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Assessment of immune parameters of the weak calf and the effect of thymosin treatment on those parameters

David Charles Flyer
The University of Montana

AN ASSESSMENT OF IMMUNE PARAMETERS OF THE WEAK CALF AND THE EFFECT OF THYMOSIN TREATMENT ON THOSE PARAMETERS

By

David Charles Flyer

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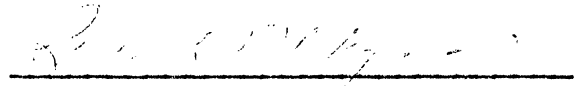

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An Assessment of Immune Parameters of the Weak Calf and the Effect of Thymosin Treatment on those Parameters (68 pp.)

Director: Dr. Richard N. Ushijima

The object of this study was to assess the status of the cellular immune response in the year-old weak calf runt, as well as the therapeutic benefits of thymosin, a protein thymic extract shown capable of inducing the maturation of thymus-derived lymphocytes.

Various assays currently available for evaluating the cellular component of the immune response, such as delayed hypersensitivity, lymphocyte proliferation, lymphokine production and humoral response to thymus-dependent antigens, were conducted. The results of these tests indicated no immunological abnormalities or deficiencies in weak calf responses when compared to the responses of normal calves, other than a minor depression in delayed hypersensitivity.

The ability of thymosin to correct the deficiency in delayed hypersensitivity and to promote normal growth and development in the weak calf was examined. Weak calves received 30,000 µg of thymosin a month for a period of four months and during this time their weights were monitored as well as their response to the tuberculin skin test antigen. After the four months, no discernible improvement in the weak calves receiving the thymus extract was observed that was not observed in the control animals. While the difference in delayed tuberculin hypersensitivity responses in thymosin treated animals were no longer significantly different from the responses of the normal animals, inconsistencies in the test results made it impossible to attribute this change to thymosin therapy.

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ABBREVIATIONS

B-lymphocyte	bone marrow-derived lymphocyte
BCG	Mycobacterium bovis, Paris strain bacille Calmette Guerin
BSS	balanced salt solution
E-rosette	erythrocyte rosette
FCS	fetal calf serum
FITC	fluorescine isothiocyanate
M.I.	migration index
MIF	migration inhibition factor
NRS	normal rabbit sera
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PHA	phytohemagglutinin
RFC	rosette forming cell
S.I.	stimulatory index
SRBC	sheep red blood cell
T-lymphocyte	thymus-derived lymphocyte
WBC	white blood cell
WCS	Weak Calf Syndrome

Chapter 1

INTRODUCTION

The Weak Calf Syndrome

Investigations into severe calf losses in the Bitterroot Valley of Montana in 1964 led to the recognition of a new disease condition of neonatal cattle (41). Because the calves afflicted with this disease appeared weak at birth and unable to rise, this condition became known as the Weak Calf Syndrome (WCS). In recent years, WCS has become a prevalent problem in parts of the Western United States and Canada and has resulted in significant economic losses to ranchers due to late abortion and a high mortality of calves less than six weeks of age (52). Mortality rates have been reported to be as high as 80% in affected herds (10). Of depressing consequence of the disease condition is that a high number of calves surviving the affliction fail to exhibit normal growth and develop into runted animals.

At or within the first few weeks after birth, weak-syndrome calves exhibit a depression along with a reluctance to stand, move or even nurse. Polyarthritis, a crusting and reddening of the muzzle and lips and calf scours are also observed. These calves exhibit a marked leukopenia at birth averaging 1×10^3 WBC/mm^3 in comparison to 2.2×10^4 WBC/mm^3

for normal calves (56). The leukopenia is transitory and shifts to a leukocytosis about four days later. Histopathology of the weak calf is characterized by subcutaneous bilateral edema and hemorrhages of the extremities, hemorrhages on the third eyelid, tongue, esophagus and trachea, involution of the thymus, enlargement and edema of the lymph nodes, petechial hemorrhages of internal organs, polyarthritis and synovitis (10, 65).

Early treatment of weak calves with intravenous injections of electrolyte solutions and transfusions of whole blood or the administration of immune sera proved beneficial for those calves less severely afflicted, but this did not alter the course of the runting syndrome associated with the disease. Later, thymosin, a protein thymic extract, was used in the treatment of weak calves (64). Again those calves less severely afflicted showed an improvement in health but no subsequent return to normal weight.

A depression in cellular immune responsiveness has been observed in weak calves skin tested with tubercle bacilli. Weak syndrome calves sensitized with Mycobacterium bovis, Paris strain bacille Calmette Guerin (BCG), failed to mount a delayed hypersensitivity response to the BCG skin test antigen (56). Other studies have shown that lymphocytes from newborn weak calves responded to a lesser degree than lymphocytes from newborn normal calves when stimulated with phytohemagglutinin (PHA), a non-specific T-lymphocyte mitogen (64).

Runting Syndrome

The induction of a runting syndrome is observed in laboratory animals after various experimental procedures. These procedures are carried out during the neonatal period and include thymectomy (35), administration of cortisone acetate (25) or sterile bacterial vaccines (13), the injection of foreign immunologically competent cells (5) and infection with certain viruses (54, 57). The resulting syndrome is usually defined by the following characteristics: 1) loss of body weight to less than 60%, 2) clinical symptoms such as lethargy, periorbital edema, ruffled fur, lymphocytopenia, diarrhea and atrophy of the thymus and other lymphoid organs, 3) subsequent early death (54).

Examination of organs of runted animals at autopsy shows many organs to be of subnormal size due to the depletion in the number of normal parenchymal cells within the organs (39, 40). This organ abnormality can be differentiated from those occurring during malnutrition which results in a decreased cytoplasmic volume and not in the number of parenchymal cells (38). In addition, a significant depletion of lymphocytes is observed in the lymphoid organs. The thymus of the runted animals lacks any histological resemblance to a normal thymus (57). The cortex is reduced to a thin perimeter of lymphocytes and the medula is replaced with histiocytes. This histological pattern is also observed in the enlarged lymph nodes of the runts. A leukocytosis due to an increase in the granulocyte population

is also present (57). The normal ratio of lymphocytes to granulocytes is completely reversed with 70-80% granulocytes observed in the differential count.

The etiology of this runting syndrome has not been completely clarified and more than one mechanism may contribute to the growth disorder of the various runts. Microorganisms appear to play some role in thymectomized and cortisone-induced runting in mice. On the other hand, axenic animals do not develop the runting syndrome after thymectomy and appear to have an increased resistance to runting induced by cortisone administration (43). Antibiotics reduce the severity of wasting that occurs following both types of treatment but not the runting caused by sterile bacterial vaccines (2).

Immunologic mechanisms also appear to play a role in runting syndromes. Runting due to the injection of foreign immunologically competent cells is believed to result from the immunological response of the donor cells against the transplantation antigens of the host (54).

Viral-induced runting may be due to the immunological destruction of lymphatic cells that acquire viral antigens as well as the direct cytopathic effect of the virus on lymphatic cells and its inhibitory effect on cell multiplication (54). Growth retardation has also been reported in human infants with congenital rubella (38) and cytomegalovirus infections (40). A subnormal number of parenchymal cells is also observed in many organs of these infants as

well as a decrease in lymphoid elements. Surviving infants are unable to mount any humoral response to these viruses until approximately one to two years of age.

Cellular Immunity

The immune response of an organism to a foreign antigen can manifest itself in two ways: the production of humoral antibody and/or the development of cellular immunity. Although the two responses are not entirely independent, the two mechanisms are distinctly different.

The study of cellular immunity began with the investigations of Landsteiner and Chase (33) in 1942. Their experiments showed that delayed hypersensitivity could be transferred to unsensitized hosts by lymphoid cells but not by serum from sensitized individuals. The delayed hypersensitivity reaction is an immunologically specific response occurring in appropriately sensitized animals after reintroduction of the antigen. The reaction occurs several hours after reinjection of the antigen and is characterized by increasing erythema and induration at the site of injection. The erythema and induration are the result of an infiltration of predominantly mononuclear lymphocytes and mononuclear phagocytes (33).

It is now recognized that cell-mediated mechanisms are responsible for certain immune responses to a variety of antigens including viral, microbial, heterologous protein antigens, transplantation antigens (organ and skin grafts)

and autologous antigens (autoimmune disease)(33).

Of the two classes of lymphocytes known today, thymus-derived lymphocytes (T-lymphocytes) and bone marrow-derived lymphocytes (B-lymphocytes), it is the T-lymphocytes that are involved in these cell-mediated immune mechanisms (33, 45). Enumeration of B-lymphocytes and T-lymphocytes can be accomplished based on their differing surface structures. B-lymphocytes have easily detectable membrane immunoglobulins as well as receptors for complement and the Fc region of IgG, all of which serve as population markers (25). These markers are absent from T-lymphocytes, which are recognized by their formation of spontaneous rosettes with sheep erythrocytes (E-rosettes). The formation of E-rosettes is non-immunological but serves as a convenient marker of T-lymphocytes. These lymphocytes have been identified as thymus-derived lymphocytes by virtue of the inhibition of rosette formation by anti-theta serum, anti-lymphocyte serum and azathioprine, but not by anti-immunoglobulin (3, 12).

E-rosettes have also been reported to serve as a marker for bovine T-lymphocytes (18). The number of bovine peripheral blood lymphocytes (PBL) has been reported in the literature to range from 0% (20) to 19% (18) with assay parameters varying from study to study. It appears that while all human and mouse T-lymphocytes form E-rosettes, only a subpopulation of bovine T-lymphocytes form E-rosettes (18)

In vivo and in vitro assays based on the immune functions of T-lymphocytes are currently being used to evaluate

cellular immunity. Delayed hypersensitivity skin testing is probably considered the most reliable *in vivo* procedure for the evaluation of cellular immune responsiveness. The development of a delayed hypersensitivity reaction at the site of injection of the skin test antigen indicates that the afferent, central and efferent portions of the immune response are functioning normally and that the ability to mount a non-specific inflammatory response is intact (51).

The migration inhibition test, an *in vitro* assay shown to correlate with *in vivo* delayed hypersensitivity, is often used in human medicine. When sensitized lymphocytes are stimulated *in vitro* by the sensitizing antigen, a soluble factor called migration inhibition factor (MIF) which retards the migration of macrophages and leukocytes from capillary tubes is produced. The production of MIF in response to the specific antigen gives an evaluation of lymphocyte function as it relates to cellular delayed hypersensitivity (44).

