1976

Thymosin therapy and the weak syndrome calf

Dru E. Willey
The University of Montana

THYMOSIN THERAPY AND THE WEAK SYNDROME CALF

by

Dru E. Willey

B.S., University of Montana, 1973

Presented in partial fulfillment of the requirements
for the degree of

Masters of Science

UNIVERSITY OF MONTANA

1976

Richard N Ockenfels
Chairman, Board of Examiners

John M Stewart
Dean, Graduate School

March 25, 1976
Date

Willey, Dru, E., M.S. March 1976 Microbiology

Thymosin Therapy and the Weak Syndrome Calf (76 pp)

Director: Richard N. Ushijima

Upon study of the calf afflicted with the weak calf syndrome, the absence of a normal thymus development was observed. It was also observed that weak syndrome calves could not elicit a delayed hypersensitivity skin reaction to purified protein derivative of tuberculin even after repeated injections of Mycobacterium bovis strain BCG. The absence of delayed hypersensitivity was hypothesised to be a result of a depressed or absent thymic lymphocyte (T-cell) population. Since neonatal thymectomized mice given thymosin were able to reconstitute a functional T-cell population, thymosin was believed to have a similar effect on weak syndrome calves. More specifically, the immunocompetence of the peripheral T-cell population in weak syndrome calves was monitored to determine if administration of thymosin reconstituted the T-cell population. The immunocompetence of the peripheral T-cell population in weak syndrome and normal calves was determined before administration of thymosin.

Uptake of tritiated thymodine by phytohemagglutinin (PHA) was used to monitor the immunocompetence of the peripheral T-cell population. This type of assay measures the stimulatory effect of the non-specific mitogen PHA on the T-cell population which can be used to determine the immuncompetence of this lymphocyte population. Whole blood was used for the blastogenesis cultures since the physical separation of peripheral lymphocytes could not be attained in calf peripheral blood samples. The immunocompetence of the T-cell population was determined prior to and after one injection of thymosin (1000 µgm). Both weak syndrome and normal calves of varying ages were used. To determine if thymosin would increase the number of lymphocytes in the peripheral blood, total and differential WBC counts were performed at the time blastogenesis cultures were done.

It was determined that the T-cells from weak syndrome calves would take up a significantly lower amount of radioactive thymidine than the T-cells from normal calves. After administration of thymosin there was no significant difference in the amount of tritiated thymidine uptake between the T-cells from both calf groups. Any increase in the total lymphocytes after thymosin was not large enough to account for the increased stimulatory effect seen in T-cells from weak syndrome calves. Therefore, it was hypothesised that thymosin induced the maturation of peripheral T-cells in weak syndrome calves. An added effect of administration of thymosin which was not fully understood was the very rapid improvement in the general health of weak syndrome calves after one injection of thymosin.

ACKNOWLEDGEMENTS

With sincere appreciation, I would like to thank Dr. Richard N. Ushijima for his friendship and guidance throughout my tenure at the University of Montana.

Also, I would like to thank the members of my Master's Degree committee, Drs. C. L. Larson, R. A. Faust and H. R. Fevold for their professional advice on my research and thesis.

Thanks is extended to Dr. Jack Ward, Hamilton, for his help and services throughout this study, as well as the Bitterroot ranchers, Mr. Del Carter and Mr. Duane Van Meter, who supplied calves for this study. I would like to extend my thanks to Mr. Steve Otto and Mr. Michael Witschen, Coulter Electronics, Inc., who arranged for the loan of a Coulter Model ZBI particle counter.

Finally, I would like to thank Mr. and Mrs. L. E. Willey for their guidance and with love, my wife Judith Kay for her devotion and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AL/mm^3	Absolute lymphocyte number per mm^3 of peripheral blood
BCG	Mycobacterium bovis, Paris strain BCG
BSA	Bovine serum albumin
C	Degrees centigrade
C.C.	Culture counts
Ci	Curie
cpm	Counts per minute
DH	Delayed Hypersensitivity
FD	Ficoll-diatrizoate
M	Molar
μ	Micro-
p	Probability
PBC	Peripheral blood culture
PHA-P	Phytohemagglutinin-P
POPOP	(1,4-bis[2-(5-phenyloxazolyl)]-Benzene
PPO	2,5-Diphenyloxazole
rpm	Revolutions per minute
S.I.	Stimulatory index
WBC	White blood cell
WS	Weak syndrome

Chapter 1

INTRODUCTION

Experimental work done prior to 1961 failed to establish the immunological function of the thymus. Thymectomies performed on a variety of adult animals resulted in little or no significant effect on antibody production. However, thymectomies performed on neonatal mice (47) and rabbits (1) resulted in a marked depletion of circulating lymphocytes. Further experiments showed that this procedure resulted in decreased antibody to some antigens, and failure to reject skin grafts. Currently the thymus is believed to be the organ in which immunocompetent thymic lymphocytes are produced or are induced to differentiate. These thymic lymphocytes migrate to peripheral lymphoid tissue where they function as effector cells of cell-mediated immunity and helper cells in the antibody response to some antigens. With the isolation and purification of thymic extracts, some workers (29, 81) attributed an endocrine function to the thymus.

The thymus is the first lymphoid organ to develop (31). Within the thymus one of the two classes of lymphocytes that are known today develops into mature immunocompetent cells. These thymic lymphocytes migrate to the interfollicular and deep cortical areas of lymph nodes and the periarteriolar areas of the white pulp in the spleen. The thymic lymphocytes (T-cells) can be differentiated from the thymic independent lymphocytes (B-cells) on the basis of surface antigens,

immune functions, and activity associated with mitogenic substances. A comparison between T-cells and B-cells is provided in table 1. At about the onset of puberty, the thymus begins to involute (7) and becomes smaller that it was at birth (31).

On the basis of structure and function, Clark (13) has divided the thymus into four distinct regions: (1) the outermost subcapsular cortex where large lymphocytes proliferate to produce new T-cells, (2) the inner-cortex into which newly produced T-cells migrate, (3) the medullar proper contained within the thymic parachyma where mature T-cells collect, and (4) the perivascular connective tissue space. The composition of the framework is anastomosing trabeculae of epithelial cells accompanied by a well defined basement membrane (30). Histologically, the cellular contents of the thymus include epithelial and mesenchymal reticular cells, small, medium and large lymphocytes, macrophages, medullary inclusion cells and fibroblasts (70, 71). Some epithelial stroma cells in the medulla develop into Hassall's corpuscles which are characteristic of the thymus.

In mammals the thymus develops embryologically from the third and fourth pharyngeal pouches. The epithelial buds of ectoderm and endoderm grow out, pinch off and migrate to the midline of the upper thorax (50). After the epithelial structures have developed, the thymus is infiltrated with precursor lymphocytes or stem cells. The derivation of these precursor lymphocytes is still controversial. Some believe that these lymphocytes are of mesenchymal origin and invade the gland secondarily from the embryonic yolk sac or liver of the fetus and the adult bone marrow after the entrance of blood vessels and connective

Table 1 Comparison of B and T lymphocytes (21)

Properties	B-cells	T-cells
Differentiation	Bursa of Fabricius (in birds) or as yet unknown in mammals	Thymus
Ag-binding receptors on cell surface	Abundant Igs	Nature of specific receptors is uncertain
Cell surface antigens		
θ	-	+
TL	-	+
Ly	-	+
PC	+	-
H-2 transplantation	+	+
Approximate frequency (%)		
Blood	15	85
Lymph (thoracic duct)	10	90
Lymph node	15	85
Spleen	35	65
Bone marrow	Abundant	Few
Thymus	Rare	Abundant
Functions		
Secretion of antibody molecules	yes	no
Helper function	no	yes
Effector cell of cell-mediated immunity	no	yes
Distribution in lymph nodes and spleen	Clustered in follicles around germinal centers	In interfollicular areas
Mitogenic substances		
PHA	no	yes
Con A	no	yes
PWM	yes	no
LPS	yes	no

tissue into the thymus (50). The second viewpoint is that the precursor thymic lymphocytes are derived by direct transformation of epithelium cells within the thymic rudiment and are of ectoderm-endodermal origin (74).

The precursor stem cells migrate into the subcapsular cortex of the thymus and become lymphoblasts. As these lymphoblasts move from the subcapsular cortex, through the inner-cortex, toward the thymus medulla, they decrease in size. From the cortical area small immunocompetent thymic lymphocytes enter the circulation of the blood and thoracic lymph duct migrating to the spleen and lymph nodes.

The maturation of T-cells involves specific changes in mitotic activity and cell surface antigens (52, 61, 80). The large and medium lymphoblasts, which compose about 10% of the thymus lymphocyte population, can be separated from small, nondividing lymphocytes by density gradient centrifugation. Using this technique, it has been shown that thymic lymphocyte immune functions are not developed until the small or medium lymphocytes are formed (86) and that the thymic lymphocyte found in the lymph nodes and spleen is similar to the small and medium lymphocyte of the thymus (15, 16). During these experiments many small lymphocytes were noted within the thymus. Some of these small lymphocytes were not immunocompetent and did not have the same surface antigens as the peripheral thymic lymphocytes. These cells were considered either to be dead-end lymphocytes or a separate subpopulation of thymic lymphocytes.

On the basis of surface antigens, the lymphocytes of the thymus can be divided into two groups. The largest group, about 85% in the

mouse strain studied, has the TL antigen, high levels of theta antigen and low levels of H-2. The antigenic properties of the smaller population are similar to those of peripheral thymic lymphocytes. These are the absence of TL antigen, low levels of theta and high levels of H-2 antigens (68), suggesting the presence of small lymphocyte subpopulations with different activities. Further evidence for this is that the thymic lymphocytes involved in the graft-vs-host reaction and tumor graft rejection are morphologically different (46).

With the more complete understanding of developmental processes of a competent immune system, some of the immunological deficiency diseases can be explained. The failure of the third and fourth pharyngeal pouches to undergo differentiation necessary to form the thymus is an example of the classic condition known as DiGeorge's syndrome (24, 43). The T-cell population is virtually absent while the B-cell line development is not impaired and functions normally, except where helper T-cell activities are required (18). Transplantation of fetal thymus tissue into patients with this syndrome has produced a correction of T-cell and cell-mediated immunity (3, 4, 14). In addition to the thymic aplasia seen in DiGeorge's syndrome, some infants with thymic dysplasia have isolated T-cell deficiencies. The thymus is small and consists almost entirely of epithelial cells with no Hassall's corpuscles (18).

A similar athymic condition has been described in "nude" mice (54). A rudimentary nonfunctional thymus which will not support thymic lymphocytes has been found in these mice and, therefore, these mice have a depressed population of thymic lymphocytes (22, 84). Although there

is a population of theta-bearing lymphocytes, these mice have been generally used to study immunological processes in which the T-cell does not partake (62, 63). These mice are highly susceptible to infections, have a retarded or runted growth and exhibit a wasting syndrome if kept in a conventional environment (65). Some endocrine functions of the nude mice have been found to be abnormal suggesting an interrelationship between the thymus and other endocrine glands (7). An artifically induced situation similar to nude mice is seen when neonatal animals are thymectomized. When thymectomies are performed during the neonatal stage mice develop a wasting syndrome with an arrest of development and eventual degeneration of thymic dependent lymphoid tissue and depression of cell-mediated immunity (48). Thymectomies in adult animals result in a gradual loss of circulating immunocompetent lymphocytes without the rapid development of a wasting syndrome (48). This would suggest that the thymus is a very important organ in the development and maintenance of a competent immune system.

In addition to the syndromes mentioned above, several viral infections of fetal and neonatal animals have deleterious effects upon the thymus and, in turn, the immune system. When reovirus type 3 is used to inject neonatal mice, a very severe disease develops and only 1% of mice survive the acute stage of the infection (85). Those mice that survive the acute stages show the wasting syndrome (73) and frequently manifest lymphomas (38, 56). The involuted thymus noted at necropsy could have been a direct result of the infection or an indirect result of stress. A depression of cell-mediated immunity has been found in children infected with measles virus as a result of a loss of

cellularity of the thymic cortex (82). An agent, possibly a herpesvirus, that produces an acute massive necrosis of the medulla and cortex of the thymus has been isolated from mice (55, 64).

Although the immunobiological effects of the thymus seem quite clear, the relation of this gland to the endocrine glands is still unclear (29, 81). In studying the anterior hypophysis of thymectomized mice, Pierpaoli and Sorkin (8, 58) found degranulation and hypertrophy of the acidophilic cells of this endocrine gland. In mice the involution and atrophy of the thymus occurred when growth hormone production was curtailed (59). This effect has also been demonstrated in rats (17). Sexual maturation and onset of puberty of female mice can be delayed when mice are thymectomized (7). Pierpaoli and Besedosky (57) found that newborn athymic "nude" mice acquired the capacity to reject skin grafts following passive transfer of immunocompetent lymphocytes. However, this therapy did not result in alleviating the retarded growth, changes in blood levels of thyroxine or delayed onset of puberty ordinarily noted in "nude" female mice. These investigators (57) hypothesize that the effects of the thymus on the thyroid, adrenal and ovaries may be a result of thymus involvement in some functions of the brain and in the programming of certain endocrine functions exerted during fetal and perinatal life.

Several different extracts of the thymus have been prepared. Table 2 lists some of the extracts, molecular weight, biochemical make-up and the principle biological effect attributed to each extract.

The protein thymic extract, thymosin, isolated by Goldstein and White (27, 28) has been studied more thoroughly than the other extracts.

Administration of this extract has resulted in reconstitution of the impaired immunological response in X-irradiated mice (26), neonatal thymectomized mice (42), and in New Zealand mice which have immunological abnormalities (25). Thymosin reconstitutes the capacity of spleen cells from congenitally thymusless "nude" mice to evoke an in vitro graft-vs-host response (44). Thymosin reduces the wasting syndrome in neonatal thymectomized mice (2) and causes an acceleration of allograft rejection (32) as well as resistance to murine sarcoma virus induced tumors (88) and Dunnings leukemia in mice (39).

Table 2 Thymic extracts

Extract	Molecular weight	Biochemical nature	Biological attributes
Thymosin (27)	12,000	protein	reconstitutes cellular immunity
Thymoprotein I & II (30)	7000	polypeptide	T-cell differentiation
Lymphocyte stimulating hormone (45)		protein	WBC differentiation & antibody production
LSH_h	15,000		
LSH_r	80,000		
Thymosterin (60)		steroid	Antiproliferative activity & increases antibody production

Recently thymosin has been used in a child with a deficient cell-mediated immune system (78). The patient had thymic hypoplasia and abnormal immunoglobulin synthesis with a depressed percentage of T-cell

rosettes. Although the patient had sepsis caused by Candida albicans during infancy, she was unable to elicit delayed hypersensitivity skin tests to mumps virus, C. albicans or purified protein derivative to tuberculin. Thymosin therapy was instituted after it was shown that in vitro incubation of her lymphocytes with thymosin increased the percentage of T-cell rosettes. During the course of treatment, which involved 23 intramuscular injections of 1 mg per kilogram per day of thymosin, the percentage of T-cell rosettes gradually increased to normal, and positive delayed hypersensitivity skin test developed. A recent report of the patient's course showed that there was an increased number of total lymphocytes and percentage of spontaneous T-cell rosette formation. Positive delayed hypersensitivity responses have been maintained for one and one-half years, and there was an increased immunoglobulin G and M levels. However, the patient's in vitro lymphocyte response to phytohemagglutinin (PHA) and mixed lymphocyte culture remained markedly depressed (77).

