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The modulation of ovine T-lymphocyte subsets and lymphocyte blastogenesis by leucine and leucine metabolites

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The modulation of ovine T-lymphocyte subsets and lymphocyte blastogenesis by leucine and leucine metabolites

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GENERAL INTRODUCTION

"The constancy of the 'milieu interieur' is the condition of free and independent existence." -Claude Bernard, 1878.

The environmental stress that is placed on an animal can evoke a range of response from minor stimulation to total exhaustion. In 1878, Claude Bernard described the "milieu interieur" of an animal, characterized by its constancy, and the external environment, characterized by its variability. This was a first description of the response of an animal to environmental stimuli. For the past 8000 years the "milieu interieur" of animals have been somewhat threatened due to the continuing practice of animal domestication. Domestication has been sucessful due to the ability of the animal to tolerate humans, to have a non-migratory nature, catholic food preferences and to adapt to a wide range of environments (Hale, 1969). An animal's degree of adaptability is challenged by changes in metabolism brought about by interactions between the animal and humans. Endocrine and neuropeptide factors elicted during a response of an animal toward environmental stress may ultimately affect the immune system to produce immunoenhancement, immunosuppression or have no effect. If we are to understand the impact of environmental stimuli on disease susceptibility, a proper understanding of the response of animals exposed to conditions of management or stress is necessary.

If stress-induced immunosuppression in livestock can be related to disease problems, then methods modulating the immune response should

benefit the animal. One approach to this problem is to give the animal substances that can augment the host defense mechanisms and, therefore, increase disease resistance. Substances which have the capability of regulating or modulating host defense mechanisms are termed immunomodulators. An effective immunomodulator is needed to relieve immunosuppression resulting from stress and thus, increase the animal's resistance to disease and infection.

The association between nutrition and disease has been well recognized. For many decades, investigators around the world have recognized that deficiencies or excesses of some dietary components lead to enhanced susceptibility to infection and disease. Studies of nutrition and immunocompetence were stimulated by the description of Kwashiorkor, observed by Cicely Williams in 1931 and 1932 in infants and young children in Africa. This observation has led to the possibility that immune defects observed in protein-calorie malnutrition may result from deficiencies or excesses of essential amino acids. In addition to the effect that total amount and quality of protein has on the immune response, excessive or deficient concentrations of specific amino acids in the diet appear to have an effect on immunity. Tryptophan (Koyanagi and Ishiguro, 1966) and phenylalanine (Ryan and Carver, 1964) must be present in adequate dietary amounts for optimal production of antibodies and reticuloendothelial system clearance functions. Branched-chain (leucine, isoleucine and valine) and sulfur-containing (methionine, cysteine and cystine) amino acid deficiencies have adverse effects on the spleen, lymph nodes, thymus and gut lymphoid tissues (Beisel, 1982;

Gross and Newberne, 1980). Immunosuppression is observed in rats fed an overload of leucine if they are on a protein inadequate diet (Chevalier and Aschkenasy, 1977). This suggests that a balanced amino acid intake (quality), as well as total amount (quantity), is important.

The use of dietary protein and amino acids as immunomodulators has not been studied extensively, although it is clear that these dietary components affect the immune system. It has been well documented that dietary proteins and amino acids interact in leucopoiesis regulation (Aschkenasy, 1975). They can act directly as building blocks or indirectly either by influencing the secretion of hormones capable of interfering with leucocyte production (e.g., glucocorticoids) or by influencing immunological reactions which mostly involve lymphocytes, macrophages and granulocytes.

REVIEW OF THE LITERATURE

Immunomodulation

Immunomodulation describes the pharmacological manipulation of the active state of the immune system. This may involve immunostimulation which is an enhancement of the host defense mechanisms, or immunosuppression which results in a decrease in the host defense mechanisms. Immunomodulators are therefore, substances that have the capacity to regulate or modulate the host defense mechanism. Immunomodulators are classified according to their origin and categorized as biological products or chemical immunomodulators (Poli, 1984). This classification can be further broken down into three subcategories: physiological products (e.g., thymic factors and glucocorticoids) substances of microbial origin (e.g., cyclosporin A and lentinan) or synthetic compounds (e.g., levamisole and isoprinosine).

Many theories of immunomodulator action have been proposed. Actions include the inhibition of prostaglandin synthesis (Nishiya and Horwitz, 1983), altering of intracellular cAMP/cGMP (Coffey and Hadden, 1985), blockage of DNA synthesis (Hinrichs et al., 1983) and interferon induction (Woodard et al., 1983). Of these proposed mechanisms it is most often possible to relate changes in immunologically active cells to cyclic nucleotide levels. Many immunosuppressive compounds have been shown to increase cAMP, for example glucocorticoids and prostaglandins of the E series. Those compounds with immunostimulatory effects increase cellular cGMP such as the thymic hormone thymopoeitin,

interleukin-1 and plant mitogens. In general, increased cGMP in lymphocytes is associated with increased activity and increased cAMP is associated with suppressed activity. The action of many immunomodulators can therefore be associated with changes in cAMP:cGMP in cells.

Immunomodulation has been used extensively in human subjects for cancer therapy, alleviation of autoimmune conditions and facilitation of organ transplantation. Immunomodulation is also used extensively to increase non-specific resistance to infection in farm animals. This is done with the aim of minimizing the deleterious effects of the less than optimal environmental conditions imposed by modern intensive agricultural practices. Many feed additives are added to livestock diets in the hopes of increasing resistance to disease and infection. For example, an antioxidant, butylated hydroxytoluene (BHT), is often added to poultry feeds and has been shown to exert an immunoenhancing effect in that it is correlated with increased resistance of chickens against Newcastle disease virus infections (Brugh, 1977). Other feed additives such as vitamins A (Stern et al., 1980) and E (Reddy et al., 1986) have been shown to enhance the immune system in vivo and in vitro. Another compound, Glucan, a particulate β-1,3 polyglucose prepared from Saccharomyces cerevisiae, has been shown to elicit a broad spectrum of immunobiological activities against infectious diseases in a variety of experimental animals. These activities include: the enhancement of macrophage phagocytic function (Riggi and Di Luzio, 1961), increased cellular and humoral immunity (Wooles and Di Luzio, 1962, 1963, 1964)

and enhancement of antigen clearance (Di Luzio and Morrow, 1971).

Two commonly used anthelmintics in cattle, sheep and pigs, levamisole and thiabendazole, have been studied widely as immunomodulators (Brunner and Muscoplat, 1980). Although these compounds produce positive immunomodulating effects in some studies, it appears that the dosage and time of administration relative to stressful, immunosuppressive stimuli restrict their use in stressed livestock. Another compound studied, sodium diethyldithiocarbamate (imuthiol; a biological respone modifier which influences T-lymphocyte function), also has been shown to exert immunostimulating effects (Renoux and Renoux, 1984). The use of this product is also limited in domestic food-type animals, since a reduction in growth is observed. Other immunomodulating exogenous substances such as avridine (CP 20,961; a lipoidal amine that has activity as an interferon inducer), isoprinosine (an inosine-containing compound used to enhance cell mediated immunity) and cytokines (e.g. interferons and interleukins) have also been investigated.

Since the use of some immunomodulators may inflict adverse effects such as reduction in growth rate or inflammation, limitations exist in their therapeutic application. These limitations can be summarized as follows: (1) the effects of immunomodulators may not be specific to particular cell subsets or types; (2) inappropriate dosage can produce undesirable results; (3) the timing of the immunomodulator administration is critical; (4) individual variation is commom; (5) side-effects are common; (6) the long-term effects are unknown (Mulcahy

and Quinn, 1986).

Stress-induced Immunosuppression

Modern day management of livestock entering feedlots often results in stress which has been shown to be associated with immunosuppression. This effect may at least partially account for the animals increased susceptibility to a variety of pathogens and subsequently the development of disease and infection. As early as 1878, animals responses toward stress were realized as Claude Bernard described the "milieu interieur" of an animal. In 1914, Cannon further defined this concept as 'homeostasis', in which a steady state is attained by the optimum interaction of counteracting processes within the host. Cannon (1929) also defined the 'flight-fight' reflex of animals exposed to stress and identified the damaging effects this may have in the individual. In 1935, Cannon presented new evidence showing that the physiological basis of the stress response was due to the activation of endocrinological factors involving the sympathetic adrenomedullary axis. Then in 1946, Selye used the response of laboratory animals exposed to experimentally noxious stimuli to describe the 'general adaptation syndrome' (GAS). Because different stimuli produced the same response, involving the pituitary-adrenal-cortical axis, he concluded that the response to stress was non-specific. Selye also concluded that stress involved the non-specific production of corticosteroids, and that the measurement of glucocorticoids could be used as the primary indicator of stress and the animal's well being. This concept was disputed by

Mason (1968), who showed that different types of stress produce different corticosteroid levels in monkeys. He explained that there exists different responses unique for each stressor and that the activation of the autonomic adrenomedullary reponse as well as the pituitary adrenocortical response occurred. It is now widely accepted that there are different endocrine responses to different physical, chemical and physiological stressors, and these responses vary within and among different species of animals (Dantzer and Mormede, 1985).

As was first decribed by Harris (1948), a major response of the body to stress is the stimulation of the hypothalamus which liberates corticotropin-releasing factor which further stimulates the anterior pituitary gland to secrete adrenocorticotropins (ACTH). The target organ affected by ACTH is the adrenal cortex. Cortical cells of the adrenal glands then synthesize and release glucocorticoids (cortisol/corticosterone) into the blood. Glucocorticoids are believed to have their dominant effect on the inflammatory and immunological systems, usually by exerting suppressive effects.

In many species glucocorticoid production is associated with adrenal hypertrophy, thymic involution, lymphocytopenia, eosinopenia and neutrophilia (Selye, 1946; Jensen, 1969; Riley 1981; Cupps and Fauci, 1982). Glucocorticoid administration markedly increases the animal's susceptibility to infectious disease and can cause activation of latent infection. In cattle glucocorticoid injections decrease resistance to infectious bovine rhinotracheitis virus (Davies and Duncan, 1974), fatal bovine viral diarrhea virus (Shope et al., 1976) and parasites (Callow

and Parker, 1979). Reduced levels of humoral immunity may also result from the administration of glucocorticoids to cattle (Gwazdauskas et al., 1978). A reduction of in vitro lymphocyte blastogenesis and neutrophil function was also seen in cattle which were treated with ACTH so that their blood glucocorticoid concentrations were elevated (Roth et al., 1982).

The systemic administration of corticosteroids has also been shown to depress the circulation of lymphocytes from lymph nodes. In sheep, the glucocorticoid methyprednisolone, given intravenously reduces the output of lymphocytes into the efferent lymph to between 16-22% of pre-administration levels (Zukoski and Montgomery, 1983). The number of lymphocytes in the lymph and blood is therefore decreased. The circulation of lymphocytes from blood to lymph is believed to be important for the selective recruitment of antigen-specific lymphocytes to a responding lymph node (Cahill et al., 1974), hence, the magnitude of the subsequent immune response may be inhibited. This decrease in lymphocytes is accompanied by a parallel increase in blood granulocytes and monocytes. These changes in sheep are kinetically and qualitatively similar to those seen in man, but are somewhat more exaggerated (Parrillo and Fauci, 1979). It has also been shown that sheep efferent lymph cells are extremely resistant to glucocorticoid-induced lysis in vitro and require glucocorticoid levels 2-4 log doses higher than those needed in murine systems to lyse the cells (Zukoski and Montgomery, 1985) which are about the same concentration as those needed for human cell lysis (Claman et al., 1971). Sheep therefore appear to be a "steroid

resistant" species like man, monkeys and guinea pigs (Claman, 1972).

Monoclonal Antibodies to T-cell Subsets

In 1975, George Kohler and Cesar Milstein combined B-cells from immunized animals with neoplastic myeloma cells and thus, developed hybridomas secreting monoclonal antibodies. This monumental achievement was made possible by a number of unrelated discoveries of other workers. Contributing to the development of monoclonal antibodies was the clonal selection theory (Burnet, 1957), the development of cell fusion techniques (Okada, 1962; Littlefield, 1964), the artificial induction of plasmacytomas (Potter and Boyce, 1962), their adaption to tissue culture (Horibata and Harris, 1970) and the demonstration of the fusion of two different plasma cell tumor lines (Cotton and Milstein, 1973).

Prior to the 1950's the concept of how antibodies were produced in response to antigens was unknown. In 1957, F. Macfarlane Burnet hypothesized the clonal selection theory. It postulated that each lymphocyte has a unique receptor specificity and was precommitted to making only one antibody after antigenic stimulation. Evidence in support of this theory was provided by Nossal and Lederberg (1958), who showed that a single antibody producing cell in rats immunized with two antigens, produced antibodies in response to only one of the antigens. The clonal selection theory remained in question until the Cold Spring Harbor Conference of 1967 in which this theory was finally accepted.

In the 1970's, Cesar Milstein had been interested in the genetic control of antibody synthesis. In a study he was performing, hybrids

between rat and mouse myeloma cells were constructed and it was shown that the synthesis of both species of immunoglobulins produced was retained but mixed molecules were formed (Cotton and Milstein, 1973). The induction of myeloma (tumor) cells in mice was described by Potter and Boyce in 1962. The development of these cells has been responsible for much of our current knowledge of immunoglobulin structure, biosynthesis and genetics.