In vitro lymphocyte proliferative responses (blastogenesis) are also used to evaluate cellular immunity. The induction of blast-like transformation of sensitized lymphocytes by the sensitizing antigen can be quantified by measuring the incorporation of tritiated thymidine into newly synthesized DNA. Certain plant mitogens can also induce lymphocyte proliferation by reacting non-specifically with the cell surface and instigating the same series of

cellular events. Phytohemagglutinin (PHA) is one such plant mitogen, but unlike antigen stimulated proliferation where only a small fraction of the lymphocytes are sensitive, PHA induces blastogenic activity non-specifically in the majority of the T-lymphocytes (45).

Immunosuppression by Corticosteroids and Viruses

Cellular immune responsiveness can be depressed non-specifically by a variety of factors such as neoplastic and infectious diseases, trauma, X-irradiation, malnutrition and elevated corticosteroid levels (54).

Corticosteroids are commonly used as chemotherapeutic agents for the suppression of undesirable immune and inflammatory responses. Although the precise mechanism of action is unknown, these steroids appear to act on many aspects of the immune response, from the inhibition of skin allograft rejection in mice to the inhibition of tuberculin skin test reactivity in man (7, 15).

The effect of corticosteroids upon circulating lymphoid cells has been described by Fauci and Dale (14). A transient lymphopenia with decreases in circulating lymphocytes and monocytes is observed after a single intravenous dose of hydrocortisone. The depletion of lymphocytes is selective with a proportionately greater decrease in the number of T-lymphocytes as measured by the E-rosette assay. This decrease in circulating lymphoid cells is believed to be due to redistribution to other body compartments from the

circulation rather than by direct destruction.

A decrease in antigen responsiveness in vitro is observed after hydroxycortisone administration (14). This decrease correlates well with the suppression of delayed hypersensitivity that occurs during corticosteroid therapy. Migration inhibition factor activity is also suppressed significantly by corticosteroids (4), which appear to disrupt inhibitory effect of MIF on the macrophage as opposed to antigen processing by the macrophage or the ability of lymphocytes to produce MIF.

Immunosuppressive effects of corticosteroids are of interest in studying immunosuppression in the weak calf due to the high levels of corticoids in newborn calves. Plasma corticoid levels in neonatal calves are extremely high, averaging 116.4 ng/ml at parturition and falling to a level of 2.2 ng/ml by day ten (22). Corticoid levels in calves with infectious calf diarrhea are generally higher than in healthy calves of the same age. Levels during the first four days of life are double that of healthy calves. Eight to 25% of calves with infectious calf diarrhea die before the age of two months, and many of those that survive are permanently stunted (22).

The ability of many viruses to suppress cellular immunity during and after infection has been demonstrated in various studies. In 1908 von Pirquet (63) reported suppression of tuberculin delayed hypersensitivity in children with measles, all of which had previously been

skin test positive. In 1919, Bloomfield and Mateer (63) reported the same suppression in patients with acute influenza infections. Many viruses known to suppress cellular immune responsiveness have since been added to the list. Mumps, rubella, Newcastle disease virus and poliovirus are among those shown to inhibit the in vitro response of normal lymphocytes to PHA while infants with congenital rubella infections also have a depressed lymphocyte response to PHA (26). This suppression of T-lymphocyte function may be the result of histological changes in thymic and other lymphoid tissue that has been observed in acute measles infections (63).

Perhaps the most noteworthy observation in suppression of cell-mediated immunity induced by viruses are those changes effected by mouse thymic virus (MTV). Neonatal infection of mice with MTV, a herpesvirus, results in thymic necrosis and cellular depletion. Inoculation of newborn mice with MTV results in a syndrome resembling neonatal thymectomy and results in functional T-lymphocyte depletion (8).

Thymic Immunodeficiencies

Besides the non-specific immunosuppressing factors mentioned, immune responsiveness can also be suppressed by primary immunodeficiency diseases. Immunodeficiency diseases result from defects in the development of normal immune responses where either the ability to produce antibodies and/or the development of cell-mediated immunity is depressed

or absent. Over thirty discrete phenotypic patterns of immunodeficiency have been described in man (9). Although classification of many of the clinical symptoms is difficult, distinct patterns have been recognized enabling the general classification of defects into three categories based on cellular response: 1) B-lymphocyte, 2) T-lymphocyte and stem cell deficiencies. Only T-lymphocyte deficiencies will be discussed in this thesis.

The role of the thymus in the development of immunity was first suggested by the frequent occurrences of thymic abnormalities in children with immunological deficiency disorders. The thymus is a lymphoid organ comprised of lymphoid and epithelial cells. During embryogenesis, the thymus along with the parathyroid gland arises from the third and fourth pharyngeal pouch epithelium and in most species is fully developed by birth (23). The thymus exerts its influence on precursor bone marrow stem cells. These cells differentiate into immunocompetent T-lymphocytes in the thymus and are transported from the thymus to peripheral lymphoid tissues, blood and lymph (62). Failure of the thymus to develop properly results in thymic immunodeficiency, under which conditions precursor stem cells fail to differentiate into T-lymphocytes responsible for cellular immunity.

Congenital thymic aplasia in man, known as Di George's Syndrome, occurs when the thymus fails to arise during the sixth week of embryonic life. Cause for this embryonic flaw

is still unknown. Autopsy reveals little or no thymus and parathyroid tissues, and those infants surviving exhibit a high susceptibility to viral, fungal and bacterial infections. In thymic aplasia, germinal center development and immunoglobulin synthesis remain normal, but the depletion of lymphocytes in the subcortical, thymus-dependent regions of the lymph nodes as well as the lymphoid sheaths of the spleen are prominent (46). Deficiencies in cellular immunity such as the loss of delayed hypersensitivity to common antigens, loss of ability for skin graft rejection, lowered response of peripheral blood lymphocytes to mitogens have been observed. Transplants of fetal thymus tissue have been successful in correcting the deficit of T-lymphocytes and cellular immunity in Di George's Syndrome patients. Normal skin graft rejection, response to intradermal skin test antigens, in vitro response to mitogens as well as increased lymphocyte count and population of thymus-dependent areas are observed as early as 48 hours after the transplant (46).

Congenital thymic dysplasia, or Nezelof Syndrome, is a genetically acquired thymic immunodeficiency in contrast to Di George's Syndrome which has no genetic involvement. In this autosomal recessive disease, the thymus is reduced in size, consists almost entirely of epithelial cells and lacks any Hassall's corpuscles. While the population of circulating lymphocytes is not depleted and immunoglobulin levels usually appear normal, cellular immune responsiveness

is impaired (9). In thymic dysplasia, transplantation of fetal thymus tissues does not correct the T-lymphocyte deficiency. An abnormality in the inductive function of the epithelial portion of the thymus is believed to be responsible for the immunologic deficiency (9).

While the preceding are examples of blatant, primary thymic immunodeficiencies, persons with chronic mucocutaneous candidiasis serve as examples of more subtle T-lymphocyte deficiency. In these cases the circulating population of T-lymphocytes is normal as well as their *in vitro* proliferative responses to mitogens, but the expression of delayed hypersensitivity and lymphokine production is impaired (9).

Effects of Thymectomy

Thymectomy has been used as a laboratory procedure to induce artificially a thymic immunodeficient condition in laboratory animals. While the effects of thymectomy in adult animals are slight, thymectomy during the neonatal period produces a severe depletion of the T-lymphocyte population, defects in the development of immune functions and gross changes in the histology of lymphoid tissue (34, 46).

The most notable result of thymectomy is the associated wasting disease characterized by decreased growth rate, listlessness, ruffled fur, hunched posture, periorbital edema, diarrhea and death (23, 36). The cause of the wasting disease has been attributed to the animals' increased susceptibility

to infectious diseases due to an inability to respond immunologically to pathogenic organisms. Thymectomized animals given either antibiotics or kept in germ-free environments exhibit a reduction or no change in the incidence of wasting or none at all.

Many of the immunological consequences of neonatal thymectomy are similar to those observed in congenital thymic aplasia. Thymectomy results in a reduction in the population of circulating lymphocytes (the immunologically competent cells responsible for cell mediated immunity) and depletion of thymus-dependent areas of the lymph nodes and spleen. Among the serious defects in cell-mediated immune responses observed are impairment of homograft rejection, inability to elicit delayed hypersensitivity reactions to tubercle bacilli and bovine serum albumin and a reduced humoral response to certain antigens (i.e. sheep erythrocytes, human gamma globulin and ova albumin) (36).

Thymic Reconstitution

Thymic deficiencies have been treated by a variety of procedures. As mentioned earlier, reconstitution of patients with congenital thymic aplasia using grafts of fetal thymus tissue has resulted in restoration of immunological competence. Restoration of immunological competence in neonatally thymectomized mice using lymphoid cell suspensions or thymic grafts have also been successful (36).

More interesting are the implantation studies using cell-tight Millipore diffusion chambers containing thymus tissue into peritoneal cavities of young thymectomized mice. These chambers permit large size molecular exchange (pore size approximately 0.1 μm in diameter) but restrict the passage of cells. Restoration of skin graft rejection and humoral antibody response to sheep erythrocytes and human gamma globulin have been observed in a majority of these animals. Circulating lymphocytes are only moderately reduced in number, and the spleen and lymph nodes are almost normal in size and cellular content (31, 40).

These studies suggest that thymic tissue produces a specific humoral factor that triggers differentiation and maturation of lymphoid precursor cells to immunologic competence. Examinations of the tissue within the diffusion chambers several weeks after their implantation shows only epithelial cells to be present. These findings suggest that the epithelial cells of the thymus are responsible for production of the humoral substance (53).

More recently, interest has centered around the use of thymus cell-free extracts. A thymic factor called thymosin has been isolated from calf thymus tissue and has been shown to be involved in the development and maturation of lymphoid tissue by a variety of *in vivo* and *in vitro* studies (16,55). Thymosin treatment of neonatally thymectomized mice has been shown to induce immunological competence. In these mice,

thymosin treatment has significantly reduced the incidence of wasting disease while increasing the number of circulating lymphocytes and conferring a partial restoration of cellular immunity as measured by homograft rejection (1).

Thymosin treatment has also been applied to human patients with thymic hypoplasis and has resulted in improved clinical appearance, the appearance of a previously depressed delayed hypersensitivity and an increased percentage of *in vitro* T-lymphocyte E-rosettes (60).