The in vitro effects of thymosin studied so far have been the ability of the preparation to stimulate the unmasking of T-cell markers on precursor cells (40, 41) and to confer on precursor cells the sensitivity to azothioprine, anti-lymphocyte serum and anti-theta serum (5). The mechanism of action for unmasking of T-cell markers by thymosin has not been completely elucidated, but it is believed to be mediated by cyclic nucleotides (67). It can be seen that the immunological properties attributed to the thymus can be reconstituted by the administration of the thymic extract thymosin. As of this date the endocrinological effects of thymosin have not been studied.

The basic concept of the immune response can be applied to cattle even though most work has been done on other mammalian models. The first circulating lymphocytes appear at the 60th day of gestation in cattle (72). Although the calf is hypogammaglobulinemic *in utero* due to the lack of transplacental transfer of maternal immunoglobulins. The bovine fetus is capable of producing antibodies as early as the 164th day of gestation (83). Most calves surviving intrauterine infection with bovine viral diarrhea virus (BVD) have been found to be producing the specific immunoglobulin prior to birth (10,12). The rise in concentration of 11 β-hydroxycorticosteroid from th 20th day prior to parturition in the fetal blood probably accounts for the depressed T-cell response to mitotic stimulation (51). With this depression of the neonatal calf's immune system, it is believed that the high concentration of maternal immunoglobulins in colostrom enhances the ability of these calves to resist infection (51). As the calf becomes older the peripheral thymic lymphocytes become responsive to mitotic stimulation with PHA (49).

During the study of the "weak calf" syndrome of western Montana, it became apparent that the cell-mediated immunity of a large number of afflicted calves was depressed. Many of those surviving the initial delayed hypersensitivity reaction to protein derivative of tuberculin or extracts of BCG (75,79). Serum immunoglobulin M and immunoglobulin G concentrations of precolostral calves afflicted with the weak calf syndrome and normal calves are not significantly different (34). Although the etiological agent has not been identified, a viral agent which will transform tissue cultures has been repeatedly isolated from

infected kidney and salivary glands of afflicted calves (35,36). Recently an experimental infection with a clinical isolate of bovine adenovirus type 5 induced lesions closely resembling those seen in naturally occurring weak calf syndrome (19,20). Upon necropsy of aborted calves or those succumbing to the infection soon after birth, the thymus gland was noted to be severely involuted or absent (53). The glands, when present, weighed only one-tenth that of normal animals of comparable age (79). Severe leukopenia with an average WBC of 1×10^3 WBC/mm^3 was characteristically measured in comparison to 2.2×10^4 WBC/mm^3 counted in normal calves. The depression was only transitory since a leukocytosis was generally detected from about the 4th day. A normal observation in these calves was their inability or unwillingness to nurse. Therefore, death was frequently directly attributed to dehydration. In past years only whole immune serum has been helpful as supportive therapy. The calves appeared not to respond directly to antibiotic therapy although the reduction of secondary bacterial infections was probably a beneficial consequence. Several calves given the whole immune serum lived for more than a year, but these calves were runted and failed to gain any appreciable weight or attain the size of normal animals in spite of being provided adequate and ample feed. These calves could not elicit a delayed hypersensitivity reaction even when injected with two doses of the Paris strain of BCG grown in the laboratory and later challenged with BCG extracts. It was surmised from the above data that although the calves were able to survive with the therapeutic treatment used, their thymus gland was severely affected by the initial <u>in utero</u> infection causing a

depression in the cell-mediated immunity and possibly resulting in the runting observed.

Statement of the problem

With the involvement of the thymus, these calves manifest a syndrome that resembles very closely the "wasting" syndrome seen in mice thymectomized neonately. Since this "wasting" syndrome in mice has been reversed by the administration of the thymic extract thymosin (42), this study was undertaken to determine whether the administration of thymosin to the "weak syndrome" calves could elicit similar responses. The study, however, was limited to the *in vitro* effects of the thymic extract of the T-cell population.

Chapter 2

MATERIALS AND METHODS

Processing of thymic extract

Thymosin was extracted from bovine thymus glands by a modification of Goldstein and White's method (27). Frozen bovine thymus glands were obtained from a local abattoir. The glands were cut into small pieces and processed as follows:

1. Homogenize thymus glands in Waring blender with 0.85% NaCl.
2. Centrifuge the blended mixture in 250 ml plastic centrifuge bottles at 3,500 x G in a Universal centrifuge for 5 minutes at 4° C.
3. Remove the fluid and place 300 ml in a 500 ml glass flask. The flask is then placed in an 80° C water bath for 15 minutes.
4. Centrifuge the fluid at 3,500 x G in a Universal centrifuge for 5 minutes and remove the clear fluid.
5. Centrifuge the clear fluid at 16,000 rpm for one hour and retain the supernatant and discard the precipitate.
6. Precipitate with acetone at -4° C. The clear supernatant is added to 10 volumes of cold acetone with constant stirring. A precipitate forms and coaleses following stirring for 5 minutes.
7. Place the precipitate in an evaporating dish overnight in the cold room.
8. Resuspend the dried precipitate in 0.1 M phosphate buffer (pH 7.2) by constant stirring for three hours. Centrifuge at 3,500 x G and

save supernatant.

9. A second precipitation is accomplished with saturated ammonium sulfate, $(NH_4)_2SO_4$. Add the $(NH_4)_2SO_4$ to the supernatant until a final concentration of 50% is attained. This precipitation is done at ambient temperatures. Centrifuge the mixture and save the precipitate.

10. Suspend the precipitate in distilled water and dialize overnight against two changes of distilled water.

11. Lyophilize the suspension. A fluffy white to whitish-brown powder is obtained with completion of the procedure.

Although chemical and physical analyses were not performed using this material extracted from the thymus glands, the extract will be referred to as "thymosin" throughout the text.

Administration of extracts

For injection into calves the lyophilized thymosin was suspended in sterile 0.1 M phosphate buffer or peanut oil. The extract was sterilized by placing under an ultraviolet lamp for at least three hours. The appropriate weights of thymic extract were suspended in the diluent to give a final concentration of 100 µgm/ml or 25,000 µgm/ml. Bovine serum albumin (BSA) was suspended in 0.1 M phosphate buffer to be used as a control. Dr. J. K. Ward, a Hamilton veterinarian administered the materials to all calves.

Collection of blood samples

Venous blood samples were collected by Dr. Ward from calves.

Blood was collected prior to any injection to establish baseline values and at selected intervals after the administration of test materials the calves were bled again. Preservative-free heparin in concentration of 50 units/ml of blood was added at the time the blood was drawn. The blood was kept at ambient temperature during transportation, or for approximately four hours. White blood cell (WBC) counts were done with a Coulter Counter (Model ZBI). At this time two blood smears were made for differential WBC counts and serum was collected for later analysis.

Blastogenesis cultures

Problems, which are explained later, in separation of peripheral calf lymphocytes from erythrocytes required the use of whole peripheral blood cultures (PBC) for blastogenesis studies (6,49).

The peripheral blood cultures were prepared in tissue culture medium composed of RPMI 1640 (Difco), table 3, with 10% fetal calf serum (Microbiological Associates) and 2% Hepes buffer (Difco).

A flow chart outlining the procedure is provided in figure 1. Briefly, a volume of whole blood was suspended in 30 ml of tissue culture medium to give a final concentration of 1 x 10^6 WBC/ml. The medium suspension was mixed thoroughly to insure an equal distribution of cells. Triplicate control samples of 1.0 ml each were removed and placed into glass tissue culture tubes prior to the addition of phytohemagglutinin-P (PHA-P). Next, the concentration of PHA-P to be used was added to the stock suspension of blood culture; triplicate samples of 1.0 ml each were placed in glass tissue culture

Table 3 Preparation of medium and stock solutions

RPMI 1640

Triple distilled water	1.0 liter
RPMI 1640 dry powder (Difco)	6.5 gm
Penicillin G	1000 units
Streptomycin sulfate	1000 μgm
$NaHCO_3$	1.5 gm
Fetal calf serum, inactivated at 56° C for 30 minutes (Microbiological Associates)	100 ml
Hepes buffer, 1.0 molar (Gibco)	20 ml

The medium was filter sterilized prior to the addition of serum and Hepes buffer. When all materials had been added together, the medium was stored at 4° C until used.

Tritiated thymidine stock solution

Thymidine, Methyl-^3H, 1mCi, Specific activity 71.5 Ci/mmole (ICN)	1 ml
RPMI 1640 with 10% fetal calf serum	99 ml

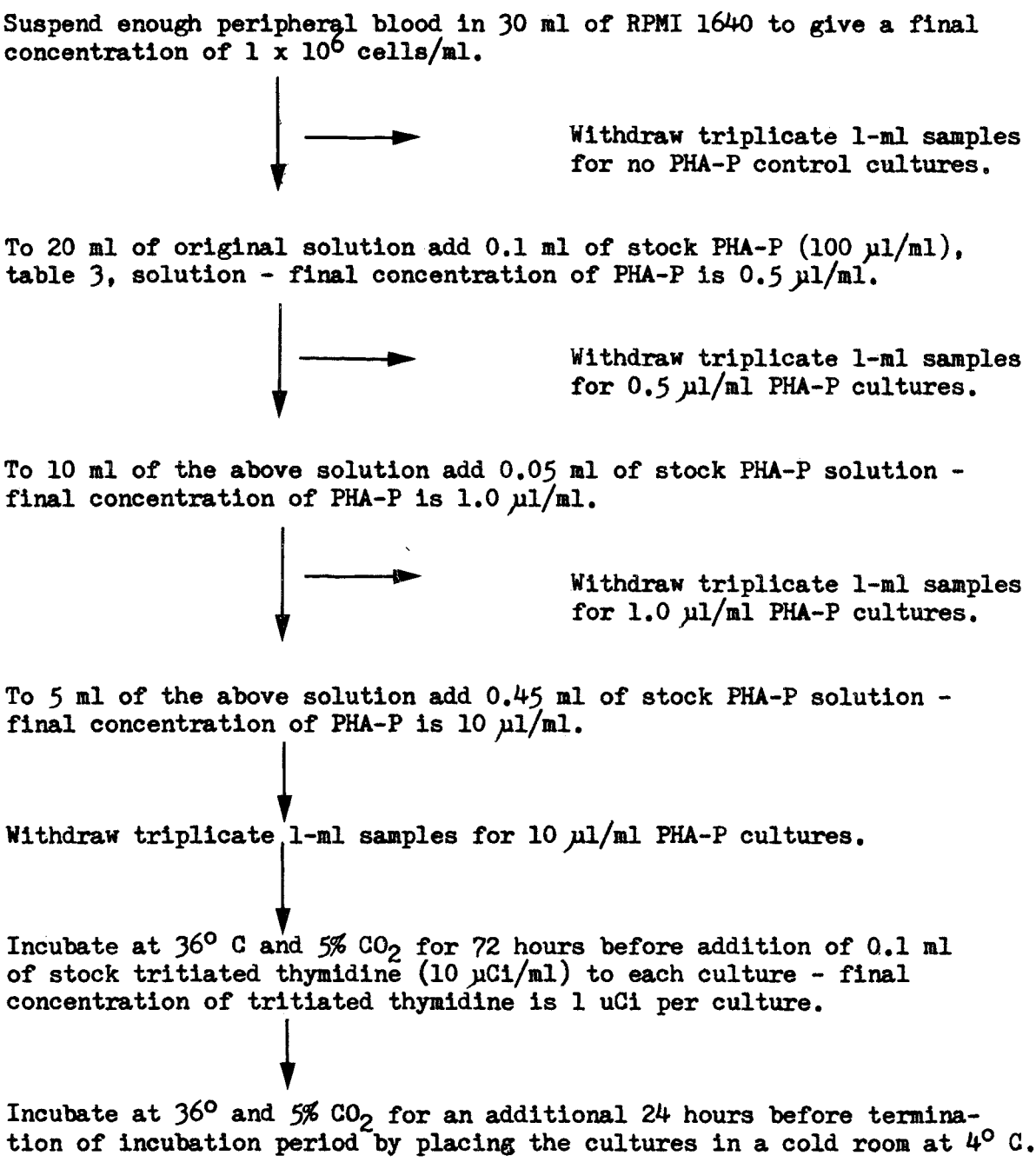
Solution at concentration of 10 μCi/ml was stored at 4° C in a serum bottle.

Stock PHA-P solution

PHA-P (Difco)	1 ml
RPMI 1640 with 10% fetal calf serum	9 ml

Solution stored in a calibrated dropper bottle (0.05 ml/drop) at -70° C.

Figure 1 Flow chart of procedures for preparation of blastogenesis cultures from peripheral blood samples

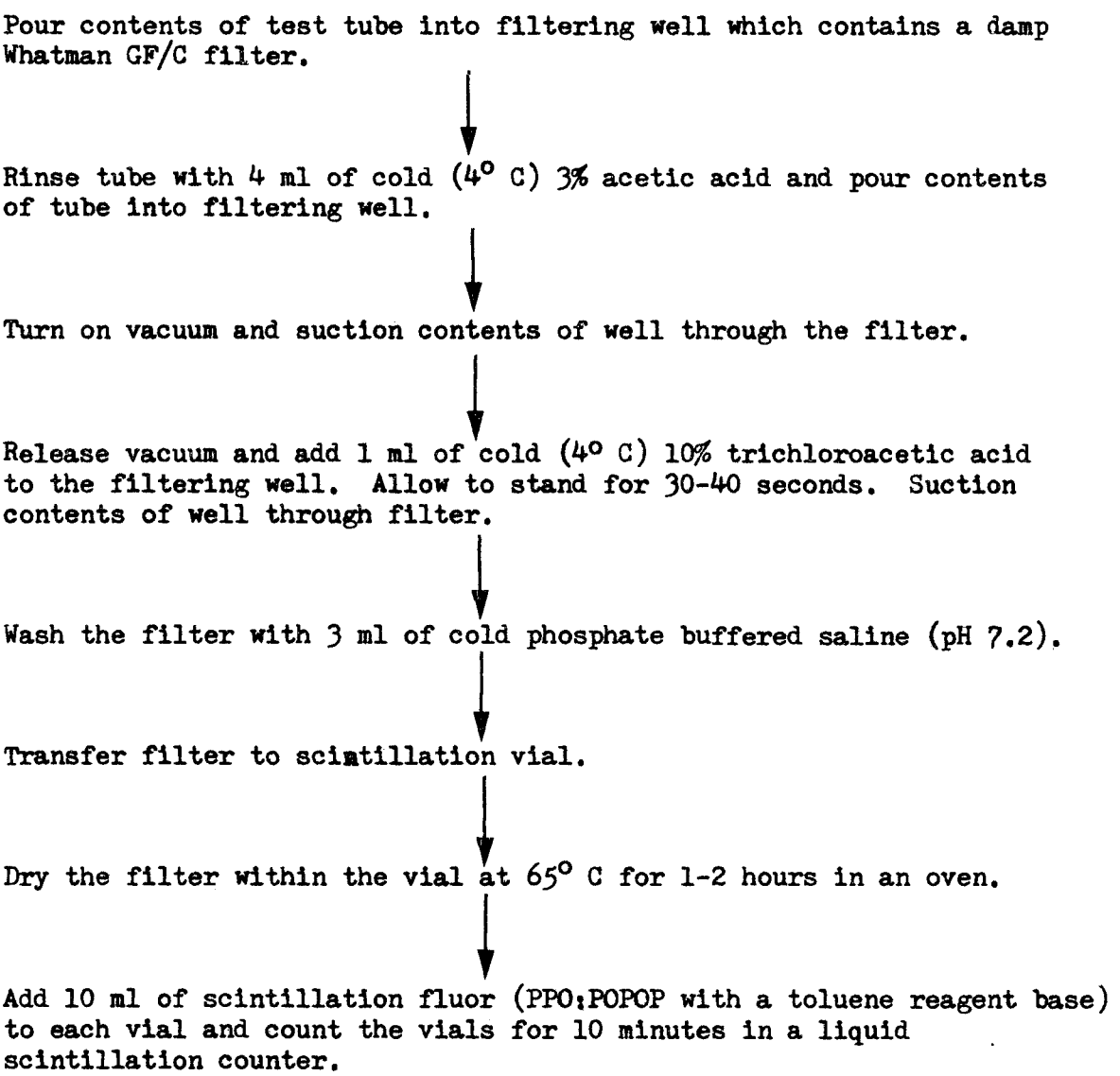


tubes. The concentrations of 0.5 µl/ml, 1.0 µl/ml and 10 µl/ml of PHA-P were used in the initial experiments, but when the 10 µl/ml of PHA-P was found to inhibitory to the mitotic activity, this concentration was abandoned. The cultures were incubated at 36° C and in 5% CO_2 in a gas chamber. After incubation for 72 hours, the tritiated thymidine (ICN) was added. A 0.1 ml volume of the stock tritiated thymidine (specific activity 71.5 Ci/mmole) was added to each culture tube. The mitotic activity was terminated 24 hours after the addition of tritiated thymidine by placing the cultures in a cold room at 4° C. The cultures were then processed for counting by liquid scintillation.