In a subsequent experiment in which somatic mutation in antibody genes was being investigated through the use of a mouse myeloma cell, it was shown that a high degree of instability in immunoglobulins existed (Cotton et al., 1973). A way of constructing continuous cell lines secreting antibody of a known specificity was needed. In 1975, Kohler and Milstein extended their previous experiments and fused a mouse myeloma cell with an antibody producing spleen cell from a mouse immunized with sheep red blood cells. The experiment worked as planned and cloned hybrid lines secreting anti-sheep red blood cell antibodies were produced. These cells were capable of forming tumors when injected into mice. These tumors are now known as "hybridomas". When hybridomas are injected intraperitoneally into mice, a large volume of ascites fluid builds up and this fluid is rich in monoclonal antibodies.

This was a revolutionary discovery. Unlimited quantities of absolutely specific and uniform antibodies recognizing only one antigenic site were produced. Since this time, improvements have been made in cell fusion and screening techniques, and monoclonal antibodies specific for various cell types and cell products are becoming readily

available.

It has been demonstrated that a high degree of evolutionary conservation exists among species in lymphocyte surface antigens. A great deal of homology in molecular size and tissue distribution is seen between the human T-cell subset monoclonal antibodies T4 and T8 and those markers for the same subsets in the rat and mouse (Ledbetter et al.,1981; Dialynas et al., 1983; Mason et al., 1983). In all three species these markers have been shown to be coexpressed on cortical thymocytes and exclusively expressed on medullary thymocytes and peripheral lymphocytes. These markers distinguish functionally distinct populations of T-cells. T4 markers distinguish T-helper cells which recognize antigens in association with major histocompatibility complex class II molecules, and T8 markers which recognize antigens in association with major histocompatibility complex class I molecules. Recently, monoclonal antibodies have been used to identify functional lymphocyte subsets in man (Lanier et al.,1983), mouse (Swain ,1983), rat (Mason et al., 1983), swine (Jonjic and Koszinowski, 1984), rabbit (Watkins et al., 1984) and some other species.

Currently, extensive research on lymphocytes in sheep is possible due to the existence of monoclonal antibodies specific for sheep T-lymphocyte subsets. Studies can now be conducted on lymphocyte recirculation, fetal lymphoid development and immunological alterations caused by various chemical or naturally occurring compounds in sheep. A panel of monoclonal antibodies specific for sheep T-cell surface antigens has been developed (Mackay et al., 1985; Maddox et al., 1985;

Mackay et al., 1986). Two of these monoclonal antibodies define the sheep T4 and T8 antigens, a third monoclonal antibody defines a T-cell antigen not previously identified in any other species. There is also a monoclonal antibody available which identifies total sheep T-lymphocytes. Table 1 and 2 summarize the properties of these sheep T-lymphocyte monoclonal antibodies.

Table 1. Properties of sheep lymphocyte antigens recognized by monoclonal antibodies

Antibody designation	Molecular mass of target antigen	Human/murine analogue
SBU-T1	67,000	CD5/Lyt-1
SBU-T4	56,000	CD4/L3T4
SBU-T8	36,000	CD8/Lyt-2
SBU-T19	215,000	unknown

(Summarized from Gorrell et al., 1986)

Table 2. Percent of mononuclear cells reacting with sheep monoclonal antibodies derived from various sheep lymphoid tissues

	Source of mononuclear cells			
Antibody designation	Peripheral blood	Lymph	Lymph nodes	Thymus
SBU-T1	62	74	62	98
SBU-T4	20	49	37	81
SBU-T8	12	18	21	77
SBU-T19	15	7	2	1

(Summarized from Mackay et al., 1986)

Leucine and Leucine Metabolites

General metabolism Leucine (Leu) is a ketogenic branched-chain essential amino acid (BCAA). The sources of Leu include protein breakdown, dietary Leu or the transamination of its α-ketoacid, α-ketoisocaproate (KIC). Leu can be reversibly transaminated to KIC via the enzyme BCAA transaminase. KIC then enters the mitochondria and can be irreversibly oxidized to isovaleryl CoA (IVACoA) by the enzyme, branched-chain ketoacid (BCKA) dehydrogenase. However, KIC can also be converted to β-hydroxy-β-methylbutyrate (HMB) by KIC oxygenase in the cytosol of the liver. The product, HMB, is believed to be an end product excreted in the urine.

IVACoA can be further metabolized in the mitochondria to β-methylcrotonyl CoA, then to β-methylglutaryl CoA and finally to β-hydroxy-β-methylglutaryl CoA (HMGCoA). HMGCoA can then be metabolized by HMG cleavage enzymes to acetoacetate plus acetyl CoA. Acetoacetate can then be converted to acetoacetyl CoA and HMGCoA or to β-hydroxybutyrate (BOHB). HMGCoA can be further metabolized to melvalonic acid which ultimately is converted to cholesterol and other steroids.

Immunological effects Feeding excess Leu has been demonstrated to adversely affect immune response. A dietary Leu overload (3% of dry weight of the diet for 2 weeks and 7% for 6 weeks) has been shown to cause a decrease in the production of rosette and plaque forming cells in the spleen and also to decrease the titer of serum antibodies in rats against sheep red blood cells (Chevalier and Aschkenasy, 1977). These suppressive effects became dramatic when the rats were subjected to a

low protein diet and Leu was then supplemented. Excess dietary Leu is known to induce a secondary drop in plasma concentrations of isoleucine and valine (Benton et al., 1956; Tannous et al., 1966). This drop in plasma concentrations may be due to an excessive activation of enzymes of the catabolic pathway of BCAA (Ichihara and Koyama, 1966). A lack of isoleucine and valine has been shown to lead to an inhibition of leucopoiesis (granulocytes and lymphocytes), with involution of the lymphoid organs, especially the thymus, and a dramatic drop in blood lymphocyte levels (Aschkenasy, 1975). Since a close relationship exists between lymphocytes and immunity, a suppressive effect on immunity is therefore induced by feeding excess leucine.

Rats fed a protein poor diet supplemented with excess Leu completely supressed immunoglobulin G production after immunization with sheep red blood cells (Chevalier and Aschkenasy, 1977). In a similar study in which chickens were fed diets supplemented with 3 levels of Leu, primary and secondary antibody responses to sheep red blood cells were depressed (Tinker and Gous, 1986). This study also measured a depression in primary and secondary serum immunoglobulins M and G. Lastly, Leu was also found to be immunosuppressive when fed to sheep in small amounts (Kuhlman et al., 1988). When ruminally by-passed Leu was fed to growing lambs at a rate of approximately 0.05% of the diet, antibody production to porcine red blood cells and ConA stimulated lymphocyte blastogenesis was depressed.

Other derivatives of Leu have also been shown to exert immunodepressive effects. It has been shown that L-leucyl-L-leucine

methyl ester, which is generated by human monocytes or polymorphonuclear leukocytes, eliminates all natural killer cell function from mixed lymphocyte populations (Thiele and Lipsky, 1985). It seems to have selective toxicity for natural killer cells, monocytes and various cytotoxic T cells but does not adversely effect other lymphoid or nonlymphoid cell types when incubated with human peripheral blood mononuclear cells in tissue culture (Thiele and Lipsky, 1986). Similar results have been seen in murine spleen cell cultures (Thiele et al., 1987).

Another derivative of Leu, cycloleucine, has been shown to inhibit primary immune responses to sheep red blood cells and allograft reactions when injected intraperitoneally to mice (Brambilla et al., 1972). In 1969, Frisch reported that in mice very high doses of cycloleucine prevent the synthesis of hemagglutinins and hemolysins for sheep erythrocytes by reducing the number of plaque-forming cells of the spleen (Frisch, 1969). Cycloleucine has since been used therapeutically as an anticancer drug (Carter, 1970).

The effects of the first catabolic product of Leu, KIC, on immune function have also been studied. When ruminally by-passed KIC was orally administered to sheep, an increase in antibody production to porcine red blood cells and lymphocyte blastogenesis was seen (Kuhlman et al., 1988). Other studies have shown that the oral supplementation of KIC to sheep for 8 weeks results in a two-fold increase in PHA stimulated lymphocyte blatogenesis (Nissen et al., 1986). The enhancement effect on immune function observed with the oral

supplementation of KIC, may be due in part to an increase of T-helper cells in these animals (Kuhlman et al., 1989). In this same study, the effects of isovalerate, a metabolite of isovaleryl CoA, was shown to have no effect on antibody production or lymphocyte blastogenesis when fed to growing lambs.

Other studies have evaluated the effects of acetone, acetoacetate, BOHB and butyrate on bovine lymphocyte blastogenesis in vitro and in vivo. In one study, bovine lymphocytes were cultured in the presence of toxic and subtoxic concentrations of these compounds ranging from 1-50 mg/dl (Targowski and Klucinski, 1983). Toxic and subtoxic concentrations of BOHB and toxic concentrations of butyrate and acetate significantly reduced the mitogenic response of the bovine lymphocytes. The reduction in mitogenic response also occurred when the lymphocytes were preincubated with BOHB or acetoacetate for 2 hours or longer. The presence of toxic concentrations of acetone in the medium did not affect the mitogenic stimulation of the lymphocytes. In a subsequent experiment, cattle were made ketotic by the addition of 1,3 butanediol so that the blood concentrations of these compounds were elevated (Targowski et al., 1985). The mitogenic response of lymphocytes collected during this ketotic period was significantly suppressed and remained supressed for 2 weeks. During this time period, all calves made ketotic showed clinical signs of upper respiratory infections. It was hypothesized that a correlation may exist between the suppressed function of lymphocytes, the increased susceptibility of the calves to infection and the increased concentrations of the Leu metabolites in the blood.

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EXPLANATION OF DISSERTATION FORMAT

This dissertation is presented in the alternate format, as outlined in the Iowa State Graduate College Thesis Manual. Use of the alternate format allows for the preparation of independent sections that are suitable for submission to scientific journals.

Three separate papers have been prepared from the data collected from research performed to partly fulfill requirements for the Ph.D. degree. Each paper is complete in itself and has an abstract, introduction, materials and methods, results, discussion and literature cited section. The closeness of the subject matter of the three papers allowed a general discussion to be prepared.

ANIMAL CARE

The animals used in the following trials were treated in accordance with the Iowa State University animal care guidelines.

SECTION I. MODULATION OF T-CELL SUBSETS IN SHEEP BY DIETARY LEUCINE AND α-KETOISOCAPROATE

Modulation of T-Cell Subsets in Sheep by Dietary Leucine and α-Ketoisocaproate

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ABSTRACT

Leucine (Leu) and its ketoacid, α-ketoisocaproate (KIC), have been shown to exert opposing effects on antibody production and lymphocyte blastogenesis when orally administered to sheep. Two experiments were conducted to determine whether these effects are associated with alterations in the percentage of circulating T-cell subsets or to hormonal changes within the animal. Mitogen stimulated lymphocyte blastogenesis and flow cytometric analysis of T1, T4, T8 and T19 lymphocyte subsets were determined. Serum cortisol, insulin and glucagon concentrations were also evaluated. In both experiments, feeding KIC resulted in increased lymphocyte blastogenic responsiveness to phytohemmagglutinin-P (PHA) and pokeweed mitogen (PWM) comparing KIC-fed animals to controls ($P < 0.05$). Feeding Leu significantly decreased responses to all three mitogens compared to KIC fed animals ($P < 0.05$) and tended to decrease mitogenic responses when compared to controls. When KIC was fed, the percentage of circulating T4 cells were significantly increased ($P < 0.05$). Leu fed animals had a significantly lower percentage of circulating T19 cells ($P < 0.05$). No significant differences were seen in serum hormone concentrations. These data indicate that the opposing effects exerted by Leu and KIC on various immune functions are associated with and may be due to alterations in T-lymphocyte subsets.

(Key words: Leucine, α-Ketoisocaproate, T-Lymphocytes, Blastogenesis)

INTRODUCTION

Previous studies indicate that the oral supplementation of leucine (Leu) to sheep decreased serum antibody response to porcine red blood cells and decreased mitogen-stimulated lymphocyte blastogenesis, however, the first catabolic product of Leu, α-ketoisocaproate (KIC), enhanced the antibody response to porcine red blood cells and increased mitogen stimulated lymphocyte blastogenesis (Kuhlman et al., 1988). The reasons for the opposing effects of Leu and KIC may involve their different sites of metabolism. Leu is primarily metabolized by the peripheral tissues (Bergman, 1986) and KIC is primarily metabolized by the liver and gut (Abumrad et al., 1982; Pell et al., 1986). Thus, the gut and liver associated lymphoid tissues would be exposed to high concentrations of KIC and/or KIC metabolites while the peripheral lymphoid tissues would be exposed to high Leu metabolite concentrations. This difference in the site of metabolism combined with the very low rate of Leu-KIC interconversion in sheep (Busboom et al., 1984; Nissen and Ostaszewski, 1985) could explain why KIC results in different metabolic effects than Leu.

The mechanism by which KIC and Leu could actually alter blastogenesis and antibody production is not clear. One possibility may be that KIC is altering the ratio of certain T-cell subsets in a manner that would enhance such immune responses, while Leu is altering the ratio of certain T-cell subsets such that antibody production and lymphocyte blastogenesis are lowered. Other possible mechanisms may involve alterations in the secretion of or responsiveness to

interleukin-2 (T-cell growth factor), or hormones such as cortisol (which has been shown to be immunosuppressive; Parrillo and Fauci 1979), glucagon and insulin (both can be affected by Leu and KIC and affect protein synthesis; Leclercq-Meyer et al., 1979) could be altered.

