activities correlate well with

in vivo DTH reactivity. They may also offer explanations for the mechanisms of the DTH reaction, though the functions measured in vitro may not correspond exactly to the roles of these lymphokines in the more complex in vivo situation (35). Three in vitro systems used most frequently to assay DTH, including DTH in recipients of transfer factor, are reviewed below.

Macrophage Migration Inhibition

Migration inhibition of macrophages is mediated by migration inhibition factor (MIF), a glycoprotein of 35,000-55,000 molecular weight, released by specifically sensitized lymphocytes incubated with antigen in vitro (12, 35, 43). Since MIF production correlates well with DTH cutaneous reactivity, it has been assumed the factor is released from T-lymphocytes. Recent studies, however, have demonstrated that MIF is produced by both T- and B-cells (25).

MIF production is assayed in laboratory animals by packing peritoneal exudate cells (PEC) into capillary tubes, incubating with and without antigen, and comparing the subsequent fan-like migration of macrophages from the end of the tube. For human studies, peripheral blood lymphocytes are usually incubated with antigen in vitro, the MIF is collected from the supernatant, and subsequently assayed on normal guinea pig PEC in capillary tubes (2, 35, 141).

Leukocyte Migration Inhibition in Agarose

Leukocyte migration inhibition in agarose (LMIA) is an assay for the production of a lymphokine, leukocyte inhibition factor (LIF), which specifically inhibits the migration of PMN's. LIF can be separated from MIF in supernatants of antigen stimulated lymphocytes

by gel filtration, and does not inhibit migration of human monocytes or guinea pig macrophages (57, 133). LIF production is assayed by placing peripheral blood leukocytes, preincubated *in vitro* with antigen, in wells punched in agarose. Migration, or inhibition of migration, of PMN's radially from the well is readily visualized (26). The assay correlates well with cutaneous DTH reactivity, requires fewer cells, and has been shown to be more sensitive than the MIF assay (26, 133).

Blastogenesis

Lymphocytes from sensitized animals or humans incubated with antigen *in vitro* undergo morphological changes accompanied by increased protein, RNA, and DNA synthesis (141). These changes can be detected by measuring the incorporation of labeled precursors added to the cultures several days after stimulation with antigen. Enhanced DNA synthesis is measured by incorporation of ^{3}H-thymidine (141) and enhanced protein synthesis by incorporation of ^{3}H-leucine (88). These lymphocyte stimulation assays correspond to the CMI status, and in general to the DTH status, of the test subjects (71, 88).

Delayed Hypersensitivity in Brucellosis

Brucellosis is an acute or chronic infectious disease caused by one of several species of *Brucella*. Each species of *Brucella* causes disease in a preferred domestic animal host, though the disease is transmissable to man and a number of laboratory animals. The diseases are characterized in the primary animal host by abortion of pregnant females, low mortality, relatively nonapparent clinical

infections, and chronicity (113).

Brucella is a facultative intracellular parasite, and studies in vitro and in vivo indicate that the host's ability to control the intracellular growth of the organism determines the course of the disease (59, 113, 121). The immune response to brucellosis is both humoral and cell mediated (114). DTH to bacterial antigens is a manifestation of the cell mediated response and will be considered here.

DTH is displayed by most humans infected by Brucella, being absent in about 5% of culturally proven cases (114). Though the prevalence of DTH in infected domestic animals is not as well known, DTH has been demonstrated in a number of experimentally infected domestic and laboratory animals including cattle (88), sheep (69), guinea pigs (8, 59, 68), rabbits (55, 142), and mice (107, 138). Experience has indicated that DTH is not usually induced by killed Brucella cells or fractions (8, 59) but requires actual multiplication of organisms within the host (113). Skin reactivity is usually detectable after one or more weeks of infection (8, 107, 113) and has been demonstrated to persist for years in humans (114) and for months in guinea pigs (8). The level of skin reactivity may diminish during acute stages of the disease, as demonstrated by Mackaness (107) in mice. In a serial study of DTH reactivity and growth of Brucella organisms in the spleens of infected mice, the level of reactivity initially rose and then diminished as the Brucella population continued to increase. In the ensuing period of bacterial inactivation the DTH reactivity rose again and remained at a high level. A similar biphasic response has been seen in BCG-infected mice (10).

DTH to Brucella is usually assayed by intradermal injection of antigen. The reaction is highly specific for the genus, but species specificity is not detected by the individual skin test. Antigen preparations from a single species have elicited reactions in animals infected by homologous or heterologous species of Brucella, (8, 68, 142), though some investigators have found the homologous reaction to be stronger (8). The skin reaction generally is not diagnostically useful, since a positive reaction does not distinguish between past and recent exposure to the organism. Rather, serum agglutination titers are used extensively in human and animal diagnosis of brucellosis (114).

A variety of antigen preparations have been used to elicit the cutaneous DTH reaction. Heat-killed suspensions of whole cells have been used in animals (59) but are not recommended for human use (114). Culture filtrates have also been used in animals (107). A variety of cell fractions prepared by physical or chemical disruption of cells and purification by extraction have been successfully used experimentally (1, 8, 9, 68, 138). A nucleoprotein fraction, Brucellergen, was developed by Huddleson (60) in the 1930's, and subsequently made available commercially for use in humans. Brucellergen is prepared by grinding ether-extracted Brucella organisms to a fine powder with subsequent purification by acid precipitation of the nucleoprotein components (60). Shortly after its development, Stahl (142) studied the chemical constitution and biological properties of the protein nucleate and of the separated protein and nucleic acid components. Protein composed about 70% of the whole protein nucleate and was shown to be the active component in eliciting the DTH cutaneous

reaction. The nucleic acid component contained guanine, adenine, and cytosine; and both pentose and desoxypentose sugars. The nucleic acid component did not elicit a DTH reaction. Antibody production in guinea pigs was stimulated by the intraperitoneal injection of relatively large amounts of either the protein nucleate or the purified protein component, but not by injection of the nucleic acid.

In vitro assays of DTH to Brucella have not been used extensively. Migration inhibition studies of macrophages and peripheral blood leukocytes have been conducted in laboratory animals and humans (34, 35, 55, 138). Blastogenesis assays have been utilized extensively in recent studies in experimental animal brucellosis (71, 88).

Delayed Hypersensitivity in the Mouse

For some time it was generally held that DTH sensitivity was not expressed by mice. Though there was available indirect evidence for DTH, investigators repeatedly failed to elicit typical cutaneous reactions by the intradermal injection of antigen (33, 52, 59). Crowle first demonstrated in 1959 that DTH reactivity could be elicited by intradermal injection of ovalbumin (30) or tuberculoprotein (31) in sensitized mice. That this was DTH was demonstrated by passive transfer of the reactivity with lymphoid cells but not serum. The cutaneous reaction was also correlated to a number of other DTH reactions in mice (31). Both of these aspects have recently been confirmed (129). Crowle suggested that some of the previous problems arose from injection of relatively large volumes of antigen suspension into mouse skin, which is very thin,and that such injections

may leak into subcutaneous tissues and fail to elicit the skin reaction (33). He describes a technique using careful intradermal injection of 0.01 ml of concentrated antigen into anesthetized mice under a stereo microscope (31). Few investigators other than Crowle have reported successful use of the skin test to detect DTH in mice, probably due to the development of the easier-to-use footpad assay.

The footpad assay as originally described by Gray and Jennings in 1955 (52) to detect DTH in mice has been used predominantly since that time. Antigen is injected into the metatarsal pads in volumes of 0.01-0.05 ml using 26- to 30-gauge needles. The subsequent swelling at 24 to 48 hours is evaluated subjectively (31, 52) or measured by calipers (107, 129), fluid displacement (160, 118), or the weight of foot replicas (144). Values are usually expressed as a comparison of the antigen injected foot to the opposite foot, which is untreated or injected with antigen solvent alone.

The macroscopic reaction, swelling and erythema, is often evident by 6 hours and reaches maximum intensity by 24 hours (33, 72, 129). The 24 hour reaction is frequently more intense than the 48 hour reaction, and is usually reported (31, 52). The histologic reaction is much like that seen in other species, with a predominantly mononuclear infiltrate evident by 24 hours and more intense at 48 hours (33, 72, 129). Significant numbers of PMN's are seen early in the reaction and are often present at 24 hours (33) particularly directly at the site of antigen injection (129) or with a preceding Arthus reaction (72).

The time course and histologic picture presented above are evidence that the footpad reaction is a DTH reaction. Further

evidence is provided by the transfer of this reaction to other mice using lymphoid cells (33, 129, 160) and transfer factor (14, 130, 131). The footpad reaction has also been shown to correspond to the skin test reaction in the mouse (31, 130).

Various explanations have been offered for the frequently observed discrepancy between skin test and footpad reactivity. Certainly the footpad injection is technically easier to perform and the reaction is more evident, since skin test reactions in the mouse are not erythematous (31, 130). Crowle has reported that the footpad reaction may be more sensitive because the antigen is retained in the footpad longer than in the skin (33). The importance of antigen retention and use of particulate antigens in the mouse has been recently pointed out (72, 129). Gershon et al. (50) have proposed that the discrepancy is due to a significantly higher proportion of mast cells in the footpad than in the flank skin of the mouse. They present evidence that expression of DTH in the mouse is dependent on mast cell activity, and suggest that in the mouse, a species deficient in circulating basophils, these mast cells may substitute for the basophil activity proposed by Dvorak (47) in DTH reactions of other species.

Other expressions of DTH have been demonstrated in mice, including contact sensitivity, systemic sensitivity, allograft rejection, and reactivity in in vitro assays (31, 33, 138). There is little doubt that DTH exists in mice, though its detection by intradermal injection of antigen may be difficult to demonstrate, and though the degree of sensitivity may be significantly less than that seen in man or guinea pigs (33).

Transfer Factor

Historical Background

The first apparent transfer of delayed-type hypersensitivity (DTH) was reported in 1909, in guinea pigs, using defibrinated blood (13). But it was not until 1942, that Landsteiner and Chase (24, 91) established the principle of passive transfer of DTH by using living cells from immune guinea pigs. Lawrence demonstrated transfer of DTH to non-sensitive humans in 1949 using viable blood leukocytes from immunized humans (92), and subsequently observed that extracts of frozen and thawed leukocytes were as effective as viable cells (93). The term transfer factor (TF) was coined to designate the material responsible for the transfer of DTH, with the acknowledgment that more than one factor might be responsible (95). The finding that TF was dialyzable (98) greatly facilitated purification and identification of the components present in TF. Dialyzed transfer factor (TFd)* could now be prepared free of non-dialyzable leukocyte constituents, including transplantation antigens.

Though TF activity was demonstrated repeatedly by Lawrence and others in experiments involving humans, the failure to demonstrate a similar phenomenon in animal models and the lack of a reliable *in vitro* model caused considerable skepticism (22, 108). Fifteen years after Lawrence first described TF, it was shown to have a therapeutic effect on patients with the Wiskott-Aldrich syndrome (140). This

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*In this review TF will refer to dialyzed or non-dialyzed transfer factor, TFd will refer specifically to dialyzed transfer factor.

and subsequent observations of benefit in other selected clinical problems sparked a renewed interest in TF research, resulting in increased activity in the analysis of TF, therapeutic trials, and attempts to develop animal models and in vitro assays for TF.