Action of Thymic Hormones

Restoration of lymphocyte activity to azothioprine, anti-lymphocyte serum and anti-theta serum in neonatally thymectomized mice by thymic hormones has been taken as an indication of its ability to induce immunocompetence in thymus-dependent lymphocytes (3, 16). This activity of thymic hormones on T-lymphocytes has been supported by the ability of thymic hormones to induce *in vitro* development of characteristic T-lymphocyte surface antigens TL, Thy-1 and Ly from populations of spleen and bone marrow cells of adult mice (27). The presence of any one of these markers indicates that the cell has undergone thymus-directed differentiation. Cells from spleen and bone marrow of nu/nu mice can also be induced to develop T-lymphocyte surface antigens. Since nu/nu mice are congenitally thymus deficient, the inducible cell must be a precursor cell that has not

undergone any thymus-directed process (26, 32).

How thymic hormones induce immunocompetence is not fully understood. Experiments monitoring activation of membrane-associated enzymes in lymphoid cells exposed to thymic hormones indicate that they exert their influence by activating membrane-associated adenyl cyclase which leads to a rise in intracellular cAMP (1). This elevation of membrane-associated enzyme activity is only observed in thymus-derived cells.

It has been suggested that these hormones act on at least three distinct classes of lymphoid cells: pre-thymic stem cells, thymus-derived cells (T_0 and T_1 cells) and mature T-lymphocytes (T_2 cells) (55). Thymic hormones appear to function by accelerating the development and maturation of stem cells into immunologically competent lymphocytes and then function in their further differentiation into specialized T-lymphocytes such as memory, killer, suppressor or effector lymphocytes.

Statement of Thesis

Weak Calf Syndrome has become a problem in the cattle industry resulting in significant economic loss to the cattle rancher. The possibility of thymic dysfunction in these animals is suggested by the absence of a normal thymus, runted appearance and failure to respond normally to tuberculin skin test antigens.

Treatment with thymic extracts in other thymic deficient systems has been shown to induce immunological competence. Applicability of treatment with thymic extracts has been demonstrated in newborn weak calves as judged by significant improvement in health and increase in uptake of tritiated-thymidine upon stimulation of lymphocytes with the T-lymphocyte mitogen PHA in an *in vitro* system (64).

The purpose of this study was designed to: 1) evaluate the status of the immune system of the weak calf by measurement of various immunologic parameters and 2) investigate the effects of treatment with thymosin, a protein thymic extract, on those parameters and on the general overall appearance of the weak calf.

Chapter 2

MATERIALS AND METHODS

Animals

Calves used in this study were made available through a loan obtained from Mr. Duane Van Meter, a cattle rancher in the Bitterroot Valley of Montana. All calves were approximately one year of age at the start of this research project. These calves were classified as either normal or weak calves by Dr. Jack Ward, a practicing veterinarian in Hamilton, Montana, who first recognized the weak calf problem in 1964. The criteria used in the classification of the calves were: comparative size for year-old calves, growth rate and hair coat texture (Illustration 1). An attempt was made to utilize in this study calves believed to have been afflicted with varied degrees and intensities of the disease condition.

Collection of Samples

Blood samples were collected from animals by Dr. Ward. Forty ml of venous blood was drawn by venipuncture from the jugular vein. Twenty ml was transferred to vacutainer collection tubes for the collection of serum and to tubes containing 1,000 units of preservative-free sodium heparin

Illustration 1. The comparative size difference of year-old normal and weak calves is illustrated in the following photograph. Other physical characteristics of the weak calf, such as rough coat texture and periorbital edema, can also be observed.

(Lilly) for whole blood samples. All samples were kept at ambient temperature during transport from Hamilton to the laboratory and were processed immediately upon arrival.

Differential White Blood Cell Counts

Blood smears were prepared from heparinized whole blood and stained with Wright's stain (Medi Chemicals Inc.) for differential WBC counts (Table 1). After staining, the blood smears were examined under oil and a total of 200 cells were counted. Cell types were identified as lymphocytes, monocytes, eosinophilic granulocytes, basiophilic granulocytes and segmented and juvenile neutrophilic granulocytes.

Table 1 Wright's staining procedure.

1) Fix blood smear in absolute methanol for 1 second.
2) Place slide in Wright's stain #1 (eosinate stain) for 20 seconds.
3) Wash slide in distilled water.
4) Place slide in Wright's stain #2 (polychrome stain) for 20 seconds.
5) Wash slide in distilled water and air dry.

Total White Blood Cell Counts

Using WBC diluting pipettes, heparinized whole blood was diluted 1:20 with Turk's diluting fluid (Table 2) for total leukocyte counts. Counting was done in Neubauer hemocytometers. All four corner mm^3 squares were counted and the

total leukocyte count was calculated using the following formula:

$$\frac{\text{number of leukocytes counted x dilution x 10}}{\text{number of mm}^3\text{ squares counted}} = \text{leukocytes/mm}^3$$

Table 2 Turk's white blood cell diluting fluid.

Glacial acetic acid	2 ml
Aqueous gentian violet	1 ml
Distilled water	100 ml

Mix and filter sterilize.

Ficoll-diatrizoate Separation of Peripheral Blood Leukocytes(PBL)

Separation of PBL on ficoll-diatrizoate density gradients was conducted by the method of Boyum (6). Twenty ml of heparinized bovine blood was centrifuged in 40 ml conical centrifuge tubes at 650 x g for 30 min at 4°C. The buffy coat was removed and resuspended in Hank's BSS and carefully layered over 5 ml of ficoll-diatrizoate (Table 3) in a 15 ml conical centrifuge tube. The blood-ficoll gradient was spun at 400 x g for 20 min at 4°C. The cells at the ficoll and Hank's interface were harvested and washed in Hank's BSS. The leukocyte pellet was resuspended and incubated in 0.83% NH_4Cl for 5 min to remove contaminating red blood cells. The leukocytes were washed two additional times in Hank's BSS and resuspended in tissue culture media.

Table 3 Ficoll-diatrizoate

1) Dissolve 9.65 g sodium diatrizoate (Winthrop Labs.) in 30 ml of distilled water and adjust the pH to 7.3.

2) Add 72 ml of distilled water.

3) Dissolve 6.35 g ficoll (Pharmacia) in the above solution.

4) Heat sterilize at 121°C for 15 min. Store at 4°C in a foil covered bottle to prevent exposure to light.

Blastogenesis

Leukocytes separated via ficoll-diatrizoate were adjusted to a concentration of 1 x 10^6 cells/ml in 10 ml of RPMI media with 10% FCS (Table 4). Triplicate 1 ml samples were removed and placed in glass tissue culture tubes and labeled "control cultures." After discarding 2 ml, 0.05 ml of a stock PHA-P solution (Table 5) was added to the remaining 5 ml of the cell suspension to achieve a final PHA-P concentration of 1 µl/ml. Triplicate 1 ml samples were removed and placed in glass tissue culture tubes and labeled "PHA-P cultures." All cultures were incubated at 37°C in 5% CO_2.

After 72 hrs, the cultures were removed from the incubator, and 0.1 ml of a stock tritiated thymidine solution (Table 6) was added to each tube to achieve a final concentration of 1 µCi/ml. The cultures were returned to the incubator for an additional 24 hrs.

After a total of 96 hrs of incubation, the cultures were processed for counting by liquid scintillation using a Millipore sampling manifold. Using suction, cells were collected on damp Whatman GF/C filter paper and washed with

Table 4 RPMI 1640 media.

RPMI 1640 dry powder (Gibco)	6.5 g
Triple distilled water	1.0 liter
Penicillin G	100,000 units
Streptomycin sulfate	100,000 µg
Fetal calf serum (Microbiology Associates) inactivated at 56°C for 30 min	100 ml

Table 5 Phytohemagglutinin-P (PHA-P) stock solution.

PHA-P (Difco)	1 ml
RPMI 1640 media with 10% FCS	9 ml

Stock PHA-P solution was stored in 0.05 ml calibrated dropper bottles at - 70°C until used. Final concentration of the PHA-P stock solution is 5 µl/ml.

Table 6 Tritiated thymidine stock solution.

Thymidine, methyl-^{3}H, 1 mCi, specific activity 71.5 Ci/mmole (ICN)	1 ml
RPMI 1640 media with 10% FCS	99 ml

Final concentration of the tritiated thymidine stock solution is 10 µCi/ml. Stock solution was stored at -70°C until used.

4 ml of cold 3% acetic acid. The cellular nucleic acids were precipitated with 6% trichloroacetic acid and then washed with 3 ml of cold PBS pH 7.2. The filters were dried for 2 hrs in a 65°C oven and transferred to scintillation vials (Beckman) which were filled with 3 ml of POP:POPOP scintillation flour (Table 7) and counted for 10 min in a Nuclear Chicago Unilex III liquid scintillation counter. The counts of the triplicate samples were averaged to give one value for each leukocyte sample. The stimulatory index (S.I.) was calculated by dividing the counts of the PHA-P sample by the counts of the control sample.

Table 7 Scintillation flour.

POPOP (1,4-bis 2-(5 phenyloxazolyl) benzene (Sigma) 0.05 g

POP (2,5-diphenyloxazole) (Sigma) . . . . . 4.0 g

Toluene . . . . . 1 liter

Store in brown bottle to prevent exposure to light.

Immunofluorescence

Two x 10^6 leukocytes that had been obtained via ficoll-diatrizoate separation were placed in 12 x 75 mm test tubes and washed twice with PBS pH 7.2. After the second wash, the cell pellets were resuspended in 0.4 ml of a 1:30 dilution of anti-bovine globulin serum of rabbit origin conjugated with FITC (Colorado Serum Co.). The cells were incubated on ice at room temperature for 45 min and then

washed four times in PBS with 3% FCS. The leukocytes were resuspended in 0.25 ml of PBS with 50% glycerin and placed on glass slides under cover slips. All slides were viewed using an AKO fluorescence microscope. A total of 200 leukocytes were counted and fluorescent cells were recorded as B-lymphocytes and non-fluorescent cells as T-lymphocytes.