Processing of radioactive samples

When the blastogenesis samples were ready for counting, each suspension was processed following the procedure outlines in figure 2. The technique used involved collecting the incorporated radioactive thymidine on a filter paper. The cells were collected on Whatman GF/C filter paper. Red blood cells were lysed and all debris washed through with cold 3% acetic acid. The cellular nucleic acids were precipitated with 10% trichloracetic acid and the filter papers were dried in an oven at 65° C prior to the addition of the scintillation fluor. The waste fluid collected in the vacuum container was disposed in a receptacle for radioactive liquid waste. After the filter papers had dried, a volume of 10 ml of scintillation fluor was added to each vial containing a single filter paper. The sample vials were counted for ten minutes in a Nuclear Chicago Unilux III liquid

Figure 2 Flow chart indicating processing procedures for tritiated thymidine labeled lymphocytes from PHA stimulated blastogenesis cultures



scintillation counter. The scintillation fluor was composed of 4 gm PPO and 0.05 gm POPOP dissolved in 1 liter of reagent grade toluene. The scintillation fluor was usually allowed to stand overnight to insure that the reagents were dissolved and then the fluor was stored in a dark brown bottle until used.

The results from the blastogenesis cultures were reported in counts per minute and are the average of triplicate cultures counted twice. The stimulatory index (S.I.) and culture counts (C.C.) were determined for each test sample. The S.I. was calculated by dividing the counts per minute for PHA-P stimulated cultures by the counts per minute for nonstimulated control cultures. The C.C. were determined by subtracting the nonstimulated culture counts per minute from the PHA-P stimulated culture counts per minute.

Because the sample sizes were small and results were compared between two groups of animals, statistical analysis of the data was performed using the Mann-Whitney U test (69). Two groups of animals were considered to be significantly different if the probability (p value) was equal to or less than 0.05.

Differential white blood cell counts

The slides prepared for differential WBC counts were stained by the polychrome staining procedure as outlined in table 4. A total of 100 cells was counted and the cell types analyzed under oil immersion to enable more accurate identification. The cell types identified were segmented and juvenile neutrophilic granulocytes, eosinophilic granulocytes, basophilic granulocytes, lymphocytes and monocytes (66).

Table 4 Staining procedure of blood smears prepared for differential white blood cell counts

1. Dip blood-smear slide into absolute methanol for one second.
2. Dip slide into eosin stain for twenty seconds.
3. Wash slide in distilled water.
4. Dip slide into polychrome stain for twenty seconds.
5. Wash in distilled water and air dry.
6. Observe under oil immersion objective lens.

The absolute number of lymphocytes per mm^3 of peripheral blood (AL/mm^3) was calculated when possible. This calculation was accomplished by multiplying the WBC count by the percentage of lymphocytes for that calf.

Washing of radioactive glassware

All glassware that came in contact with radioactive substances was decontaminated by soaking in 2% solution of Isoclean (Isolab) overnight. The glassware was then rinsed three times in tap water prior to running through standard laboratory washing procedures for tissue culture glassware as follows:

1. Place in dilute acid (HCl) bath overnight.
2. Rinse three times in tap water.
3. Rinse three times in distilled water.
4. Dry and sterilize in autoclave.

Chapter 3

RESULTS

Lymphocyte separation

Purified T-lymphocytes are desirable for measuring their capacity to respond to mitotic stimuli. Three methods of obtaining purified lymphocytes were compared. The separation of human and bovine peripheral blood was analyzed by the following: (a) ficoll-diatrizoate (9), (b) gelatin (87), and liquid silicone oil (37).

(a) Ficoll-diatrizoate (FD) - FD was prepared by dissolving 9.84 gm of sodium diatrizoate in 30 ml of distilled water (pH 7.3). To this solution an additional 72 ml of distilled water was added followed by 6.28 gm of Ficoll. The FD (specific gravity 1.080) was then heat sterilized at 121° C and 15 psi for 15 minutes. The sterile solution was stored in a foil-covered container at 4° C. Prior to the separation procedures, FD was warmed to ambient room temperature of 24° C. Heparinized peripheral blood was diluted 1:2 with 0.85% saline, and 10 ml was carefully layered over 5 ml of FD. The sample was centrifuged with a swing head for 20 minutes with a force of 400 x G at ambient temperature. Lymphocytes of human blood aggregated at the interface and were removed with a Pasteur pipette. These cells were then washed three times with tissue culture medium prior to suspension. However, in following the

same protocol, the lymphocytes from bovine calf blood could not be separated from the erythrocytes. After centrifuging bovine calf blood, the lymphocytes remained dispersed throughout the FD with the erythrocytes. Muscoplat et al. (49) reported that they were able to separate adult bovine lymphocytes with FD at a specific gravity of 1.080. Even by varying the specific gravity of FD above and below 1.080 as well as the force applied to the interface, the separation of calf lymphocytes still could not be attained. The reasons for the discrepancies were not established.

(b) Gelatin - Ten ml of heparinized peripheral blood was mixed with an equal volume of 3% gelatin dissolved in TC 199 medium. After standing at 37° C for one hour, human erythrocytes collected at the bottom of the test tube and leukocytes remained in suspension. However, the same results could not be obtained with bovine blood, which failed to separate even after reducing the gelatin concentration to 2%. The erythrocytes and leukocytes continued to remain in suspension.

(c) Liquid silicone - Heparinized bovine peripheral blood was centrifuged at 2,500 rpm, and the buffy coat was removed along with 2 to 3 ml of serum. A 0.5 ml volume of liquid silicone, specific gravity of 1.075, (Diffusion pump oil grade) was first placed in the bottom of a 1-ml plastic centrifuge tube. A portion (1 ml) of the leukocyte-rich buffy coat was then layered carefully on top of the oil. After centrifugation at 2,500 rmp for 15 minutes, the leukocytes were dispersed in the diffusion oil, and the erythrocytes were pelleted in the bottom of the

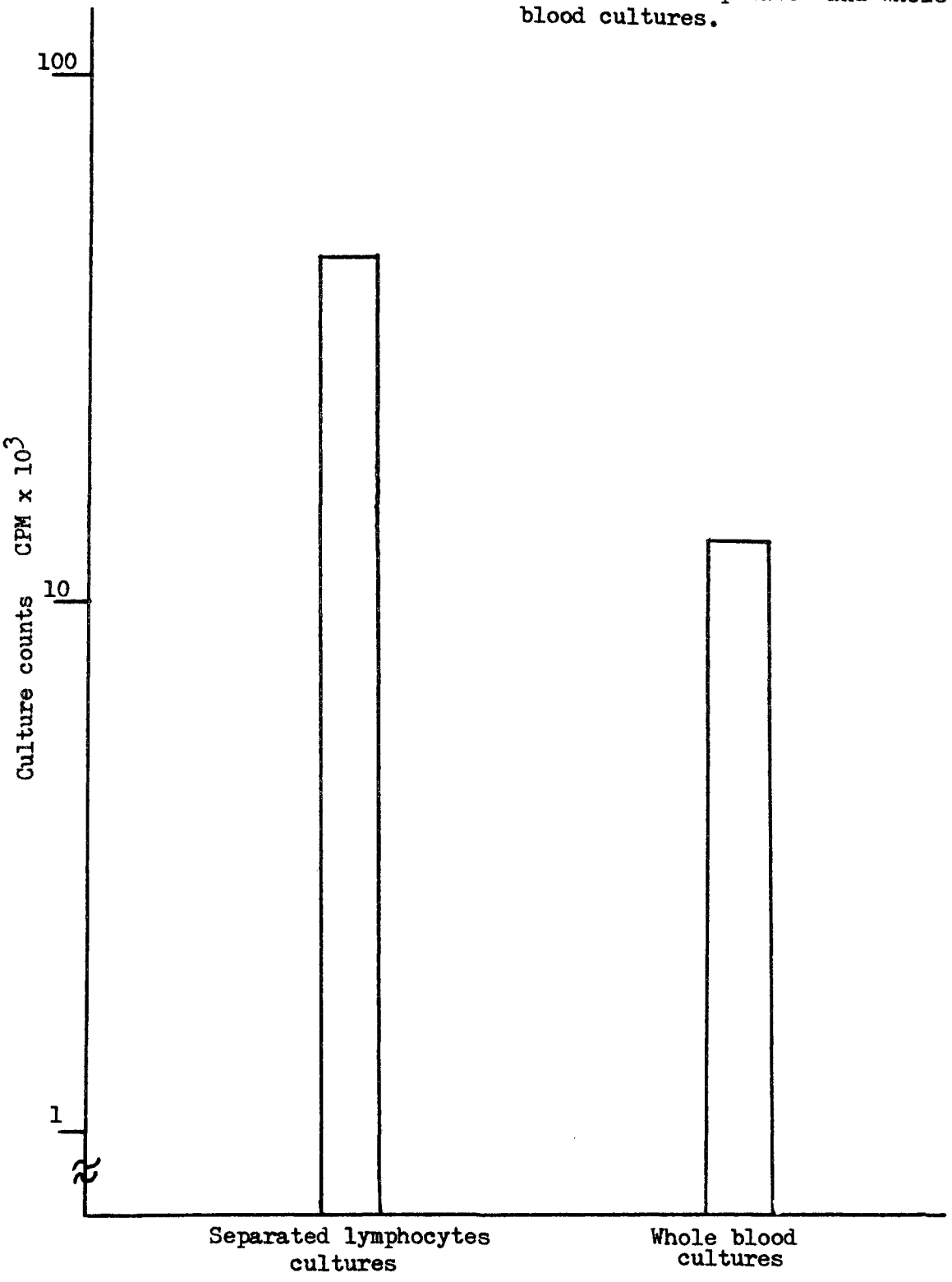
centrifuge tube. The leukocytes were removed with a Pasteur pipette, washed free of silicone with medium and suspended in RPMI 1640. This method accomplished the separation of peripheral bovine leukocytes from erythrocytes, but the cost of liquid silicone was prohibitive for the large number of samples anticipated.

Comparison of blastogenesis between whole and separated peripheral blood

In view of the problems involved in preparing preparations of bovine lymphocytes, an alternative culture technique to measure blastogenic activity of bovine lymphocytes in the presence of PHA was desired. Therefore, the use of nonseparated peripheral blood was attempted (6, 49). A comparison of the blastogenic response to PHA of lymphocytes in cultures of whole human blood and of FD separated peripheral lymphocytes was made.

Figure 3 shows the culture counts (C.C.), in log scale, for nonseparated blood and FD separated cultures stimulated with PHA. The C.C. for separated lymphocytes was approximately four times higher than that of the whole blood. Because the concentration of leukocytes in each group was maintained at 1×10^6 WBC/ml, the higher C.C. with separated blastogenesis cultures were not surprising since these cultures contained a higher population of lymphocytes. The leukocyte population of FD-treated blood is almost 95% lymphocytes (49) whereas, the percentage of lymphocytes in normal peripheral human blood is approximately one half the total leukocytes. This would suggest that the C.C. shown using nonseparated whole blood in the blastogenic assays was comparable to the C.C. obtained by using FD separated lymphocytes.

Figure 3 Bar graph representing a comparison between the culture counts from blastogenesis cultures of stimulated lymphocytes in ficoll-diatrizoated separated and whole blood cultures.



With the problems encountered in separation of peripheral bovine calf lymphocytes, the above results demonstrated that with human blood equivalent data could be attained using nonseparated peripheral blood. Therefore, whole blood blastogenesis cultures could be used as an indication for stimulation of T-lymphocytes by PHA.

Selection of filter paper and scintillation fluor

The separation of residual radioactive thymidine in the culture medium was essential to measure the amount of the isotope that had been taken up by the cells during blastogenesis activity. One of the most common methods of attaining the separation is by filtration (76), through Millipore 45 μ membrane filter or Whatman GF/C filter paper. The retention of radioactive materials by the two filter types was compared by separating the blastogenesis cultures into two equal portions and filtering the appropriate samples through each of the filters. Although both filter types were found to retain similar amounts of radioactive materials, the Whatman GF/C filter paper was selected because of availability and considerably lower cost.

The scintillation fluor was the PPO:POPOP combination dissolved in toluene indicated earlier, which had been suggested by New England Nuclear. Since the filter papers were dried prior to the addition of the scintillation fluor, the addition of absolute methanol was avoided.

Determination of incubation period for blastogenesis cultures

To determine the optimal incubation time for the maximum thymidine uptake by stimulated bovine blood cultures, an incubation-time

experiment was performed. Triplicate cultures of control and PHA-P blastogenesis cultures from three "normal" calves were incubated for periods of time varying from 24 hours to 144 hours. The tritiated thymidine was introduced into each culture 24 hours prior to the termination of the incubation period. At the end of each incubation time period, the cultures were precipitated with trichloroacetic acid and collected on filter papers, which were stored until the entire group was ready for counting. The scintillation fluor was added to each vial immediately before the samples were all counted.

The raw data presented in table 5 shows that the highest S.I. and C.C. were achieved when cultures were incubated for a total of 96 hours. Therefore, since the maximum uptake of tritiated thymidine occurred after 96 hours of incubation, this time interval was used throughout the study.

Studies involving newborn calves

The selection of calves was made by Dr. J. K. Ward. The criteria used for the diagnosis of weak syndrome (WS) calves were as follows: rough hair, slow to stand with stiff arthritic knee joints, failure to nurse and a red muzzle.