Physical Properties of Transfer Factor

Transfer factor is a soluble dialyzable moiety of leukocyte extracts, generally held to be less than 10,000 molecular weight (95). The TF activity is resistant to enzyme treatment with ribonulcease or deoxyribonuclease, trypsin, and lysosomal hydrolases (76, 93, 95, 130), but is destroyed by pronase and snake venom phosphodiesterase (76, 130). Rifkind has recently demonstrated that mouse TFd activity, though resistant to monomeric ribonuclease, is sensitive to dimerized ribonuclease, an enzyme active against double- as well as single-stranded RNA (131). Other investigators have shown that a guinea pig TFd is destroyed by RNase III, an enzyme that specifically degrades double-stranded RNA, but is not affected by RNase enzymes active against single-stranded RNA (38).

Transfer factor has been found to be quite stable under various storage conditions. It is labile to treatment at 56°C for 30 minutes but stable at 25°C or 37°C for as long as 6 hours (95). TF in frozen whole leucocyte preparations stored at -20°C for 4 years and subsequently prepared as usual for TFd, was found to retain its activity (99). Lyophilized, TFd can be stored as a powder for at least 5 years at 4°C without loss of potency (99).

The dialysate of crude leukocyte lysates contains a complex mixture of substances, including products of enzymatic breakdown of

cellular materials (22). It is probable that only some of these are responsible for the immunologic transfer activities, other substances being inactive or acting to suppress or modify these activities (20, 51). Though much work has been devoted to analyzing these substances in an effort to identify the active components, the material(s) responsible for *in vivo* activity have not to date been identified.

Most investigations have shown the presence of ribose, polypeptides, and polynucleotides (6, 84, 95). Others have reported the presence of RNA bases, hexose, hypoxanthine, tyrosine, nicotinamide, serotonin, histamine, ascorbate, and lipid phosphorous (20, 78, 80, 90, 131). Some of these components have been shown by selective elimination not to mediate the immunologic *in vivo* activity of TF, while others have been shown to be responsible for non-specific phenomena (19, 20, 80, 155).

Lawrence demonstrated that TFd does not contain detectable immunoglobulin (95). He added either Bence-Jones protein or papain-digested gamma-globulin fragments to the lysate before dialysis in a procedure to monitor the integrity of the dialysis membrane. Analysis of the dialysate by 10% trichloroacetic acid and by immunodiffusion tests revealed no detectable albumin, alpha- or gamma-globulin, or other protein.

The presence of antigenic substances is not expected in TF preparations that have been dialyzed. Attempts by Lawrence to raise antibody to TFd injected in Freund's adjuvant have been unsuccessful. Lawrence also demonstrated the failure of TFd to sensitize recipients to the HLA antigens of the TFd donor, as measured by either skin allograft rejection or humoral antibody determinations (95). This,

in view of the fact that TFd can transfer accelerated allograft rejection from an actively sensitized donor (95), indicates the absence of HLA antigens in the dialysate. Burger has also reported the absence of superantigen properties in guinea pig TFd (17).

Several investigators have demonstrated serial transfer of DTH by TF (93, 79, 87). TF prepared from the cells of one individual transferred DTH reactivity to an anergic recipient and TF subsequently prepared from that recipient transferred DTH reactivity to a third individual. The dilutional aspects of these experiments argue against the role of antigen or immunoglobulin in TF preparations.

Preparation of Transfer Factor

Human

Lawrence and associates established the basic methods for preparation of dialyzable transfer factor from human sources in their early work (99). Refinements of these techniques have been introduced by others (18, 141). There are six stages in the preparation procedure: (1) leukocyte preparation, (2) leukocyte lysis, (3) dialysis, (4) lyophilization, (5) reconstitution, and (6) filter sterilization prior to clinical use. As will be seen below there is considerable variation of technique in each of these stages, which has however not led to obvious differences in *in vivo* activity.

Leukocyte Preparation

Transfer factor donors are selected on the same criteria as donors for blood transfusion: good health, adequate hematocrit, free of blood-borne diseases and no evidence of hepatitis (99, 141). An additional criterion, the most important condition for successful

transfer, is that the donor have a marked degree of delayed cutaneous reactivity to the antigen(s) under study (99, 141). This reactivity may be due to natural sensitization or elicited by active immunization.

Human TF is usually prepared from peripheral blood leukocytes. Venous blood (up to 500 ml) is drawn into syringes or tubes containing heparin or EDTA anticoagulant (49, 99, 141). A 500 ml blood-donor bag (Fenwal Bag) may be more convenient for larger volumes of blood, but it has been shown that the leukocyte cell yield is significantly lower per volume of blood (141). Leukopheresis may be used when a large number of leukocytes are desired, as from a donor with a rare immunity. This procedure can be repeated on the same donor more frequently and yields a much higher number of leukocytes (49, 141), which are predominantly mononuclear (80). The safety, convenience, and efficiency of this process, in fact, makes it a more practical method of cell collection for all forms of TF (49).

Leukocytes are separated from other blood components by one of several methods. Whole cell suspensions containing leukocytes, erythrocytes, and platlets are occasionally used (78), though the leukocyte preparations are usually more purified. Lawrence (99) and other investigators (104, 141) mix whole blood with high molecular weight substances, such as dextran, bovine fibrinogen fraction I, or polyvinylpyrrolidone, to accelerate erythrocyte sedimentation. The leukocyte-rich, erythrocyte-free plasma is then further processed. More recently whole blood has been layered over Hypaque-Ficoll density gradients to yield leukocyte suspensions containing 60-90% lymphocytes and 10-30% monocytes, with few granulocytes (78). After

processing, the leukocytes are suspended in either distilled water (4, 80, 104) or saline (99, 141) prior to freeze-thaw lysis.

Lysis of Leukocytes

Lawrence demonstrated that TF could be released from leukocytes by distilled water lysis or by 7 to 10 cycles of freezing and thawing, in a dry-ice-alcohol bath and a 37°C water bath respectively (93). Since that time the freeze-thaw method has been universally used as a convenient, reproducible method to lyse leukocyte preparations. This process releases intracellular DNA which forms a large gelatinous mass. DNAse and magnesium sulfate are added by most investigators (49, 99), though not all (49, 80) to liquify this mass for easier handling. This treatment has been shown to not affect the *in vivo* activity of TF (93). The enzyme may be added before or after the freeze-thaw procedure. The resulting lysate contains TF activity and represents the preparation originally used by Lawrence to transfer DTH in humans.

Dialysis

Lawrence found in 1963 that dialysates of TF were as effective as the whole lysate in transferring DTH (98). This has been confirmed by several groups of investigators (see 99) and TF preparation now usually includes dialysis, which yields a product free of the host of large molecular weight substances in cells, including immunoglobulins and HLA antigens (95, 99). TFd is thus more amenable to chemical characterization and represents a pharmacologically safer agent for clinical use.

Various methods of dialysis have been employed. Though some

variation in fractionation profiles on gel chromatography has resulted, no change in *in vivo* activity has been detected due to these variations (7).

In methods employing dialysis tubing, the lysate is placed inside a dialysis sac, avoiding contamination of the outside surface. Dialysis is then carried out against sterile distilled water (49, 99, 141), saline (99), NH_4HCO_3 (78), or tissue culture medium (4). The latter procedure was found to yield a preparation free of a factor found in water-dialyzed preparations to be toxic to lymphocytes *in vitro* (4). Dialysis is usually done at 4°C with agitation for time periods of 18-24 hours.

A procedure utilizing a vacuum dialysis apparatus (18) has been successfully used to prepare TFd. This process takes less time (4-8 hours) and eliminates the need to concentrate the dialysate by lyophilization and reconstitution. Ultrafiltration techniques have also been successfully utilized in the processing of large quantities of TFd (7, 54).

Lyophilization, Reconstitution, and Filter Sterilization

Preparations dialyzed into distilled water, saline, or NH_4HCO_3 (see above) are concentrated by lyophilization. The resultant powder can be conveniently stored at 4°C for an extended time (99), or reconstituted, filter sterilized and stored frozen until use (4, 80, 141). The powder may be reconstituted in water (4, 99, 80) or saline (141) and can be reconstituted to an arbitrary lymphocyte-equivalent concentration (80).

Animal

Preparation methods for TF from animals do not vary greatly from those used for human TF with the exception of cell source and release of TF from the cells.

Peripheral leukocytes have been utilized from animals, especially in those species large enough to provide adequate volumes of blood (64, 67, 109). Other lymphoid organs however are frequently used as sources of animal TF. These include: lymph nodes (14, 15, 41, 86), spleen (130), peritoneal exudate (15, 41) and alveolar washings (15).

The release of TF from these cells has been achieved by several methods other than the freeze-thaw procedure of Lawrence, though this method remains in wide usage (67, 130). Burger found that TF activity was released from guinea pig (15) or rabbit (16) lymphoid cells when these cells were incubated in Hank's balanced salt solution at 37°C for four hours, with no control on the pH of the medium. Concomitant with decrease in pH and cellular viability, TF activity could be detected in the supernatant fluids and was found to decrease in the cellular material. Klesius has applied this procedure to bovine lymph node cells (86), and found it to be a practical method to produce large quantities of TF. The supernatant fluid can be dialyzed and processed as any cell lysate preparation (15, 86). Jeter's group has also demonstrated the release of a TF-like activity into the plasma of guinea pigs treated *in vivo* with anti-lymphocyte serum (117).

Manifestations of Transfer Factor

Specificity and De Novo Sensitization

The question of de novo sensitization of an immunologically naive recipient by TF and the antigen specificity of that transfer has not been unequivocally resolved. A major problem in the investigation of these aspects of TF has been the predominant use of human subjects, whose history of disease and exposure to antigenic substances and whose immunocompetance are often unknown (2, 137). Studies have also been criticized because of uncertainty regarding the specificity of test antigens, cross reactions of antigens, and the possibility of actively sensitizing TF recipients by skin testing before the administration of TF (49, 115, 141). The transfer of DTH to microbial antigens prevalent in the environment presents problems in that the TF recipient has likely been exposed to these, and subsequent conversion by TF may actually represent a non-specific stimulation of previously non-detectable reactivity (13, 21, 141). Evidence for this has been presented by Green et al. (53), who restored DTH reactivity in mice that had lost previously demonstrable DTH using a lymphoid cell lysate that did not transfer DTH to antigenically naive mice.

Lawrence has cited two studies as evidence of de novo sensitization and antigen specificity (95). In one study (127), TF from coccidioidin-sensitive California residents was prepared and administered to residents of New York who had not traveled to an area endemic for coccidioidomycosis. Twenty-three of 27 recipients developed positive reactions. These conclusions have however been criticized because several recipients also developed positive responses after

receiving TF from donors initially not showing reactivity, and because the recipients were pre-skin tested (49, 141).

In another study, TF was prepared from an individual (A) who had been sensitized to the tissue antigens of individual (B) by repeated skin grafts. Administration of this TF to individual (C) caused accelerated rejection of a skin graft from (B) but not of skin grafted, at the same time, from other individuals (95). This study is still regarded by many to be evidence for de novo sensitization and antigen specificity in TF activity (11, 21, 49).