The objectives of the studies reported here were: (1) to confirm the effects of Leu and KIC seen previously on lymphocyte blastogenesis, (2) to determine whether the oral administration of small doses of Leu and KIC can alter the proportions of circulating T-cell subsets in lambs, and (3) determine if hormonal changes occur and if these changes could be responsible for the alterations of lymphocyte blastogenesis caused by Leu and KIC.

MATERIALS AND METHODS

Animals and Experimental Design. Two separate experiments were conducted using 21 mixed-breed ram lambs in the first experiment and 24 mixed-breed ewe lambs in the second experiment. Animals were allowed free access to water throughout the study and housed individually in fiberglass pens (1.15 m^2). Animals were randomly assigned to one of three dietary treatments: Leu (n=15), KIC (n=15) or limestone (control; n=15). Feed consumption was recorded and animals were weighed at the beginning and at the end of the 46-52 d studies.

Diets. The diets fed to the sheep in experiment 1 and experiment 2 are shown in Table 1. Diets were formulated using the metabolizable protein system (Burroughs et al., 1975) and contained approximately 70 g metabolizable protein/kg. Dietary treatments were supplemented to the basal diet (Table 1) in the form of zein coated pellets whose composition and method of preparation have been previously described (Kuhlman et al., 1988). Lambs were fed their respective pellets at a rate of approximately 3 g/d, which corresponds to approximately 5 mmol of LEU or KIC per total kg of diet. Leu was purchased from Sigma Chemical, St. Louis, Mo. KIC was purchased as calcium-$(KIC)_2$ from SOBAC, Paris, France and 325 mesh limestone was purchased from Iowa Limestone, Des Moines, Iowa.

Table 1. Composition of basal diet fed to all sheep in experiment 1 and 2

Ingredient	kg Dry Matter	
	Experiment 1[1]	Experiment 2[2]
Ground corn	84.4	52.5
Dehydrated alfalfa	0	31.2
Expeller soybean meal[3]	4.9	10.2
Molasses	7.2	4.4
Trace mineral & vitamin premix[4]	0.57	0.57
Salt	0.57	0.57
Limestone	1.84	0.57
Urea	0.57	0

[1] Formulated to contain 1.9 Mcal net energy/kg for maintenance, 1.3 Mcal net energy/kg for gain, 10.6% crude protein, 70.98 g metabolizable protein/kg and 87% dry matter.

[2] Formulated to contain 1.7 Mcal net energy/kg for maintenance, 1.2 Mcal net energy/kg for gain, 16% crude protein, 68.64 g metabolizable protein/kg and 88.5% dry matter.

[3] Purchased from West Central Cooperative, Ralston, IA.

[4] Purchased from Feed Specialties Co., Des Moines, IA. Contains 1.64% Mg, 4949 mg Mn/kg, 371 mg Zn/kg, 2600 mg Fe/kg, 0.05 mg Cu/kg, 47.4 mg Co/kg, 128 mg I/kg, 25 mg Se/kg, 3.25% S, 772727 USP Units vitamin A/kg, 102273 USP Units vitamin D3/kg, 773 IU vitamin E/kg.

Blood Sampling and Lymphocyte Preparation. In the first experiment, animals were bled for lymphocyte blastogenesis assays and T-cell subset analysis 38, 45 and 52 d after dietary treatments began. In the second experiment, animals were bled 14, 33 and 46 d after dietary treatments began. Blood (50 ml) was collected, aseptically, via jugular venapuncture into 10 ml acid-citrate-dextrose anticoagulant (44 g trisodium citrate, 16 g citric acid and 50 g dextrose per 1 of triple distilled water). Blood was centrifuged, the buffy coat removed, and 1 volume of buffy coat was diluted with one half volume of plasma and was added to 2 volumes of Sepracell-MN (Sepratech Corporation, Oklahoma City, OK). After further centrifugation at 1500 x g for 25 min, the mononuclear layer was removed and washed twice with Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) by centrifuging at 150 xg for 15 min. The remaining white blood cells were resuspended in HBSS and the total number of white blood cells were determined using an automated cell counter (Coulter Electronics, Hialeah, FA). The concentration of the cells was adjusted to 1×10^6 cells/ml with HBSS.

Lymphocyte Blastogenesis Determinations. Blastogenesis assays were performed on isolated lymphocytes by using a 3-d culture procedure as described previously (Roth et al., 1982). Briefly, isolated lymphocytes were cultured in microtiter plates with 2×10^5 cells in 0.15 ml of culture medium/well. Culture medium consisted of RPMI media-1640 (Gibco, Grand Island, NY) containing 15% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY).

Unstimulated and mitogen stimulated cultures were assayed in triplicate. Mitogens used were phytohemagglutinin-P (PHA), concanavalin A (ConA) and pokeweed mitogen (PWM). PHA was purchased from Difco Laboratories, Detroit, MI; ConA from Miles Laboratories, Elkhart, IN; and PWM from Gibco, NY. Microtiter plates were incubated at 37° C in a humidified 5% CO_2 atmosphere. After 48 h, 0.25 uCi of ^{3}H-thymidine (New England Nuclear, Boston, MA) was added to each well. Eighteen h later, the cultures were harvested onto glass-fiber filters with an automated sample harvester (Flow Laboratories, Rockville, MO), filters were placed in 10 ml CytoScint scintillation solution (ICN Biomedicals, Irvine, CA) and radioactivity was counted in a liquid scintillation counter.

Antibodies. A panel of monoclonal antibodies directed against sheep T-lymphocyte surface determinants (Table 2) was used. Monoclonal antibodies, in the form of cell-free culture supernatants were obtained from Dr. M.R. Brandon, University of Melbourne, Parkville, Victoria, Australia. Additional properties of the antigens recognized by the monoclonal antibodies have been reviewed elsewhere (Mackay et al., 1985 and 1986; Maddox et al., 1985). A fluoresceinisothiocyanate-conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulin (FITC-RAM) was purchased from DAKO Corporation, Carpinteria, CA. Monoclonal antibodies SBU-T4, T8 and T19 were used in both experiments. Monoclonal antibody SBU-T1 was used in experiment 2 only.

Table 2. The target specificity of monoclonal antibodies used

Antibody designation	Molecular weight of target antigen	Human/murine analogue	Percentage of stained peripheral lymphocytes
SBU-T1	67,000	T1/Ly1	60-80
SBU-T4	56,000	T4/L3T4	20-50
SBU-T8	36,000	T8/Ly2	10-20
SBU-T19	215,000	?	15

Immunofluorescent Staining. Sheep lymphocytes (10^6) in 50 ul phosphate-buffered saline, 0.1% sodium azide (PBS/Az), 0.5% BSA were incubated for 30 min at 4° C with 50 ul monoclonal antibody culture supernatant. Additionally, cells were incubated with monoclonal antibody culture supernatant only and with FITC-RAM only so that any nonspecific fluorescent staining could be determined. Nonspecific labelling averaged approximately 1-2% so was not considered an important variable in this study. The cells were washed twice with PBS/Az by centrifuging at 150 x g for 15 min and incubated with 40 ul of FITC-RAM for 30 min at 4° C. Cells were washed 3 times with PBS/Az, resuspened in 200 ul 1% formalin and stored overnight at 4° C until analyzed.

Flow Cytometric Analysis. Fluorescent stained cells were detected using an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FA). Light passing to the fluorescence detector was split using a 488 nm dichroic filter. FITC fluorescence was collected through a 515 long pass filter and the presence or absence of a range of cell surface

markers was determined. Only cells containing the lymphocyte populations indicated by the forward angle light and 90^{o} light scatter histogram were analyzed for fluorescence using the EASY88 computer system with Intgra software (Coulter Electronics, Hialeah, FL). A minimum of ten thousand events were collected per sample. All data are presented as percent positive cells.

Determination of Serum Cortisol, Glucagon and Insulin Concentrations. Serum cortisol and insulin concentrations were determined by coated tube single antibody radioimmunoassay using commercial kits (Diagnostic Products Corporation, Los Angeles, CA). Serum glucagon concentrations were determined using a double antibody radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). Samples were assayed in duplicate and all samples were processed concurrently. Blood for these asays was collected prior to starting dietary treatments and on the last day of each study (d 52 in experiment 1 and d 46 in experiment 2). All kits gave parallel standard curves when added to sheep plasma.

Statistical Analysis. Blood was collected for the determination of lymphocyte blastogenesis and monoclonal antibody analysis 3 times during each experiment. In the first experiment, animals were bled during wk 5, 6 and 7. In experiment 2, animals were bled during wk 2, 5 and 6. A mean and SEM was determined for each parameter for each group from these 3 blood collections. An analysis of variance

procedure was performed to determine the level of significance of any differences between groups. Duncan's multiple range test (Steel and Torrie, 1960) was used to determine the differences between dietary treatments if the overall analysis of variance was significant ($P < 0.05$).

RESULTS

Average daily gain and feed consumption are shown in Table 3. In experiment 1 animals consumed approximately 1.34 ± 0.05 kg/d and gained approximately 0.61 ± 0.10 kg/d and in experiment 2 animals ate 1.37 ± 0.07 kg/d and gained 0.22 ± 0.04 kg/d. No significant differences were seen among dietary treatments.

Serum hormone concentrations for both experiments are shown in Table 4. No significant differences were seen in glucagon, insulin or cortisol serum concentrations in either experiment; however, feeding KIC did tend to increase serum insulin concentrations.

The lymphocyte blastogenic response to mitogens is summarized in Table 5. Feeding KIC resulted in significantly increased blastogenic response to PHA and PWM (KIC vs control; $P < 0.05$). KIC-fed animals had significantly higher ConA stimulated blastogenesis when compared to Leu-fed animals ($P < 0.05$) and ConA stimulation tended to be higher when KIC-fed animals were compared to controls. Feeding Leu significantly decreased blastogenic responses to all 3 mitogens when compared to KIC-fed animals ($P < 0.05$) and tended to decrease lymphocyte blastogenesis when compared to controls. No changes were seen in background (unstimulated) blastogenesis.

Table 6 summarizes the effects of feeding Leu or KIC on T-cell subset in sheep. KIC fed animals had a significantly greater percentage of T4 cells when compared to control and Leu fed animals ($P < 0.05$). Leu-fed animals had a signifcantly lower percentage of T19 cells when compared to KIC-fed animals ($P < 0.05$) and this number also tended to be

lower than the control animals. The percent of T1 cells from experiment 2 are also shown here. No significant differences were seen in the percentage of T1 cells. The percentages of T4, T8 and T19 cells were subtracted from the percent of T1 cells resulting in a T-null (T-unknown) subset. KIC fed animals had a significantly lower percentage of T-null cells when compared to the other dietary treatments ($P < 0.05$).

Table 3. Average daily gain and feed consumption of sheep fed control, KIC or LEU diets (n=15)

	Average daily gain (kg)		Feed consumed/day (kg)	
Diet	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Control	0.577	0.220	1.41	1.35
KIC	0.623	0.210	1.35	1.38
Leu	0.632	0.220	1.36	1.39
SEM[1]	0.10	0.04	0.05	0.07

[1] Pooled standard error of the mean from ANOVA

Table 4. Mean serum hormone concentrations of sheep fed control, KIC or LEU diets (n=15)

Diet	Cortisol (ug/dl)	Insulin (uU/ml)	Glucagon (pg/ml)
Control	2.7	26.1	75.8
KIC	2.6	32.6	66.6
Leu	2.2	26.3	65.9
SEM[1]	.39	3.6	6.9

[1] Pooled standard error of the mean from ANOVA

Table 5. The effects of oral supplementation of Leu or KIC on lymphocyte blastogenesis in sheep [1]

	Mitogen			
Diet	None[2]	PHA[2]	ConA[2]	PWM[2]
Control	700[4,a]	18900[b]	17960[ab]	17320[b]
KIC	776[a]	24870[a]	24130[a]	29780[a]
LEU	730[a]	16160[b]	11840[b]	14520[b]
SEM[3]	92	1975	2461	2504

1 Means are of 3 repeated measures (n=15).

2 Expressed as counts per min per 200,000 lymphocytes

3 Pooled standard error of the mean from ANOVA

4ab Means within a column not sharing a common superscript letter are significantly different at $P < 0.05$

Table 6. The effects of oral supplementation of LEU or KIC on T-cell subsets in sheep

	Subset				
	% stained peripheral lymphocytes				
Diet	T1[2]	T4[1]	T8[1]	T19[1]	T-null[2]
Control	56.5[4,a]	18.6[a]	9.5[a]	17.9[a]	12.6[a]
KIC	52.2[a]	21.3[b]	10.5[a]	18.2[a]	6.4[b]
LEU	55.0[a]	18.1[a]	10.8[a]	14.9[b]	11.5[a]
SEM[3]	3.1	1.0	.59	1.3	2.2

1 Means are of 3 repeated measures (n=15) in both experiments 1 and 2

2 Mean of three repeated measures (n=8) in experiment 2 only

3 Pooled standard error of the mean from ANOVA

4ab Means not sharing a common superscript letter are significantly different at $P < 0.05$

DISCUSSION

No significant effects were seen on average daily gain and feed conversion in the present studies. In previous experiments in which KIC was fed at 1% of the diet to lambs, an 11% increase in gain and an 8% increase in feed efficiency was seen (Flakoll et al., 1986). In other feeding trials in which steers were fed 1% KIC resulted in a 14% increase in daily gain and an 8% increase in feed efficiency (Flakoll et al., 1987). In the present study sheep were fed a diet containing approximately 10% crude protein, while in earlier studies animals consumed diets containing 17-18% protein. These two levels of protein intake may be responsible for the differences in average daily gain and feed conversion.