E-rosette Assay

The E-rosette assay was carried out with modification by the method of Jondal et al (25). Leukocytes separated via ficoll-diatrizoate were resuspended in Hank's BSS with 10% FCS at a concentration of 1×10^7 cells/ml. One tenth ml of this suspension was added to 0.1 ml of a 1% suspension of sheep red blood cells (SRBC). The cells were gently mixed and incubated at 37°C for 10 min. Following incubation, the cells were centrifuged at 200 x g for 5 min and allowed to sit overnight at 4°C. The following day the pelleted cells were gently resuspended by rotating the conical centrifuge tube on a horizontal axis until all cells were in suspension. The cells were added to a cold Neubauer hemocytometer and allowed to settle. Two hundred cells were counted and the number of rosette forming cells determined. A cell with three or more SRBC adhering to its surface was considered to be a rosette.

Humoral Response to a T-dependent Antigen

To assay for production of antibody to a T-dependent antigen, all calves received 5.0 ml intravenous injections

of a 5.0 mg/ml preparation of human albumin fraction V (Sigma). Serum samples were collected on day 0, 7 and 14. Anti-human albumin antibody was measured by indirect hemagglutination.

Indirect hemagglutination: Using "U" bottom microtiter plates (CECO), each test well in columns 1-12 received 50 µl of PBS pH 7.2 with 2% normal rabbit sera (NRS). Fifty µl of whole sera was added to well 1 and titrated out through well 12 using 50 µl microdiluters. All wells then received 50 µl of a 0.5% suspension of SRBC sensitized with human albumin. Controls of tanned and sensitized SRBC were included in the experiment. The plates were incubated at room temperature for 2 hrs and the titers determined.

Sensitization of SRBC's: A 3% suspension of SRBC in PBS pH 7.2 was mixed with an equal volume of a freshly prepared 1:40,000 dilution of tannic acid (Baker) and incubated in a 37°C water bath for 15 min. After incubation, the SRBC were washed once in PBS and resuspended in a 3% suspension. The SRBC suspension was then mixed with an equal volume of a 5.0 mg/ml preparation of human albumin fraction V and incubated at room temperature for 15 min. The SRBC were washed twice in PBS and resuspended at a concentration of 0.5% in PBS pH 7.2 with 2% NRS.

Immunoelectrophoresis

Immunoelectrophoresis was performed using 1% agarose (Seakem) in veronal buffer (Table 8) on a glass microscope

slide. Electrophoretic separation of bovine serum was carried out at 3 mamps/slide over a 3 hr period. Anti-bovine sera (rabbit origin) was added to troughs cut into the agarose slides and the slides were incubated in a humid chamber at room temperature for 48 hrs. Evaluation of normal and weak calf sera was done by comparing the immunoelectrophoretic pattern (precipitin arc shapes, intensities, general appearance and electrophoretic location) of the various sera after precipitation with anti-bovine sera.

Table 3 Veronal buffer pH 8.4.

0.2 M sodium barbitol (Mallinckrodt Inc.)	50 ml
0.2 M HCl	9 ml
Distilled water	141 ml

Delayed Hypersensitivity to BCG

The Paris strain of BCG vaccine (43) was obtained from stock maintained in the Stella Duncan Institute, University of Montana, Missoula, Montana. The initial stock cultures were diluted in Dubos broth to achieve an optical density of K-100 on a Klett-Summerson photoelectric colorimeter and stored as the working solution in vials at -70°C until ready to use.

All calves were vaccinated with 2.5 ml of the BCG vaccine via intramuscular injections. Two weeks after vaccination, the calves were skin tested in the caudal fold with 800 μg of BCG protoplasm. Skin tests were measured 72 hrs after

being administered.

The BCG protoplasm was obtained from the Stella Duncan Institute and was prepared by resuspending twice-washed BCG bacilli in distilled water at a concentration of 10-15 g (wet weight) of bacilli per 100 ml. Cells were ruptured in a Sorval Ribi cell fractionator at 45,000 psi, and the effluent was centrifuged at 27,000 x g for 1 hr to remove cellular debris. The supernate, referred to as BCG protoplasm, was lypholyzed and stored at -20°C.

Macrophage Migration Inhibition Assay

Production of MIF: Leukocytes separated via ficoll-diatrizoate were adjusted to a concentration of 5 x 10^6 leukocytes/ml in M-199 media with 10% FCS (Table 9). Two ml of the leukocyte suspension was placed in duplicate tissue culture tubes. One tube received 100 µg of the BCG skin test antigen and the control tube received none. The cultures were incubated at 37°C for 48 hrs in 5% CO_2. After incubation, the leukocytes were centrifuged at 400 x g for 10 min, and the supernate fluids containing the MIF were removed. The supernates were stored at -70°C until used.

Collection of guinea pig macrophages: Normal guinea pigs received intraperitoneal injections of 30 ml of sterile mineral oil to increase the number of macrophages in the peritoneal cavity. Approximately 72 hrs later, the animals were sacrificed and tacked on an autopsy board. After the abdomen was gently kneaded, 50 ml of cold, sterile Hank's BSS

with 10 units of preservative-free heparin (Lilly)/ml was injected into the peritoneal cavity. An incision was made into the peritoneal wall, and the fluid withdrawn with a 50 ml syringe and placed in a cold separatory funnel. Upon standing, the layers soon separated and the aqueous layer was decanted into cold, siliconized, 40 ml centrifuge tubes and were centrifuged at 200 x g for 10 min at 4°C. The cells were collected and washed twice with Hank's BSS without heparin. After the final wash, the cells were resuspended to a final concentration of 10% by volume in M-199 media containing 15% guinea pig serum.

MIF assay: Capillary tubes were filled to 3/4 volume with the macrophage suspension and one end plugged with warm paraffin wax. The capillary tubes were then placed in sterile tubes with cotton plugs on the bottom and centrifuged at 200 x g for 5 min to pack the cells. The capillary tubes were cut at the interface between the cells and the supernate with a diamond pencil, and the portion with the cells was attached to the bottom slide in sterile Mackness chambers with silicon grease. The Mackness chambers were incubated at 37°C for 24 hrs. The results were recorded by drawing scale drawings of the migration patterns, and the percent inhibition was calculated using the following formula:

$$\% \text{ inhibition} = 1.0 - \frac{\text{test area}}{\text{control area}} \times 100$$

Table 9 M-199 media with Earl's salts

M-199 powder (Gibco)	9.9 g
Triple distilled water	1 liter
Penicillin G	100,000 units
Streptomycin sulfate	100,000 µg
$NaHCO_3$ (Baker)	1.5 g
Fetal calf serum (Microbiological Associates) inactivated at 56°C for 30 min	100 ml

Processing of Thymus Extract

Thymosin was extracted from bovine thymus glands by a modification of the method of Goldstein and White (17). Bovine thymus glands obtained from a local abattoir were homogenized in Waring blenders with 0.85% NaCl. The homogenate was centrifuged in 250 ml plastic centrifuge bottles at 3,500 x g for 5 min at 4°C. The supernatant fluid was decanted into 500 ml glass flasks and placed in 80°C water baths for 15 min. Most of the precipitate was removed by centrifugation at 3,500 x g and then cleared further by centrifugation at 16,000 rpm for 1 hr in a Sorval high speed centrifuge. The clear supernatant fluid was decanted and precipitated with acetone at 4°C. Acetone precipitation was accomplished by adding the clear supernate to 10 volumes of cold acetone with constant stirring. The precipitate formed was placed in an evaporating dish and allowed to sit overnight in the cold room. The dried precipitate was resuspended in 0.1 M phosphate buffer pH 7.2 by constant stirring for 3 hrs

and then centrifuged at 3,500 x g for 15 min. The supernate was collected by decanting and then precipitated with saturated $(NH_4)_2SO_4$ at room temperature. The final precipitate was resuspended in distilled water and dialyzed overnight against two changes of distilled water to remove the $(NH_4)_2SO_4$. The suspension was lypholyzed and stored at -20°C.

Administration of Thymus Extracts

Calves numbered 10-15 and 25-28 received 30,000 µg of thymosin monthly in two intramuscular injections over a period of four months. The first injection was administered in 5.0 ml of PBS at a concentration of 1,000 µg/ml. The second injection was administered in 5.0 ml of a 50% PBS - 50% peanut oil (Planters) mixture at a concentration of 5,000 µg thymosin/ml.

Statistical Analysis of Data

Statistical analysis of the data was done using the Mann-Whitney U test. The U test is a nonparametric test that is used to determine whether two independent groups have been drawn from the same population (30). The level of significance is obtained by calculating the value of the statistic U in each experiment.

To apply the U test, the data from the two groups are combined and ranked in order of decreasing size. The lowest score is assigned the lowest rank. The sum of the ranked

scores in each group is calculated and used in the following formula:

$$\underline{U} = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1 \quad \text{or} \quad \underline{U} = n_1 n_2 + \frac{n_2(n_2 + 1)}{2} - R_2$$

n_1 = number of items in the smaller group

n_2 = number of items in the larger group

R_1 = sum of the ranks of the items in the smaller group

R_2 = sum of the ranks of the items in the larger group

The smallest value of $\underline{U}$ is calculated and used to find the level of significance in the appropriate table (50). When the statistic is assigned a p value (probability value) less than 0.05, the two groups are considered to be significantly different and are considered to be two separate populations.

Chapter 3

RESULTS

Total and Differential Leukocyte Values

Comparisons of differential leukocyte values (Table 10) between normal and weak calves showed no significant difference. All values fell within the range of normal bovine values as reported by Schlam (47). The average lymphocyte differential of normal and weak calves were 74.1% and 74.2% respectively. The average total leukocyte count was found to be higher in normal calves (7,833 WBC/mm^3) than in weak calves (6,609 WBC/mm^3), but this difference was not found to be significant ($p > 0.05$).

Separation of Peripheral Blood Leukocytes

Ficoll-diatrizoate density-gradient centrifugation was found to be an effective method for the isolation of a reasonably pure population of mononuclear lymphocytes and monocytes. The lymphocyte population was found to sediment at the interface between the ficoll-diatrizoate and the Hank's BSS while the granulocytes were distributed throughout the ficoll-diatrizoate, directly above the erythrocyte pellet. Granulocyte contamination was found to be less than 5% as determined by differential staining of the purified lymphocyte preparation and lymphocyte viability, measured by trypan blue

Table 10 Total and differential leukocyte values of year-old normal and weak calves.