Other studies have shown evidence for antigen specificity and de novo sensitization both in vivo and in vitro (see for example 41, 48, 104, 130, 139, 158, 164). Two that do not suffer from the criticisms outlined earlier are the demonstration by Burger et al. (21) of specific transfer of sensitivity to a neo-antigen, keyhole limpet hemocyanin, by TF preparations made from donors after but not before specific immunization of the donors, and the demonstration by Maurer (110) of DTH transfer using TF from donors sensitized to ethylene oxide-treated serum. TF recipients were not pretested by skin test in either study.

Bloom has proposed that TF is not antigen-specific, but rather acts as a non-specific immunologic adjuvant (11). He cites the fact that TF recipients rarely demonstrate reactions to all of the specificities of the donor, occasionally showing reactivity to specificities for which the donor is negative, and a theoretical difficulty in explaining the ability of such low-molecular-weight molecules to carry specific information for all antigens for which successful transfer has been reported. Other investigators have suggested that TF restores

defects in non-specific expression of DTH rather than the antigen-specific recognition phase of the DTH response (90).

Demonstration of TF activity in a guinea pig model (154) and an *in vitro* lymphocyte stimulation assay (19, 28) has proved to be independent of TF donor specificity and in fact dependent on recipient (or recipient-lymphocyte donor) reactivity. The *in vitro* activity has also been demonstrated in dialyzable extracts from nonlymphoid as well as lymphoid organs (153). A number of other antigen-independent phenomena, both *in vitro* and *in vivo*, have been attributed to TF or fractions of TF (20, 75, 78, 80).

The available evidence indicates that TF can be antigen specific, transferring the reactivities of the TF donor, but that TF probably represents a mixture of specifically and non-specifically acting moieties (11, 97, 108). Part or all of these components may contribute to the cell mediated immune response as evidenced by clinical improvement, conversion of skin DTH reactivity, and change in other immunologic parameters (89, 104).

In Vivo Manifestations

As outlined above TF has a number of manifestations *in vivo* and *in vitro*, demonstrating antigen specificity and non-specificity. The originally described manifestation, by which TF is functionally defined, is the transfer of delayed cutaneous hypersensitivity exhibited by the TF donor to a recipient previously anergic to the specificities transferred. The transferred DTH appears in the recipient as early as four hours, and generally by 18 hours after TF administration. Sensitivity has been shown to persist for months to

two years. Successful transfer requires marked sensitivity in the TF donor and is lymphocyte-equivalent dose dependent (95). Transfer of sensitivity to bacterial (93, 155), fungal (23, 67, 130), viral (96), parasitic (86, 109), and chemical (21, 155) antigens has been demonstrated.

Transfer of DTH skin reactivity has been accomplished by both local techniques (intradermal injection of TF followed 24-96 hours later by antigen at the same site) and systemic techniques (intradermal, intramuscular, subcutaneous, or intravenous injection of TF followed 24-96 hours later by intradermal antigen injection at a separate site) (99). The local technique requires a ten-fold lower TF dose, but the systemic technique is more meaningful because the possible contribution of non-specific local inflammatory responses to TF is excluded from the DTH reaction site (99).

The DTH reactivity in TF recipients demonstrated by intradermal injection of antigen, has been confirmed by <u>in vitro</u> assays performed on cells of TF recipients. Conversion has frequently been demonstrated by macrophage migration inhibition assay (5, 7, 79, 104), but inconsistently by lymphocyte blastogenesis (49, 86, 95).

A number of antigen-independent phenomena have been reported in recipients of TF. These include increase in PHA responsiveness, mixed lymphocyte culture responsiveness, ability to respond to active sensitization with dinitrochlorobenzene (DNCB), and E-rossette formation (97, 75). These have been primarily demonstrated in recipients who exhibited deficiencies in cell mediated immunity. Kirkpatrick and Smith (78) have demonstrated an antigen-independent chemotactic activity in TFd for PMN's and monocytes. The activity was demonstrated

both *in vivo* and *in vitro*. Gottlieb *et al*. found that TFd fractionated on Sephadex G-10 contained a fraction that elicited an antigen-independent intradermal reaction resembling DTH (51). Further purification of this fraction revealed two active sub-fractions, one of which augmented existing recipient sensitivity to an antigen when injected intradermally with the antigen. Vandenbark and Burger (155) reported a similar activity in a Sephadex G-25 fraction which was also active in transferring cutaneous sensitivity. They were however able to separate these two activities by modification of the fractionation procedure.

Transfer Factor Therapy

Since the early 1970's, when Levine and Spitler demonstrated clinical improvement in patients with the Wiskott-Aldrich Syndrome following administration of TF (140), there has been a great deal of interest in the treatment of other diseases of the cellular immune system with TF. Dialyzable TF is especially useful in this regard, since it does not contain viable cells or antigenic material, and thus avoids the possibility of the often fatal graft-versus-host reaction seen with bone marrow transplantation and can be administered repeatedly without sensitizing the recipient to donor leukocyte antigens (141). Dialyzable transfer factor (TFd) can also be conveniently stockpiled and administered when needed.

There is a low frequency of adverse reactions to TFd administration, even when large doses have been given over a long period of time (49). Local inflammation and mild pyrexia and malaise may occurr (49) and there is one report of transient immunosuppression

following TFd administration (77). Possible hazards of TFd therapy include the transmission of hepatitis, induction of autoimmunity due to TFd-donor sensitivity to HLA antigens of the recipient, and initiation of an overwhelming reaction following a sudden conversion of DTH reactivity in patients with disseminated infection or malignancy (141).

TF has been administered in the treatment of patients with congenital and acquired immunodeficiencies; chronic infections caused by mycobacteria, fungi, and viruses; and a variety of malignancies. An attempt will not be made here to review all of the clinical trials of TF treatment of these syndromes. Representative examples in each area will be cited. Several recent review articles are available (49, 96, 97, 100).

Infectious Diseases

The bulk of data concerning TF treatment of infectious diseases has been accumulated from trials on patients with chronic mucocutaneous candidiasis. Patients with this disease display an array of clinical and cell-mediated immune abnormalities (5, 78, 141). This may account for the variations in reported success rates, though most investigators report better than 50% of treated patients demonstrate some clinical improvement (95, 108, 124). Clinical improvement is generally preceded or accompanied by correction of the observed immunological abnormalities (5, 104, 108), an indication that TF functions to convert the patients to immunological competency.

Studies by Kirkpatrick (78, 81) and others (104) have shown

that TF will not effect clinical improvement without prior or concomitant anti-fungal drug therapy. After initiation of TF therapy, drug therapy may be terminated, and TF functions to maintain clinical remission. A study by Littman et al. (104) provides evidence that the clinical response is antigen specific. Several interesting investigations in mucocutaneous candidiasis patients with thymic deficiencies, have demonstrated the need for thymus-derived cells in recipients of TF. No response was seen in these patients when TF alone was administered, but clinical and immunological improvements were seen when fetal thymus tissue was transplanted prior to or concomitant with the TF (5, 81).

Patients with chronic disseminated coccidioidomycosis display abnormalities in cell mediated immunity much like those seen with chronic mucocutaneous candidiasis. The cumulative experience of a number of investigators in treatment of this disease-state with TF has recently been reported (23). Thirty of 49 patients with chronic coccidioidomycosis refractory to Amphotericin therapy who were treated with TF, showed evidence of clinical improvement. Amphotericin treatment was continued during TF administration. Significant conversion of skin test reactivity and migration inhibition were also noted.

Successful treatment of patients with progressive tuberculosis refractory to chemotherapy has been reported. Rubinstein et al. (136) demonstrated sustained clinical recovery from tuberculosis after TF treatment in a patient displaying marked cutaneous skin reactivity to tuberculin and normal macrophage and leukocyte migration inhibition reactivity, but low T-cell numbers and abnormalities in other

in vitro lymphocyte functions. Rocklin (134) reported a similar successful treatment of a tuberculous patient with an undiagnosed cellular immune deficiency.

There are several reports of successful treatment of viral infections with TF (97, 96). Children with giant-cell measles pneumonia and with subacute sclerosing panencephalitis (SSPE) have been treated with TFd from rubeola-immune donors, with subsequent clinical and immunological improvement. Neonatal herpes infection and cases of herpes zoster in immunosuppressed patients have likewise been treated. Earlier reports showed that disseminated vaccinia could be treated with whole viable leukocytes, indicating this disease could be treated with TF. Some of the beneficial effects seen may be due to enhanced interferon levels, which have been demonstrated following TFd administration (74).

Lawrence has noted that caution should be exercised in treating viral infections located in certain areas of the body (97). Local inflammatory responses have been detected at viral infection sites following systemic TFd administration. In cases such as cytomegalovirus retinitis, serious damage to the affected tissue could result.

The studies of Klesius' group, though not involving trials in humans, should be noted. Using a bovine TFd preparation, Klesius has transferred partial protection to coccidiosis, a parasitic disease, in calves (86) and mice (87). The same preparation failed to confer protection to rabbits, as did a similar TFd prepared from rabbits (86). Liburd *et al.* have demonstrated a similar phenomenon using treatment of rats with rat TF (103).

Immune Deficiencies

Transfer factor has been administered to patients with a variety of deficiencies of the cellular immune system, in the hope that TF would reconstitute the deficient components and render the patients immunocompetant. As noted above, the earliest experimental therapy in this area was carried out by Levin and Spitler (140, 141) on patients with the Wiskott-Aldrich Syndrome. This syndrome, an x-linked disease, is characterized by recurrent pyogenic infections, eczema, splenomegaly, thrombocytopenia, and failure to display DTH immune responses in vivo or in vitro (49, 140). Since that time more than 30 patients with this condition have been treated. A good clinical response, as evidenced by freedom from infections, regression of splenomegaly, and clearing of eczema, has been recorded in about 50% of these patients (49, 100, 140). Appearance of DTH skin reactivity and MIF responsiveness have also been noted (49, 141) and appear to correlate with the clinical response. Maintenance of the improved clinical and immunological conditions is dependent on continued TF administration to these patients.

Reports of beneficial effects following TF therapy in other immunodeficiency conditions are less extensive. Clinical and immunological improvement have been noted in patients with Swiss-type agammaglobulinemia (141), ataxia teleangiectasia (95, 100), a monocyte defect (2), and a variety of other combined immunodeficiency diseases (95, 100).

Transfer factor may also have a role in the treatment of infections in immunosuppressed patients. Lawrence has recently reviewed this subject (97). Response to TF therapy has been seen in patients

with internal immunosuppression as a result of disseminated intracellular infections and secondary to diseases such as sarcoidosis, lymphocytic leukemia, established cancer, kwashiorkor, and Hodgkin's disease (97) though the response in the last syndrome has been quite low (22). Several reports have also indicated benefit by TF administration to patients on external immunosuppressive therapy (97). These have included patients with Hodgkin's disease, rheumatoid arthritis, disseminated lupus erythematosus, and a patient undergoing renal transplantation.