None of the calves were moved to other locations to simplify the study but were retained on their respective ranches to minimize any stress that could aggravate the existing conditions. When the syndrome was diagnosed, a blood sample was taken and sent from Hamilton to Missoula. Unfortunately, under this system the samples were frequently received six hours later. It should be noted that the health of some

Table 5 Tabulated data for incubation period determination from normal calf PHA stimulated blastogenesis cultures

Calf	Age (wks)	Total incubation time	No PHA culture counts	PHA culture counts	S.I.(a)	C.C.(b)
1	4	24	448(c)	1289(c)	2.9	841
		48	694	12,776	18.4	12,082
		72	415	17,682	42.6	17,267
		96	409	19,982	48.9	19,573
		120	458	339	0.7	-119
2	2	48	4006	17,463	4.4	13,457
		72	383	16,245	42.4	15,862
		96	253	25,610	101.2	25,357
		120	224	20,109	89.9	19,885
		144	302	29,971	99.2	29,669
3	6	48	573	6146	10.7	5573
		72	477	13,861	29.0	13,384
		96	No data available			
		120	316	8560	27.1	8244
		144	193	3374	17.5	3181

(a) S.I. = stimulated culture counts/control culture counts
(b) C.C. = stimulated culture counts - control culture counts
(c) Each value expressed in counts per minute is the average of triplicate cultures counted twice.

severely afflicted calves continued to deteriorate, often succumbing within 48 hours even after the administration of thymosin. The data obtained from blastogenesis studies of these calves were not included in the analysis. Since the incidence of WS calves is believed to be very high in the Bitterroot Valley and the onset of the syndrome is sometimes insidious, the normal calves were selected from another area. There were instances in which "normal" appearing calves developed the syndrome even up to three week after birth, but this did not occur with any of the normal calves used in this study. The normal calf blood was obtained from animals located two miles from Missoula. Dr. E. M. Pruyn, a Missoula veterinarian, selected and drew the blood samples from the normal calves. Table 6 list the calves used in this portion of the test of the therapeutic value. Thymosin in 0.1 M phosphate buffer was administered to WS calves after the initial blood sample was drawn.

A table comparing the total WBC count of calves in this age group is given in table 7. The average number of WBC for normal newborn calves was 8763 and for WS calves of a comperable age, 6530. The WBC counts of normal calves used in this study are within the range for calves less than one year old as established by Schalm (66), who reported the average WBC count for Hereford calves to be 11,140 with a range of 5750 to 17,800. The average WBC count found in WS calves was in the low range.

In view of the low respone of T-lymphocytes to stimulation observed in perinatal calves by Osburn and co-workers (51), the T-cell blastogenesis response of newborn "normal" calves was investigated. Although

Table 6 Pertinent information for newborn calves used in study

Owner	Identification number	Age	Comments
Carter	918	1 day	WS calf
Carter	817	4 days	WS calf
Carter	W10	1 day	WS calf
Carter	904	1 day	WS calf
Ward	SD47	1 day	WS calf
Ward	W3	1 day	WS calf
Ward	1054	1 day	WS calf
Ward	K1324	3 days	WS calf
Pruyn	1	1 hour	Normal calf
Pruyn	2	10 hours	Normal calf
Pruyn	3	12 hours	Normal calf
Pruyn	4	1 day	Normal calf
Pruyn	5	1 day	Normal calf
Pruyn	6	4 days	Normal calf
Pruyn	7	7 days	Normal calf
Pruyn	8	8 days	Normal calf

Table 7 White blood cell counts of calves

Calf type	Number of animals	Age	Ave. counts/mm^3	Range
Normal, newborn	8	0-10 days	8763	12,600 - 5800
Normal	5	1-2 months	8946	10,930 - 6600
Normal	6	8-9 months	8767	11,400 - 7400
WS, newborn	8	0-10 days	7239	14,400 - 2200
WS	9	1-2 months	7010	11,740 - 4500
WS	8	8-9 months	7775	9200 - 6700

the number of test animals was limited (a total of eight calves), the T-cell response to PHA-P was low during the first hours postpartum. The counts for control and stimulated cultures as well as S.I. and C.C. for each calf is given in table 8. When the S.I. is plotted in relationship to age (figure 4), the T-cell population is depressed in relation to stimulation with PHA-P during the first hours postpartum. The ability to stimulate T-cells with PHA-P increased after 48 hours to a level that was maintained up to 8 days.

The results of blastogenesis studies on newborn calves afflicted with the weak calf syndrome is tabulated in table 9. The average S.I. for newborn WS calves prior to injection of thymosin was 1.5 compared to an average S.I. of 12.9 for newborn normal calves. Following one injection of thymosin (1000 μgm) in phosphate buffer, the average S.I. of WS calves increased to 57.8. However, this increase is misleading since the blood blastogenesis cultures for the WS calves given thymosin were not prepared until two months after the first S.I. were determined. A bar graph of the S.I. for each test group is presented in figure 5. The C.C. for each test group are shown in the bar graph in figure 6. The average C.C. for normal newborn calves was 404 cpm with a range of -3 cpm to 853 cpm. The C.C. for WS calves prior to the administration of thymosin ranged from -225 cpm to 522 cpm with an average C.C. of 101 cpm. After the administration of thymosin the C.C. for the same WS calves rose to an average C.C. of 1233 cpm with a range of 729 cpm to 1722 cpm.

When comparing the newborn normal calf and WS calf, the S.I. ($p<.05$) and C.C. ($p<.05$) are significantly different. Since the data

Table 8 Tabulated data for PHA stimulated blastogenesis cultures for normal newborn calves

Calf	Age	No PHA culture counts	PHA culture counts	S.I.(a)	C.C.(b)
1	1 hour	11(c)	8(c)	0.7	-3
2	10 hours	20	191	9.5	171
3	12 hours	44	478	10.9	434
4	24 hours	150	1003	6.7	853
5	24 hours	14	388	27.7	374
6	4 days	24	442	18.4	418
7	7 days	38	600	15.8	562
8	8 days	34	453	13.3	419

(a) S.I. = stimulated culture counts/control culture counts
(b) C.C. = stimulated culture counts - control culture counts
(c) Each value expressed in counts per minute is the average of triplicate cultures counted twice.

Figure 4 Scatter graph showing the stimulatory index for PHA stimulated blastogenesis cultures of newborn normal calves

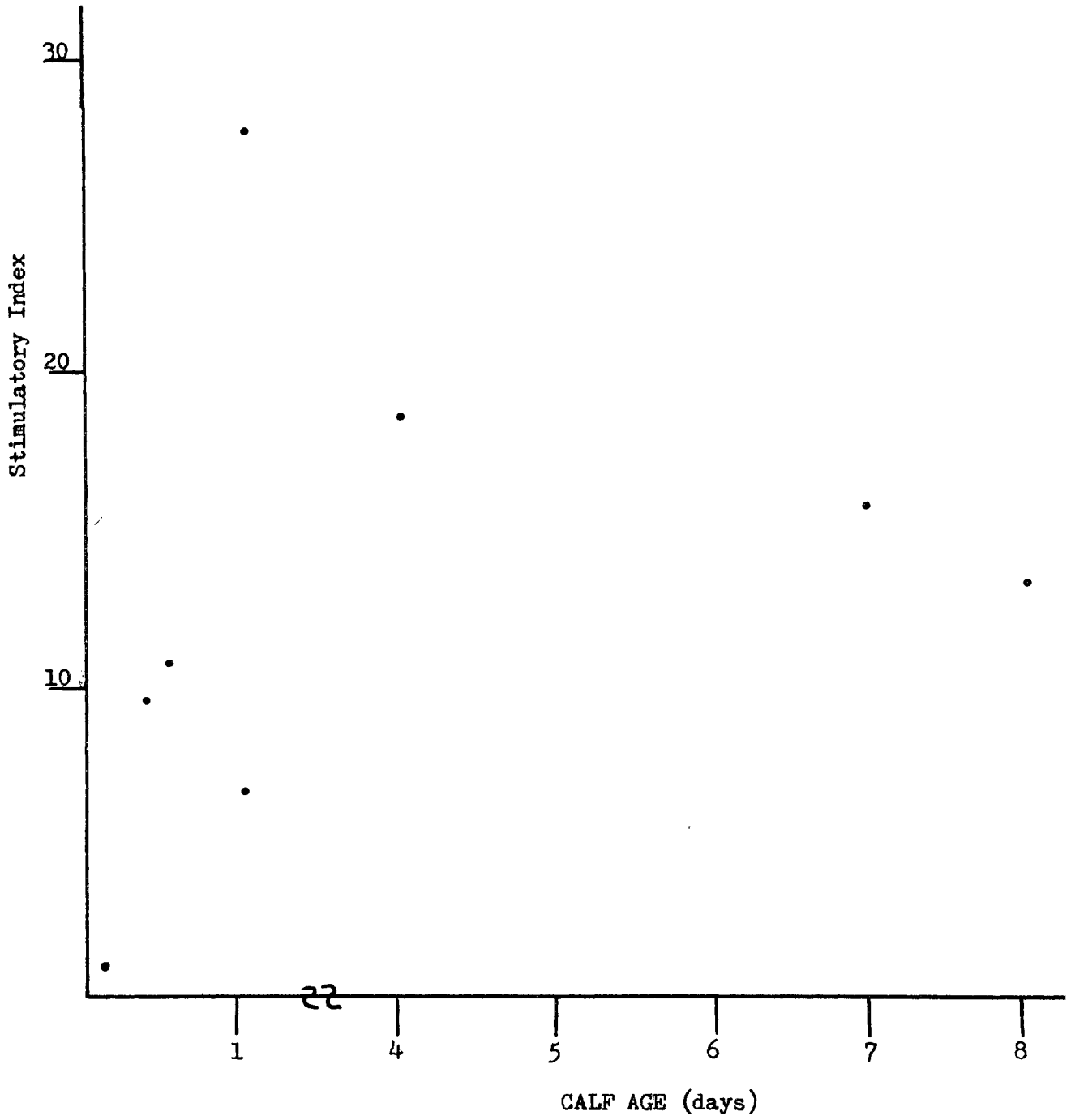


Table 9 Tabulated data for PHA stimulated blastogenesis cultures for WS calves prior to and after administration of thymosin

Calf	Age	No PHA culture counts	PHA culture counts	S.I.(a)	C.C.(b)
Prior to administration of thymosin					
Carter 918	1 day	465(c)	705(c)	1.5	240
" 817	4 days	1401	1176	0.8	-225
" W10	1 day	522	331	0.6	-191
" 904	1 day	1957	2479	1.3	522
Ward SD47	1 day	492	730	1.5	238
" W3	1 day	94	169	1.8	75
" K1328	1 day	5	149	2.6	144
" 1054	1 day	282	288	1.0	6
After administration of thymosin (d)					
Carter 918	2 months	27	1749	64.8	1722
" 817	2 "	12	1370	114.2	1358
" W10	2 "	20	1610	80.5	1590
" 904	2 "	20	1370	68.5	1350
Ward SD47	2 "	34	763	22.4	729
" W3	Not available				
" K1328	Not available				
" 1054	2 months	69	1035	15.1	966

(a) S.I. = stimulated culture counts/control culture counts
(b) C.C. = stimulated culture counts - control culture counts
(c) Each value expressed in counts per minute is the average of triplicate cultures counted twice.
(d) The blastogenesis cultures for this portion of the study were not performed until two months after the first cultures were done.

Figure 5 Bar graph representing the stimulatory index from blastogenesis cultures from PHA stimulated cultures of newborn normal and WS calves. Bar lines represent range.