Number	% lymphocytes	% monocytes	% neutrophils segmented	% neutrophils juvenile	%eosinophils	Total WBC/mm^3
Normal calves						
12	69.0	7.5	18.0	1.0	4.0	5,625
16	58.0	1.0	38.5	0.5	1.0	5,025
19	86.0	3.5	8.0	1.0	1.5	8,000
21	82.0	1.0	15.0	0.5	1.5	9,988
26	80.0	5.5	9.0	1.5	2.5	6,400
28	68.5	4.5	18.0	3.0	6.0	11,158
average	74.1	3.8	17.8	1.3	2.8	7,833
Weak calves						
10	74.0	6.5	13.0	3.0	3.5	5,475
11	71.5	5.0	14.0	3.5	5.0	5,025
13	58.5	2.0	23.5	0.5	15.5	5,300
14	64.5	1.0	22.5	4.0	7.0	6,200
15	84.5	3.5	5.0	1.5	5.5	6,300
17	61.0	2.5	24.5	7.0	5.0	6,650
18	80.5	0.5	14.5	2.0	2.5	7,660
20	84.0	2.0	11.0	1.0	2.5	5,425
22	80.5	2.5	11.5	1.5	4.0	6,213
23	71.5	1.5	13.5	4.0	9.5	3,425
24	81.0	3.5	11.0	3.5	1.0	10,125
25	76.0	1.5	16.5	2.5	3.5	6,325
27	82.5	4.5	9.0	1.5	2.5	6,100
average	74.2	2.8	14.6	2.7	5.2	6,609

exclusion, was greater than 99%. The purified lymphocyte preparation was used in all subsequent *in vitro* assays. Occasional difficulties arose in the separation of PBL when agglutination of buffy coat layers occurred. These samples were discarded and new blood samples obtained and processed whenever possible.

Determination of Lymphocyte Subpopulations

Lymphocytes were divided into two major subpopulations based on immunofluorescence after incubation with FITC conjugated anti-bovine globulin serum (Colorado Serum Co.). No difference was observed between normal calves and weak calves in the percentage of B-lymphocytes (fluorescent cells) and T-lymphocytes (non-fluorescent cells) within the total population of lymphocytes (Table 11). Weak calves were found to have 31.9% B-lymphocytes and 69.1% T-lymphocytes whereas normal calves had 33.0% B-lymphocytes and 67.0% T-lymphocytes.

A further division of the population of T-lymphocytes was made using the E-rosette assay. This subpopulation of T-lymphocytes was also found to be consistent in both groups. The population of rosette forming cells (RFC) was 14.9% in normal calves and 15.2% in weak calves.

Blastogenesis

Lymphocyte stimulation by PHA-P was observed in both normal calves and weak calves. The results of the blastogenesis study are shown in Table 12. Although weak calves

Table 11 Lymphocyte subpopulations present in peripheral blood of normal and weak calves.

Number	% E-rosettes	% B-lymphocytes	% T-lymphocytes
Normal calves			
12	13.0	32.5	67.5
16	15.5	33.0	67.0
19	12.0	36.5	63.5
21	17.0	34.0	66.0
26	13.5	26.5	72.5
28	16.5	34.5	65.5
average	14.9	33.0	67.0
Weak Calves			
10	12.5	28.0	72.0
11	13.5	27.5	72.5
13	13.0	35.0	65.0
14	16.0	35.0	65.0
15	18.0	30.5	69.5
17	14.5	34.0	66.0
18	18.0	29.5	70.5
20	14.0	36.5	63.5
22	16.5	32.0	68.0
23	14.5	34.5	65.5
24	16.0	28.0	72.0
25	18.0	34.0	66.0
27	13.0	30.0	70.0
average	15.2	31.9	68.1

Table 12 Blastogenic response of normal and weak calf peripheral blood lymphocytes after stimulation with PHA-P.

Number	Control cpm	PHA-P cpm	S.I.*
Normal calves			
12	1,711.9	37,824.4	22.1
16	918.5	13,968.5	15.2
19	1,074.8	78,652.9	73.2
21	1,444.6	36,180.4	25.0
26	1,273.8	14,449.0	11.3
28	2,134.5	89,012.2	41.7
average	1.426.4	45,014.6	31.4
Weak calves			
10	816.2	8,078.4	9.9
11	1,870.5	11,609.0	6.2
13	1,085.9	78,340.5	72.1
14	1,924.5	84,806.7	44.1
15	1,577.8	25,839.0	16.4
17	739.8	55,741.0	75.3
18	1,219.4	64,055.4	52.5
20	1,352.9	81,988.0	60.6
22	1,727.7	74,730.0	43.3
23	1,398.7	54,062.6	38.7
24	1,532.0	11,166.3	7.3
25	1,756.4	68,502.0	39.0
27	1,663.6	73,295.9	44.1
average	1,435.8	53,248.1	39.2

*Stimulatory index (S.I.) = PHA-P cpm/Control cpm.

had an overall higher rate of tritiated thymidine incorporation than normal calves as indicated by the higher average stimulatory index (S. I.), this was not found to be significant ($p < 0.05$). The average S.I. of normal calves was 31.4 with S.I. values ranging from 11.3 to 73.2. The average S.I. of weak calves was 39.2 with S.I. values ranging from 6.2 to 75.3.

Humoral Reponse to a T-dependent Antigen

A humoral response to human albumin fraction V, a T-dependent antigen was observed in both weak calves and normal calves. Day 14 antibody titers measured by hemagglutination ranged from 1:16 to 1:28 in weak calves and from 1:8 to 1:64 in normal calves. No difference in the ability of normal and weak calves to respond to human albumin fraction V was observed.

Immunoelectrophoresis

Since the only bovine anti-sera available was anti-whole bovine sera (rabbit origin), comparative immunoelectrophoresis was done rather than quantitative immunoelectrophoresis. Evaluation of normal and weak calf sera was done by comparing the immunoelectrophoretic patterns of the various sera after precipitation by anti-whole bovine serum.

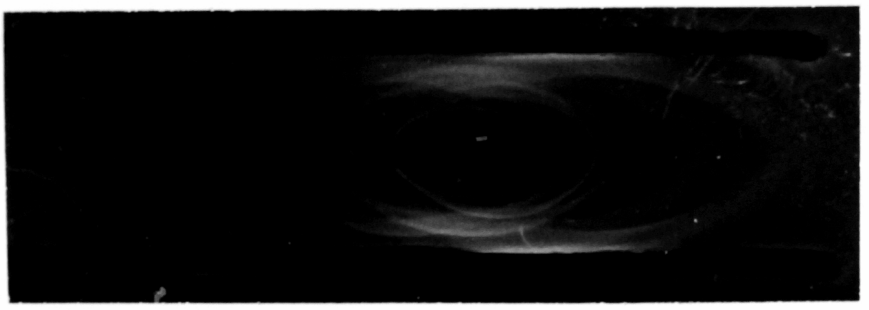
The results of the immunoelectrophoresis study showed no discernible differences in the electrophoretic patterns of normal and weak calves (Illustration 2). No precipitin arcs observed in normal calf patterns were absent in the patterns of weak calves and no additional arcs were present either.

Table 13 Humoral response to human albumin, a T-dependent antigen, in normal and weak calves.

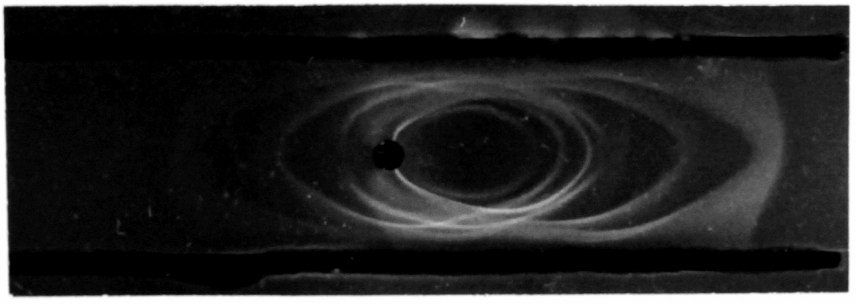
Number	Day 0	Day 7	Day 14
Normal calves			
12	< 1:4	1:16	1:16
16	1:4	1:16	1:64
19	1:4	1:16	1:32
21	< 1:4	1:32	1:32
26	< 1:4	1:16	1:8
28	1:4	1:16	1:16
Weak calves			
10	1:4	1:32	1:32
11	< 1:4	1:32	1:32
13	< 1:4	1:32	1:16
14	1:4	1:16	1:16
15	< 1:4	1:32	1:32
17	1:4	1:32	1:64
18	1:4	1:32	1:32
20	< 1:4	1:32	1:128
22	< 1:4	1:64	1:64
23	< 1:4	1:16	1:16
24	< 1:4	1:16	1:32
25	< 1:4	1:32	1:32
27	< 1:4	1:32	1:32

Illustration 2. Sample immunoelectrophoretic patterns of normal calves (#21 and 28) and weak calves (#17 and 22).

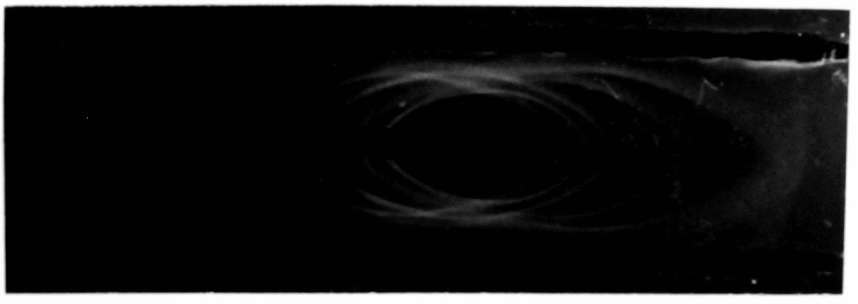
21



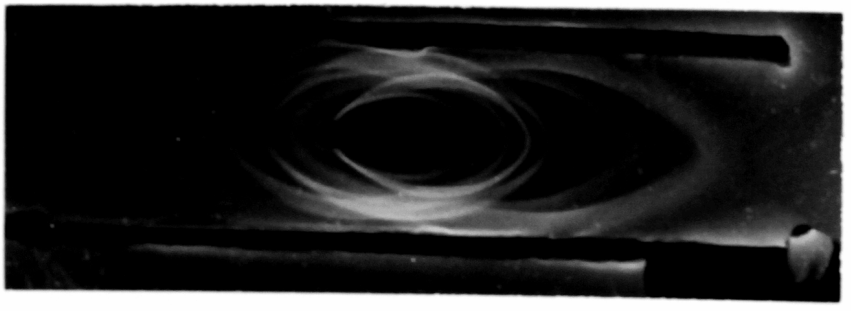
28



17



22



Delayed Hypersensitivity to BCG

All calves immunized with the BCG vaccine were found to be capable of mounting a delayed hypersensitivity response when injected with 0.2 ml of the BCG skin test antigen (BCG protoplasm). Skin tests were administered in the caudal fold two weeks after immunization with the BCG vaccine, and the area of induration was measured 72 hrs after the injection was given. The results of the skin tests are given in Table 14. A positive delayed hypersensitivity response is normally considered to be indicated when the area of induration is 5 mm or greater at the test site 24 hrs after injection of the skin test antigen (51). The 72 hr time period was used in this study since the responses were greater than the 48 hr responses and this permitted better resolution between the various responses.