Malignancy

There is extensive evidence from studies in animals and tumor-bearing patients that cellular immunity is involved in the anti-tumor response of the host (95). It is assumed that there are specific antigenic determinants, "tumor-associated antigens," on the surface of malignant cells to which the host's lymphocytes mount a response. This response may be adversely affected by suppressor cells, blocking antibodies or antigen-antibody complexes, and by the inability of the host immune system to adequately contain a tumor (49).

The rationale for use of TF in anti-tumor therapy is that TF from donors displaying CMI to specific tumors will induce specific anti-tumor activity in the recipient. This has been evidenced by lymphocytic and monocytic infiltration and regression or control of certain tumors (97, 100, 156). TF has been prepared from household contacts, who have been found to display tumor specific immunity to certain malignancies (49, 100, 156). Cancers with suspected viral association might be effectively treated by TF from donors previously exposed to the viral agent (49).

A list of some malignancies that have shown clinical or immunological benefit from TF therapy is included below. Two tumors treated most frequently are melanoma and osteogenic sarcoma (49).

Tumor Type	References
melanoma	49 156 97 80
osteogenic sarcoma	49 156 97 100
breast carcinoma	49 95 100
nasopharyngeal carcinoma	49 156
Wilms' renal cell carcinoma	49 156
lymphosarcoma	49 156
neuroblastoma	49
rhubdomyosarcoma	156
leiomyosarcoma	156
epidermoid carcinoma	156
adenocarcinoma	156
alveolar sarcoma	80
lymphocytic leukemia	80
vulvar carcinoma	49

Reported results vary greatly, and may be attributed to the inherent variations in patient health status, tumor load, and concurrent anti-tumor therapy (49). Clinical improvement has ranged from total regression to arrest of metastasis. Concurrent immunological improvement has been measured by conversion or enhancement of DTH skin reactivity, MIF release, and lymphocyte-mediated cytotoxicity as well as non-specific parameters (49, 156).

TF may be optimally used as an adjunct to other anti-tumor therapy, such as surgery, radiotherapy, and chemotherapy. TF is more effective in treatment or maintenance of patients with minimal tumor-load, which can be accomplished by surgical removal of macroscopic tumor (49, 100). Radiotherapy and chemotherapy have numerous undesirable side affects. TF therapy may be instituted to directly remedy one of these, transient immunosuppression, and indirectly remedy others by decreasing the need for these treatment modalities (49).

Recent studies by Pizza et al. (120), are of interest, in that they may provide a means of large scale TF production for use in therapy of malignancy and other diseases. These investigators have demonstrated the in vitro production of a biologically active TF specific for transitional cell carcinoma of the bladder (TCCB) by continuous lymphoblastoid cell lines induced in vitro by TFd from TCCB patients. In vivo immunologic activity of this in vitro produced TF has been demonstrated, but studies in clinical application are still in progress.

Animal Models

The development of a reliable animal model for TF would contribute much to the study and characterization of TF. Controlled experiments could be performed on large numbers of genetically similar animals whose prior exposure to antigenic substances and disease are more fully known. Procedures such as the injection of tumor cells or induction of disease for TF protection studies and the external manipulation of immune systems to determine the role(s) of TF could be performed. Recent studies showing evidence for activity of animal TF in humans (66) provides an interesting possibility of large scale production in domestic animals of specific TF for human use.

The transfer of DTH by living whole cells was demonstrated in animals (24, 91) before the same phenomenon was demonstrated in humans (92). Repeated attempts to confirm Lawrence's work with cell lysates (TF) in animals however produced conflicting results (13). Jeter et al. showed evidence of transfer of DTH to

2,4-dinitrochlorobenzene (DNCB) in guinea pigs by extracts of disrupted cells in 1954 (63). Other investigators reported confirmation of these results, but Bloom and Chase by their studies refuted transfer of DTH in the guinea pig model by preparations other than viable leukocytes (13). Burger and Jeter later reported that DTH to chemicals (DNCB and DNFB) could be transferred in guinea pigs by dialyzable supernatant fluids from sensitive leukocyte cultures incubated without antigen (15). The authors suggested that earlier difficulties may have been due to loss of TF from cells into the suspending medium during preparation (62). These observations were followed by isolated reports of TF activity in primates (95, 109) and rodents (95, 103). In recent years the number of animal models reported has expanded rapidly. These reports are organized below by species.

Non-Human Primates

Transfer of DTH by TFd to two antigens, tuberculin (PPD) and Schistosomal extracts, has been demonstrated by the group of Maddison (109) in Rhesus monkeys. Transfer was seen using TFd derived from monkey or human leukocytes. Attempts to assess protection to infection by these preparations, however, yielded equivocal results. Dumonde (39) has reported conflicting results in Rhesus monkeys using KLH antigen. Steele et al. (143) demonstrated TFd activity in three other species of monkeys using either human or monkey TFd. Human TFd has also been shown to be active in monkeys by other investigators (80, 95, 143) using a variety of antigens. Klesius has shown a similar phenomenon using bovine TFd (83).

Bovine

The bovine has been shown by several investigators to be a promising model and source of TFd. Klesius has prepared large amounts of TFd from bovine lymph node cells incubated *in vitro*. Using this preparation, his group has demonstrated transfer of DTH to calves (85, 86), rabbits (86), mice (87), dogs (83), and Rhesus monkeys (83). DTH to PPD, KLH, diptheria toxoid, and coccidia antigens was transferred. These results have correlated with *in vitro* lymphocyte stimulation studies and in some species with partial protection to infection by the coccidia species of that host (86, 87). Demonstration of activity in alcohol precipitation fractions has been also reported (85).

Jeter *et al.* (29, 67) have reported the transfer of DTH to cattle by TFd prepared from peripheral leukocytes of actively sensitized bovine donors. DTH to PPD and coccidioidin was transferred. This TFd has been reported to show *in vivo* activity in guinea pigs (102) and humans (66).

Dogs

Jeter has reported successful systemic transfer to dogs of DTH to tuberculin by TFd prepared from sensitized dogs (64). Tomar has reported less success systemically, but reported local transfer, though the specificity of the phenomenon was suspect (145, 147).

Guinea Pigs

Most early attempts to develop an animal model involved guinea pigs (13). The problems associated with these attempts were outlined above. In addition to the early reports cited above, successful

transfer of DTH to DNCB and PPD to guinea pigs by guinea pig TFd has recently been reported by Jeter et al. (65, 102).

It was initially hoped the guinea pig could serve as an animal model to assay human TF activity. Attempts were unsuccessful until the apparent development by several investigators (154, 157) of a technique using antigen primed guinea pigs. The obvious need for antigen priming, injection of antigen with TF, and subsequent demonstrations of non-donor specificity however indicated this was not a true model for TF activity (154). Recent reports indicate that purified preparations of human TFd may transfer DTH to guinea pigs with no antigen priming (101, 102).

Rabbits

Klesius et al. have demonstrated the transfer to rabbits of DTH to coccidian and PPD antigens by bovine TFd and to coccidian antigen by rabbit TFd (86). Protection to infection by the coccidial parasite (Eimeria stiedai) was not afforded by either preparation. Burger et al. (16) demonstrated rabbit-to-rabbit transfer of DTH using a TFd prepared by the technique developed earlier for guinea pig TFd (15).

Rats

Partial immunity in rats to infection by the coccidial parasite (E. nieschulzi) was induced by Liburd et al. (103) using TFd prepared from lymphoid tissues of immune rats.

Mice

Crowle demonstrated in 1959 that DTH could be transferred to mice by whole lymphoid cells from immunized mice (30). Transfer with TF has not been accomplished in the mouse until recently, but the mouse could provide a convenient, inexpensive model and assay for TF activity.

Rifkind et al. (130, 131) have demonstrated that TFd prepared from spleen cells of immunized mice will transfer DTH to recipient mice. The transfer demonstrated specificity for Coccidioides, Candida, and Mycobacterial antigens. Lawrence's group has confirmed mouse-to-mouse transfer using TFd prepared from lymph node cells (14).

The possible use of the mouse as an assay for TFd prepared from other species is indicated by recent reports. Both Rifkind's (119) and Lawrence's (14) group have reported transfer to mice using human TFd. Klesius has reported a similar transfer with bovine TFd (87).

MATERIALS AND METHODS

Animals

Albino ICR female adult mice were purchased initially from Spartan Research Animals, Inc., Haslett, Michigan, and later from Harlan Industries, Inc., Cumberland, Indiana. All mice used in a single experiment were purchased from one source. Mice weighed 25-30 grams at use.

New Zealand white female rabbits weighing 8-10 pounds (Spartan Research Animals, Inc., Haslett, Michigan.) were used in procedures to standardize the skin test antigen (Brucellergen).

Holstein heifers used in these experiments were part of a brucellosis research project being conducted at Michigan State University, under a grant from the United States Department of Agriculture. The animals were housed in an isolation barn. Non-infected controls were kept separated from infected cattle.

Heifer #221, #230, and #249 were 2-year old non-infected heifers. Heifer #221, a negative control animal, had not been actively or passively immunized. Heifer #230 and #249 were immunized at about 5 months of age by subcutaneous injection of 5 ml/each of the commercially available attenuated Brucella abortus strain 19 vaccine (Colorado Serum Co., Denver, Colorado). This treatment was repeated 3 months later.

Heifer #7, a pregnant test animal, had received 1.6×10^9 viable

peripheral mononuclear leukocytes from heifer #230 thirty days preceding challenge with Brucella abortus strain 2308 (9.5×10^5 cells). Subsequent active Brucella infection was demonstrated by rise in agglutination titers and isolation of the organism from placental membranes at partuition.

Microorganisms

Two strains of Brucella abortus were used in these experiments. Strain 19, an attenuated vaccine strain, was subcultured from the commercially supplied vaccine (Colorado Serum Co., Denver, Colorado) and maintained on Trypticase Soy agar (TSA) (Difco, Detroit, Mich.) slants. Strain 2308, a fully virulent non-CO_2 requiring strain, was maintained as a stock culture on TSA slants in our laboratory. This strain was checked for maintenance of smooth characteristics before use by the oblique lighting method of Henry (56). Organisms used for immunization of mice or rabbits were prepared by washing 48-hour TSA slants with peptone-saline (0.1% peptone-0.85% NaCl) and diluting the suspension to a reading of 78% light transmittance at 650 nm on a Perkin-Elmer spectrophotometer, Model 139 (Coleman, Maywood, Ill.). This reading has been determined to equal 1×10^9 bacteria/ml. The suspension was further diluted in peptone saline to the desired concentration and the actual cell count established by plate counts on TSA.

Immunization of Rabbits and Mice

Rabbits ST1 and 74H were immunized by intravenous injection of 1.0 ml of a 1:100 dilution of the growth washed from a 48-hour TSA slant culture of Brucella abortus strain 2308, according to the methods originally described by Huddleson (60) for the standardization

of Brucellergen. In the experiments outlined here, this was equivalent to 5.8×10^7 organisms per dose. Rabbits from a previous project, which had been infected 1 year earlier with Brucella abortus strain 2308 (1×10^6 organisms), were also skin tested to assay antigen activity.

Mice were immunized by intraperitoneal injection of (unless otherwise noted) 4.8×10^6 to 5.3×10^6 viable organisms in 0.5 or 1.0 ml peptone saline. Both strain 2308 and strain 19 were used in respective experiments.