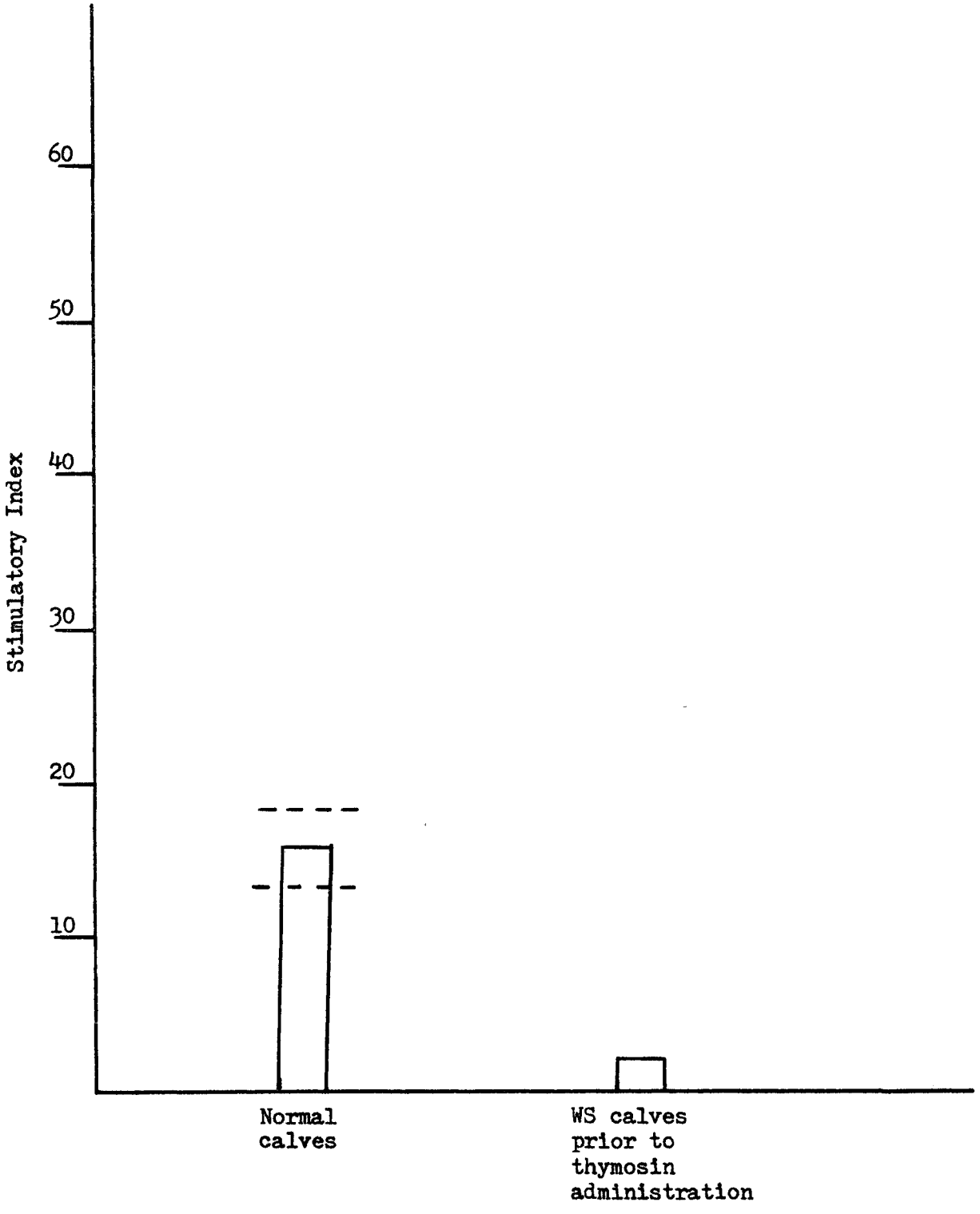
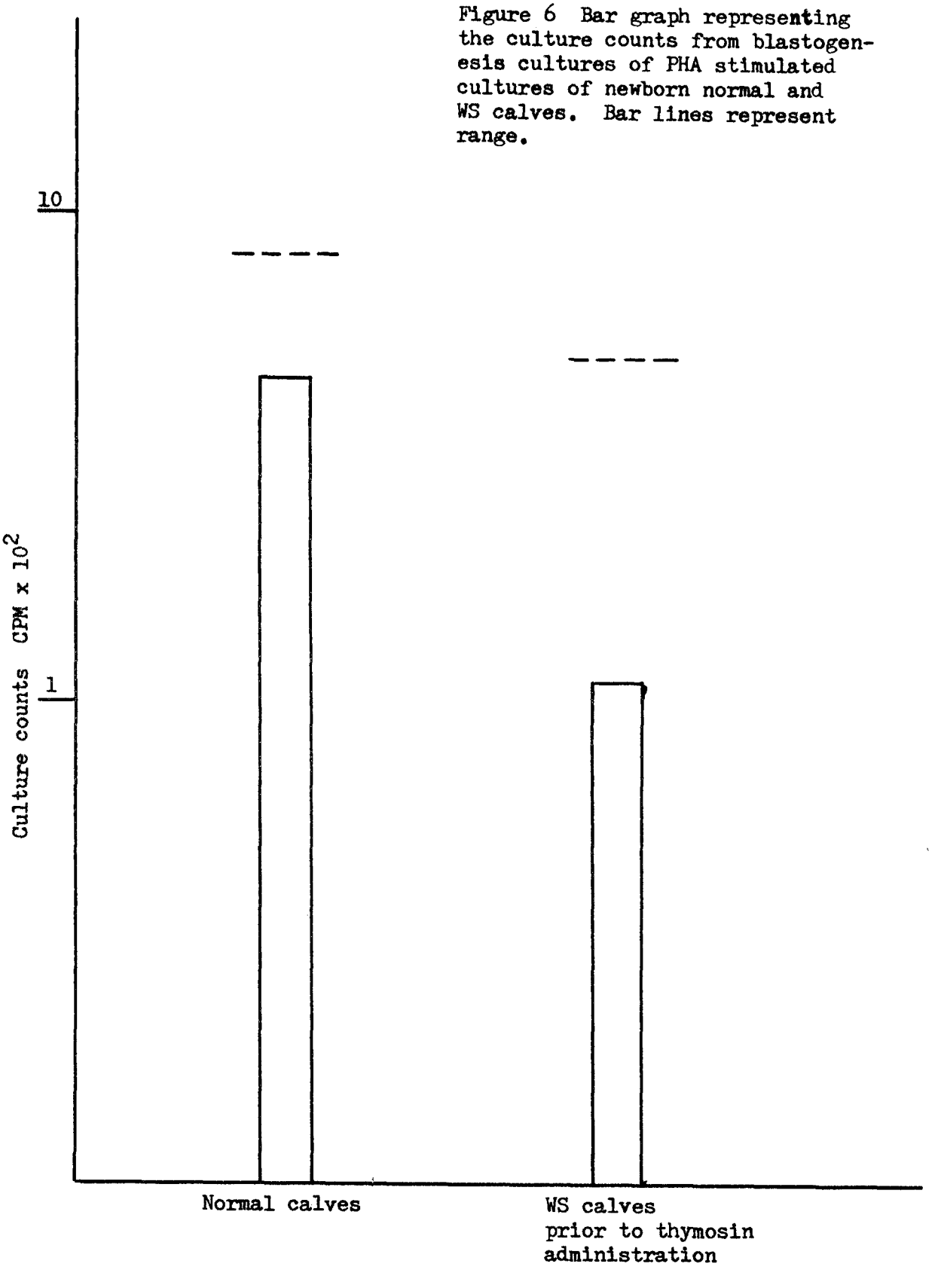


Figure 6 Bar graph representing the culture counts from blastogenesis cultures of PHA stimulated cultures of newborn normal and WS calves. Bar lines represent range.



gathered on treated WS calves was misleading, statistical comparison of the treatment was not computed between prior and after administration of thymosin.

Studies involving calves 1-2 months old

In order to determine the effects of the thymic extract on chronic WS calves, two test groups were selected from the same herd within the Bitterroot Valley. The first group consisted of calves 1-2 months old and the second group selected from the same calf crop were 8-9 months old. Mr. Van Meter, who ranches approximately twelve miles south of Hamilton, allowed his herd to be used after the initial arrangements were made by Dr. Ward. The test calves were kept in an open pasture with the other cattle belonging to the rancher. All animals were caught by Mr. Van Meter at the times the blood samples were obtained.

Mr. Van Meter experienced a calf loss rate of approximately 25% in his herd during the spring of 1975. Dr. Ward stated that this is about the average loss rate for herds affected with the weak calf syndrome for the first time in contrast to about 5 to 7% in herds having experienced this condition previously. The selection of calves to be included in the "normal" and "WS" calf groups was made by Dr. Ward using the same criteria stated previously. A summary of the calves used in this portion of the test is given in table 10.

The blood samples were drawn by Dr. Ward from the test calves. With the blood collected on the initial visit, WBC count, differential WBC count and blastogenesis studies were performed. The results of these

Table 10 Pertinent information for calves 1-2 months old

Owner	Identification Number	Age(days)	Comments
Van Meter	Y84	42	Normal, given thymosin in phosphate buffer
Van Meter	Y26	42	Normal, given thymosin in phosphate buffer
Van Meter	Y89	46	Normal, given thymosin in phosphate buffer
Van Meter	W34	39	Normal, given BSA
Van Meter	Y96	45	Normal, given BSA
Van Meter	Y75	39	WS, given thymosin in phosphate buffer
Van Meter	W89	29	WS, given thymosin in phosphate buffer
Van Meter	Y1	62	WS, given thymosin in phosphate buffer
Van Meter	Y121	21	WS, given thymosin in phosphate buffer, expired
Van Meter	Y66	22	WS, given thymosin in phosphate buffer
Van Meter	Y103	35	WS, given thymosin in peanut oil
Van Meter	W78	33	WS, given thymosin in peanut oil
Van Meter	W32	39	WS, given BSA
Van Meter	Y63	32	WS, given BSA

tests will be given later. During this initial visit one injection of (1000 µgm/calf) in 0.1 M phosphate buffer, thymosin (1000 µgm/calf) in peanut oil or BSA (1000 µgm/calf) in 0.1 M phosphate buffer was administered. The proper dose of each was given by intramuscular injection into alternate normal and WS calves. Neither Dr. Ward or Mr. Van Meter was aware of which injection was given to a particular calf. The subsequent visit to the ranch was made one week later. At that time a sample of blood was drawn from each test calf for comparison studies. During the week one calf, Y121, that had been given thymosin in phosphate buffer expired. Therefore, the results of this calf were not included.

The average WBC counts for each group are given in table 7. The average WBC count for normal calves was 8946 compared to 7134 for the WS animals. After the administration of thymosin in phosphate buffer, the average WBC count for WS calves was 7887 and 9717 for normal calves. The average WBC count was 6800 for WS calves given thymosin in peanut oil. The values obtained during the initial visit are below the average WBC counts of 11,400 for Hereford calves less than one year old (66) but are within the range of 5750 to 17,800. The differential counts, table 11, for normal and WS calves showed little variation between the groups in relation to percentages of lymphocytes and polymorphic neutrophils. The average percentage of lymphocytes was 77 for normal calves and 70 for WS calves prior to the administration of an extract. The average polymorphic neutrophils percentages were 16 and 23 respectively. One week after the administration of thymosin in phosphate buffer the lymphocytes and polymorphic neutrophils average

Table 11 Differential WBC average counts for 1-2,month old calves

Group	Number	Neutrophils Juvenile	Neutrophils Segmented	Lymphocytes	Monocytes
Normal calves					
Before admin.	5	4.2	12.2	76.6	7.0
After thymosin	3	1.0	35.3	77.0	3.6
After BSA	2	1.5	12.5	84.5	2.0
WS calves					
Before admin.	7	4.0	18.8	69.6	3.8
After thymosin in phosphate buffer	4	2.3	28.9	61.2	6.3
After thymosin in peanut oil	2	4.5	21.5	72.0	6.5
After BSA	1	1.0	15.0	82.0	1.0
Normal values (66)	26	23.7		68	3.2
Range		12 - 38		12 - 80	1 - 5

percentages for WS calves were 62 and 31 percent respectively and 72 and 26 respectively for calves given thymosin in peanut oil. One of the two WS calves given BSA had a differential count of 22 percent lymphocytes and 77 percent polymorhpic neutrophils. The high percentage of polymorhpic neutrophils indicates an infection, probably of bacterial or protozoan etiology. Therefore, the resutls for this calf were not included. The other WS calf injected with BSA had 82 percent lymphocytes and 16 percent polymorhpic neutrophils. The high percentage of lymphocytes is normal for cattle. The values obtained are within the range for lymphocytes of 42 and 80 percent with an average of 68 and for polymorhpic neutrophils the range is 12 to 38 percent with an average of 23 percent (66).

The absolute number of lymphocytes per mm^3 of peripheral blood (AL/mm^3) can be determined by multiplying the WBC count by the percentage of lymphocytes. To determine if there is an increase in absolute number of lymphocytes with the administration of thymosin, the AL/mm^3 was determined for the calves of this test group. The AL/mm^3 for each test animal of normal and weak calf groups is tabulated in table 12. The average AL/mm^3 for normal calves and WS calves was 6846 and 5072 repsectively. There was a significant difference ($p=.05$) between normal calf and WS calf groups prior to any administration of test materials.

The average AL/mm^3 for those normal calves given thymosin increased from 7318 to 7448 and for WS calves the average decreased from 4768 to 4759. The AL/mm^3 dropped in two normal calves, W26 and Y89, and in one WS calf, Y75. For calves given thymosin in peanut oil, there

Table 12 Tabulation of absolute lymphocytes per mm^3 (AL/mm^3) for normal and WS calves 1-2 months old

Group	Calf	WBC count	% lymphocytes	AL/mm^3
Normal				
Prior to any administration of thymosin or BSA				
	Y84	10,350	78	8073
	W26	8050	85	6243
	Y89	8800	80	7040
	W34	10,930	70	7651
	Y96	6600	70	4620
After administration of thymosin in phosphate buffer				
	Y84	11,300	90	10,170
	W26	7400	84	6216
	Y89	10,450	57	5957
After administration of BSA				
	W34	8600	86	7396
	Y96	5600	83	4648
Weak syndrome				
Prior to administration of thymosin or BSA				
	Y75	11,740	61	7161
	W89	4800	77	3696
	Y1	6100	60	3660
	Y66	6800	67	4556
	Y103	8400	64	5376
	W78	3980	90	3582
	W32	8120	92	7470
After administration of thymosin in phosphate buffer				
	Y75	7000	62	4340
	Y89	7050	66	4653
	Y1	9600	47	4512
	Y66	7900	70	5530
After administration of thymosin in peanut oil				
	Y103	9000	60	5400
	Y78	4600	84	3864
After administration of BSA				
	W32	6900	82	5658

was an increase in the average AL/mm^3 from 4479 to 4632. The calves of both groups given BSA showed a decrease in average AL/mm^3. The administration of thymosin did not increase the AL/mm^3 in WS calves to a level where this group would not be significantly different ($p < .05$) from the normal calves prior to treatment.

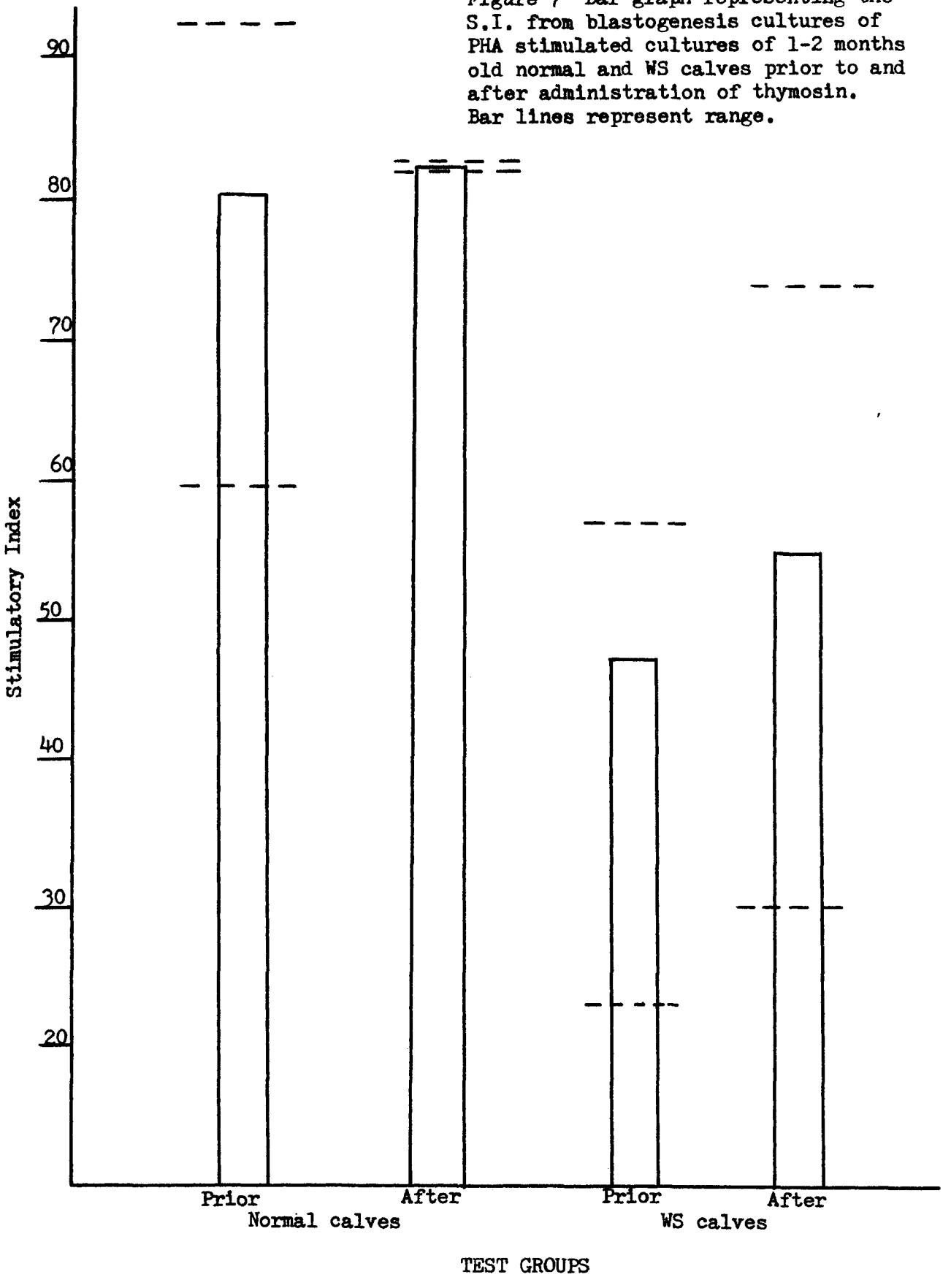
The blood collected during the initial visit was studied to obtain the baseline value of blastogenesis. The results of these studies are tabulated in table 13. Because of technical problems the blastogenesis culture samples for the two calves, W78 and Y103, given thymosin in peanut oil were not included. The average S.I. shown in figure 7 for normal calves was 80.9 ranging from 59.5 to 92.7, whereas, the WS calf average S.I. was 47.5 ranging from 23.1 to 57.6. These data indicate that there is a significant difference ($p < .05$) between the S.I. of normal calves and WS calves prior to any administration of test materials. The S.I. for normal calf group after treatment shown in figure 7 rose to 106 but the results from calf W34 and W26 were disregarded; the low S.I. of 10 and 8 respectively for these calves appeared to result from nonspecific stimulation of the T-cells of control samples. The two normal calves, Y84 and Y89, given thymosin in phosphate buffer showed an average S.I. of 153. This very high S.I. resulted from the very low uptake of tritiated thymidine by the lymphocytes of the control cultures. The average S.I. for WS calves indicated in figure 7 given thymosin was 55 with a range of 30.3 to 77.5. The one WS calf given BSA showed a S.I. of 102, but the other calf, Y63, was not included because of an apparent infection. After the WS calves were treated with tymosin, there was no significant