In the previous steer and sheep studies, KIC feeding decreased serum cortisol concentrations. In the present studies, no changes were seen in serum cortisol concentrations but only one sample was collected. Repeated measures of cortisol concentrations may have led to significant results since the time and method of blood collection and the activity and metabolic state of the animal will influence cortisol concentrations. Similarly no significant changes were seen in glucagon and insulin concentrations, although KIC feeding tended to increase serum insulin concentrations ($P < 0.10$). Again, repeated measures may have been more suitable in evaluating hormonal changes.

As reported in previous experiments (Kuhlman et al., 1988) mitogen stimulated lymphocyte blastogenesis was increased with KIC supplementation. PHA and ConA are known to stimulate T-cell division,

while PWM stimulates both T- and B-cell mitosis in humans (Oppenheim and Schecter, 1980). If sheep respond to mitogens as do humans and feeding KIC increases the percentage of circulating T4 cells, it is consistent that lymphocyte blastogenesis is also increased with KIC feeding. In contrast to initial studies, PWM stimulated blastogenesis was also enhanced by KIC feeding suggesting that KIC may also stimulate B cell activity. This is probably the case since it was shown that in previous studies, KIC enhanced antibody responses to porcine red blood cells (Kuhlman et al., 1988).

In sheep, peripheral T-cells are divided into three groups. The T4, T8 and T19 molecules mark subsets of sheep lymphocytes comprising 15-25%, 8-12% and 20-50% of peripheral blood lymphocytes respectively (Mackay et al., 1989). The T4 subset represents T-helper cells which recognize antigen in association with major histocompatibility complex (MHC) class II molecules and the T8 subset represent T-cytotoxic/suppressor cells that recognize antigen in association with MHC class I molecules. Both these T-cell subsets have been identified to use the $\alpha\beta$ form of the T-cell receptor. While the function of the T19 subset is currently unknown, the T19 monoclonal antibody marks a specific subset of T-cells which use the $\gamma\delta$ form of the T-cell receptor (Mackay et al., 1989).

In these experiments, the T-cell subsets defined by T4, T8 and T19 did not comprise the entire T-cell population, as there was often a small population of cells which were identified when T1 was used, but was not when the sum of T4, T8 and T19 was considered. A T-null subset was therefore reported.

The results of these experiments suggest that dietary Leu and KIC alter the percentage of circulating T-lymphocyte subsets and these alterations may be responsible for the changes previously observed in lymphocyte blastogenesis and antibody responses. In the case of KIC, the percentage of T4 cells was increased approximately 15% which could be responsible for the increase in blastogenesis and antibody production. Additionally, the T-null subset was significantly decreased which could also relate to the observed effects of KIC on immune function. In the case of Leu, the only observed change was a 17% decrease in T19 cells for which a function has not yet been described. The fact that these two compounds affect two distinct T-lymphocyte subsets in opposing ways, may partially explain the mechanism of the opposite effects that Leu and KIC exert on immune function.

In conclusion, these data suggest that the dietary supplementation of KIC may increase blastogenesis by increasing the number of T-helper lymphocytes, whereas Leu decreases blastogenesis by decreasing the number of T19 lymphocytes in the peripheral blood circulation.

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SECTION II. THE EFFECTS OF LEUCINE AND α-KETOISOCAPROATE ON ACTH-INDUCED IMMUNOSUPPRESSION IN SHEEP

The Effects of Leucine and α-Ketoisocaproate on ACTH-induced Immunosuppression in Sheep[1]

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ABSTRACT

The effects of oral administration of leucine (Leu) and its first catabolic product, α-ketoisocaproate (KIC), on adrenocorticotrophic hormone (ACTH) induced immunosuppression in sheep were determined. Immune status was monitored by lymphocyte blastogenic responsiveness to mitogens and enumeration of the percentage of T-cells and T-cell subsets in the peripheral blood by using monoclonal antibodies and a flow cytometer. Twenty-four mixed-breed ewe lambs were fed diets supplemented with 0.10% ruminally protected; limestone (control), KIC or Leu for 60 d. Animals were then injected with either saline or ACTH (100 IU) twice daily for 3 consecutive d, at which time they remained on appropriate dietary treatments. Serum cortisol, insulin and glucagon concentrations were determined. ACTH injections increased both serum cortisol and insulin concentrations. No effects were seen in serum glucagon concentrations. Mitogen stimulated lymphocyte blastogenesis was determined by adding phytohemmagglutinin P (PHA), concanavalin A (ConA) or pokeweed mitogen (PWM) to isolated lymphocytes and measuring ^{3}H-thymidine incorporation. ACTH administration significantly suppressed mitogen stimulated lymphocyte blastogenesis by approximately 50% regardless of the mitogen used ($P < 0.05$). KIC-fed animals had greater PHA and ConA stimulated blastogenic responses when compared to Leu-fed or control animals given corresponding injections ($P < 0.05$). ACTH injections tended to decrease the percentage of total circulating T- lymphocytes when compared to saline injected animals. Leu-fed animals injected with ACTH had a significantly lower percentage of T-

cells when compared to Leu-fed animals injected with saline ($P < 0.02$). KIC-fed animals tended to have an increased percentage of stained T4 cells when compared to control and Leu-fed animals ($P < 0.07$). These data indicate that ACTH decreased in vitro lymphocyte blastogenesis and altered the subset ratios of circulating peripheral lymphocytes in sheep. These changes were partially reversed by feeding KIC, although KIC could not completely prevent the suppressive effects of ACTH on T-lymphocyte function.

(Keywords: Sheep, Leucine, α-Ketoisocaproate, Immunosuppression, T cell subsets, Adrenocorticotophic Hormone.)

INTRODUCTION

Stress is believed to be associated with immunosuppression and therefore can contribute to disease development in a number of domestic food animals such as cattle (Hoerlin and Marsh, 1957) and pigs (Westly and Kelley, 1984). An important response of the body to stress is the secretion of pituitary-derived adrenocorticotrophic hormone (ACTH) which stimulates the adrenal gland to release glucocorticoids (Stephens, 1980). It is believed that this increase in plasma corticosteroid concentration is an important factor in stress-induced immunosuppression, in that glucocorticoids are known to impair lymphocyte and neutrophil functions (Roth et al., 1982; Westly and Kelley, 1984). Therefore, to reduce immunosuppression caused by stress, either glucocorticoid secretion could be prevented or the action of glucocorticoids on these cell types could be prevented.

Dietary leucine (Leu) supplementation has been shown to suppress various indices of immune function (Chevalier and Aschkenasy, 1977; Tinker and Gous, 1986; Kuhlman et al., 1988), its first catabolic product, α-ketoisocaproate (KIC) has been shown to exert an enhancing effect on T-cell dependent antibody production and lymphocyte blastogenesis when orally administered to sheep (Nissen et al., 1986; Kuhlman et al., 1988). KIC has also been shown to stimulate lymphocyte blastogenesis when fed to growing steers for eight wk (Flakoll et al., 1987). However, the effects of KIC on lymphocyte function in immunosuppressed animals is not known.

The purpose of the present study was to determine if ACTH

administration would alter T-lymphocyte number and function and to determine if the α-keto acid of leucine (α-ketoisocaproate) could overcome any immunosuppressive effects caused by ACTH administration.

MATERIALS AND METHODS

Animals, Diets and Experimental Design. Twenty four mixed-breed ewe lambs, approximately 5 mo of age, were randomly assigned to one of three dietary treatments: Leu (n=8), KIC (n=8) or limestone (control; n=8). Dietary treatments were supplemented to the basal diet (Table 1) in the form of zein coated pellets. The composition and method of preparation of these pellets have been previously described (Kuhlman et al., 1988). Lambs were fed their respective pellets at a rate of approximately 3 g/d, which corresponds to approximately 5 mmol of Leu or KIC per kg of diet. Lambs were housed individually in fiberglass pens (1.15 m^2) in which they were allowed free access to water and food.

Animals were fed their appropriate diets for approximately sixty d. At this time each group of eight lambs was randomly divided into two subgroups of four lambs, a saline-injected or an ACTH-injected subgroup. The ACTH-injected subgroup was given 100 IU of ACTH (Sigma Chemical Co., St. Louis, MO.), intramuscularly, every 12 h for 3 consecutive d. The ACTH (1000 IU) was suspended in 2 ml 0.1M hydrochloric acid (HCl) and 10 ml saline/0.5% bovine serum albumin (BSA). The saline injected ewes were given injections of the HCl/saline/BSA solution (without ACTH) at corresponding times. Blood was collected prior to ACTH treatment and then daily, in the morning before injections, for 3 d.

Table 1. Composition of basal diet fed to all sheep[a]

Ingredient	kg Dry Matter
Ground corn	52.5
Dehydrated alfalfa	31.2
Expeller soybean meal[b]	10.2
Molasses	4.4
Trace mineral & vitamin premix[c]	.57
Salt	.56
Limestone	.57

[a] Formulated to contain 1.7 Mcal net energy/kg for maintenance, 1.2 Mcal net energy/kg for gain, 16% crude protein and 88.5% dry matter.

[b] Purchased from West Central Cooperative, Ralston, IA.

[c] Purchased from Feed Specialties Co., Des Moines, IA. Contains 1.64% Mg, 4949 mg Mn/kg, 371 mg Zn/kg, 2600 mg Fe/kg, 0.05 mg Cu/kg, 47.4 mg Co/kg, 128 mg I/kg, 25 mg Se/kg, 3.25% S, 772727 USP Units vitamin A/kg, 102273 USP Units vitamin D3/kg, and 773 IU vitamin E/kg.

Lymphocyte Preparation. Blood (50 ml) was collected via jugular venapuncture aseptically into 10 ml acid-citrate-dextrose anticoagulant (44 g trisodium citrate, 16 g citric acid and 50 g dextrose per 1 of triple distilled water). Blood was centrifuged and 1 vol of buffy coat was diluted with one half vol of plasma and was added to 2 vol of Sepracell-MN (Sepratech Corporation, Oklahoma City, OK). After further centrifugation at 1500 x g for 25 min, the mononuclear layer was removed and washed twice with Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) by centrifuging at 150 x g for 15 min. The remaining white blood cells were resuspended in HBSS and the total number of white blood cells were determined using an automated cell counter (Coulter Electronics, Hialeah, FL). The concentration of the cells was adjusted to 1 x 10^6 cells/ml with HBSS.

Lymphocyte Blastogenesis Determinations. Blastogenesis assays were performed on isolated lymphocytes by using a 3-d culture procedure as described previously (Roth et al., 1982). Briefly, isolated lymphocytes were cultured in microtiter plates with 2 x 10^5 cells in 0.15 ml of culture medium/well. Culture medium consisted of RPMI medium-1640 (Gibco, Grand Island, NY) containing 15% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (Grand Island Biological, Grand Island, NY). Unstimulated and mitogen stimulated cultures were assayed in triplicate. Mitogens used were phytohemagglutinin-P (PHA), concanavalin A (ConA) and pokeweed mitogen (PWM). PHA was purchased from Difco Laboratories, Detroit, MI; ConA from Miles Laboratories,

Elkhart, IN; and PWM from Grand Island Biological, Grand Island, NY. Microtiter plates were incubated at 37° C in a humidified 5% CO_2 atmosphere. After 48 h, 0.25 uCi of ^{3}H-thymidine (New England Nuclear, Boston, MA) was added to each well. Eighteen h later, the cultures were harvested onto glass-fiber filters with an automated sample harvester (Flow Laboratories, Rockville, MO), filters were placed in 10 ml CytoScint scintillation solution (ICN Biomedicals, Irvine, CA) and radioactivity was counted in a liquid scintillation counter.

Antibodies. A panel of monoclonal antibodies directed against sheep T-lymphocyte surface determinants (Table 2) was used. Monoclonal antibodies, in the form of cell-free culture supernatants were obtained from Dr. M.R. Brandon, University of Melbourne, Parkville, Victoria, Australia. Additional properties of the antigens recognized by the monoclonal antibodies have been reviewed elsewhere (Mackay et al., 1985; Maddox et al., 1985; Mackay et al., 1986). A fluoresceinisothiocyanate-conjugated rabbit F(ab')$_2$ anti-mouse immunoglobulin (FITC-RAM) was purchased from DAKO Corporation, Carpinteria, CA.

Table 2. Properties of monoclonal antibodies used

Antibody designation	Molecular wt of target antigen	Human/murine analogue	% stained peripheral lymphocytes
SBU-T1	67,000	T1/Ly1	60-80
SBU-T4	56,000	T4/L3T4	20-50
SBU-T8	36,000	T8/Ly2	10-20
SBU-T19	215,000	?	15

Immunofluorescent staining. Sheep lymphocytes (10^6) in 50 ul phosphate-buffered saline, 0.1% sodium azide (PBS/Az), 0.5% BSA were incubated for 30 min at 4° C with 50 ul monoclonal antibody culture supernatant. Additionally, cells were incubated with monoclonal antibody culture supernatant only and with FITC-RAM only so that any nonspecific fluorescent staining could be determined. Nonspecific labelling averaged approximately 1-2% and was therefore not considered an important variable in this study. The cells were washed twice with PBS/Az by centrifuging at 150 x g for 15 min and incubated with 40 ul of FITC-RAM for 30 min at 4° C. Cells were washed 3 times with PBS/Az, resuspended in 200 ul 1% formalin and stored overnight at 4° C until analyzed.