Tests for Delayed-Type Hypersensitivity

Brucellergen Preparation

Brucellergen was prepared as originally described by Huddleson (60). Smooth Brucella abortus strain 2308 was grown at 37°C on liver infusion agar prepared as described by McCullough (114) in flat 32-ounce bottles. The growth at 72 hours was washed from the surface of the agar with distilled water and the cells recovered by centrifugation. The cell pellets from 27 bottles were combined and extracted with three successive changes of anhydrous ether (total 1500 ml) over a 30 day period to remove lipids. The cells were dried in vacuo over H_2SO_4 at 37°C and subsequently ground to a fine powder in a ball mill for seven days. One gram of this powder was suspended in 200 ml of phosphate buffered distilled water (pH 7.0) and allowed to stand at 4°C overnight (17 hours). Insoluble material was removed by centrifugation, and the protein nucleate was precipitated from the supernatant at pH 4.0 by addition of 1:2 glacial acetic acid. Precipitation was allowed to continue for 24 hours at 4°C. The precipitate

was recovered by centrifugation, resuspended in 100 ml cold distilled water, and redissolved at pH 6.8 by addition of 1 N NaOH. Insoluble material was again removed by centrifugation. The precipitation at pH 4.0 and resolution at pH 6.8 was repeated twice more. Each centrifugation was done at 3,000 rpm (2,000 x g) on a IEC-PR 6000 Centrifuge (Damon/IEC, Needham Hts., Mass.). The final precipitate was centrifuged in graduated centrifuge tubes so that the volume could be estimated. The precipitate was redissolved at approximately 1% (vol/vol) in distilled water at pH 6.8. Phenol was added to a concentration of 0.5% and the solution was filter sterilized (0.2 μm) (Nalgene, Rochester, N.Y.).

Brucellergen Standardization

The allergic potency of the Brucellergen preparation was determined by skin tests in sensitized rabbits, the method previously used to standardize Brucellergen for human use (60, 114). An aliquot of the 1% stock solution was adjusted with 1 N HCL until a fine particulate precipitate of the antigen was visible. Serial dilutions of this suspension were made in sterile phenolized saline (0.5% phenol-0.85% NaCL) and tested by intradermal injection of 0.1 ml into separate skin sites on the shaved abdomen of the rabbits. In these experiments, the 1% stock solution was taken to be a 1:100 starting dilution. Non-sensitized rabbits were included as controls. Reactions at the injection sites were read at 24, 48, and occasionally 72 hours by two right-angle measurements of the diameter of induration and erythema using Vernier calipers (supplied by T. H. Conger, D.V.M.). Although erythema was noted, the measurement of palpable

induration was used as indication of a DTH reaction.

DTH Tests in Cattle

DTH skin tests in cattle were applied in the lateral cervical area by intradermal injection of 0.1 ml of a 1:1,000 dilution of Brucellergen, prepared as described above. Hair was clipped from the test area before injection. Induration was measured at 48 and 72 hours in the manner described for rabbits.

DTH Tests in Mice

DTH was tested in mice which had been actively immunized by Brucella infection or passively immunized with TFd, by the footpad assay as originally described by Gray and Jennings (52). Injections were made into the ventral pads of the hind feet using a 30-gauge needle and a 1 ml tuberculin syringe calibrated in 0.01 ml increments. Preliminary experiments indicated that injection of 0.05 ml but not 0.025 ml occasionally caused excessive trauma. The latter volume was therefore used throughout. Brucellergen, prepared as described for the rabbit skin tests, was injected into one footpad. An equal volume of phenolized saline, acidified and diluted in the same manner as the antigen, was injected into the opposite footpad. Reactions were read at 24 and 48 hours both by subjective evaluation and by measurement of footpad swelling using Vernier calipers. The dorsoventral thickness of each foot was measured three times and the result expressed as the difference between the mean of these three measurements for the antigen-injected foot and the mean of the three measurements for the saline-injected foot (test minus control). Subjective evaluation was limited to the descriptions below:

negative(-)	No observable difference between test and control foot
slight (sl)	Observable, but insignificant swelling in test foot
plus (+)	Observable difference in swelling between test and control foot. Positive reaction

Preparation of Transfer Factor

Dialyzable transfer factor (TFd) was prepared from three sources: bovine peripheral blood cells, bovine lymph node cells, and murine spleen cells.

Bovine Peripheral Blood Transfer Factor

TFd was prepared from the peripheral blood mononuclear leukocytes of heifer #230, a strain 19 hyperimmunized animal displaying positive DTH skin reactivity to Brucellergen. Blood (1,500 ml) was collected from the jugular vein into flasks containing a 10% EDTA (J. T. Baker Chemical Co., Phillipsburg, N.J.) solution. The blood was centrifuged in 250 ml centrifuge bottles at 2300 rpm (1,400 x g) (IEC-PR 6000 centrifuge) for 30 minutes at 4°C. The clear plasma layer was decanted and the leukocyte-rich buffy coat layer was carefully aspirated and mixed in an equal volume of EDTA-phosphate buffered saline (EPS). This suspension was centrifuged at 2,000 rpm (800 x g) for 15-20 minutes and the buffy coat layer aspirated and mixed with EPS as before. Ten to 12 ml of the buffy coat suspension was carefully layered over 3 ml of Ficoll-Hypaque solution in plastic screw-cap tubes (Corning, Corning, N.Y.), and centrifuged at 2,000 rpm for 30 minutes at room temperature. This method of density-gradient centrifugation yields a distinct white layer at the Ficoll-Hypaque-

supernatant interface consisting of greater than 95% mononuclear leukocytes. Erythrocytes and granulocytes are centrifuged through the Ficoll-Hypaque gradient to the bottom of the tube. The mononuclear cell layer, and part of the Ficoll-Hypaque layer if it contained cells (indicated by cloudiness), was carefully removed and mixed with phosphate buffered saline (PBS) (Gibco, Grand Island, N.Y.). The cells were washed free of Ficoll-Hypaque by two cycles of centrifugation and resuspension in PBS. Cell viability was determined by trypan blue exclusion (123), cell counts were made with a hemacytometer, and an air-dried smear was stained by Wright's stain for a differential cell count. The cells were then sedimented by centrifugation at 1,300 rpm (350 x g) and resuspended in sterile distilled water to a total volume of 8.5 ml. This suspension was frozen and stored at -20°C. A total of 2.2×10^9 viable mononuclear cells were isolated from 1,500 ml of blood.

The cell lysate was prepared by adding 1 mg/ml bovine pancreatic DNase (Nutritional Biochemical Co., Clevelend, Ohio) and 75 mg/ml $MgSO_4 \cdot 7H_2O$ to the cell suspension and submitting the suspension, in plastic screw-cap tubes, to 10 freeze-and-thaw cycles in a dry ice-90% alcohol bath and 37°C water bath respectively. Cell lysis was confirmed by observing a wet mount microscopically.

The lysate was dialyzed against 25 volumes of sterile distilled water using 7/8" dialysis tubing (A. H. Thomas Co., Philidelphia, Pa.). Dialysis was carried out at 4°C with gentle agitation provided by a magnetic stirring bar. The dialysate was replaced at 24 hours with fresh sterile distilled water and dialysis was continued another 24 hours. The dialysates were then combined, shell frozen, and

lyophilized (New Brunswick Lyophilizer, New Brunswick, N.J.).

The lyophilized powder from an original suspension of 2×10^9 viable mononuclear cells was dissolved in 6.0 ml saline. The preparation was filter sterilized (0.22 μm) (Millipore, Bedford, Mass.) and stored at -20°C until use.

Bovine Lymph Node Transfer Factor

TFd was prepared from lymph nodes collected from heifer #7 at necropsy post-partuition. The major lymph nodes, stored at -20°C since necropsy (one month), were thawed, trimmed of extraneous fatty tissue, and coarsely minced with scissors. Weighed portions were mixed with saline and ground to a fine slurry using a Waring blender. This slurry was expressed through a metal screen to remove gross material and diluted to a measured total volume with additional saline to contain 0.1 grams lymph node/ml.

The lymph node slurry was pipetted to screw-cap plastic tubes (6-7 ml per tube), 25 mg/ml $MgSO_4 \cdot 7H_2O$ and a few crystals of DNase were added to each tube, and the cells were further lysed by 10 freeze-thaw cycles as before. The lysates were combined and dialyzed against approximately 10 volumes of sterile distilled water for 24 hours at 4°C with slight agitation. Because of the large volumes of lysate and dialysate involved, a second 24 hour dialysis was not done.

The dialysate was shell frozen and lyophilized as before. The lyophilized powder was reconstituted in sterile distilled water to yield a final concentration of 0.5 gram-equivalents/ml in one preparation and 0.1 gram-equivalents per ml in a second preparation. A gram-equivalent is defined as the lyophilized powder obtained from one

gram of original lymph node material. Experience in our lab indicates that 1 gram of freshly harvested lymph node will yield roughly 1×10^9 cells. One gram of lymph node prepared as above yielded approximately 200 mg of lyophilized powder. The TFd preparations were filter sterilized (0.20 μm) (Nalgene) and stored at -20°C until use.

Murine Transfer Factor

TFd was prepared from the spleen cells of sensitized mice by the method of Rifkind et al. (130). Lot #1 was prepared from mice used for the initial evaluation of the footpad test and reinfected with strain 19 three weeks before spleens were harvested. Lot #2 and Lot #3 were prepared from mice infected four weeks earlier with strain 2308 and strain 19 respectively. These mice had been footpad tested for DTH 21 days after infection, and only those mice showing DTH reactivity were used. Lot #4 was prepared from mice that had been sensitized by repeated footpad tests. A negative control TFd was prepared from unsensitized mice.

Spleens were aseptically collected and kept in cold M-199 tissue culture medium (Gibco, Grand Island, N.Y.) during processing. Initially (Lot #1) cells were dispersed by expressing coarsely minced spleens through a Ten Broeck glass tissue grinder. Since this treatment could conceivably release transfer factor activity from the cells, a modified procedure was used in subsequent preparations (Lots #2, 3, 4). Spleen cells were dispersed by injection of 1 ml M-199 into the spleen, followed by manipulation with forceps to tease the cells free from the spleen capsule. The spleen cells were further dispersed by gently drawing the suspension several times

through a 23-gauge needle. The cells were washed three times in M-199 by centrifugation, 1,000 rpm (200 x g), and resuspension. Viability was determined by trypan blue exclusion and cell count determined using a hemacytometer. The cell suspensions were adjusted to 5 x 10^8 cells/ml in M-199 and stored frozen at -20°C.

Lysates were prepared as before by 10 freeze-thaw cycles following the addition of 50 mg/ml $MgSO_4 \cdot 7H_2O$ and a few crystals of DNase. The lysates were dialyzed at 4°C against 10 volumes of sterile distilled water with slight agitation. The dialysate was replaced at 24 hours with fresh sterile distilled water and dialysis was continued another 24 hours. To avoid filter sterilization of the small volume of final reconstituted product, the dialysates were filter sterilized (.22 μM) (Millipore) prior to shell freezing and lyophilization. The lyophilized powder was reconstituted with sterile distilled water to the same volume as the original lysate, therefore containing 5 x 10^8 spleen cell equivalents/ml. One ml of M-199 containing 5 x 10^8 spleen cells yielded approximately 50 mg of lyophilized powder. Reconstituted TFd preparations were stored at -20°C.