Table 13 Tabulated data for PHA stimulated blastogenesis cultures for normal and WS 1-2 month old calves prior to and after administration of thymosin or BSA

Group	Calf	No PHA culture counts	PHA culture counts	S.I.(a)	C.C.(b)
Normal	Prior to administration of thymosin or BSA				
	Y84	45(c)	4168(c)	92.6	4123
	Y89	61	5811	95.9	5750
	W26	40	2359	59.5	2319
	W34	56	4268	74.2	4212
	Y96	65	5330	82.1	5265
	After administration of thymosin				
	Y84	69	5694	82.5	5625
	Y89	37	3030	81.2	2993
	W26	200	1539	7.7	1339
	After administration of BSA				
	W34	181	1827	10.1	1746
	Y96	20	3056	152.8	3036
Weak syndrome	Prior to administration of thymosin or BSA				
	Y75	126	7229	57.3	7103
	W89	285	6607	23.1	6322
	Y1	38	2077	55.4	2039
	Y66	105	4054	38.5	3949
	Y121	18	977	53	959
	W32	81	4660	57.6	4579
	After administration of thymosin				
	Y75	94	2844	30.3	2750
	W89	98	4494	45.8	4396
	Y1	21	1628	77.5	1607
	Y66	38	2586	68.1	2548
	Y121	Calf expired during the study			
	After administration of BSA				
	W32	57	5798	101.7	5741

(a) S.I. = stimulated culture counts/control culture counts
(b) C.C. = stimulated culture counts - control culture counts
(c) Each value expressed in counts per minute is the average of triplicate cultures counted twice.

Figure 7 Bar graph representing the S.I. from blastogenesis cultures of PHA stimulated cultures of 1-2 months old normal and WS calves prior to and after administration of thymosin. Bar lines represent range.



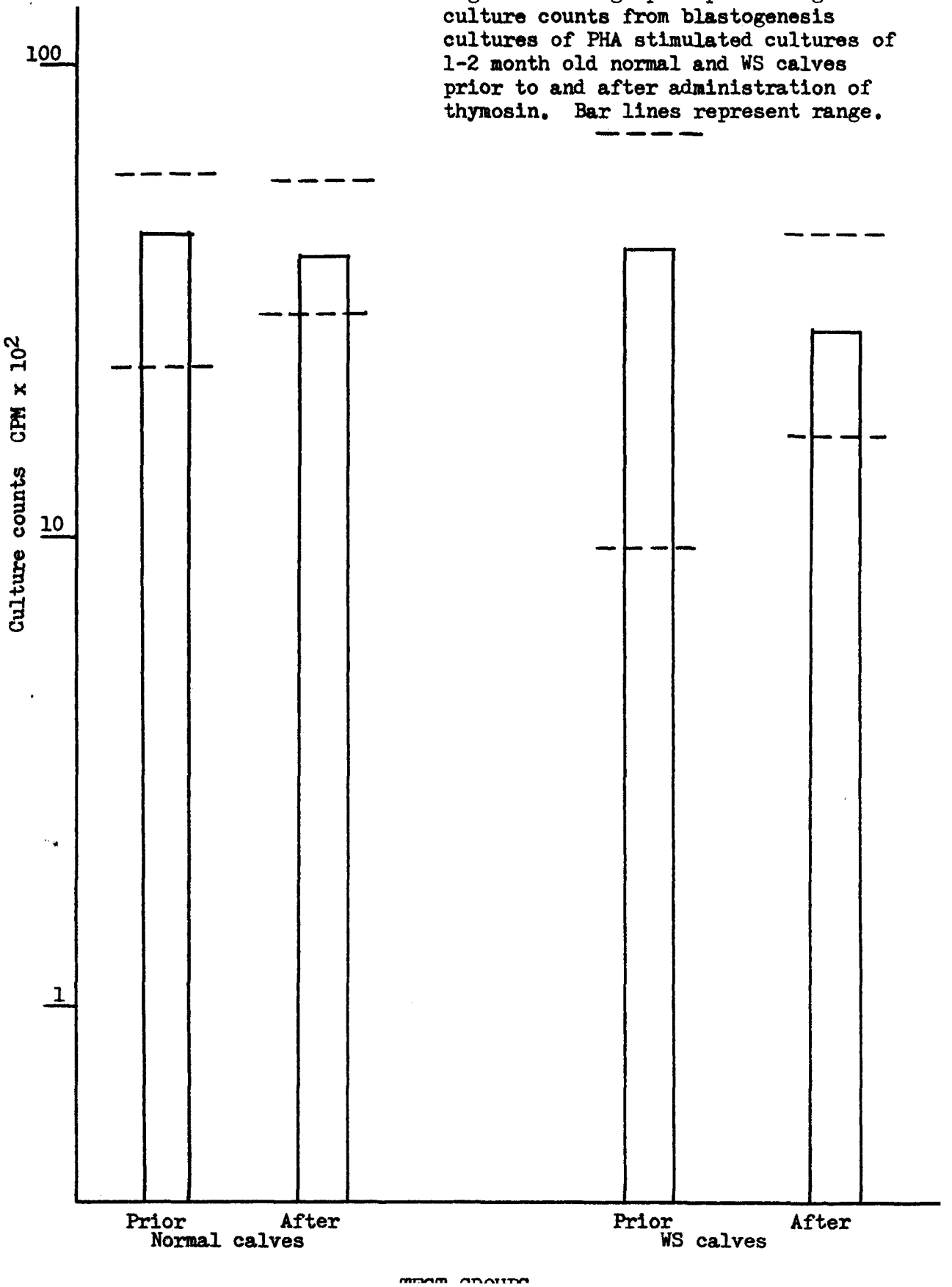
difference ($p > .05$) between the S.I. of this group and the S.I. of normal calves prior to any treatment. There was no significant difference ($p > .05$) between the S.I. of normal and WS calves after administration of thymosin. However, the data of calf W32 was not considered and the S.I. for the remaining two calves was higher than the S.I. of any weak calf. Because of the small sample size for calves treated with BSA, statistical analysis could not be computed.

There was adequate uptake of tritiated thymidine by PHA-P stimulated lymphocytes shown by the fact that most cultures had around 2000 cpm. The average C.C. for normal calves indicated by the bar graph in figure 8 was 4334 cpm with a range of 2319 cpm to 5750 cpm. The WS calf average C.C. was 4159 with a range of 959 cpm to 7103 cpm. Using the Mann-Whitney statistical analysis, there was no significant difference between the two groups of test animals. The C.C. for the two normal calves, Y84 and Y89, after administration of thymosin in phosphate buffer was 4308 cpm. The C.C. of calf Y96 given BSA measured 3036 cpm. The WS calves given thymosin in phosphate buffer exhibited an average C.C. of 2825 cpm with a range of 1607 cpm to 4396 cpm, and the calf W32 given BSA exhibited a C.C. of 5741.

Studies involving calves 8-9 months old

The effect of thymosin on 8-9 month old calves was tested in order to establish an understanding of the effect of thymosin in older calves. The animals were selected from Mr. Van Meter's herd. Care was taken to select calves about the same age and not to include calves previously used. Although the date of birth for each calf

Figure 8 Bar graph representing the culture counts from blastogenesis cultures of PHA stimulated cultures of 1-2 month old normal and WS calves prior to and after administration of thymosin. Bar lines represent range.



could not be established, the calves were all born in February and March 1975 and were, therefore, 8 to 9 months old when tested. The calves were kept separated from the herd and fed daily. The blood was drawn by Dr. Ward while the calves were held in a squeeze chute. The administration of test materials was done by intramuscular injection while the calves were in the chute. The selection of calves shown in table 14 was made by Dr. Ward using the criteria outlined previously. A total of six "normal" calves and eight "weak syndrome" calves was included in the testing. To show the significant difference between the size of normal and WS calves, the calves were weighed. The weight for normal calves ranged from 410 pounds to 530 pounds with and average weight of 450 pounds, whereas, the average weight of WS calves was 236 pounds with a range from 185 to 275 pounds. There was a significant difference ($p < .05$) between the two groups of aminals.

The schedule and dose of injected materials were changed from previous tests. An injection of BSA (20,000 µgm/calf) was administered after the initial blood sample was drawn. The second blood sample was drawn a week later as well as the administration of thymosin (20,000 µgm/calf) in 0.1 M phosphate buffer to each calf. One week later the final blood sample was collected. With each blood sample collected, WBC count, differential WBC counts and blastogenesis studies were completed.

Table 7 shows the average WBC count obtained for each group of calves during the initial visit. The average WBC count and range for normal and WS calves was 8767 (7400 - 11,400) and 7775 (6700 - 9200) respectively. The average WBC count for normal calves increased to

Table 14 Pertinent information for calves 8-9 months old

Owner	Identification number	Weight	Comments
Van Meter	R62	430	Normal calf
Van Meter	R43	530	Normal calf
Van Meter	R84	410	Normal calf
Van Meter	W7	455	Normal calf
Van Meter	W127	450	Normal calf
Van Meter	Y106	425	Normal calf
Van Meter	S9401	225	WS calf
Van Meter	W38	205	WS calf
Van Meter	W18	275	WS calf
Van Meter	W108	265	WS calf
Van Meter	W120	185	WS calf
Van Meter	W111	260	WS calf
Van Meter	Y119	250	WS calf
Van Meter	Y46	220	WS calf

9232 (7300 - 11,200) after the administration of BSA and to 8967 (7200 - 12,600) after the administration of thymosin. For WS calves the average WBC count after the injection of BSA decreased to 7538 (6300 - 8900) and after thymosin increased to 8571 (5700 - 9800). The differential showed that the distribution of polymorphic neutrophils and lymphocytes was similar in both test groups. There was no significant increase in the percentage of lymphocytes after administration of BSA or thymosin. The data is summarzied in table 15.

The absolute lymphocyte number per mm^3 of peripheral blood (AL/mm^3) was determined as previously stated. The data for each animal is tabulated in table 16. The average and range for normal calves was 6898 (5328 - 8778) AL/mm^3 prior to any injection of test materials. The average increased for normal calves after BSA administration 7570 (5986 - 10,192) AL/mm^3; after thymosin administration the average AL/mm^3 was 7071 (6192 - 8820). There was in increase in AL/mm^3 in WS calf average to 6181 (4810 - 8064). Prior to the administration of BSA, there was a significant difference ($p < .05$) between AL/mm^3 for normal and WS calves. However, after BSA administration there was no significant difference ($p < .10$) as was also seen after administration of thymosin ($p > .10$), and the probability factor (p factor) was larger after administration of thymosin than after administration of BSA.

Technical problems with the incubation system for blastogenesis caused the data obtained to be disregarded. Although blastogenesis cultures were established, there was too much variability in the results obtained for the results to be useful.

Table 15 Differential WBC average counts for 8-9 month old calves

Group	Number	Neutrophils		Lymphocytes	Monocytes
		Juvenile	Segmented		
Normal calves					
Before admin.	6	2.8	10.7	78.7	4.3
After BSA	6	1.2	10.5	81.8	2.5
After thymosin	6	1.3	11.5	80.0	3.6
WS calves					
Before admin.	8	3.6	15.3	72.5	5.9
After BSA	8	2.5	11.0	81.5	2.3
After thymosin	7	3.0	14.1	74.0	5.0

Table 16 Tabulation of absolute lymphocytes per mm^3 (AL/mm^3) for normal and WS calves 8-9 months old

Group	Calf	WBC count	% lymphocytes	AL/mm^3
Normal	Prior to administration of BSA			
	R62	11,400	77	8778
	R43	9800	80	7840
	R84	8000	77	6160
	W7	8000	82	6560
	W127	7400	72	5328
	Y106	8000	84	6720
	After BSA administration			
	R62	11,100	79	8769
	R43	11,200	91	10,192
	R84	9500	81	7695
	W7	7700	85	6545
	W127	8600	73	6278
	Y106	7300	82	5986
	After thymosin administration			
	R62	12,600	70	8820
	R43	9000	74	6600
	R84	8600	83	7138
	W7	7300	88	6424
	W127	7200	86	6192
	Y106	9100	79	7189
Weak syndrome	Prior to administration of BSA			
	S9401	9200	67	6164
	W38	6700	81	5427
	W18	7100	71	5041
	W108	8100	81	6561
	W120	6700	66	4422
	W111	8300	69	5727
	Y119	8800	64	5632
	Y46	7300	81	5913
	After BSA administration			
	S9401	8600	80	6880
	W38	6300	80	5040
	W18	6500	74	4810
	W108	8400	96	8064
	W120	7300	75	5475
	W111	8900	73	6497
	W119	7700	86	6622
	Y46	6600	88	5808
	After thymosin administration			
	S9401	8200	82	6724
	W18	6500	59	3835
	W108	9200	77	7084
	W120	7600	62	4712
	W111	9200	72	6624
	W119	9500	78	7410
	Y46	9800	88	8624
	W38	5700	Not available	

Chapter 4

DISCUSSION

Selection of calves

Since the University of Montana does not have a bovine test herd or facilities for housing large animals, this study had to be done with calves on the owner's ranches within the Bitterroot Valley. Dr. Ward made the initial arrangements with the ranchers to allow their calves to be used in these experiments. Although inherent problems were encountered, these arrangements were necessary to obtain any data.

By using a group of animals fifty miles from the laboratory, however, transportation of samples created a major problem. The blood samples requently arrived at the laboratory six hours after collection. On several occasions the samples were received on the following day after being drawn. To add to the complication, these samples were left either in a cooler in the truck used by Dr. Ward or in the clinic at unknown temperatures until transported to the laboratory. The variability of some of the data could have been due to these conditions. The most ideal situation was to be present when the blood samples were drawn as was done during the bleeding of one-month and older calves. However, samples from newborn weak syndrome (WS) calves were another matter since the birth of such calves could not be predicted. Dr. Ward drew blood from these diseased animals only when the opportunity arose.