Flow Cytometric Analysis. Fluorescent stained cells were detected using an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL). Light passing to the fluorescence detector was split using a 488 nm

dichroic filter. FITC fluorescence was collected through a 515 long pass filter and the presence or absence of a range of cell surface markers was determined. Only cells containing the lymphocyte populations indicated by the forward angle light and 90° light scatter histograms were analyzed for fluorescence using the EASY88 computer system with Intgra software (Coulter Electronics, Hialeah, FL). A minimum of ten thousand events were collected per sample. All data are presented as percent positive cells or ratios of these percents.

Determination of Serum Cortisol Concentrations. Serum cortisol concentrations were determined by a coated tube single antibody radioimmunoassay using a commercial kit (Diagnostic Products Corporation, Los Angeles, CA). Samples were assayed in duplicate and all samples were processed concurrently. The cortisol antiserum used in the radioimmunoassay was specific for cortisol with an extremely low cross-reactivity to other naturally occurring steroids.

A subsequent experiment was conducted to verify the time course of cortisol appearance after ACTH injection. Sheep were injected with either ACTH (n=3) or saline (n=2) every 12 h for 3 consecutive d. Blood samples were collected once/d for the 3 d period, serum was removed and cotisol concentrations were determined.

Serum insulin and glucagon concentrations were also determined. Insulin concentrations were measured using a coated tube single antibody radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). The insulin antiserum was highly specific for insulin, with particularly

low crossreactivity to compounds other than insulin and proinsulin. Serum glucagon was measured with a double antibody radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA). This procedure is highly specific for intact glucagon and glucagon fragments with no crossreactivity for related compounds. All kits gave parallel standard curves when added to sheep plasma.

Statistical Analysis. Blood was collected from both groups for the determination of lymphocyte blastogenesis and for monoclonal antibody analysis immediately before beginning the ACTH treatment. A mean was determined for each parameter for each group and a two-way analysis of variance procedure was performed to determine the level of significance of any differences among groups before ACTH administration. Pooled SEM were taken from ANOVA. These blood samples were collected to assure that no significant differences were seen between injection subgroups within the same dietary treatment. Blood was collected three times (once/d) during the ACTH administration treatment. A mean was determined for each parameter for each group from these three blood collections. A two-way ANOVA procedure was performed to determine the level of significance of any differences between the two groups during ACTH treatment. Pooled SEM were taken from ANOVA. Conservative degrees of freedom were used in the F tests. The interactions between dietary and injection treatments were also determined for pre- and post-injection groups.

RESULTS

No significant differences were seen between animals in serum cortisol concentrations prior to injections within dietary treatments. The data in Table 3 indicate that serum cortisol concentrations were elevated within 2 h after ACTH administration and returned to basal values within 10-12 h. In the present study, serum hormone concentrations were determined on blood samples collected once/d for 3 consecutive d. Each sample was therefore collected 12 h after ACTH administration. Although no significant differences were observed in serum cortisol concentrations at 12 h after ACTH administration, ACTH-injected animals tended to have higher serum cortisol concentrations regardless of the dietary regime (Table 4). Serum cortisol concentrations of the ACTH-treated group averaged 2.3 ug/dl and averaged 1.6 ug/dl for the saline treated groups. When compared to control animals, the KIC-fed animals tended to have lower serum cortisol concentrations regardless of the type of injection.

ACTH-injected animals fed control diets had increased serum insulin concentrations when compared to all other dietary treatments without ACTH ($P < 0.09$). Although not significant, ACTH-injected groups had increased serum insulin concentrations when compared to saline-injected groups. No significant differences were seen in serum glucagon concentrations (Table 4).

Before the administration of the injections, there was no significant differences observed between injection groups in any of the parameters measured. The results obtained from the lymphocyte

blastogenesis assays are shown in Table 5. Each value represents the average of 3 collections (1/d). Mitogen-stimulated blastogenesis responses generally tended to be increased when KIC was fed and decreased when Leu was fed.

ACTH administration significantly suppressed lymphocyte blastogenesis by up to 52%, 50% and 63%, respectively, when PHA, ConA or PWM were used ($P < 0.05$) when compared to saline injected groups. KIC-fed animals not injected with ACTH had significantly increased blastogenesis responses when all 3 mitogens were used. KIC-fed animals injected with ACTH had a greater blastogenesis response when compared to Leu-fed or control animals given ACTH. No significant changes were observed in unstimulated lymphocyte blastogenesis.

In the preinjection analysis, no significant differences were seen in T-cell subsets between injection groups within the same dietary treatment. Table 6 shows the results obtained from the flow cytometric analysis of T-lymphocyte subsets. The total percentage of each subset was used for statistical analysis. KIC-fed animals tended to have increased T4 cell percentages when compared to animals fed control or Leu supplemented diets. Also, ACTH treatment of Leu-fed animals significantly lowered the percentage of T-lymphocytes ($P < 0.005$). This decrease in total T-cell percentage was due to a decrease in T19 lymphocytes ($P < 0.02$).

Table 3. The effects of ACTH administration on serum cortisol concentrations in sheep

Hours after injection	Mean ± SEM Saline (ug/dl) (n=2)	ACTH (ug/dl) (n=3)
0	1.09 ± 0.23	1.22 ± 0.57
2	0.86 ± 0.24	15.60 ± 2.50
4	0.83 ± 0.41	15.06 ± 1.98
6	0.69 ± 0.22	8.84 ± 2.10
8	0.53 ± 0.20	5.20 ± 1.80
10	0.45 ± 0.15	2.76 ± 1.20
12	0.76 ± 0.33	1.69 ± 0.72

Table 4. The effects of ACTH administration[a] on mean serum hormone concentrations in sheep (n=4) fed control, KIC or Leu diets for 60 days

Diet	Cortisol (ug/dl)	Insulin (uU/ml)	Glucagon (pg/ml)
Control			
Saline	1.81	28.07	127.78
ACTH	2.91	157.71	123.54
KIC			
Saline	1.21	34.73	111.20
ACTH	1.80	50.23	97.92
Leu			
Saline	1.67	28.21	108.60
ACTH	2.11	79.99	103.40
SEM[b]	.48	36.01	15.39
Probability values for main effects and interactions			
Diet	.34	.32	.33
Injection	.67	.09	.55
Diet * Injection	.54	.31	.94

[a] Average of 3 days

[b] Pooled standard error of the mean from ANOVA

Table 5. The effects of ACTH administration[a] on lymphocyte blastogenesis in sheep (n=4) fed control, KIC or Leu diets for 60 days

	Mitogen Counts/minute/$2x10^5$ cells			
Diet	None	PHA	ConA	PWM
Control				
Saline	471	19700	18600	23400
ACTH	678	11200[t]	10300[t]	14300[t]
KIC				
Saline	430	33200[*]	38800[*]	41000[*]
ACTH	497	16000[t]	19400[t]	23300[t]
Leu				
Saline	746	14300	18100	28300
ACTH	750	9700[t]	9790[t]	10400[t]
SEM[b]	158	3600	5300	6700
Probability values for main effects and interactions				
Diet	.22	.008	.016	.012
Injection	.48	.003	.013	.015
Diet* Injection	.81	.23	.50	.77

[a] Average of 3 days

[b] Pooled standard error of the mean from ANOVA

[*] $P < 0.05$, as compared to all other diet and ACTH injection treatments

[t] $P < 0.05$, as compared to the same diet without ACTH

Table 6. The effects of ACTH administration[a] on T-cell subsets in sheep fed control, KIC or Leu diets for 60 days

	Subset % stained peripheral lymphocytes			
Diet	T1	T4	T8	T19
Control				
Saline	56.4	15.4	10.3	20.7
ACTH	46.7	14.4	11.4	19.1
KIC				
Saline	57.6	21.3	11.1	23.1
ACTH	49.3	18.8	11.4	19.6
Leu				
Saline	63.7	17.6	12.7	25.8
ACTH	47.4*	15.5	13.9	15.3*
SEM[b]	4.4	2.1	1.1	2.6
Probability values for main effects and interactions				
Diet	.66	.07	.09	.86
Injection	.005	.29	.38	.02
Diet* Injection	.63	.93	.91	.22

[a] Average of 3 days

[b] Pooled standard error of the mean from ANOVA

* $P < 0.05$, as compared to same diet without ACTH

DISCUSSION

The present study suggests that the treatment regime of 100 IU of ACTH injected intramuscularly every 12 h stimulates the secretion of cortisol. Blood was collected 12 h after ACTH injections so that serum cortisol, insulin and glucagon concentrations could be determined. These values may not reflect the acute effects of ACTH administration due to the time of blood collection. As shown in the subsequent experiment, cortisol concentrations almost returned to baseline values by 12 h after injection. Cortisol values did, however, tend to be higher in the ACTH injected groups than those of the saline injected groups. Similarly, blood insulin increased with ACTH which has been shown to occur in rats (Sussman and Vaughan, 1967) and mice (Genuth and Lebovitz, 1965). The mechanism of the marked suppression in mitogen-stimulated blastogenesis seen in this study with the administration of ACTH is currently unknown. It may be due to a direct effect of ACTH on the lymphocytes to inhibit mitosis, to a corticosteroid-induced inhibition of T-cell growth factor production by mononuclear cells or to a redistribution of a proportion of the lymphocytes out of circulation and into an extravascular lymphocyte compartment. The latter possibility is supported in the present study since two distinct peripheral blood T-cell subsets, T4 and T19, tended to decrease after ACTH administration. T4 cells represent a subset of T-helper cells bearing the $\alpha\beta$ T-cell receptor (Maddox, 1985) and T19 cells represent a subset of T cells bearing the $\gamma\delta$ T-cell receptor (Mackay et al., 1989).

In man, the administration of glucocorticoids causes the

recirculating lymphocytes to leave the intravascular space and to enter the tissues, but nonrecirculating lymphocytes are not affected (Parrillo and Fauci, 1979). This selective depletion of T-lymphocytes from the peripheral blood may be responsible for the decreased responsiveness of peripheral blood lymphocytes in man to T-cell mitogens. This may also be the case in sheep. It has been shown that in sheep a 16-22% reduction of the output of lymphocytes into the efferent lymph results from giving an intravenous infusion or a bolus injection of a cortisol analog, methylprednisolone (Zukoski and Montgomery, 1983).

These data support the argument that in sheep, as in man, ACTH induces a selective sequestering of T-lymphocytes in the lymphoid tissues and this sequestering may be responsible for the decrease seen in lymphocyte blastogenesis following ACTH administration. These data also suggest that KIC may partially decrease the sequestering of T4 and T19 peripheral blood lymphocytes seen in control and Leu-fed animals given ACTH in this study. KIC-fed animals tended to have larger percentages of circulating T4 cells after ACTH injection ($P < 0.07$). KIC feeding suceeded in partially overcoming the depression in mitogen-stimulated lymphocyte blastogenesis seen after ACTH administration. In a study in which insulin was added to the culture media, insulin enabled T-cell proliferation in a murine one-way mixed lymphocyte reaction (Snow, 1985). Therefore, insulin may be involved in the regulation of lymphocyte blastogenesis. However, in the present study, cortisol may have interferred with insulin action and thus the cultured lymphocytes may have been resistant to the action of insulin. In other

studies, the administration of excess ACTH to rats resulted in a state of insulin resistance (Kahn et al., 1978). Thus, the suppression of blastogenesis by ACTH may have been the result of a direct action of cortisol on lymphocyte function and may have interferred with the indirect action of insulin on lymphocyte function.

In conclusion, ACTH injections partially stimulate the endocrine pattern of acute stress and lymphocyte blastogenesis which may be due to a selective depletion of T4 and T19 peripheral lymphocytes in sheep. Also, KIC was partially effective in preventing or reversing the suppressive effects of ACTH on T-lymphocyte function when fed at a rate of 5 mmol/kg total diet.

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SECTION III. α-KETOISOCAPROATE ENHANCES OVINE LYMPHOCYTE BLASTOGENESIS THROUGH PRODUCTION OF β-HYDROXY-β-METHYLBUTYRATE

α-Ketoisocaproate Enhances Ovine Lymphocyte Blastogenesis Through Production of β-Hydroxy-β-methylbutyrate

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ABSTRACT

Experiments were conducted to determine if the in vivo suppression of lymphocyte blastogenesis by leucine (Leu) and the in vivo stimulation of lymphocyte blastogenesis by α-ketoisocaproate (KIC) were due to direct effects of these compounds on blastogenesis. Peripheral blood lymphocytes were isolated and cultured in the presence of 1 and 10 mM Leu, KIC, isovalerate (IVA), β-hydroxy-β-methylbutyrate (HMB), β-hydroxy-β-methylglutarate (HMG), butyrate, acetate, acetone, acetoacetate (AcAc) and β-hydroxybutyrate (BOHB). Cells were then incubated with the mitogen phytohemagglutinin-P (PHA) and the incorporation of ^{3}H-thymidine into the DNA of the dividing lymphocytes was measured. Leu suppressed blastogenic responses by approximately 25% at a concentration of 10 mM, while KIC and IVA did not alter blastogenesis. BOHB significantly enhanced blastogenesis by approximately 50% while HMB increased blastogenesis by approximately 100% at a concentration between 1-10 mM. HMB increased responses by approximately 2 fold more than BOHB, although both metabolites enhanced responses at all concentrations used. The maximum change over the control in blastogenesis occurred at low doses of both HMB and BOHB. In conclusion, the effects of Leu in vivo can be explained by a direct effect of the compound, but the effects of KIC in vivo may be due to a hydroxy acid metabolite of the compound.