Assay of Transfer Factor Activity

TFd was administered to mice by intraperitoneal injection, the route used by both Rifkind (130) and Klesius (87). Doses were determined by cell-equivalents for bovine peripheral-blood TFd and murine spleen-cell TFd, and by gram-equivalents for the bovine lymph-node TFd. Whole viable cells were administered to mice by intraperitoneal injection of bovine cells and by intravenous injection of murine cells.

Initial experiments indicated that large doses of TFd were toxic to mice, causing death in some. Dose-response studies however clearly revealed that the toxicity was due to the magnesium sulfate concentration in the preparations. Mice could be killed by a single intraperitoneal dose of 20 mg $MgSO_4 \cdot 7H_2O$. Subsequent large TFd doses were administered by several injections over a period of time to avoid this toxic effect. This problem was not encountered when normal doses of TFd were administered.

Recipient mice were tested for DTH reactivity using the footpad assay 48 hours, 72 hours, or 7 days following the administration of TFd or whole cells. The footpad test was usually repeated one week later. Mice were not footpad tested before receiving TFd or whole cells.

Histological Studies

Footpads submitted for histological study were fixed in formalin. Paraffin-embedded, hematoxylin-and-eosin stained sections were prepared by the Animal Health Diagnostic Laboratory at Michigan State University, East Lansing, Michigan. Both saline- and antigen-injected footpads were examined.

Preparation of Chemical Reagents

EDTA-phosphate buffered saline (EPS)

3.58 gm	EDTA - trisodium salt (Sigma Chemical Co., St. Louis, Mo.)
9.0 gm	NaCl
1.08 gm	KH_2PO_4
1,000 ml	distilled H_2O

The solution was filter sterilized (0.20 μm) (Nalgene) and adjusted to pH 7.2 - 7.4 before use.

Ficoll-Hypaque Density Gradient

72.8 gm	Ficoll 400 (Pharmacia, Piscataway, N.J.)
480 ml	Hypaque (sodium diatrizoate) 25% aqueous solution (Winthrop Labs., New York, N.Y.)
720 ml	distilled H_2O

Specific gravity was determined to be 1.076 to 1.079 using a hydrometer (Arthur Thomas Co., Philadelphia, Pa.) and the solution was filter sterilized (0.20 μm) (Nalgene) and stored at 4°C.

RESULTS

Brucellergen Standardization in Rabbits

The allergic potency of the Brucellergen preparation was assayed by intradermal injection of serial dilutions of the preparation into rabbits infected with Brucella abortus strain 2308. Two rabbits (ST1 and 74H) were skin tested 30 days after infection, as outlined originally by Huddleson for standardization of Brucellergen for human use (60). They were skin tested again 7 weeks after infection. Two rabbits infected 1 year earlier and two non-infected rabbits were also tested. The results are shown in Table 1.

The rabbits infected for 30 days showed positive, indurated DTH responses at 1:1,000 and 1:4,000 dilutions, though the reactions at 48 hours were less than the 5 mm standard set by Huddleson (60). The reactivity of the rabbits infected a year earlier was stronger, showing positive reactions at 1:8,000 and reactions of 5.0 mm at the 1:4,000 dilution. Skin tests applied to ST1 and 74H at 7 weeks following infection showed enhanced reactivity. Positive reactions were seen to persist for 72 hours, when measurements were taken at that time. In addition to palpable induration, erythema and edematous swelling were frequently noted in infected rabbits, indicating concurrent Arthus-type hypersensitivity. Negative control rabbits showed no inflammatory skin reactions.

Table 1. Delayed Hypersensitivity Responses in Rabbits to Brucellergen[1]

Antigen Dilution		Blank			1:1,000			1:4,000			1:8,000			1:16,000		
Time of Measurement (hours)		24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
Rabbit	Interval after Infection[2]															
ST 1	30 days	0	0		5.5	4.6		4.3	4.3		sl	sl				
	7 weeks	0	0	0	sl	5.6	5.5									
74 H	30 days	0	0		5.0	3.6		4.4	sl		3.9	0		0	0	
	7 weeks	0	0	0	5.3	5.1	4.0									
119 - J	1 year	0	0	0	+	6.1	5.8	+	5.0	4.9	3.5	4.7	3.4			
126 - J	1 year	0	0	0	3.4	5.6	5.7	sl	5.0	5.0	sl	4.0	3.2			
Controls	Not infected	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		0	0	0	0	0	0	0	0	0	0	0	0	0	0	

[1]Measured as induration in mm at 24, 48 and 72 hours after intradermal injection of 0.1 ml antigen dilution.

[2]Infected with Brucella abortus strain 2308.

+ = Positive induration but obscured by Arthus-type reaction; sl = slight reaction

DTH Response in Cattle to Brucellergen

Brucellergen was injected intradermally into immunized (strain 19 vaccine) and non-immunized cattle to determine the ability of the antigen preparation to detect DTH reactivity in these animals. Typical indurated DTH responses were seen in the immunized cattle, but not in the non-immunized cattle, when examined at 48 and 72 hours. The results are shown in Table 2.

Table 2. Delayed Hypersensitivity Responses in Cattle to Brucellergen[1]

Cattle	Diameter of Induration	
	48 hours	72 hours
Immunized[2]		
#230	19.5 mm	14.3 mm
#249	12.2 mm	8.2 mm
Non-Immunized		
#221	0	0
#10	0	0

[1]Measured 48 and 72 hours after intradermal injection of 1:1,000 Brucellergen (0.1 ml).

[2]*Brucella abortus* Strain 19 vaccinated.

Footpad Reaction in Mice to Brucellergen

The footpad assay was used to test the DTH response of infected mice to Brucellergen. Antigen was injected into the footpad of the right hind foot and the antigen solvent (phenolized saline) was injected into the left footpad. At 24 and 48 hours, and in one study

at 6 hours, the swelling of the two footpads was compared and measured. Values were expressed as swelling of right footpad minus swelling of left footpad (test minus control).

Table 3 shows the results obtained using 3 dilutions of Brucellergen. The 1:500 dilution elicited maximal swelling in infected mice and no response in control mice. This dilution was therefore used throughout this study in the footpad assay. In preliminary studies, a 1:100 dilution was found to cause non-specific footpad swelling in non-sensitized mice. The 1:4,000 dilution elicited only weak reactions in infected mice.

Table 3. Delayed Hypersensitivity Response in Mice to Three Dilutions of Brucellergen[1]

Antigen Dilution	Footpad Reaction at 24 Hours[2]		
	1:500	1:1,000	1:4,000
Infected[3] Mice	69(10/10)	52 (8/9)	15 (1/5)
Non-Infected Mice	4 (0/7)	7 (0/2)	4 (0/5)

[1]Elicited by injection of 0.025 ml antigen dilution into hind footpad.

[2]Expressed as the mean footpad swelling, in 0.01-mm increments, of all mice tested (number positive reactions/total mice tested). Values for 1:500 and 1:1,000 combined from two experiments.

[3]Infection with Brucella abortus strain 19 or 2308.

The footpad reactions of strain 19 infected and strain 2308 infected mice are shown in Tables 4 and 5 respectively. The footpad assay was applied to these mice at 21 days after infection, a time when Brucella-infected mice have been shown to exhibit strong DTH

Table 4. Delayed Hypersensitivity Response to Brucellergen in Strain 19 Infected Mice[1]

Mice[3]	Footpad Reaction[2] at		
	6 hours	24 hours	48 hours
Group A			
Infected	58 (4/6)	54 (6/6)	30 (6/6)
Non-Infected	17 (0/6)	-3 (0/6)	-3 (0/6)
Group B			
Infected	not done	39 (11/11)	15 (8/11)
Non-Infected	not done	6 (0/6)	7 (0/6)

[1]Elicited by injection of 0.025 ml antigen (1:500) into hind footpad.

[2]Expressed as the mean footpad swelling, in 0.01-mm increments, of all mice tested (number positive reactions/total mice tested).

[3]Group A mice infected with 1.8×10^6 cells/mouse.
Group B mice infected with 5.3×10^6 cells/mouse.

Table 5. Delayed Hypersensitivity Response to Brucellergen in Strain 2308 Infected Mice[1]

Mice	Footpad Reaction[2] at	
	24 hours	48 hours
Infected[3]	51 (23/24)	40 (22/24)
Non-Infected	4 (0/14)	3 (0/14)

[1]Elicited by injection of 0.025 ml antigen (1:500) into hind footpad.

[2]Expressed as the mean footpad swelling, in 0.01-mm increments, of all mice tested (number positive reactions/total mice tested)

[3]Infected with 4.8×10^6 - 5.0×10^6 cells/mouse.

reactivity (107). Good DTH responses, characterized by swelling and erythema at 24 and 48 hours, were seen in mice infected by either strain of Brucella. Swelling persisted in positive reactions for 48 hours, although the swelling at 48 hours was quantitatively less than at 24 hours. In one group where reactions were measured at 6 hours, significant swelling was observed in 4 of 6 mice tested, possibly due to an Arthus-type reaction. A small degree of swelling was also evident at 6 hours in non-infected controls, a non-specific early response also reported by Youdim et al. (160). The macroscopic findings correspond to those reported for footpad DTH reactions by others (33, 72, 129).

Sensitization of Control Mice by Repeated Footpad Tests

When the footpad test was repeatedly applied to mice that had received no other treatment, it was noted that a number of mice showing no reactivity in the initial (1°) test, showed positive reactions in a second (2°) footpad test one week later. When data was compiled from several experiments (Table 6), it was evident that the 1° footpad test had sensitized a considerable number of mice. An even greater proportion of the mice showed reactivity in a third (3°) consecutive footpad test, applied 1 week after the 2° test. These reactions were of equal intensity and could not be distinguished from the reactions displayed by infected mice.

Since antigen had been injected into the same footpad in both 1° and 2° tests, two experiments were done in which the opposite footpad was injected with antigen in the 2° test. The results (Table 6) show that not only was a response elicited in the opposite

Table 6. Sensitization of Control Mice[1] by Repeated Footpad Tests[2]

Location of Footpad Tests	Footpad Reaction[3] at			
	1° test	2° test	3° test	4° test
All tests in same footpad[4]	2 (1/57)	21 (25/57)	31 (19/28)	46 (6/8)
Primary test in right footpad	2 (0/8)			
Secondary test in left footpad		34 (6/8)		
Primary test in right footpad	7 (0/8)			
Secondary test in right footpad		37 (3/4)		
Secondary test in left footpad		31 (4/4)		

[1]All mice served as negative controls in transfer factor experiments.

[2]Injection of 0.025 ml antigen (1:500) into hind footpad at 1 week intervals.

[3]Expressed as the mean footpad swelling at 24 hours, in 0.01-mm increments, of all mice tested, (number positive reactions/total mice tested).

[4]Values combined from 8 experiments.

footpad one week after the 1° test, but that the response was of relatively the same intensity as that elicited when the 2° test was applied to the same footpad.