The data in Table 14 indicates that, whereas both normal and weak calves were capable of mounting positive delayed tuberculin hypersensitivity responses, the area of induration was significantly greater in normal calves ($p < 0.001$).

Thymosin Therapy

Thymosin therapy in 10 of the 19 calves (3 normal and 7 weak calves) consisted of monthly injections of 30,000 µg of thymosin. The remaining 9 calves (3 normal and 6 weak calves) served as controls and received no thymosin or placebo. During the course of thymosin therapy, no outward physical changes or improvements were noted in weak calves

Table 14 Delayed tuberculin responses in normal and weak calves sensitized with BCG.

Number	Delayed Hypersensitivity Response in cm.
Normal calves	
12	---
16	2.0 x 2.5
19	2.0 x 2.0
21	1.5 x 2.0
26	2.0 x 2.0
28	2.0 x 2.5
Weak calves	
10	2.0 x 1.5
11	0.75 x 0.5
13	1.0 x 1.0
14	1.0 x 1.0
15	0.75 x 1.0
17	1.5 x 1.5
18	1.25 x 1.25
20	1.5 x 1.0
22	1.5 x 1.5
23	1.0 x 1.5
24	1.5 x 2.0
25	1.0 x 1.5
27	2.0 x 2.0

treated with thymosin that were observed in the controls. General improvement in the outward appearance of the weak calves, such as hair coat texture and periorbital edema, were noticed in all calves during the course of this study but was not confined to those weak calves receiving thymosin.

The weights of all calves were recorded at the commencement of thymosin therapy and are tabulated in Table 15. The initial average weight of the normal calves was 546 lbs compared to 350 lbs for the weak calves. After three months of thymosin therapy the calves were reweighed. The average weight of the normal calves had increased to 682 lbs and the average weight of the weak calves had increased to 500 lbs. The percent weight increase of the weak calves during the three months was found to be significantly greater than that of the normal calves ($p < 0.001$), but the overall weight increase was not. Comparisons of the thymosin treated and control weak calves as well as thymosin treated and control normal calves showed no difference in the percent weight gained or the net weight gained.

Re-evaluation of delayed tuberculin hypersensitivity of thymosin treated and control animals was conducted after each month of thymosin therapy. The results are given in Table 16. In those calves receiving the thymus extract, no significant difference was observed in the ability of normal and weak calves to respond to the skin test antigen. In two of the three re-evaluations conducted in control animals, normal calves exhibited a significantly greater

Table 15 A comparison of the weights of normal and weak calves before and after thymosin therapy.

Number	Weight #1*	Weight #2**	Δ Weight	% Wt. Increase
Normal calves - thymosin treated				
12	510	625	115	22.5
26	400	455	55	13.7
28	640	775	135	21.5
average†	575	700	125	21.7
Normal calves - controls				
16	520	700	180	34.6
19	495	635	140	28.3
21	535	655	120	22.4
average	517	663	147	28.4
Weak Calves - thymosin treated				
10	450	570	120	26.7
11	270	390	120	44.4
13	335	490	155	46.3
14	305	455	150	49.2
15	455	640	185	40.6
25	420	550	130	43.5
27	345	495	150	42.4
average	368	513	145	39.4
Weak calves - controls				
17	305	425	120	39.3
18	315	490	175	55.5
20	335	480	145	43.3
22	315	500	185	58.7
23	350	515	165	47.1
24	380	520	140	36.8
average	333	488	155	46.5

* Weight #1 represents the weight of the calves as of 7/27/77. The day on which thymosin treatment began.

** Weight #2 represents the weight of the calves as of 10/24/77. All thymosin treated calves had received three monthly treatments with thymosin by this date.

† The average of the normal calves treated with thymosin does not include data from calf #26. Calf 26 became ill shortly before thymosin treatment and lost considerable weight but was carried through the experiment with the other calves.

Table 16 Delayed tuberculin responses in normal and weak calves during thymosin therapy.

Number	Test A*	Test B**	Test C***
Control calves			
Normal calves			
16	2.0 x 2.0	2.0 x 2.0	1.5 x 1.5
19	1.25 x 1.25	0.5 x 0.5	1.0 x 1.0
21	0.75 x 0.75	1.0 x 1.0	1.5 x 1.5
Weak calves			
17	0.75 x 0.75	0.5 x 0.5	N.R.
18	0.5 x 0.5	0.25 x 0.25	N.R.
20	0.25 x 0.25	1.0 x 1.0	0.25 x 0.25
22	1.5 x 1.0	1.5 x 1.25	0.75 x 0.75
23	0.25 x 0.25	1.0 x 1.0	N.R.
24	0.5 x 0.5	0.25 x 0.25	0.25 x 0.25
Thymosin treated calves			
Normal calves			
12	0.75 x 0.75	1.0 x 1.0	1.0 x 1.0
26	1.0 x 1.0	0.5 x 0.5	1.0 x 0.5
28	1.5 x 1.5	2.0 x 2.0	1.5 x 2.0
Weak calves			
10	1.25 x 1.25	1.0 x 1.0	0.5 x 0.5
11	0.5 x 0.5	N.R.	0.5 x 0.5
13	1.5 x 1.5	1.5 x 1.0	0.75 x 0.75
14	0.75 x 0.75	1.25 x 0.25	N.R.
15	1.5 x 1.5	0.5 x 0.5	1.0 x 1.0
25	1.25 x 1.25	1.25 x 1.25	0.75 x 0.75
27	1.5 x 1.5	1.0 x 1.0	1.5 x 1.0

* Test A - 8/20/77 skin test
** Test B - 9/23/77 skin test
*** Test C - 10/24/77 skin test

N.R. = no reaction

capacity to respond to the skin test antigen ($p < 0.05$). The significance of the difference in the normal and weak calf controls to respond to the skin test antigen was not found to be as great as that observed in the initial delayed tuberculin hypersensitivity tests ($p < 0.001$). It should be noted that large fluctuations were observed in the delayed hypersensitivity responses of individual animals (both normal and weak calves) from test to test.

An *in vitro* macrophage migration inhibition assay was performed to evaluate the *in vitro* sensitivity of normal and weak calf lymphocytes to the BCG skin test antigen. The inhibition of guinea pig macrophages was assayed by indirect MIF tests using supernates from bovine lymphocyte cultures incubated with and without the skin test antigen. All supernates tested displayed positive inhibition of macrophage migration (Table 17). A 0.80 migration index (20% inhibition) is normally interpreted as a positive response (21). No differences were noted when comparing the average migration indices of normal calf controls (0.70) to either weak calf controls (0.67) or to normal calves that had received the thymus extract (0.67) or when comparing weak calf controls (0.67) to weak calves that had received the thymus extract (0.65). The migration indices of normal and weak calves did not show any significant differences when compared as did the initial skin tests.

Table 17 Inhibition of macrophage migration by MIF produced by lymphocytes from normal and weak calves sensitized with BCG.

Number	Migration Index*	% Inhibition
Control calves		
Normal calves		
16	0.69	31
19	0.59	41
21	0.74	26
average	0.67	33
Weak calves		
17	0.72**	28
18	--	-
20	0.74	26
22	0.62	38
23	--	-
24	0.61	39
average	0.67	33
Thymosin treated calves		
Normal calves		
12	0.70	30
26	0.65	35
28	0.72	28
average	0.70	30
Weak calves		
10	0.48	52
11	0.64	36
13	1.62	38
14	0.76	24
15	0.57	43
25	0.66	34
27	0.79	21
average	0.65	35

* Migration Index = $\frac{\text{area of migration with antigen}}{\text{area of migration without antigen}}$

** Contamination of these chambers prevented the calculation of the % macrophage migration inhibition for these samples.

The Effect of Thymosin on E-rosette Formation

The *in vivo* and *in vitro* effects of thymosin on E-rosette formation of peripheral blood lymphocytes of normal and weak calves were examined. PBL separated from blood samples of thymosin treated and control animals were assayed for E-rosette formation in the absence and in the presence of thymosin (100 µg/ml). These results are tabulated in Table 18.

No increase in the percentage of the RFC population present in PBL was observed in normal and weak calves that had received thymosin therapy when compared to control calf values or their own pre-thymosin values. *In vitro* incubation of PBL with thymosin also resulted in no significant increase in the percentage of RFC.

Table 18 E-rosette formation of peripheral blood lymphocytes of normal and weak calves after *in vivo* thymosin therapy and *in vitro* incubation with thymosin.

Number	pre-thymosin	post-thymosin	in vitro thymosin
Control calves			
Normal calves			
16	15.5	15.0	19.0
19	12.0	18.0	16.5
21	17.0	14.5	15.0
average	14.8	15.8	16.8
Weak calves			
17	14.5	15.0	15.5
18	18.0	14.0	15.5
20	14.0	16.5	17.0
22	16.5	13.0	18.5
23	14.5	14.0	16.0
24	16.0	17.5	19.5
average	15.7	15.0	17.1
Thymosin treated calves			
Normal calves			
12	15.0	17.0	18.0
26	13.5	14.5	15.5
28	16.5	14.0	19.0
average	15.0	15.2	17.5
Weak calves			
10	12.5	13.0	15.5
11	13.5	15.5	19.0
13	13.0	15.0	16.0
14	16.0	12.0	14.5
15	18.0	13.5	17.5
25	18.0	16.5	20.0
27	13.0	14.5	12.5
average	14.9	14.6	16.5

Chapter 4

DISCUSSION

While a specific etiologic agent of the Weak Calf Syndrome has yet to be ascertained, evidence available suggests that the syndrome is the result of an infectious process. After recognition of the syndrome as a possible new disease entity of neonatal cattle in 1964, inquiries at several ranches in the Bitterroot Valley revealed that once the disease was experienced in a herd, the problem became enzootic, occurring each year with a varied incidence. The disease was particularly evident among ranches operating an open herd and routinely bringing in replacement heifers. Ranches with a closed herd did not appear to suffer these losses.