(Keywords: Sheep, Leucine, α-Ketoisocaproate, Hydroxy-methylbutyrate)

INTRODUCTION

Previous studies indicate that the oral supplementation of leucine (Leu) suppressed in vitro lymphocyte blastogenesis and production of antibodies in sheep while the first catabolic product of Leu, α-ketoisocaproate (KIC), enhanced ovine lymphocyte blastogenesis and antibody production (Kuhlman et al., 1988). In the same study another metabolite of Leu, isovalerate (IVA), did not affect these same parameters. In other studies, it was determined that feeding KIC to lambs at 1% of the diet for 8 wk resulted in a two-fold increase in PHA stimulated lymphocyte blastogenesis (Nissen et al., 1986). In cattle, feeding KIC at 0.07% of the diet resulted in a significant increase in unstimulated and mitogen stimulated lymphocyte blastogenesis (Flakoll et al., 1987). Since these studies involved the feeding of these compounds, a direct effect of the compound itself or an indirect effect of a metabolite of the compound could be possible.

To determine if these compounds are directly responsible for the observed changes in lymphocyte blastogenesis, the effects of various concentrations of Leu and Leu metabolites on the mitogenic stimulation of ovine lymphocytes were determined. The metabolites used were Leu, KIC, IVA, β-hydroxy-β-methylbutyrate (HMB), β-hydroxy-β-methylglutarate (HMG), β-hydroxybutyrate (BOHB), butyrate, acetate, acetone and acetoacetate (AcAc). Dose response curves were therefore constructed for HMB and BOHB because they significantly stimulated lymphocyte blastogenesis when incubated with the mitogen stimulated lymphocytes. To assure that these enhanced effects were due

specific compounds and not to all hydroxy acids, α-hydroxybutyrate (AOHB), γ-hydroxybutyrate (GOHB), α-hydroxyisovalerate (AOHI) and citramalic acid (Cit) were also included as metabolites in the lymphocyte blastogenesis assays.

MATERIALS AND METHODS

Animals. Mature mixed-breed ram and ewe sheep were used. All animals were fed a corn-soybean meal based diet with adequate protein. Water was provided at all times.

Lymphocyte Preparation. Blood (50 ml) was aseptically collected via jugular venapuncture into 10 ml acid-citrate-dextrose anticoagulant (44 g trisodium citrate, 16 g citric acid and 50 g dextrose per 1 of triple distilled water). Blood was centrifuged, the buffy coat was removed and 1 vol of buffy coat was diluted with one half vol of plasma and was added to 2 vol of Sepracell-MN (Sepratech Corporation, Oklahoma City, OK). After further centrifugation at 1500 x g for 25 min, the mononuclear layer was removed and washed twice with Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) by centrifuging at 150 x g for 15 min. The remaining white blood cells were resuspended in HBSS and the total number of white blood cells were determined using an automated cell counter (Coulter Electronics, Hialeah, FL). Cells were adjusted to a concentration of 1×10^6 cells/ml with RPMI media-1640 (Gibco, Grand Island, NY).

Lymphocyte Blastogenesis Determinations. Blastogenesis assays were performed on isolated cells by using a 3-d culture procedure as described previously (Roth et al., 1982). Briefly, isolated lymphocytes were cultured in microtiter plates with 2×10^5 cells in 0.2 ml of culture medium/well. Culture medium consisted of RPMI medium-1640

containing 15% heat-inactivated fetal bovine serum (Flow Laboratories, McLean, VA) and 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY). Unstimulated and mitogen stimulated cultures were assayed in triplicate. The mitogen used was phytohemagglutinin-P (PHA), which was purchased from Difco Laboratories, Detroit, MI. Metabolites were added to each well in 25 ul aliquots. The final concentration of each metabolite in the well was 0 (control), 1.0 or 10 mM. Leu concentrations, however, were slightly higher since RPMI medium-1640 contains approximately 0.38 mM Leu. Microtiter plates were incubated at 37° C in a humidified 5% CO_2 atmosphere. After 48 h, 0.25 uCi of ^3H-thymidine (New England Nuclear, Boston, MA) was added to each well. Eighteen h later, the cultures were harvested onto glass-fiber filters with an automated sample harvester (Cambridge Technology, Watertown, MA), filters were placed in 10 ml Scintiverse scintillation solution (Fisher Scientific, Springfield, NJ) and radioactivity was counted in a liquid scintillation counter.

Leucine and Leucine Metabolites. Figure 1 illustrates the metabolic pathway of Leu including only the Leu metabolites used in this study. Metabolites were prepared in RPMI medium-1640. The l-Leu, IVA, HMG, dl-BOHB, n-butyrate, AcAc, dl-AOHB, GOHB and dl-AOHI were purchased from Sigma Chemical Company, St. Louis, MO. Acetate and acetone were purchased from Fisher Scientific, Springfield, NJ and the (s)-(+)-Cit was purchased from Aldrich Chemical Co., Milwakee, WI. The KIC was purchased from SOBAC, Paris, France and the HMB was synthesized in our

laboratory (Coffman et al., 1957) and the resulting calcium salt was purified by crystallization in 95% ethanol. The calcium salt was converted to a sodium salt form and used in subsequent assays. Purified HMB was derivatized with N-methyl-N-(t-butyldimethylsilyl) trifluoroacetamide (Regis Chemical Company, Morton Grove, IL) and analyzed by gas chromatography/mass spectrometry. The pattern of fragmentation indicated HMB to be the major product. High performance liquid chromatography indicated the purity of this compound to be greater than 90%. All metabolites were purchased or prepared in the form of sodium salts except the AcAc which was purchased as a lithium salt. Since lithium has been shown to augment lymphocyte proliferation (Gelfand et al., 1980) the AcAc was converted to the sodium salt form using a cation exchange resin (Sigma Chemical Co., St. Louis, MO). The pH of all metabolites was adjusted to approximately 7.3. Metabolites were passed through a 0.2 um acrodisc filter assembly (Ann Arbor, MI) to assure sterility.

Dose Response Curves. The sodium salt forms of HMB and BOHB were diluted with RPMI medium-1640 so that the final concentration of each metabolite in the lymphocyte culture would be 0, .0001, .001, .010, .100, 1.0, 10.0 mM. Twenty five ul aliquots were added to the appropriate wells. Quadruplicate wells were set up for each metabolite at each concentration.

Statistical Analysis. The experimental results are expressed as the percent change from the control ± standard error (SE). The percent change was calculated from each assay and the mean percent change is reported. The significance of the changes was determined by student's t-tests (Steel and Torrie, 1960).

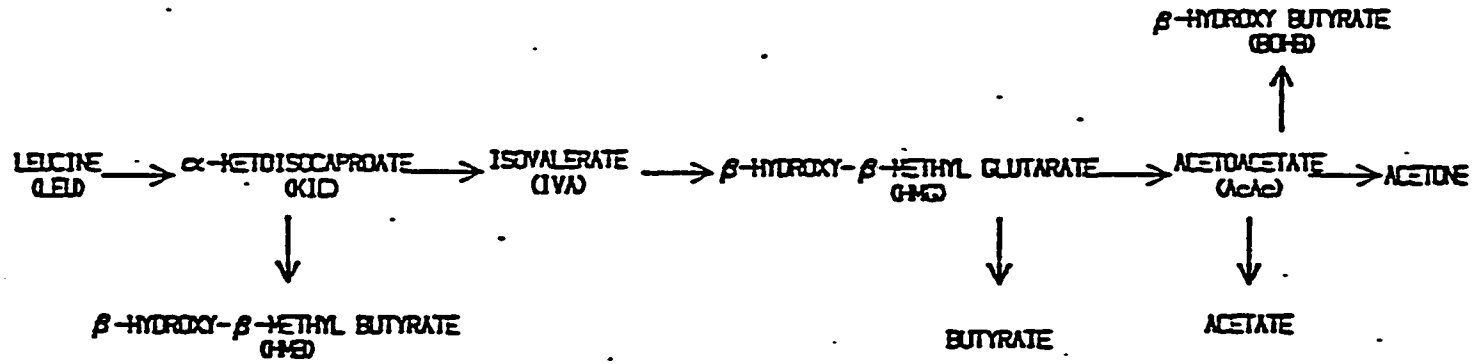

Figure 1. The metabolic pathway of leucine (including only the metabolites used in this study).

RESULTS

The results for the mitogen stimulated blastogenesis assays are shown in Table 1. The PHA-stimulated blastogenic response for lymphocytes cultured in the absence of metabolites was 29212 ± 2324 counts per min per 200,000 lymphocytes. When Leu was added to lymphocytes, a decrease in lymphocyte blastogenesis of approximately 25% was observed at 10 mM Leu when compared to the control value. The next two metabolites in the Leu pathway, KIC and IVA, did not alter the mitogenic stimulation. AcAc also did not significantly alter blastogenic responses. The addition of acetate and HMG at 10 mM significantly increased blastogenesis while acetone did not alter blastogenesis. BOHB and HMB also significantly increased blastogenesis at both 1 and 10 mM with HMB being approximately 2 fold as active as BOHB. Unstimulated lymphocytes cultured in the presence of each metabolite followed the same trends as those lymphocytes cultured with PHA. HMB and BOHB enhanced unstimulated blastogenic responses by 70 and 30 % respectively. These results, however, were not significant due to the large day to day variations.

The dose response curve for HMB and BOHB is shown in Figure 2. Both metabolites increase blastogenesis, again, HMB increased responses approximately 50% more than BOHB at all concentrations. When evaluating the mitogenic stimulatory responses for both compounds, the greatest percent change from the control occurred between 1 and 10 uM. Blastogenesis did, however, slightly increase continually as the concentration of each metabolite increased.

The incubation of lymphocytes with PHA and the other hydroxy acid compounds (AOHB, GOHB, AOHI and Cit) at concentrations of 1 and 10 mM did not significantly alter blastogenic responses and, in most instances tended to decrease lymphocyte transformation by approximately 3-7% (data not shown).

Table 1. Effect of addition of Leu and Leu metabolites to PHA-stimulated cultures of normal ovine lymphocytes

Metabolite	Concentration	Percent change over control ± SE	P <	Number of replicates
Leu	1 mM	-12 ± 6	0.10	8
	10 mM	-24 ± 5	0.01	8
KIC	1 mM	- 3 ± 7	0.60	8
	10 mM	- 8 ± 12	0.60	8
IVA	1 mM	- 1 ± 9	1.00	6
	10 mM	-17 ± 8	0.30	6
HMB	1 mM	+78 ± 13	0.01	13
	10 mM	+91 ± 12	0.01	13
HMG	1 mM	+28 ± 9	0.20	6
	10 mM	+46 ± 11	0.01	6
Butyrate	1 mM	+52 ± 12	0.20	8
	10 mM	-21 ± 17	0.50	8
Acetate	1 mM	+22 ± 7	0.50	4
	10 mM	+47 ± 8	0.05	4
Acetone	1 mM	+31 ± 9	0.60	4
	10 mM	+27 ± 6	0.70	4
AcAc	1 mM	- 6 ± 9	0.70	6
	10 mM	-14 ± 11	0.50	6
BOHB	1 mM	+25 ± 6	0.01	15
	10 mM	+49 ± 8	0.01	15

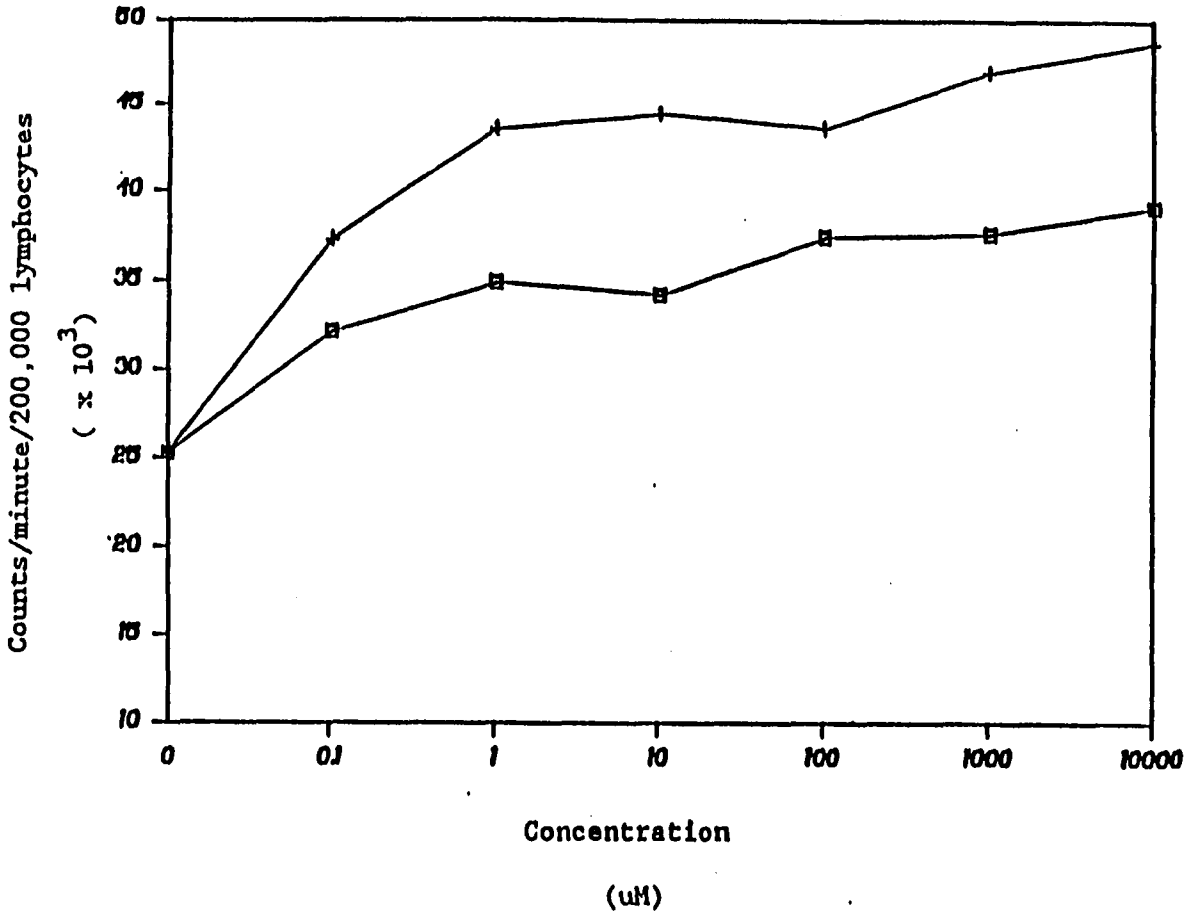

Figure 2. The effect of β-hydroxy-β-methyl butyrate (+) and β-hydroxybutyrate (□) at ten fold concentrations on the proliferation of PHA stimulated normal ovine lymphocytes

DISCUSSION

Leu and KIC are metabolized following ingestion, therefore, a direct effect of the compound or an indirect effect of a metabolite of the compound on lymphocyte blastogenesis is possible. From the present study, it is clear that the stimulatory effect of KIC on lymphocyte blastogenesis is not due directly to KIC itself. However, findings from these studies suggest that a metabolite of KIC, HMB, which markedly stimulated blastogenesis may indirectly be responsible for the effects of KIC seen in vivo.