Two other experiments offer data relevant to these unexpected findings. In one, mice were primed by a footpad test using a 1:4,000 dilution of Brucellergen. No footpad reactivity was demonstrated one week later by a second footpad test at 1:4,000. In another experiment, mice were primed by subcutaneous injection of 0.1 ml of a 1:2,000 dilution of Brucellergen, a total antigen dose equivalent to that used in the footpad assay (0.025 ml of 1:500 Brucellergen). No response was seen in 10 of 12 mice when footpad tested one week later. Two mice showed reactions with quantitative values of 20 and 34 respectively.

Histology of the Footpad Reaction

Histological studies of the footpad reaction in mice sensitized by either infection or repeated footpad testing were done on sections of the footpads taken at 24 and 48 hours after antigen injection. A marked cellular infiltrate was seen in both reactions. At 24 hours the infiltrate was composed of PMN's and mononuclear cells in approximately equal numbers. At 48 hours mononuclear cells were predominant, with few PMN's seen.

Transfer Factor Activity in Mice

The TFd preparations described in Materials and Methods were used in an attempt to transfer DTH to Brucella from sensitized animals to non-sensitized mice. Transfer was also attempted in several experiments using whole cells, collected and isolated in the same

manner as the cells used for preparation of TFd. These cells were not lysed, but injected as intact viable cells.

The results of transfer experiments using bovine (#230) peripheral leukocytes are shown in Table 7. Preliminary experiments using whole cell preparations in a small number of mice showed no footpad reactivity in recipient mice at the 1° test applied 72 hours after administration of the cells. Positive responses were seen in two of these recipient mice at a 2° footpad test one week later. In an attempt to induce a greater response, a larger dose (3×10^8 cells) was administered. No significant response was seen in the recipient mouse at the 1° or 2° footpad test. To test the possibility that an interval of more than 72 hours was necessary for transferred whole cells to render the recipient mice DTH reactive, an experiment was done in which the 1° footpad test was not applied until 7 days after injection of cells. In this set of experiments, using a larger number of mice, there was no apparent evidence of DTH transfer. Experiments using several doses of TFd prepared from bovine (#230) peripheral leukocytes also failed to demonstrate transfer of DTH to recipient mice in either the 1° footpad test at 72 hours or the 2° test one week later (Table 7).

The results of transfer experiments using bovine lymph node TFd are shown in Table 8. TFd was administered to mice in doses ranging from 0.5 to 0.005 gram-equivalents of original lymph node material. There was no demonstration of transfer of DTH as assayed by the 1° footpad test at 2, 3, or 7 days following administration of TFd. The apparent transfer demonstrated by the 2° footpad test in one experiment was not confirmed in an experiment using a larger

Table 7. Bovine Peripheral Blood Transfer Factor Activity in Mice.[1]

Dose[2]	Time[3] 1° Test Done	Footpad Reactions[4] 1° test	2° test
Whole Cells			
1.5×10^7	72 hr.	4 (0/3)	30 (2/3)
control		-7 (0/1)	8 (0/1)
3×10^8	72 hr.	7 (0/1)	18 (0/1)
control		-3 (0/1)	0 (0/1)
1×10^7	7 days	5 ± 2.7 (1/15)	8 ± 4.4 (4/15)
control		0 ± 2.2 (1/15)	15 ± 3.7 (5/15)
TFd			
1×10^8	72 hr.	5 ± 3.2 (0/6)	Not Done
5×10^7		0 ± 1.7 (0/10)	14 ± 4.3 (3/9)
5×10^6		3 ± 2.3 (0/10)	25 ± 3.6 (7/10)
control		5 ± 1.5 (0/10)	42 ± 10.9 (6/10)

[1]Elicited by injection of 0.025 ml antigen (1:500) into hind footpad.

[2]Dose of whole cells or cell-equivalents of TFd administered by intraperitoneal injection to test mice. Controls received no cells or TFd.

[3]Interval between administration of TFd and injection of antigen for primary test. Secondary test applied one week following primary test.

[4]Expressed as the mean footpad swelling at 24 hours, in 0.01-mm increments, of all mice tested $\pm$ standard error (number positive reactions/total mice tested).

Table 8. Bovine Lymph Node Transfer Factor Activity in Mice[1]

TFd Dose[2]	Time[3] 1° Test Done	Footpad Reactions[4] 1° test	2° test
0.50	72 hr.	2 ± 4.7 (0/3)	1 ± 1.8 (0/3)
0.05		6 ± 2.1 (0/4)	14 ± 6.5 (4/4)
0.005		2 ± 4.7 (0/4)	24 ± 10.3 (2/4)
control		-3 ± 2.9 (0/4)	-7 ± 4.0 (0/4)
0.05	7 days	-2 ± 3.6 (0/10)	
0.005		1 ± 2.2 (0/10)	Not Done
control		0 ± 3.8 (0/5)	
0.50	48 hr.	0 ± 2.2 (0/10)	38 ± 6.1 (9/10)
0.10		4 ± 2.0 (0/9)	25 ± 5.5 (7/9)
0.05		0 ± 1.0 (0/10)	34 ± 7.4 (8/10)
0.005		5 ± 2.7 (0/10)	23 ± 7.4 (3/10)
0.05 heat treated		4 ± 2.6 (0/9)	26 ± 7.6 (4/9)
control		5 ± 1.5 (0/10)	42 ± 10.9 (6/10)

[1]Elicited by injection of 0.025 ml antigen (1:500) into hind footpad.

[2]Dose of gram-equivalents administered by intraperitoneal injection to test mice. Controls received no TFd.

[3]Interval between administration of TFd and injection of antigen for primary test. Secondary test applied one week following primary test.

[4]Expressed as the mean footpad swelling at 24 hours, in 0.01-mm increments, of all mice tested ± standard error (number positive reactions/total mice tested).

number of mice. One preparation was heated at 56°C for 30 minutes, a treatment reported to destroy transfer factor activity (95). Mice receiving this preparation showed no significantly different reactivity at either the 1° or 2° footpad test.

Since the failure to demonstrate transfer of DTH in these experiments could be attributed to difficulty of cross species transfer and/or the unknown DTH status of heifer #7, transfer factor was prepared from Brucella infected mice showing strong footpad reactivity to Brucellergen. Only those mice showing positive reactions were used as TFd donors; the mean footpad reaction being 61 overall and less than 50 in only one preparation (Lot #3). The results of experiments using these TFd preparations are shown in Table 9.

Experiments using a TFd preparation (Lot #1) from strain 19 infected mice showed some evidence of DTH transfer activity. In one experiment using doses of 5×10^7 and 5×10^6 cell-equivalents, statistically significant (student's t test, $P \leq .01$) DTH reactivity was detected by the 1° footpad test on mice receiving 5×10^7 cell-equivalents, but not on mice receiving 5×10^6 cell-equivalents. No significant difference was demonstrated in the DTH reactivity of test and control mice in the 2° footpad test applied one week later, though the relative number of "positive" reactions, determined by subjective evaluation, was higher in mice that had received TFd. In a second experiment, a 5-fold higher dose of TFd (2.5×10^8 cell-equivalents) was administered in an attempt to induce greater reactivity in the 1° footpad test. No significant difference between test and control animals was demonstrated, however, by the 1° test. A single mouse in the TFd treated group showed reactivity (value 51) in the 2° test, but the mean reactivity was not

Table 9. Murine Transfer Factor Activity in Mice[1]

Preparation* and Dose[2]	Time[3] 1° Test Done	Footpad Reactions[4] 1° test	2° test
TFd Lot #1 Prep. #1			
5×10^7	48 hr.	16 ± 3.3 3/8)	29 ± 5.4 (5/6)
5×10^6		7 ± 3.4 (1/7)	26 ± 8.7 (4/6)
control		-1 ± 3.0 (0/8)	19 ± 8.7 (2/5)
2.5×10^8	48 hr.	4 ± 2.3 (0/3)	25 ± 15.3 (1/3)
control		10 ± 1.6 (0/2)	18 ± 0.7 (0/2)
TFd Lot #1 Prep. #2			
5×10^7	48 hr.	6 ± 2.3 (0/7)	65 ± 9.5 (6/7)
control		-3 ± 1.8 (0/12)	29 ± 6.2 (8/12)
TFd Lot #2			
5×10^7	48 hr.	1 ± 1.5 (0/9)	29 ± 6.6 (6/9)
5×10^6		-1 ± 2.5 (0/10)	38 ± 8.3 (8/10)
5×10^5		3 ± 1.9 (0/9)	35 ± 7.9 (5/9)
5×10^7 heat treated		-2 ± 1.6 (0/10)	22 ± 6.3 (5/10)
control		-3 ± 1.8 (0/12)	29 ± 6.2 (8/12)

*Lot #1 prepared from strain 19 infected mice;
Lot #2 prepared from strain 2308 infected mice;
Lot #3 prepared from strain 19 infected mice;
Lot #4 prepared from non-infected mice sensitized by repeated footpad tests.
Negative TFd prepared from non-sensitized mice.

Table 9. (Continued)

Preparation* and Dose[2]	Time[3] 1° Test Done	Footpad Reactions[4] 1° test	Footpad Reactions[4] 2° test
TFd Lot #3			
5 x 10^7	48 hr.	1 ± 3.3 (0/7)	33 ± 9.2 (4/7)
control		2 ± 2.5 (0/7)	34 ± 6.3 (5/7)
TFd Lot #4			
5 x 10^7	48 hr.	-1 ± 2.8 (0/7)	42 ± 7.6 (6/7)
control		2 ± 2.5 (0/7)	34 ± 6.0 (5/7)
Whole Cells Lot #4			
5 x 10^7	6 days	0 ± 2.6 (0/7)	
control		7 ± 2.6 (0/7)	
Negative TFd Prep.			
5 x 10^7	48 hr.	3 ± 2.3 (0/10)	30 ± 4.0 (7/10)
control		-3 ± 1.8 (0/12)	29 ± 6.2 (8/12)

[1]Elicited by injection of 0.025 ml antigen (1:500) into hind footpad.

[2]Dose of whole cells or cell-equivalents of TFD administered by intraperitoneal injection to test mice. Controls received no TFd.

[3]Interval between administration of TFd and injection of antigen for primary test. Secondary test applied one week following primary test.

[4]Expressed as the mean footpad swelling at 24 hours, in 0.01-mm increments, of all mice tested ± standard error (number positive reactions/total mice tested).

significantly different between test and control animals.

A second TFd preparation was made from an aliquot of the non-dialyzed cell lysate that had been stored at -20°C. Mice receiving 5×10^7 cell-equivalents of this preparation did not show evidence of DTH transfer in the 1° footpad test. Strong DTH reactions were seen in the 2° footpad test in 6 of 7 mice that had received TFd.

A TFd preparation (Lot #3) from a second group of strain 19 infected mice failed to transfer DTH to recipient mice as assayed by 1° and 2° footpad tests (Table 9). Similarly, a preparation (Lot #2) from strain 2308 infected mice administered at several doses failed to transfer DTH. In the latter experiment, a group of mice received a heat treated (56°C for 30 minutes) TFd preparation. These mice showed no significant difference in reactivity in the footpad tests.