An inspection of herd management records from ranches with the Weak Calf problem revealed that dams appeared to abort or give birth to weak calves only once (61) suggesting that the dams develop an immunity to the disease. The apparent immunity prompted the treatment of afflicted calves by transfusions with whole blood obtained from selected dams. While this approach appeared to have a beneficial therapeutic value, the favorable response was short lived. Since the administration later of cell-free serum from the same

selected dams was reported to be much more effective as a long term benefit, the outcome of a graft-versus-host reaction has been suspected in severely afflicted calves receiving the complete cellular components.

A comparative study between animals surviving the disease condition and normal calves of comparable ages show weak calves to be considerably smaller than their counterparts and appear to exhibit varying degrees of a "wasting disease." The condition is similar to the runting and wasting observed in laboratory mice after neonatal thymectomy (35) and other forms of stress (5,54). The calves demonstrating this "wasting" are characterized by a runted appearance, periorbital edema and a rough coat which becomes dull, shaggy and matted as the disease progresses. In view of the thymic atrophy observed at necropsy in severely afflicted calves succumbing to the disease, the runted condition of these calves was assumed to be related to the thymus.

The possibility of immunoincompetence occurring in weak calves is suggested by the depressed lymphocyte proliferation, as measured by the standard blastogenic response to PHA *in vitro*, in newborn calves (64) as well as by the inability of weak calves to mount delayed hypersensitivity responses to tuberculin skin test antigens after appropriate sensitization (56). The depression in the immune response prompted earlier studies with thymosin, a protein thymic extract

shown capable of restoring immunocompetence and reducing the incidence of wasting in neonatally thymectomized mice (1,17). The regimen of thymosin therapy used in the treatment of newborn weak calves resulted in an improvement in their clinical appearance and general health but was not long lasting (64), probably due to the short duration of the treatment.

Because of the problem the Weak Calf Syndrome represents to the cattle rancher, this study was designed to evaluate the treatment of weak calves with thymosin over a longer period of time in an attempt to correct the "runting" associated with the disease. In order to monitor the immune status of the weak calf during and after thymosin therapy, an assessment of several immune parameters was conducted prior to thymosin therapy.

The assessment of the immune status of the weak calf indicates that while weak calves are afflicted with a runting syndrome, these animals do not possess gross immunologic abnormalities. Except for a depression in delayed tuberculin hypersensitivity, no significant differences were noted in either the population of circulating lymphocytes or the *in vitro* measurement of cellular immune responsiveness. The immunologic depression reported in newborn weak calves was not observed in those weak calves surviving the initial year. These calves appear to have overcome the immunosuppressing factor(s). During the period of time that separates the two groups of weak calves in age, a return to normal immunologic

responsiveness, with the exception of delayed hypersensitivity, has occurred.

Using delayed tuberculin hypersensitivity to monitor immune responsiveness in the weak calf, thymosin was administered to both normal and weak calves for a period of three months. During this time weight gain as well as delayed tuberculin hypersensitivity was monitored in thymosin treated and control groups of normal and weak calves.

At the conclusion of thymosin therapy, no discernable effects of treatment with the thymic extract were noted in the weak calves' general appearance or in their ability to attain normal weight. It was noted, however, that the general appearance of weak calves did improve during this time regardless of whether they received thymosin or not. Also, comparative weights of the weak calves to normal calves increased from 64% to 74% suggesting that the initial effects of the Weak Calf Syndrome resulting in the runted appearance may be overcome to some degree given sufficient time.

The effect of thymosin therapy on delayed hypersensitivity in year-old weak calves appears inconclusive at this point. While the significant difference that did exist between the two groups prior to treatment with the thymus extract disappeared in thymosin treated calves, the decreased statistical difference observed in control animals and the inconsistencies in responses of individual animals make interpretation of the data difficult. Possible explanations for these inconsistencies are that the sensitization with

the BCG vaccine was inadequate to achieve a lasting cutaneous delayed hypersensitivity or that delayed hypersensitivity may not be an effective method for measuring cellular immune responsiveness in cattle. In order to elicit a delayed tuberculin response, 300 ug of the skin test antigen is required to elicit a response in contrast to the maximum 5 ug dosage used in man. The ability to mount constant and uniform delayed hypersensitivity responses may thus not be a suitable testing parameter of the bovine immune system.

The ability of thymosin to increase the number of rosette forming cells (RFC) in peripheral blood lymphocytes (PBL) obtained from patients with immunological deficiency diseases after *in vitro* incubation with thymosin and after *in vivo* thymosin therapy has been reported in the literature (59,60). These patients include those with primary cell-mediated immunodeficiencies, thymic hypoplasia, acute systemic lupus erythematosus and certain cancer patients. *In vitro* incubation of PBL with thymosin has been shown to be useful in identifying immunodeficiency patients who may be candidates for *in vivo* thymosin therapy (50).

In this study, no increase in the number of RFC could be attributed to thymosin treatment, either *in vivo* or *in vitro*. The percentage of RFC in PBL of normal and weak calves before and after thymosin treatment and after *in vitro* incubation with thymosin remained relatively unchanged.

The inability of thymosin to affect any changes in the percentage of RFC in weak calf peripheral blood or the growth and appearance of year-old weak calves indicates that the regimen of thymosin therapy used here had no apparent beneficial effect.

In order to assess fully the therapeutic ability of thymosin, additional studies appear warranted. These studies should include a longer period of thymosin therapy and a higher concentration of the thymic extract. Because of the rapid improvement reported in young calves treated with thymosin, it seems probable that the results of thymosin therapy are due to an effect other than an increase in immunological responsiveness. The possibility of thymosin acting in hormonal regulation must also be investigated.

The common embryological origin of the thymus, thyroid and parathyroid glands suggest that the impairment of these and other glands may also occur. Thyroidectomized animals fail to exhibit normal growth and are characteristically smaller than normal animals (11). Manifestations of hypothyroidism largely depend on the age and extent to which the deficiency develops. As age progresses, delayed growth and development are seen. Early treatment with thyroid therapy in hypothyroid animals can restore normal growth and development.

The exact mechanism by which thymosin therapy has produced beneficial results in younger weak calves is unknown,

but interrelationships between thymosin and endocrine hormones appear likely. If thymosin plays some role in hormonal regulation of the growth processes, thymosin treatment of older weak calves may be ineffective since they have already attained the majority of their adult growth and development. Additional studies should then be concentrated on younger calves and the prevention of the associated "runting" rather than trying to correct the effects of the disease in older calves.

Chapter 5

SUMMARY

An assessment of the immunological status of the year-old weak calf was conducted using *in vivo* and *in vitro* assays. With normal calf data serving as baseline values, no immunologic abnormalities or deficiencies, with the exception of a depressed cutaneous delayed tuberculin hypersensitivity, were observed in the weak calf.

An examination of the total and differential leukocyte values as well as lymphocyte subpopulations present in peripheral blood showed no abnormalities in the number of circulating lymphocytes to exist in weak calves. All data fell within the accepted range of normal bovine values (57).

Responses indicating normal immunological responsiveness were observed in weak calves in studies of *in vitro* lymphocyte proliferation, *in vitro* lymphocyte production of lymphokines and *in vivo* humoral responses to thymus-dependent antigens.

The *in vivo* measurement of delayed tuberculin hypersensitivity showed weak calves to have a depressed ability to respond to the BCG skin test antigen. Since lymphocyte production of the lymphokine MIF appears to be normal, it is possible that this depression in delayed tuberculin hypersensitivity was due to depressed macrophage function or

a depression in the ability to mount non-specific inflammatory responses.

Previous studies which describe depression of cellular responsiveness in young weak calves, as characterized by depressed lymphocyte proliferative responses (64) and the inability of weak calves to respond to the BCG skin test antigen, even after repeated sensitization with the BCG vaccine (56), were not observed in this study. These contrasting observations may be due to the age difference between the two groups of experimental animals. During the time that separates the two groups in age, a return to normal immunological responsiveness appears to have taken place.

Thymosin therapy, which proved beneficial to newborn and 1-2 month old calves (64) was also examined in year-old calves. The regimen of thymosin therapy used consisted of two monthly injections of thymosin, the first (5,000 μg) administered in saline and the second (25,000 μg) administered in a 50% saline and 50% peanut oil emulsion.

During and after thymosin therapy, a reevaluation of delayed tuberculin hypersensitivity was conducted. The significantly lower responses initially observed in weak calves was not seen in weak calves receiving thymosin. To attribute this change to thymosin would be premature at this point because of the inconsistencies observed during the repeated skin testing in both control and experimental animals.

Weight gain and general appearance of weak calves was also monitored during thymosin therapy and no improvement was observed in calves receiving the thymus extract that was not observed in control animals. The beneficial therapeutic activity observed in new born and 1-2 month old weak calves may in some way be due to the endocrine involvement of thymosin. Because of the age of the calves used in this study, these calves may have passed the age where correction of endocrine imbalances may be effective in promoting normal growth.

LITERATURE CITED

1. Asanuma, Y., A. L. Goldstein and A. White. 1970. Reduction in the incidence of wasting disease in neonatally thymectomized CBA/W mice by the injection of thymosin. Endocrinology 86:600-610.

2. Azar, H. A. 1964. Bacterial infection and wasting in neonatally thymectomized rats. Proc. Soc. Exp. Biol Med. 116:817-823.

3. Bach, J-F. 1973. Evaluation of T-cells and thymic serum factors in man using the E-rosette technique. Transplant. Reviews 16:196-217.

4. Balow, J. E. and A. S. Rosenthal. 1973. Glucocorticoid suppression of macrophage migration inhibitory factor. J. Exp. Med. 137:1031-1041.

5. Billingham, R. E., V. Defendi, W. K. Silvers and D. Steinmuller. 1962. Homograft tolerance and runt disease in rats. J. Natl. Cancer Instit. 28(2):366-414.

6. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21(suppl. 97):1-109.

7. Clamen, H. A. 1972. The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. New Eng. J. Med. 53:240-246.

8. Cohen, P. L., S. S. Cross and D. E. Mosier. 1975. Immunologic effects with mouse thymic virus. J. Immunol. 115(3):706-710.