In the present study, HMG at the 10 mM concentration also stimulated blastogenesis. The metabolic relationship between KIC, HMB and HMG is not clear. KIC has been shown to be converted to HMB by the enzyme KIC oxygenase (Sabourin and Bieber, 1982). Thus, KIC could act in vivo via conversion to HMB. Older isotopic studies, using paper and column chromatography, demonstrates that HMG and HMB can be interconverted in vivo (Adamson and Greenberg, 1957). This supports the present study in which high concentrations of HMG enhanced lymphocyte blastogenesis. More recent literature in which newer methodology was used, however, does not mention this metabolic interconversion. Thus, it is unclear if a relationship exists in vivo. Assuming the most potent metabolite is the bioactive compound, then HMB is the product stimulating blastogenesis in vitro and in vivo. Other stimulators such as BOHB, acetate and HMG may directly affect blastogenesis or may be converted to HMB and have an indirect effect on blastogenesis.

In the present study, Leu significantly suppressed lymphocyte

blastogenesis when added to PHA stimulated lymphocytes at a final concentration of 10 mM. It seems that the suppressive effect of Leu on immune function in vivo could, therefore, be due to a direct effect of Leu on lymphocyte function. Further studies will be necessary to document the importance of this observation and its relationship to Leu nutrition.

Although most metabolites of Leu have not been examined relative to blastogenesis, suppression of lymphocyte blastogenesis has been demonstrated (Targowski and Klucinski, 1983) when BOHB was added to bovine lymphocyte cultures at similar concentrations (.01 - .5 mM). The culture medium used in the studies with bovine lymphocytes was Eagle's minimal essential medium supplemented with 0.1% fetal bovine serum. In the present study, RPMI medium-1640 supplemented with 15% fetal bovine serum was used as the culture media for the ovine lymphocytes. RPMI medium-1640 contains approximately .38mM Leu while Eagle's media contains twice this much Leu which could interact with BOHB effects. The other possible explaination for the discrepancies between studies could be the amount of fetal bovine serum used. In the study with the bovine lymphocytes, only 0.1% fetal bovine serum was used and no enhancement was seen with the addition of any metabolite tested (butyrate, acetone, AcAc, BOHB). In the present study, 15% fetal bovine serum was used and enhancement, suppression and no effect were seen with the addition of metabolites. The BOHB used in both studies was dl-BOHB. Since the d isomer is the natural metabolite, it is unknown whether the l isomer is inert or if it affects metabolism.

Both HMB and BOHB stimulated blastogenesis at .1 uM, although blastogenic responses tended to increase as the concentration of each metabolite increased. The stimulatory effects of these two hydroxy acids are not common to all hydroxy acids because AOHB, GOHB, AOHI and Cit did not alter blastogenesis. It, therefore, seems that HMB and possibly BOHB may have specific direct effects on lymphocyte blastogenesis.

In conclusion, the stimulatory effects of KIC on lymphocyte blastogenesis in vivo can not be explained by a direct effect in vitro. However, the production of a specific hydroxy acid metabolite, HMB, could be reponsible for the observed effects. Conversely, the suppression effects of Leu on blastogenesis and immune response in vivo could be explained by a direct suppressive effect of Leu on lymphocyte function.

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GENERAL DISCUSSION

The major findings from these experiments are that: (1) the oral administration of leucine (Leu) and α-ketoisocaproate (KIC) to sheep affect lymphocyte blastogenesis in opposing manners and that these effects are associated with alterations in the ratios of specific circulating T-cell subsets; (2) the effects exerted by Leu on lymphocyte blastogenesis may be caused by a direct effect of Leu on lymphocyte blastogenesis while the effects exerted by KIC could be due to another Leu metabolite, β-hydroxy-β-methylbutyrate (HMB); and (3) KIC partially overcomes the adverse effects of ACTH-induced immunosuppression in sheep.

When evaluating the possible mechanism of Leu and KIC on immune function, the effects of these compounds on T-cell subsets must be given foremost consideration since alterations in T-cell subsets have been shown to be associated with a variety of disease and immunodeficiency states. KIC has been shown to increase the percentage of circulating T4 cells when orally administered. This could explain the enhancement that KIC exerts on lymphocyte blastogenesis and the positive effects that KIC also exerts on ACTH-induced immunosuppression in sheep. However, the in vitro addition of KIC to mitogen-stimulated lymphocyte blastogenesis did not cause enhancement. Another metabolite of KIC, HMB, enhanced mitogen-stimulated lymphocyte blastogenesis by 100% when added to lymphocyte cultures in vitro. Two other metabolites of Leu also enhanced blastogenic responses. These metabolites were β-hydroxy-β-methylglutarate (HMG) and β-hydroxybutyrate (BOHB). Both

of these metabolites, however, enhanced the blastogenesis to a much lesser degree than HMB. It has, therefore, been concluded that the effect which KIC exerts on immune function in sheep, both in vitro and in vivo, is due to the further catabolism of KIC to HMB. KIC has been shown to be converted to HMB by the enzyme, KIC oxygenase. BOHB and HMG may also be converted to HMB and, therefore, the in vitro addition of these compounds also resulted in enhanced blastogenic responses.

Leu suppresses lymphocyte blastogenesis both in vivo and in vitro. It has, therefore, been concluded that the effects which Leu exerts on immune function is due to a direct effect of Leu and not to further catabolism of this compound. Leu has been shown to decrease the percentage of circulating T19 lymphocytes and this may explain the immunosuppressive effects seen with the oral supplementation of Leu.

It could be argued that since HMB, HMG and BOHB are hydroxy-acids, β-oxidation of these compounds may occur during lymphocyte transformation and the energy equivalent formed may be responsible for the increase in lymphocyte blastogenesis. However, the data from the current research indicate that this is not so. First of all, other hydroxy-acids were added to lymphocyte cultures and they did not effect lymphocyte blastogenesis. Secondly, other compounds tested in this research (butyrate and acetoacetate), which could alter lymphocyte blastogenesis due to their associated energy effects, did not significantly alter lymphocyte blastogenesis. This supports the contention that the actions of HMB, HMG and BOHB were not due to energy alterations by these compounds. Therefore, the actions of KIC, in vivo, on lymphocyte

blastogenesis must be due to an indirect effect of HMB not to KIC directly.

Conclusions drawn from these experiments are based on two separate in vitro lymphocyte assays. Although these assays have substantial validity, several problems exist with in vitro cell assays. Some of these problems involve sample collection, cell separation, culture methods, the standardization of cell numbers and the variation between animals. In turn, these minor problems make it much more difficult to extrapolate in vitro and in vivo cellular immune response to animal health response.

Peripheral blood samples were used in all T-cell evaluations reported here. It is, however, important to note that the relative proportions and functional behavior of T-cells from different lymphoid tissues vary, and normal controls established for peripheral blood lymphocytes may be not applicable to other lymphocyte populations. Blood samples were collected in the morning, prior to feeding which could affect the number and relative proportions of circulating lymphocytes due to the diurnal patterns of some hormones and circulation patterns of lymhpocytes. In addition to animal variation, the physical method of separation may also vary. Lymphocytes were isolated using Sepracell-MN, a colloidal silica based medium, which works on a continuous density gradient system by centrifugation. When comparing this method with other separation techniques such as Ficoll-Hypaque, this method was perferred. No layering of the blood over the gradient was necessary, a larger number of lymphocytes containing a low

percentage of monocytes (1-2%) were recovered and there was very little red blood cell contamination. This is important because erythrocyte aggregation polymers have been shown to decrease mitogen responsiveness of isolated lymphocyte preparations.

The in vitro stimulation of lymphocytes by specific mitogens has become one of the most widely used screening assays for cellular immune responsiveness. This assay was used in the current research as an indicator of lymphocyte function. A medium was selected which contained a relatively low concentration (0.38 mM) of Leu since Leu was a variable studied in the reported experiments. It is necessary to supplement cultures with animal serum for maximal lymphocyte transformation and cell viability. Fetal bovine serum was added in these ovine cell cultures since it was commercially available and since fetal ovine serum could not be obtained. An electronic cell counter was used to standardize cell numbers and an electronic pipette was used to plate the cell suspensions to increase the accuracy of pipetting. Initially, a dose response curve was constructed for each mitogen so that the proper amount of each mitogen necessary for maximum stimulation could be determined. Lymphocytes were cultured in the presence of ^{3}H-thymidine for 18 hours. Since exposure to ^{3}H-thymidine at very high levels is detrimental to cell growth, cells were incubated with low levels of ^{3}H-thymidine for a relatively long period of time (18 hours) to minimize DNA damage. Also, the 18 hour incubation period provided enough time for all lymphocytes to divide.

To evaluate T-lymphocyte subset percentages, monoclonal

antibodies directed against specific T-lymphocyte surface receptors were used. Again, the problems associated with in vitro cell assays were encountered here. Since no total blood lymphocyte counts were taken, only the percentages of each subset could be reported which did not allow for any changes in total lymphocyte numbers to be quantitated. An additional problem encountered in evaluating T-cell subsets, was the interpretation of the T19 cell-type histograms. For all other monoclonal antibodies used, peaks obtained were clearly defined and integrated. However, histograms obtained from T19 cell subsets were somewhat broad and not easily defined (see Appendix E). The most significant problem was encountered when trying to determine where the negative control range ended and the T19 range began. It may be that the maturing T19 cell may bear two distinct phenotypes which may have different levels of receptors or may have different receptor binding affinities. Either of these possibilities could lead to a broad peak range on the histogram. All histogram peak ranges obtained from the use of these sheep monoclonal antibodies were, however, repeatable between animals and weeks.

While the mechanisms are not entirely clear, it is evident that Leu and KIC exerted opposing effects on immune function in sheep. Both these compounds can modulate immune function when fed in relatively small quantities (0.1% of the diet). Leu has been shown to also alter immune parameters in non-ruminants when fed in much higher amounts. The difference in amounts needed to elicit these responses may be partially explained by the fact that ruminants have lower concentrations of the

enzymes necessary to metabolize these compounds. The fact that these enzyme concentrations are lower in ruminants may be related to the fact that sheep and cattle are primarily grazing animals. Most forages contain relatively low Leu as compared to grains. Cattle and sheep therefore, may have not had to adapt to metabolizing large amounts of Leu. These relatively low enzyme concentrations would then be a type of an "adaptation mechanism" developed throughout evolution.

Domestication and intensification of modern day farm management practices can stress animals and this in turn can result in disease outbreak. Thus, a role for immunomodulators in livestock practices becomes more important as these practices intensify further. The observation that KIC improves immune function in sheep suggests that supplementation could augment the immune system during the stressful times of weaning, handling, transport, castration and parturition. This in turn, has the potential for economic returns by the sheep producer. Further studies will be necessary to determine if beneficial effects of KIC are manifest in field "disease" states.

The immunosuppressive effect of small amounts of Leu is surprising. It is hard to imagine that the supplementation of 1 g of Leu a day in addition to the 10 g normally absorbed could adversely affect immune function. Again, the marginal degradation pathways of Leu in the gut and liver of sheep may result in direct immunosuppressive effects in gut and liver associated lymphoid tissues. The practical implication of this is unknown but could be of importance. For example, if Leu is immunosuppressive, it may be of importance to use a feedstuff containing

relatively low amounts of Leu when weaning, transporting or handling animals. Our domestication practices have taken grazing livestock and subjected them to stressful handling and housing conditions. Currently in the United States, these grazing species are given high corn containing diets and corn is high in Leu. This may be a contributing factor in the large economical losses of animals observed in our current livestock production practices.

There is still much to learn about the use of KIC and Leu in ruminant animals. One of the foremost questions to be answered is: Does the feeding of the apparent active metabolite of KIC, HMB, have any affect on the immune system? In any event, the addition or depletion of nutrients and/or additives which enhance the immune system of ruminants is certainly warranted so that livestock can better adapt to stressors of modern day production systems. Immunomodulators can then be adjuncts to the homeostasis mechanism.