Transfer of DTH was also attempted using spleen cell preparations (Lot #4) from mice that had been sensitized by repeated footpad testing. These donor mice showed strong reactivity in a footpad test applied one week earlier (mean 85, range 68-130). One aliquot of the spleen cells was injected intravenously as intact viable cells and a second aliquot was used to prepare TFd. Mice receiving either of these two preparations failed to demonstrate transfer of DTH (Table 9).

A TFd preparation from spleen cells of non-sensitized mice was administered as a negative control. The footpad reactivity of mice receiving this preparation was not significantly different when compared either to non-treated controls or to mice, in a concurrent experiment, that had received TFd (Lot#2) from Brucella infected mice (Table 9).

DISCUSSION

A model for testing the DTH status of mice to Brucella antigens was developed to measure the transfer of DTH from sensitized animals to non-sensitized mice with TFd. It was hoped this model could be used as a research tool to study transfer factor. The mouse, a readily available and easily maintained animal, would also provide a convenient in vivo assay of the biological activity of TFd preparations before their use in other animals.

The ability to use the Brucellergen preparation to assay DTH was demonstrated by typical DTH reactions to intradermal injection of the antigen in sensitized rabbits and cattle, and by positive footpad reactions in mice. No macroscopic reactions were seen in unsensitized animals. The reactivity in rabbits 30 days after infection with Brucella was not as great as that reported in the standardization techniques of Huddleson (60, 114), where 5 mm of induration was usually seen with the 1:8,000 dilution. It is of interest that rabbits infected nearly a year earlier showed better DTH responses than the 30-day infected rabbits. The enhanced DTH response seen in rabbits 7 weeks after infection may indicate that maximum DTH reactivity is not attained by 30 days.

The footpad assay, using Brucellergen as the test antigen, was shown to be a convenient measure of DTH reactivity to Brucella in mice in accordance with earlier reports using other antigen preparations

(107, 138). The DTH response was characterized macroscopically by swelling and erythema at 24 and 48 hours and microscopically by a mixed PMN-mononuclear infiltrate in the 24-hour reaction which was replaced by a predominant mononuclear component at 48 hours. An Arthus-type reaction may also have been displayed as evidenced by the swelling at 6 hours and the PMN component at 24 hours. This macroscopic and histological picture in the footpad of a DTH reaction preceded by an Arthus reaction has recently been described by Katsura et al. (72). Since humoral antibody production is part of the immune response in brucellosis, Arthus reactivity in infected mice could be expected.

Positive reactions were readily evident by subjective observation, and this method could be used where exact measurements are not required or where large numbers of mice are being used. Although measurement of the reaction with calipers is more tedious, consistent measurements can be achieved and may be more useful in analysis of experimental data.

An unexpected result was the apparent DTH sensitization of mice by footpad testing with the Brucellergen preparation. Earlier studies indicated that an antibody response could be stimulated in guinea pigs by the injection of relatively large amounts of Brucellergen (142), and occasionally in man by a single Brucellergen skin test, whether that test was positive or negative (60, 112). Repeated skin tests using normal human doses (1:8,000 to 1:10,000) did not result in positive skin tests (60). Stimulation of antibody production by repeated skin tests has also been demonstrated in this study in rabbits using a 1:1,000 dilution of Brucellergen. A DTH response

was not observed in these rabbits.

Mice however displayed a delayed-type footpad reaction, characterized by swelling and erythema, following repeated footpad testing with Brucellergen. About 40% of the negative control mice showed a reaction one week after the first footpad test and most of the mice showed reactions one week after the 2° test. The time course of the reaction resembled a DTH reaction with swelling at 24 and 48 hours. Histologically the 24 hour reaction contained a mixed PMN-mononuclear infiltrate with a much more predominant mononuclear component at 48 hours. The sensitization was shown to be more than a local phenomenon by the ability to elicit the reaction in the opposite foot in the 2° footpad test. A weak antibody titer ($\pm$ 1/20) was occasionally seen in serum drawn one week after the 1° test. A number of mice showing strong footpad reactions at the 2° test had no detectable antibody. A rise in the antibody titers was seen following repeated footpad testing (data not shown).

The discrepancy between these results and results in other animal species is probably due to the high concentration of antigen used in the mouse footpad assay. Though the total antigen in 0.025 ml of a 1:500 dilution is equivalent to that in 0.1 ml of a 1:2,000 dilution, the mouse itself is significantly smaller than these other species. DTH was not induced or detected in an experiment using a 1:4,000 antigen dilution in the footpad test. It should also be noted that the intradermal route of antigen injection has been found to be the most efficient route for DTH sensitization in the mouse (32) and that injection into the footpad probably mimics intradermal injection. In one experiment, subcutaneous injection of an equivalent

amount of Brucellergen used in the footpad test (0.1 ml of a 1:2,000 dilution) failed to significantly sensitize mice as assayed by the normal footpad test ten days later.

Whether the delayed reaction described above is a classic tuberculin-type DTH reaction can not be ascertained. It is possible that this is an example of cutaneous basophil hypersensitivity (CBH). The early appearance (7 days) of the reaction and the induction by injection of a non-viable protein antigen without adjuvant, in fact favors this interpretation. In one experiment (data not shown) no footpad reaction was seen in control mice that had been footpad tested 2 months previously. Though this is a relatively long interval, it does indicate the sensitivity is not long lasting, a characteristic of CBH. Dvorak et al. (45, 46) have reported that basophils are not detected by the tissue preparation methods used in this study.

In experiments using the footpad assay to evaluate transfer of Brucella DTH from sensitized animals to nonsensitized mice, there was evidence of transfer activity in one TFd preparation, Lot #1, prepared from strain 19 infected mice. Administration of this TFd to mice provided the only evidence of transferred sensitivity detectable in the 1° footpad test. Mice having received 5×10^7 cell-equivalents 48 hours earlier displayed weak, but statistically significant responses in the 1° test compared to control mice. Subsequent experiments using a higher dose of the same preparation and a second preparation from the same cell source did not show significant difference in 1° test footpad reactivity of test and control mice, although there was evidence of enhanced reactivity in test mice in

the 2° footpad test, especially in those that received preparation #2.

Enhanced reactivity in the 2° footpad test was also evident in TFd- or whole cell-recipient mice in several other experiments. One of these involved mice given bovine (#230) peripheral leukocytes and another involved mice given bovine (#7) lymph-node TFd. Evidence for transfer of DTH provided by the 2° footpad test must however be evaluated with caution in light of the demonstrated sensitization of mice by the 1° footpad test. A number of unknown variables are involved in this sensitization. A possible explanation of the enhanced reactivity seen in some of these cases is that TFd treatment may in fact have an adjuvant effect on the sensitization induced by the 1° footpad test. Although this would represent a TF activity proposed by some authors (11), it would not be the classic activity of TF this study has attempted to demonstrate; that is the conversion of DTH reactivity in antigen-naive mice demonstrated by the 1° footpad test a short time (48-72 hours) following TFd treatment. As mentioned above, this type of reactivity was seen in mice receiving one murine TFd preparation, but not in mice receiving other murine or bovine TFd and whole cell preparations.

The failure to consistently demonstrate transfer of DTH was disappointing in light of several recent reports of successful transfer of DTH to mice. Klesius et al. (87) have transferred sensitivity to PPD using a bovine lymph-node TFd preparation. Rifkind et al. (130, 131) have transferred sensitivity to Coccidioides, Candida, and Mycobacteria with a murine TFd prepared in the same manner as the murine TFd in this study, and Lawrence's group has reported transfer of sensitivity to several antigens using a murine lymph-node TFd

preparation (14). Successful mouse-to-mouse transfer with whole lymphoid cells has also been reported (33, 129, 160), although we have found no report of transfer of DTH to Brucella by either whole cells or transfer factor.

Several explanations may be offered for the failure to consistently demonstrate transfer. Cross-species transfer, though reported by Klesius, has in general been difficult to demonstrate. TF donors also need to display strong DTH reactivity, an unknown factor in heifer #7. Both of these problems should not be factors in the experiments involving mouse-to-mouse transfer with TFd prepared from mice displaying strong (mean value 61) DTH reactivity. The authors cited above have not, however, reported the DTH reactivity of TFd-donor animals, so direct comparisons can not be made.

Methods of preparation may be questioned, especially in the bovine preparations which represented modifications of previously reported techniques. Methods of cell collection and isolation were evaluated by the viability of the cell suspensions after processing. Whole cell or cell-equivalent TFd doses were then calculated as viable cell numbers, on the assumption that viable cells had retained their transfer factor moieties. The failure to transfer DTH with either whole bovine mononuclear peripheral cells or whole murine spleen cells indicates that the primary problem, at least, is not due to methods involved in preparation of TFd after the harvesting of cells.

The doses of TFd used were in accord with those reported by others (87, 129, 130, 160). Doses both higher and lower than these were employed, ranging from 2.5×10^8 cell-equivalents to 5×10^5

cell-equivalents. Youdim et al. (160) demonstrated that 5×10^7, but not 1×10^7 mouse spleen cells, would transfer DTH to Listeria. This (5×10^7) is the TFd and whole cell dose regularly employed by Rifkind et al. (129, 130).

Primary footpad tests were applied 48 hours, 72 hours, and 7 days following TFd administration. Classically, transferred DTH is demonstrable in humans within 48 hours (95). Rifkind et al. (130) demonstrated transferred DTH in mice as early as 24 hours after TFd administration.

The use of the footpad assay to test DTH conversion may be questioned. Although others have used this assay to test DTH to Brucella antigens (107, 138) and the evidence in this study indicates that DTH reactivity is responsible for the footpad swelling, a comprehensive study of the use of the footpad assay in mice sensitized to Brucella has not been conducted. Such a study would be beneficial in the interpretation of the negative transfer data presented above.

One factor in the experiments reported here that is not present in any of the reports of successful DTH transfer to mice cited above, is the use of Brucella antigens. Klesius et al. have reported transfer of reactivity to Brucella antigens in mice as assayed by an in vitro lymphocyte stimulation assay (82) but not by footpad reactivity (Klesius-personal communication). A significant non-specific reactivity has been noted under certain conditions in this assay (82).

Brucella organisms and the immune response they elicit may be significantly different than other organisms for which successful DTH transfer has been achieved. The role of these differences has not been resolved in this study. Spleen cells were harvested for TFd

preparation at a time following Brucella infection when footpad reactivity has been shown to be maximal (107). This may not have been, however, the optimal time in the immune response to prepare TFd from the donor animals. It is conceivable that a suppressor moiety or suppressor cell stimulating moiety could have been present in cells collected at this time. The role of recipient priming by environmental exposure to microbial antigens prior to administration of TFd may be important in comparing transfer of DTH to relatively rare organisms such as Brucella to transfer of DTH to more commonly encountered organisms such as Candida or Mycobacteria. It should also be noted that mice are relatively resistant to Brucella infection, showing few signs of disease when infected with doses that cause serious illness in some other species. The mouse, therefore, may not be an optimal model to study certain aspects of brucellosis, among these transfer of DTH reactivity.

Transfer of DTH to Brucella was not consistently demonstrated by the methods or preparations used in this study. It should be noted that initial studies in transfer factor research have often yielded negative data, until new methods or models were developed (13, 62, 95). Modification of one or several aspects of the procedures used here might lead to unequivocal demonstration that DTH to Brucella can be transferred using TFd in mice.

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