Calves are obviously of economic value to the rancher, but the depressed cattle market decreased the attempts of many ranchers to save animals with severe afflictions. In the case of the WS calves, the problem was magnified since previous therapy with whole immune serum that had "saved" numerous calves did not alleviate the runting condition associated with the syndrome. Therefore, weight gain of normal calves was approximately 100% greater after one year that in WS calves. Since the runted animals consumed almost as much feed as the normal ones, the financial return was considerably less. In view of the above conditions, the rancher was reluctant to incur the additional expense of procuring the services of a veterinarian. Since Dr. Ward was depended upon to collect the materials from the diseased animals, samples became increasingly difficult to obtain.

The pool of available calves that could be used was further reduced since calves from previous herds given thymosin or passive immune therapy were not suitable for the study of chronic calves. Thymosin had been administered to a number of animals during the spring of 1974. Thymosin and passive immune therapy was given to a large population of calves belonging to several ranchers who had been particularly cooperative during the studies of the WS calf by this laboratory. Therefore, herd animals without previous exposure to thymosin or immune serum was desired, but locating these types of herds was difficult.

Just prior to the spring of 1974, Mr. Van Meter moved into the valley from eastern Montana. He brought several head of cattle from elsewhere and purchased cattle from other ranchers in the Bitterroot

area to establish his herd. Not aware of the disease problem prevalent in the area, he was perplexed during the first year when his pregnant heifers began to abort or give birth to calves unable to stand and nurse. After losing a large number of his calf crop the first year, Mr. Van Meter contacted Dr. Ward who diagnosed the problem as the weak calf syndrome. The next spring, 1975, Mr. Van Meter lost 25% of his calf crop. When he learned of our interest in conducting studies with thymosin using a herd not previously exposed to any test materials, he expressed his desire to provide the necessary animals from his herd for our limited studies.

During the testing of thymosin on calves under one month of age, there was a tendency to treat all WS calves with thymosin and not to include control animals injected with a placebo. Since the administration of thymosin to some calves during the spring of 1974 resulted in dramatic improvements, the ranchers understandably desired to treat all, including normal, calves with thymosin. The desired procedure was to use one herd to test all materials, a situation provided by Mr. Van Meter. Since neither the rancher nor Dr. Ward was aware of which injections each calf received, proper control measures were maintained.

Scheduling of the bleeding and administration of test materials were made by Dr. Ward. Since young calves were still nursing, all animals were retained with the herd during the study. The arrangement had its difficulties in that the calves desired for bleeding or injection were intermingled with the other non-testing cattle. Therefore, in addition to sorting the test animals, reliance had to be upon

Mr. Van Meter to rope the calves.

Older calves did not create as much difficulty in that they were corralled and kept separate from the herd. This arrangement was more suitable since less help was needed and the collection time was reduced, but Mr. Van Meter also absorbed the additional expense of feeding these animals for the three-week period.

On occasion the scheduling of all the persons necessary for the bleeding and injections was difficult to coordinate. For example, the time intervals required for bleeding and injection of animals could not always be coordinated, and delays were the frequent consequences. The blastogenesis assays for newborn WS calves given thymosin should have been performed within two weeks after the injection instead of two months later.

Visual observation of thymosin therapy

Although the serum therapy used previously was undoubtedly responsible for the survival of numerous diseased calves, their general health did not necessarily improve. The calves were susceptible to secondary infections especially those leading to scours. Although the treated calves were frequently able to stand and to nurse, several of these animals had to be bottle fed or had to have their heads held while nursing. Even six months later when lymph node excision was done on two of these animals, the lesions required over a month to heal whereas, the lesions due to lymph node excision in normal calves healed within 48 hours.

Since immune serum was tedious to prepare and, as stated earlier, the WS calves showed thymic atrophy and involution, the administration of thymosin was attempted. With three injections of thymosin (1000 µgm each) on alternate days dramatic improvements were observed. An example of the observations made by Dr. Ward following this schedule of injections during preliminary studies during the spring 1974 is as follows:

day of 1st injection:	calf docile, unable to nurse or stand, typical WS calf
day of 2nd injection:	calf able to stand, began to nurse, increased mobility since able to walk
day of 3rd injection:	calf nursed vigorously, can not be approached, had to be roped to administer the injection, running with increased agility

After thymosin administration, calf scours were either reduced or controlled, particularly upon continued therapy.

As stated above the general health of most WS calves improved within a couple days after the first injection. The rapid improvement seen when the WS calves were given thymosin suggested that the agent does more than reconstitute the T-cell mediated immune functions. Although the immunocompete peripheral T-cells increased with thymosin, an effect upon the hormonal balance of WS calves is suggested in view of the dramatic and rapid improvement observed. The thymus had been implicated as affecting the production of growth hormone and thyroxine (57). With injection of thymosin the production and utilization of these and other hormones could have been stimulated in the WS calves. If the thymus is involved in regulation of growth hormone and thyroxine in calves, then the administration of thymosin could reverse the runting

syndrome of WS calves and result in more rapid weight gain. Beneficial effects in normal calves such as increased weight gain per pound of feed may also occur. Further investigation over several years would be required to establish the validity of this hypothesis. However, the improvement of WS calves had regressed in some animals after one week if only one injection of thymosin was administered. The regression of improved health observed when thymosin injections were not continued gives further support to the hypothesis that the thymus has a hormonal function, at least in calves.

Several calves severely afflicted still died even after thymosin was administered. The condition of these animals had deteriorated beyond the point that therapy was unavailable. On the other hand, whether more concentrated and frequent injections of thymosin could have improved the prognosis of these types of calves is speculative.

Blastogenesis study

The mitogen, phytohemagglutinin (PHA), induces T-cells to undergo blast-like transformation. The stimulation can be measured by the uptake of tritiated thymidine during mitosis and can be used as a basis for evaluating the population of immunocompetent T-cells in the peripheral blood. Since the thymus of WS calves has been shown to be involuted (53), the response of the peripheral T-cells to PHA stimulation was used as an index of the status of the cellular immune system.

A summary of the blastogenesis data is given in table 17. Newborn normal calves have a lower or depressed response as compared to calves 1-2 months old. A statistically significant difference ($p<.05$) was

Table 17 Summary of S.I. and C.C. data for normal and WS newborn and 1-2 month old calves

Calf group	Number calves	Average S.I. (a)	Average C.C. (b)
Normal, newborn	8	12.9	404
Normal, 1-2 months old	5	80.9	4334
After thymosin (c)	2	81.9	4309
After BSA	1	152.8	3036
Weak syndrome, newborn	8	1.5	101
Weak syndrome, 1-2 months old	6	47.5	4158
After thymosin (c)	4	55.4	2852
After BSA	1	101.7	2118
Thymosin at birth (d)	6	60.9	1319

(a) S.I. = stimulated culture counts/control culture counts
(b) C.C. = stimulated culture counts - control culture counts
(c) Thymosin and BSA (1000 µgm/injection) were given one week prior to the blood samples being taken.
(d) Calves of this test group were given thymosin (1000 µgm) at birth. Blood samples were taken when the calves were 2 months old.

measured when the data was analyzed by the Mann-Whitney U test. The depression was also noted when the mitogenic response of newborn WS calves was compared to 1-2 month old WS calves. The generalized depression observed in newborn animals could be a result of the high concentration of corticosteriods reported to be present in the circulatory system of fetuses prior to parturition (51).

The S.I. and C.C. calculated from the mitogenic response data of newborn normal calves were significantly different from that of newborn WS calves. Although no significant difference was measured in the C.C. between the two groups of 1-2 month old calves, a difference was noted in calculating the S.I. data. The results show that suppressed peripheral T-cell population is an indication of involuted thymuses in WS calves. The involution could be the result of a direct invasion of the organ by an infectious agent or a stress response induced by the infectious process. In any case, susceptibility of these animals to secondary infections was no doubt increased by the involution and depressed peripheral T-cell population.

The competence of the cell-mediated immune system is routinely determined by the ability of sensitized animals to elicit a delayed hypersensitivity (DH) reaction to skin test antigens. In cattle a DH reaction to protein derivative of tuberculin in animals naturally infected with Mycobacterium bovis or given the BCG strain of M. bovis requires a dose of 400 tuberculin units. In contrast, the maximum of 5 tuberculin units is used in man. A DH reaction could not be elicited in WS calves even though several injections of BCG had been administered previously. With failure to elicit a DH reaction, the WS

calves were thought to be depleted of immunocompetent T-cells. However, the results from calves tested at the Van Meter ranch indicated the presence of T-cells responding to PHA stimulation in chronic WS calves. Therefore, the chronic WS calves do have immunocompetent T-cells although this population is depressed.

After the administration of thymosin the average S.I. of 1-2 month old WS calves, as shown in table 17, increased. When compared to the S.I. of normal calves prior to treatment, the increased S.I. for WS calves was not significantly different ($p>.05$). There was also no significant difference between the S.I. of normal and WS calves after the administration of thymosin. It should be kept in mind that the normal group consisted of only two calves, which obviously make the comparison of the data questionable. Since no significant difference was noted between the WS group after treatment and the normal group prior to treatment with thymosin, the results indicate that thymosin induced mature immunocompetent T-cells to appear in the peripheral blood circulation of WS calves. Two mechanisms could be responsible for this increase in immunocompetent T-cells. However, it is not possible to distinguish if the increase was caused by a release of mature T-cells from the thymus or if thymosin actually induced the maturation of T-cells in the circulating blood. The fact that thymosin has been shown to restore cell-mediated immunity to neonatal thymectomized mice (42) would seem to indicate that the latter hypothesis is appropiate.

As newborn WS calves given thymosin were approximately two months old before the second blastogenesis study was completed, this group

can be compared to calves tested at the Van Meter ranch. The Mann-Whitney U test showed no significant difference between the S.I. of newborn WS calves given thymosin immediately after birth and normal or WS calves 1-2 months old prior to treatment. The observation indicated that thymosin administration did induce a mature T-cell population in these newborn calves, but one injection of 1000 µgm was inadequate to increase the level of response to that seen in normal calves. Based on these results, the therapy should include more concentration of the preparation and increased frequency of treatment.

Some of the technical problems encountered during the blastogenesis study included the incubation temperature and variability of the amount of radioactive thymidine uptake. When the incubation temperature of the lymphocyte cultures was increased to 39° C, blast-like transformation of the cells in the presence of PHA could not be measured. During the blastogenesis studies of 8-9 month old calves, the incubator failed to operate properly. The temperature of over 39° C was noted in the incubator. Variability of thymidine uptake by "stimulated" lymphocytes was observed. At times a ten-fold difference was recorded between triplicate cultures of the same test sample, and these counts were not used in the calculations. Since the concentration of PHA used was in the high range, some of the variability could be attributed to the high concentration of PHA (23).

Effects of thymosin on lymphocyte numbers

To determine the effects of thymosin administration on the number

of lymphocytes per volume of blood, an absolute lymphocyte number analysis was performed using two groups of chronic calves at the Van Meter ranch. The first group consisted of 1-2 month old calves and were also used in the blastogenesis study. The second group of calves 8-9 months old was selected from Van Meter's spring 1975 calf crop seven months later.

The absolute lymphocytes number per mm^3 of peripheral blood (AL/mm^3) for both age groups showed a significant difference between normal and WS calves prior to any injection of thymosin. After thymosin was administered to 1-2 month old calves an increase in the AL/mm^3 was observed. This increase in AL/mm^3 was also noted in most normal 8-9 month old calves given BSA, but a decrease in AL/mm^3 was observed for 8-9 month old WS calves when administered BSA. With the administration of thymosin the AL/mm^3 increased in most calves, but some showed a decrease in AL/mm^3.

The increase of AL/mm^3 was only about 100 lymphocytes in most cases after injection of either BSA or thymosin. The small increase could possibly be attributed to differences in the differential. The difference would be magnified since the lymphocyte count was multiplied by the total WBC count. Brown (11) stated that there could be a 10-15% difference in the cell counts when performing the differential WBC count. Therefore, the small increase of 100 lymphocytes does not seem sufficiently large to account for the increase in S.I. noted after injection of thymosin in the 1-2 month old WS calves. This would tend to support the hypothesis that thymosin causes the maturation of T-cells in peripheral blood rather than the release of mature

T-cells from the thymus. At the present time sufficient data is not available to support either mechanism.

The rosette formation by peripheral lymphocytes has been used to assay the T-cell population of peripheral blood (78). In a human patient there had been reported an increase in the number of lymphocytes as well as an increase in the percentage of T-cell rosettes with thymosin injections (77). This assay system could not be performed during the course of the study on WS calves because of the problems encountered when lymphocytes were separated from erythrocytes. When the separation and purification of bovine calf peripheral lymphocytes can be perfected, the rosette formation would probably be a more effective means of ascertaining whether the administration of thymosin increased the circulating peripheral T-cells.

Chapter 5

SUMMARY

Upon study of the calf afflicted with the weak calf syndrome, the absence of a normal thymus development was observed. It was also observed that weak syndrome calves could not elicit a delayed hypersensitivity skin reaction to purified protein derivative of tuberculin even after repeated injection of Mycobacterium bovis strain BCG. The absence of delayed hypersensitivity was hypothesised to be a result of a depressed or absent thymic lymphocyte (T-cell) population. Since neonatal thymectomized mice given thymosin were able to reconstitute a functional T-cell population, thymosin was believed to have a similar effect on weak syndrome calves. More specifically, the immunocompetence of the peripheral T-cell population in weak syndrome calves was monitored to determine if administration of thymosin reconstituted the T-cell population. The immunocompetence of the peripheral T-cell population in weak syndrome and normal calves was determined before administration of thymosin.

Uptake of tritiated thymidine by phytohemagglutinin(PHA) was used to monitor the immunocompetence of the peripheral T-cell population. This type of assay measures the stimulatory effect of the non-specific mitogen PHA on the T-cell population which can be used to determine the immunocompetence of this lymphocyte population. Whole blood was used for the blastogenesis cultures since the physical separation of peripheral lymphocytes could not be attained in calf peripheral

blood samples. The immunocompetence of the T-cell population was determined prior to and after one injection of thymosin (1000 µgm). Both weak syndrome and normal calves of varying ages were used. To determine if thymosin would increase the number of lymphocytes in the peripheral blood total and differential WBC counts were performed at the time blastogenesis cultures were done.

It was determined that the T-cells from weak syndrome calves would take up a significantly lower amount of radioactive thymidine than the T-cells from normal calves. After administration of thymosin there was no significant difference in the amount of tritiated thymidine uptake between the T-cells from both calf groups. Any increase in the total lymphocytes after thymosin was not large enough to account for the increased stimulatory effect seen in T-cells from weak syndrome calves. Therefore, it was hypothesised that thymosin induced the maturation of peripheral T-cells in weak syndrome calves. An added effect of administration of thymosin which was not fully understood was the very rapid improvement in the general health of weak syndrome calves after one injection of thymosin.

LITERATURE CITED

1. Archer, O. K. and J. C. Pierce. 1961. Role of thymus in development of the immune response. Fed. Proc. Fedn. Am. Soc. Exp. Biol. 20:26.

2. 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.

3. August, C. S., R. H. Levey, A. I. Berkel and F. S. Rosen. 1970. Establishment of immunological competence in a child with congenital thymic aplasia by a graft of fetal thymus. Lancet 1:1080-1083.

4. August, C. S., F. S. Rosen and R. M. Filler. 1968. Implantation of a foetal thymus restoring immunological competence in a patient with thymic aplasia (DiGeorge's syndrome). Lancet ii:1210-1211.