9. Cooper, M. D., R. G. Keightley, D-Y. F. Yu and A. R. Lawton III. 1973. Developmental defects of T and B-cell lines in humans. Transplant. Reviews 16:51-84.

10. Cutlip, R. C. and A. . McClurkin. 1975. Lesions and pathogenesis of disease in young calves experimentally induced by bovine Adeno type 5 isolated from a calf with Weak Calf Syndrome. Amer. J. Vet. Res. 36:1095-1098.

11. Dillon, R. S. 1973. Handbook of endocrinology: Diagnosis and management of endocrine and metabolic disorders. p.207-271. Lea and Febiger. Philadelphia, Pa.

12. Dardenne, M. and J-F. Bach. 1973. Studies on the thymus products I. Modification of rosette-forming cells by thymus extracts. Determination of the target RFC subpopulations. Immunology 25:343-352.

13. Ekstedt, R. D. and L. L. Hays. 1967. Runt disease induced by non-living bacterial antigens. J. Immunology 98:110-118.

14. Fauci, A. S. and D. C. Dale. 1974. The effect of in vitro hydrocortisone on subpopulations of human lymphocytes. New Eng. J. Med. 53:240-246.

15. Gabrielson, A. E. and R. A. Good. 1967. Chemical suppression of adoptive immunity. In F. J. Dixon and J. H. Humphery (ed.) Adv. Immunology 6:92-229.

16. Goldstein, A. L. and A. White. 1971. The role of thymosin and other thymic factors in the development, maturation and function of lymphoid tissue. In Current topics in experimental endocrinology vol. 2 p. 122-150.

17. Goldstein, A. L., A. Guha, M. M. Zatz, H. A. Hardy and A. White. 1972. Purification and biological activity of thymosin, a hormone of the thymus gland. Proc. Nat. Acad. Sci. 69:1800-1803.

18. Grewal, A. S., B. T. Rouse and L. A. Babiuk. 1976. Erythrocyte rosettes - A marker for bovine T-cells. Can. J. Comp. Med. 40:298-305.

19. Gutoff, S. P. 1972. Lymphocytes in congenital and immunological deficiency diseases. Clin. Exp. Immunology 3:843-851.

20. Higgens, D. A. and M. J. Stack. 1977. Bovine lymphocytes: Recognition of cells forming spontaneous (E) rosettes. Clin. Exp. Immunology 27:348-356.

21. Hoffman, P. N., L. E. Spittler and M. Hsu. 1976. Leukocyte migration in guinea pigs: I. Correlation with skin test reactivity and macrophage migration inhibition. Cell. Immunology 21(2):358-363.

22. Hudson, S., M. Mullord, W. G. Whittlestone and E. Payne. 1976. Plasma corticoid levels in healthy and diarrhoeic calves from birth to 20 days of age. Br. Vet. J. 132:551-556.

23. Humphrey, J. H. and R. G. White. 1970. Immunology for students of medicine. p. 277-337. Blackwell Scientific Publications. Oxford, England.

24. Ioachim, J. 1971. Cortisone induced wasting disease of new born rats. J. Pathology 104:201-205.

25. Jondal, M., G. Holm and H. Wigzell. 1972. Surface markers on human T and B-lymphocytes. I: A large population of lymphocytes forming non-immune rosettes with sheep red blood cells. J. Exp. Med. 136:207-215.

26. Kantzler, G. B., S. F. Lauteria, C. Cusumano, J. D. Lee, R. Ganguly and R. H. Waldman. 1974. Immunosuppression during influenza virus infection. Infection and Immunity 10(4):996-1002.

27. Komuro, K. and E. A. Boyse. 1973. In vitro demonstration of thymic hormones in the mouse by conversion of precursor cells into lymphocytes. Lancet 1:740-744.

28. Komuro, K. and E. A. Boyse. 1973. Induction of T-lymphocytes from precursor cells in vitro by a product of the thymus. J. Exp. Med. 138:193-207.

29. Kook, A. I. and N. Trainin. 1974. Hormone-like activity of a thymus humoral factor on the induction of immune competence in lymphoid tissue. J. Exp. Med. 139:193-207.

30. Lamelin, J-P. 1971. Inhibition of macrophage migration. In Cell Mediated Immunity. In vitro correlates p. 75-102.

31. Levey, R. H., N. Trainin and L. W. Law. 1963. Evidence for function of thymic tissue in diffusion chambers implanted in neonatally thymectomized mice. Preliminary report. J. Natl. Cancer Instit. 31:199-206.

32. Lonai, P., B. Mogilner, V. Rotter and N. Trainin. 1973. Studies on the effect of a thymic humoral factor on differentiation of thymus-derived lymphocytes. Eur. J. Immunology 3: 21-26.

33. McCluskey, R. T. and P. D. Leber. 1974. Cell-mediated reactions in vivo. p. 1-24. In R. T. McCluskey and S. Cohen (ed.) Mechanisms of Cell-mediated Immunity. John Wiley and Sons. New York, N. Y.

34. Miller, J. F. A. P. 1961. Immunological function of the thymus. Lancet 1:748-749.

35. Miller, J. F. A. P. 1962. Effect of neonatal thymectomy on the immunological responsiveness of the mouse. Proc. Royal Soc. 156:415-428.

36. Miller, J. F. A. P. and D. Osoba. 1967. Current concepts of the immunological function of the thymus. Physiological Rev. 47:437-520.

37. Naye, R. L. 1965. Malnutrition, a probable cause of fetal disorder. Amer. J. Clin. Pathol. 79:284-291.

38. Naye, R. L. 1967. Cytomegalic inclusion disease: The fetal disorder. Amer. J. Clin. Pathol. 47(6): 738-744.

39. Naye, R. L. and W. Blanc. 1965. Pathogenesis of congenital rubella. J. A. M. A. 194(12):1277-1286.

40. Osoba, D. 1965. The effects of thymus and other lymphoid organs enclosed in Millipore chambers on neonatally thymectomized mice. J. Exp. Med. 122:633-639.

41. Page, L. A., H. L. Frey, J. K. Ward, F. S. Newman, R. K. Gerloff and O. H. Stalheim. 1972. Isolation of a new serotype of Mycoplasma from a bovine placenta. J. A. V. M. A. 161:919-924.

42. Reed, N. D. and J. W. Jutila. 1965. Wasting disease induced with cortisol acetate: studies in germ free mice. Science 150:356-357.

43. Ribi, E., C. Larson, W. Wicht, R. List and G. Goode. 1966. Effective non-living vaccine against experimental tuberculosis in mice. J. Bacteriology 91(3):975-983.

44. Rocklin, R. E. 1976. Production and assay of Human migration inhibition factor. In Manual of Clinical Microbiology. p. 95-100. American Society of Microbiology Wash. D. C.

45. Roitt, I. 1974. Essential Immunology. p. 176-180. Blackwell Scientific Publications. Oxford, England.

46. Rosen, F. 1972. Immunological deficiency disease. p. 271-289. In Clinical Immunobiology. Academic Press. New York, N. Y.

47. Schalm, O. W., N. C. Jain and E. J. Cattol. 1975. Veterinary Hematology. p. 122-144. Lea and Febiger, Philadelphia, Pa.

48. Scheis, E. P., E. K. Hoffman, K. Komuro, U. Hammerling, J. Abbott, E. A. Boyse, G. H. Cohen, J. A. Hooper, R. S. Schulof and A. L. Goldstein. 1973. Differentiation of T-cells induced by preparations from thymus and non-thymic agents. J. Exp. Med. 138:1027-1032.

49. Shortman, K. and H. Jackson. 1973. The differentiation of T-lymphocytes. I. Proliferation kinetics and interrelationships of subpopulations of mouse thymus cells. Cellular Immunology 12:230-246.

50. Siegel, S. 1956. The Mann-Whitney U test. In Nonparametric statistics for behavioral sciences. p. 116-126. McGraw-Hill. New York, N. Y.

51. Spitler, L. E. 1976. Delayed hypersensitivity skin testing. In Manual of Clinical Microbiology. p. 51-63. American Society of Microbiology. Wash. D. C.

52. Stauber, E. H. 1976. Weak Calf Syndrome: A continuing enigma. J. Amer. Vet. Med. Asso. 168:223-225.

53. Stutman, O., R. A. Good. 1973. Thymus Hormones. In Contemporary topics in immunobiology. vol. 2 p. 299-319. Plenum Press. New York, N. Y.

54. Takeichi, N., N. Kuzumaki, T. Kodama, F. Sendo, H. Hosokawa and H. Kobayashi. 1972. Runting syndrome in rats inoculated with Freind virus. Cancer Research 32(3):445-449.

55. Thurman, G. B. and A.L. Goldstein. 1975. The role of thymosin in lymphocyte maturation. Boll. Inst. Sieroter. Milan 54:203-210.

56. Ushijima, R. N. Unpublished data.

57. Vandeputte, M. and P. DeSomer. 1965. Runting syndrome in mice inoculated with Polyoma virus. J. Natl. Cancer Instit. 35:237-250.

58. Walters, M. L., N. F. Stanley, R. L. Dawkins and M. P. Alpers. 1973. Immunological assessment of mice with chronic jaundice and runting induced by Reovirus type 3. Br. J. Exp. Pathol. 54:329-345.

59. Wara, D. W. and A. J. Amman. 1975. Activation of T-cell rosettes in immunodeficient patients by thymosin. Ann. N. Y. Acad. Sci. p. 308-314.

60. Wara, D. W., A. L. Goldstein, N. E. Doyle and A. J. Amman. 1975. Thymosin activity in patients with cellular immunodeficiency. New Eng. J. Med. 292:70-74.

61. Ward, J. K. Personal communication.

62. Weiss, L. and A. G. Osler. 1972. The cells and tissues of the immune system. p. 79-99. Prentice-Hall, Inc. Englewood Cliffs, New Jersey.

63. White, R. G. and J. F. Boyed. 1973. The effect of measles on the thymus and other lymphoid tissues. Clin. Exp. Immunology 13:343-357.

64. Willey, D. E. 1973. Thymosin therapy and the Weak Syndrome calf. Master of Science thesis, University of Montana. Missoula, Montana.

65. Woldman, D. G., R. F. Hall, W. A. Meinershagen, G. S. Card and F. W. Frank. 1974. Haemophilus somnus infection in the cow as a possible contributing factor to Weak Calf Syndrome: Isolation and animal inoculation studies. Amer. J. Vet. Res. 35(11):1401-1404.