5. Bach, J. -F. and M. Dardenne. 1972. Antigen recognition by T lymphocytes II. similar effects of azathoprine, antilymphocyte serum and anti-theta serum on rosette-forming lymphocytes in normal and neonatal thymectomized mice. Cell. Immuno. 3:11-21.

6. Baker, R. E. 1975. Personal communication.

7. Besedovsky, H. O. and E. Sorkin. 1974. Thymus involvement in femal sexual maturation. Nature 249:356-358.

8. Bianchi, E. W., W. Peipaoli and E. Sorkin. 1971. Cytological changes in the mouse anterior pituitary after neonatal thymectomy: a light and electron microscopical study. J. Endocrin. 51:1-6.

9. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow: isolation and removal of lymphocytes from bone marrow of rats and guinea pigs. Scand. J. Clin. Lab. Invest. Suppl. 21:97-10(

10. Braun, R. K., B. I. Osburn and J. W. Kendrick. 1973. Immunological response of bovine fetus to bovine viral diarrhea virus. Am. J. Vet. Res. 34:1127-1132.

11. Brown, B. A. 1973. Hematology: principles and procedures. Lea and Febriger, Philadelphia, PA. p. 72.

12. Casaro, A. P. E., J. W. Kendrick and P. C. Kennedy. 1971. Response of the bovine fetus to bovine viral diarrhea-mucosal disease virus. Am. J. Ve. Res. 32:1543-1562.

13. Clark, S. L., Jr. 1973. The intrathymic environment. p. 77-99. In A. J. S. Davies and R. L. Carter (ed.), Contemporary topics in immunobiology Vol. 2, thymus dependency. Plenum Press, New York, NY.

14. Cleveland, W. W., B. J. Fogel, W. T. Brown and H. E. M. Kay. 1968. Foetal thymic transplant in a case of DiGeorge's syndrome. Lancet ii:1211-1214.

15. Colley, D. G., A. Malakian and B. H. Wakeman. 1970. Cellular differentiation in the thymus II. thymus-specific antigens in rat thymus and peripheral lymphoid cells. J. Immuno. 104:585-592.

16. Colley, D. G., A. Shih Wu and B. H. Wakeman. 1970. Cellular differentiation in the thymus III. surface properties of rat thymus and lymphnode cells separated on density gradients. J. Exp. Med. 132:1107-1121.

17. Comsa, J., J. A. Schwarz and H. Neu. 1974. Interaction between thymic hormone and hypophyseal growth hormone on production of precipitating antibodies in the rat. Immunol. Commun. 3:11-18.

18. Copper, M. D., R. G. Keightley, L. F. Wu and A. R. Lawton III. 1973. Developmental defects of T and B cell lines in humans. Transplant. Rev. 16:51-84.

19. Coria, M. F., A. W. McCurkin, R. C. Cutlip and A. E. Ritchie. 1975. Isolation and characterization of bovine adenovirus type 5 associated with "weak calf syndrome." Arch. Virol. 47:309-317.

20. Cutlip, R. C. and A. W. McClurkin. 1975. Lesions and pathogenesis of disease in young calves experimentally induced by a bovine adenovirus type 5 isolated from a calf with weak calf syndrome. Am. J. Vet. Res. 36:1095-1098.

21. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, W. B. Wood and M. McCarty. 1973. Microbioloby, p. 459. Harper and Row, Haggerstown, MD.

22. de Sousa, M. A. B., D. M. V. Parrott and E. M. Panelcuris. 1969. The lymphoid tissue in mice and congenital aplasia of the thymus. Clin. Exp. Immunol. 4:637-644.

23. Faguet, G. B. 1974. Lymphocyte responsiveness to phytohemagglutinin (PHA): quantitive aspects and reproducibility. J. Reticuloendothel. Soc. 16:114-121.

24. Gatti, R. A., J. J. Gershanik, A. H. Levkoff, W. Wertelecki, R. A. Good. 1972. DiGeorge syndrome associated with combined immunodeficiency. J. Pediatrics 81:920-926.

25. Gershwin, M. E., A. Ahmed, A. D. Steinberg, G. B. Thurman and A. L. Goldstein. 1974. Correction of T cell function by thymosin in New Zealand mice. J. Immunol. 113:1068-1071.

26. Goldstien, A. L., S. Banerjee, G. L. Schneebeli, T. F. Dougherty and A. White. 1970. Acceleration of lymphoid tissue regeneration in X-irradiated CBA/W mice by injection of thymosin. Radiation Res. 41:579-593.

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

28. Goldstein, A. L, F. D. Slater and A. White. 1966. Preparation, assay and partial purification of a thymic lymphocytopoietic factor (thymosin). Proc. Nat. Acad. Sci. USA 56:1010-1017.

29. Goldstien, A. L. and A. White. 1970. The thymus as an endocrine gland: hormones and their actions, p. 465-502. In G. Litwack (ed.), Biochemical actions of hormones. Academic Press, New York, NY.

30. Goldstein, G. 1975. The isolation of thymoprotein (thymin), p. 177-185. In H. Friedman, Annals New York Acad. Sci. Vol. 249. New York, NY.

31. Good, R. A., C. Martinez and A. E. Gabrielsen. 1964. Clinical considerations of the thymus in immunobiology, p. 3-47. In R. A. Good and A. E. Gabrielsen (ed.), The Thymus in immunobioloby. Hoeber Medical Division, New York, NY.

32. Hardy. M. A., J. Quint, A. L. Goldstein, D. State and A. White. 1968. Effect of thymosin and an antithymosin serum on allograft survival in mice. Proc. Nat. Acad. Sci. USA 61:875-882.

33. Hwang, W. S., T. Y. Ho, S. C. Luk and G. T. Simon. 1974. Ultrastructure of the rat thymus; a transmission, scanning electron microscope and morphometric study. Lab. Invest. 31:472-487.

34. Ivanoff, M. R. and H. W. Renshaw. 1975. Weakcalf syndrome: serum immunoglobin concentrations in precolostral calves. Am. J. Vet. Res. 36:1129-1131.

35. Jannke, C. C. 1972. In vitro oncogenicity of a virus isolate from sheep and cattle afflicted with weak calf and lamb disease. Master's of Science thesis, University of Montana.

36. Januszewski, T. C. 1972. Isolation and characterization of a virus associated with the weak calf-lamb syndrome. Master's of Science thesis, University of Montana.

37. Joel, D. D., E. R. Adamik, A. D. Chanana, E. P. Cronkite, L. M. Schiffer and C. R. Sipe. 1969. Separation of lymphocytes from blood of calves and goats. Am. J. Vet. Res. 30:1099-1104.

38. Keast, D., N. F. Stanley and P. A. Phillips. 1968. The association of murine lymphoma with reovirus type 3 infection. Proc. So. Exp. Biol. Med. 128:1033-1038.

39. Khaw, B. A. and A. H. Rule. 1973. Immunotherapy of the dunning leukemia with thymic extracts. Brit. J. Cancer 28:288-292.

40. Komuro, K. and E. A. Boyse. 1973. In vitro demonstration of thymic hormone in the mouse by conversion of precursor cell into lymphocytes. Lancet i:740-743.

41. Komuro, K. and E. A. Boyse. 1973. Induction of T lymphocytes from precursor cells in vitro product of the thymus. J. Exp. Med. 138:479-482.

42. Law, L. W., A. L. Goldstein and A. White. 1968. Influence of thymosin on immunological competence of lymphoid cells from thymectomized mice. Nature 219:1391-1392.

43. Lischner, H. W. and A. M. DiGeorge. 1969. Role of the thymus in humoral immunity. Observations in complete or partial congenital absense of the thymus. Lancet ii:1044-1049.

44. Lowenberg, B., H. T. M. Nieuverkerk and D. van Bekkem. 1972. Annual report of Radiobiological Institute, THO, Ryswijk, Netherland. p. 105-106. As sited in Goldstien, A. L., J. A. Hooper, R. S. Schulof, G. H. Cohen, G. B. Thurman, M. C. McDaniel, A. White and M. Dardenne. 1974. Thymosin and the immunopathology of aging. Fed. Proc. Fedn. Am. Soc. Exp. Biol. 33:2053-2056.

45. Luckey, T. D., W. G. Robey and B. J. Campbell. 1973. LSH, a lymphocyte stimulating hormone, p. 167-183. In T. D. Luckey (ed.), Thymic hormones. University Park Press, Baltimore, MD.

46. Matter, A. 1974. The differentiation pathway of T lymphocytes: evidence for two differentiated cell types. J. Exp. Med. 140:566-577.

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

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

49. Muscoplat, C. C., A. W. Chen, D. W. Johnson and I. Alhaji. 1974. In vitro stimulation of bovine peripheral blood lymphocytes: standardization and kinetics of the response. Am. J. Vet. Res. 35:1557-1561.

50. Norris, E. H. 1938. The morphogenesis and histogenesis of the thymus gland in man: in which the origin of Hassall's corpuscles of the human thymus is discovered. Carnegie Inst. Wash. Pub. 496, Contrib. Embryology 27:191-207.

51. Osburn, B. I., G. H. Stabenfeldt, A. A. Ardans, C. Trees and M. Sawyer. 1974. Perinatal immunity in calves. J. Am. Vet. Med. Assoc. 164:295-298.

52. Owen, J. J. T. and M. C. Raff. 1970. Studies on the differentiation of thymus-derived lymphocytes. J. Exp. Med. 132:1216-1232.

53. Page, L. A., M. 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. Am. Vet. Med. Assoc. 161:919-925.

54. Pantelouris, E. M. 1968. Absense of thymus in a mouse mutant. Nature 217:370-371.

55. Parker, J. C., M. L. Vernon and S. S. Cross. 1973. Classification of mouse thymic virus as a herpesvirus. Infect. Immunity 7:305-308.

56. Phillips, P. A., D. Keast, M. N. -I. Walters and N. F. Stanley. 1971. Murine lymphoma induced by reovirus 3. Pathology 3:133-138

57. Pierpaoli, W. and H. O. Besedovsky. 1975. Role of the thymus in programming of neuroendocrine functions. Clin. Exp. Immunol. 20:323-338.

58. Pierpaoli, W. and E. Sorkin. 1967. Cellular modification in the hypophysis of neonatal thymectomized mice. Brit. J. Exp. Pathol. 48:627-631.

59. Pierpaoli, W. and E. Sorkin. 1968. Hormones and immunological capacity I. effects of hetrologous anti-grown (ASTH) antiserum on thymus and peripheral lymphatic tissue in mice. Induction of a wasting syndrome. J. Immunol. 101:1036-1043.

60. Potop, I. and S. M. Milcu. 1973. Isolation, biological activity and structure of thymic lipids and thymosterin, p. 205-273. In T. D. Luckey (ed.), Thymic hormones. University Park Press, Baltimore, MD.

61. Raff, M. C. 1971. Surface antigenic markers for distinguishing T and B lymphocytes in mice. Transplant. Rev. 6:52-80.

62. Raff, M. C. 1973. θ-bearing lymphocytes in nude mice. Nature 246:350-351.

63. Raff, M. C. and H. H. Wortis. 1970. Thymus dependence of θ-bearing cells in peripheral lymphoid tissue of mice. Immunology 18:931-942.

64. Rowe, W. P. and W. I. Capps. 1961. A new mouse virus causing necrosis of the thymus in newborn mice. J. Exp. Med. 113:831-844.

65. Rygaard, J. 1973. Thymus and self. Immunobiology of the mouse mutant nude. F. A. D. L., Copenhagen, Denmark. p. 77-89.

66. Schalm, O. W. 1965. Veterinary hematology. Lea and Febiger, Philadelphia, PA. p. 219-237.

67. Scheid, M. P., M. 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 the T cells induced by preparations for thymus and by nonthymic agents. J. Exp. Med. 138:1027-1032.

68. Shortman, K. and H. Jackson. 1974. The differentiation of T lymphocytes I. proliferation kinetics and interrelationships of subpopulation of mouse thymus cells. Cell. Immunol. 12:230-246.

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

70. Simpson, J. G., E. S. Gray and J. S. Beck. 1975. Age involution in the normal human adult thymus. Clin. Exp. Immunol. 19:261-265.

71. Smith, C. 1964. The microscopic anatomy of the thymus, p. 71-84. In R. A. Good and A. E. Gabrielson (ed.), The thymus in immunology. Hoeber Medical Division, New York, NY.

72. Soloman, J. B. 1971. Ontogeny of defined immunity in mammals, p. 355. In Foetal and neonatal immunology. North-Holland Publishing Co., Amsterdam, Netherlands.

73. Stanely, N. F., P. J. Leak, M. N-I. Walters and R. A. Joske. 1964. Muring infection with reovirus: II. the chronic disease following reovirus type 3 infection. Brit. J. Exp. Pathol. 45:142-149.

74. Tachibana, F., J. Imai and M. Kojima. 1974. Development and regeneration of the thymus: the epithelial origin of the lymphocytes in the thymus of the mouse and chick. J. Reticulo-endothel. Soc. 15:476-496.

75. Ushimima, R. D. 1975. Personal communication.

76. Waithe, W. I. and K. Hirshhorn. 1973. Lymphocyte response to activators, p. 25.1-25.10. In D. M. Weir (ed.), Handbook of experimental immunology, Vol. 2, Cellular immunology. Blackwell Scientific Publications, Oxford, England.

77. Wara, D. W. and A. J. Ammann. 1975. Effects of thymosin on B-lymphocyte function. N. Engl. J. Med. 293:507.

78. Wara, D. W., A. L. Goldstein, N. E. Doyle and A. J. Ammann. 1975. Thymosin activity in patients with cellular immunodeficiency. N. Engl. J. Med. 292:70-74.

79. Ward, J. K. 1975. Personal communication.

80. Warner, N. L. 1972. Differentiation of immunocytes and the evolution of immunological potential, p. 467-537. In F. Borek (ed.), Frontiers in Biology, Vol. 25. Immunogenicity. North-Holland Publishing, Co., Amsterdam, Netherlands.

81. White, A. and A. L. Goldstein. 1968. Is the thymus an endocrine gland? Old problem, new data. Prespectives in Biol. Med. 11:475-489.

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

83. Wilkie, B. N. 1971. Review of bovine immunology for veterinary practitioners. Can. Vet. J. 15:243-248

84. Wortis, H. H., S. Nehlsen and J. H. Owens. 1971. Abnormal development of the thymus in "nude" mice. J. Exp. Med. 134:681-692.

85. Walters, N. N-I., R. A. Joske, P. J. Leaks and N. F. Stanley. 1963. Murine infection with reovirus: I. pathology of the acute phase. Brit. J. Exp. Pathol. 44:427-436.

86. Wu, A. S. and B. H. Wakeman. 1972. Cellular differentiation in the thymus IV. response of rat thymic subpopulation to anti-thymus serum and other nonspecific mitogens. Cell. Immunol. 3:516-528.

87. Yamumura, M. 1973. Standardization of lymphocytes transformation to phytohemagglutinin. Clin. Exp. Immunol. 14:457-467.

88. Zisblatt, M., A. L. Goldstein, F. Lilly and A. White. 1970. Acceleration by thymosin of the development of resistance to murine sarcoma virus-induced tumor in mice. Proc. Nat. Acad. Sci. USA. 66:1170-1174.