"All the vital mechanisms, varied as they are, have only one object, that of preserving constant the conditions of life in the internal environment." -Claude Bernard, 1878.

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APPENDIX A

Isolation of Ovine Mononuclear Cells Using Sepracell-MN

Use aseptic techniques.

1. Collect 50 ml of whole blood using a sterile 60 ml syringe and 14 gauge (1.5 inch) needle filled with 10 ml 2 X ACD (acid-citrate-dextrose) solution. Transfer to a sterile 50 ml conical centrifuge tube. Mix well after collection.

   2 X ACD:

   22.0 g trisodium citrate

   8.0 g citric acid

   25.0 g dextrose

   500.0 ml distilled water

   (Filter sterilize using a .22 micron disposable sterile filter system)

2. Centrifuge at 1000g for 25 minutes.
3. Transfer 3 ml buffy coat and 1.5 ml plasma (use a 5 ml sterile glass volumetric pipet) into a 15 ml centrifuge tube containing 6 ml Sepracell-MN (Sepratech Corporation, Oklahoma City, OK). Mix well.
4. Centrifuge at 1500g for 25 minutes.
5. Aspirate mononuclear cell layer (using a 1 ml sterile glass volumetric pipet) and transfer to a sterile, 25 ml glass tube containing 10 ml Hank's Balance Salt Solution without Ca^{++} or Mg^{++} (HBSS; Gibco, Grand Island, NY) or phosphate buffered saline (PBS) which has been warmed in a 37^{o} C water bath. Rinse the pipet with the HBSS to get all cells. Invert to suspend cells.

Phosphate buffered saline:

16.5 ml solution A

33.5 ml solution B

8.0 g sodium chloride

Solution A: 2.76 g/10 ml $NaH_2PO_4 \cdot H_2O$

Solution B: 5.37 g/10 ml $Na_2HPO_4 \cdot 7H_2O$

(Filter sterilize)

6. Spin at 150g for 15 minutes.
7. Decant supernatant carefully (cells don't stick well at this stage) and resuspend in 10 ml HBSS or PBS.
8. Spin at 300g for 10 minutes.
9. Decant supernatant and resuspend in 1 ml HBSS or PBS (if large pellet, may want to use more HBSS to resuspend).

APPENDIX B

Isolation of Ovine Mononuclear Cells Using Histopaque-1077

Use aseptic techniques.

1. Collect blood in same manner as stated in appendix A.
2. Remove buffy coat and dilute 1:2 with HBSS or PBS. Mix by gently inverting.
3. Layer 10 ml of diluted buffy coat on 6 ml Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) which has been previously pipetted into 25 ml sterile centrifuge tubes.
4. Centrifuge at 400g for for 30 minutes.
5. Remove lymphocyte band and transfer to another sterile 25 ml tube.
6. Wash cells with HBSS or PBS as in Sepracell-MN separation method.

APPENDIX C

Outline for a 3-day Lymphocyte Blastogenesis Culture

Use aseptic procedures and keep all reagents at 37^{o} C in a water bath.

1. Transfer approximately 0.1 ml of isolated lymphocytes in HBSS or PBS (use above procedures for isolation of lymphocytes) into a glass 12 x 75 mm tube.
2. Count each sample in glass tube on automated cell counter (Coulter Electronics, Hialeah, FA). Multiply each count from the Coulter counter by 10^3 to obtain the number of cells per ml.

   Example: Count from Coulter = 7500. Therefore the number of cells/ml = $7.5 \times 10^3 \times 10^3 = 7.5 \times 10^6$.
3. Calculate the amount of cells needed. Need 2×10^5 cells per well. If you were to set up one row for each animal: (2×10^5 cells/well) (12 wells) = 2.4×10^6 cells required from each animal.
4. Calculate the total volume required. Use 200 ul/well. So: (0.2 ml/well) (12 wells) = 2.4 mls minimum needed. Add approximately 0.4 mls extra, so need 2.8 mls.
5. prepare medium.

   RPMI-1640 + 15% Fetal calf serum + 1% Penn strep + 1% Fungizone. (RPMI: Gibco, Grand Island NY; Fetal calf serum: Flow Labs Inc., McLean, VA and Antibiotic/antimycotic: Sigma Chemical Co., St. Louis, MO). Fetal calf serum is heated at 56^{o} for 30 minutes and diluted 1:4 with medium prior to use.
6. Calculate the amount of cell suspension you need to add to medium to get final concentration.

Example: Count from one animal 10 x 10^6 cells/ml and you want to set up one row, to calculate:

2.4/10 = .24 - 2.8 = 2.56

Cells needed divided by cells/ml equals ml of cell suspension you need to add to medium for final concentration. Subtract that amount from total volume desired to arrive at the amount of medium you need to mix up for that animal.

7. First mix up the medium solution in a tube or bottle. Add the appropriate amount of medium solution to a 12 x 75 mm snap-cap tube for each sample. Next, add the necesary amount of cell suspension from each animals into the appropriate tubes. Add one animal's cell at a time, gently invert tube to mix and then transfer 0.2 ml of suspension into each well.
8. After the cell suspensions have been added to the plates, the various mitogens are added to the wells. Phytohemmagglutin-P (PHA; Difco Labs, Detriot, MI) and added at a dilution of 1:100 prepared with medium; ConcanavalinA (ConA; Miles Labs, Elkhart, IN) was diluted 1:200 and Pokeweed mitogen (PWM; Gibco, Grand Island, NY) was diluted 1:10. Do each in triplicate. Add aliquots of 25 ul. Add 25 ul of medium to control plates to keep volume constant.
9. Place plates at 37 ^{o} C in a humidified 5% CO_2 atmosphere for approimately 48 hr.
10. Add .25 uCi of ^{3}H-thymidine (New England Nuclear, Boston, MA) to each well. Place back in incubator for approximately 18-20 hr.
11. Harvest cells with an automated cell harvester (Flow Laboratories,

McLean, VA), place filter pads in scintillation vials, add 10 ml CytoScint scintillation solution (ICN Biomedicals, Irvine, CA) and count for four minutes each.

APPENDIX D

Procedures for use of SBU-T1, SBU-T4, SBU-T8 and SBU-T19 Monoclonal Antibodies with Isolated Ovine Peripheral Lymphocytes

Keep all reagents ice cold.

1. Isolation ovine peripheral blood lymphocytes using above isolation procedures (The author's experiences show that Sepracell-MN works best since no red blood cell contamination is apparent). If red blood cells are a problem use the following solution:

   Red blood cell lysis solution:

   8.3 g ammonium chloride

   .037 g EDTA

   1.0 g Potassium Bicarbonate

   per 1.00 1 triple distilled water

   pH = 7.2

   Keep ice cold, use 1:1 with whole blood, gently invert until a color change is seen, centrifuge at 400g for 15 minutes, decant and wash white blood cell pellet three times with ice cold PBS/Az.

2. Count cells on a automated cell counter (Coulter Counter Electronics Hialeah, FA).

3. Adjust concentration with PBS containing 0.1% sodium azide (PBS/Az) and 0.5% BSA so that 1×10^6 cells/well can be plated in 50 ul of PBS/Az.

4. Plate enough cells so that each monoclonal can be done in duplicate. Use U-bottom microtiter plates. Prepare enough wells so that cells can be incubated with first and second antibodies only.

5. Prepared monoclonal antibody supernatant as directed by adding 5 ml distilled water (Dr. M.R. Brandon, University of Melbourne, Parkville, Victoria, Australia).
6. Add 50 ul of appropriate supernatant to each well and incubate at $4^{o}C$ for 30 minutes.
7. Wash cells twice with PBS/Az by centrifuging at 300 x g for 15 minutes.
8. Incubate cells for 30 minutes at $4^{o}C$ with 40 ul of a 1:10 dilution of a fluoresceinisothiocyanate (FITC)-conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulin (DAKO Corporation, Carpinteria, CA). Cover plates with aluminum foil so that no light can exhaust the fluorescence.
9. Wash cells 3 times with PBS/Az.
10. Resuspend in 200 ul if cells are to be run immediately, if not, resuspend in 200 ul 1% formalin (Electron Microscopy Sciences, Fort Washington, PA) and store at $4^{o}C$ overnight. Centrifuge and resuspend in PBS/Az prior to sorting.

APPENDIX E

Cytofluorographic Analysis Used in the Analysis of Ovine T-cell Monoclonal Antibodies

An EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FA) was used for detection of fluorescent stained cell. Cells were excited at 488 nm. Light passing to the fluorescence detector was split using a 488 nm dichroic filter. Fluorescence was collected through a 515 long pass filter and the presence or absence of a range of cell surface markers was determined. Only those cells containing the lymphocyte populations indicated by forward angle light scatter and 90^{o} light scatter histograms were analyzed for fluorescence using the EASY88 computer system with Intgra software (Coulter Electronics, Hialeah, FA). A minimum of ten thousand events were collected per sample. Monocytes and debris were excluded by gating out on the basis of forward angle light scatter and 90^{o} light scatter.

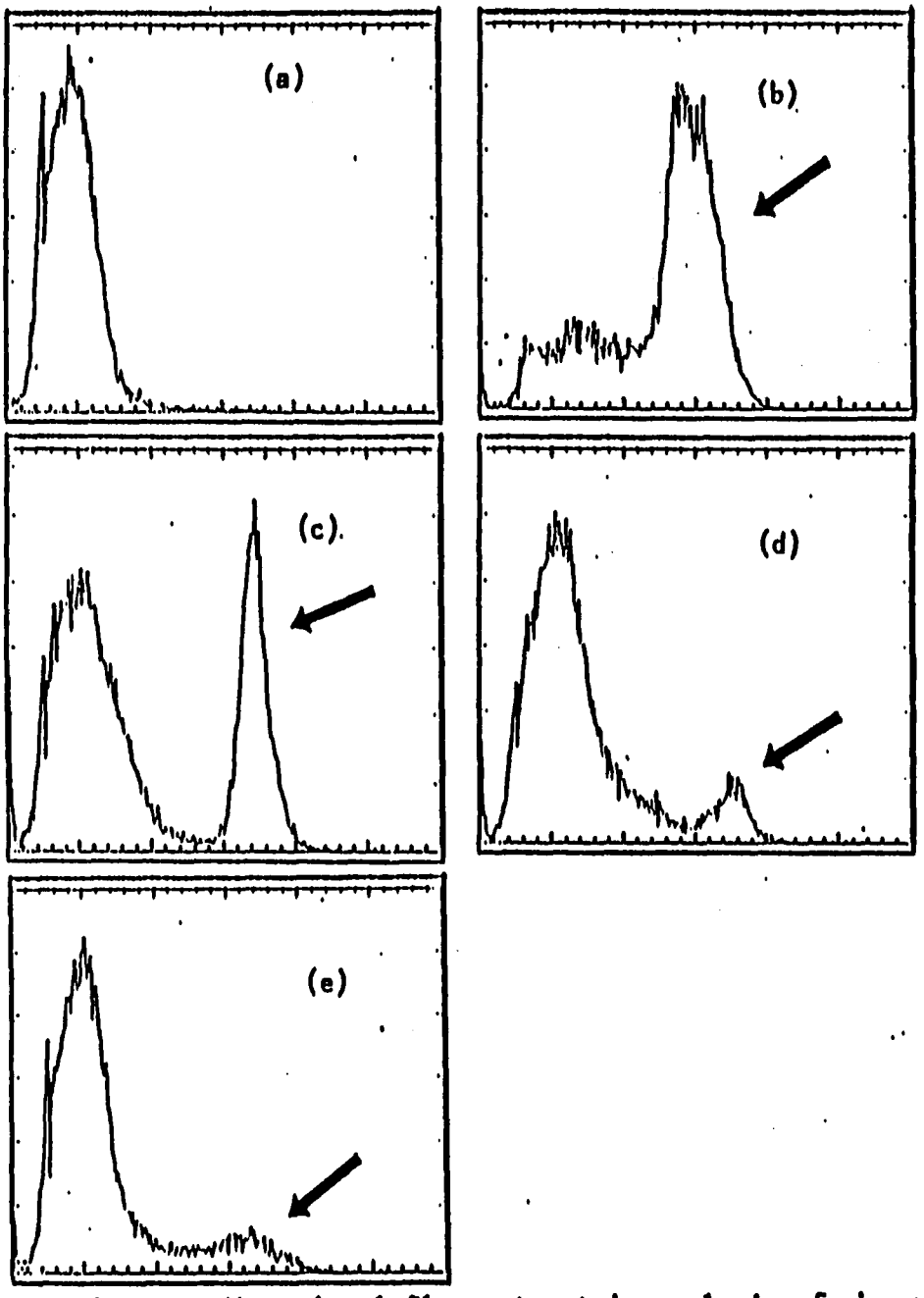

Figure 1. Two dimensional flow cytometric analysis of sheep peripheral blood lymphocytes labelled with the monoclonal antibodies SBU-T1, SBU-T4, SBU-T8 and SBU-T19. Sheep lymphocytes were reacted with monoclonal antibody supernatant and then with FITC-rabbit anti-mouse IgG. Fluorescence intensity is plotted on the X axis in arbitary units and cell frequency on the Y axis. The controls for each cell type consists of PBS/Az followed by FITC-conjugated second antibody and in each case shows only negligible fluorescence. Profiles are (a) control; (b) SBU-T1; (c) SBU-T4; (d) SBU-T8, and (e) SBU-T19.

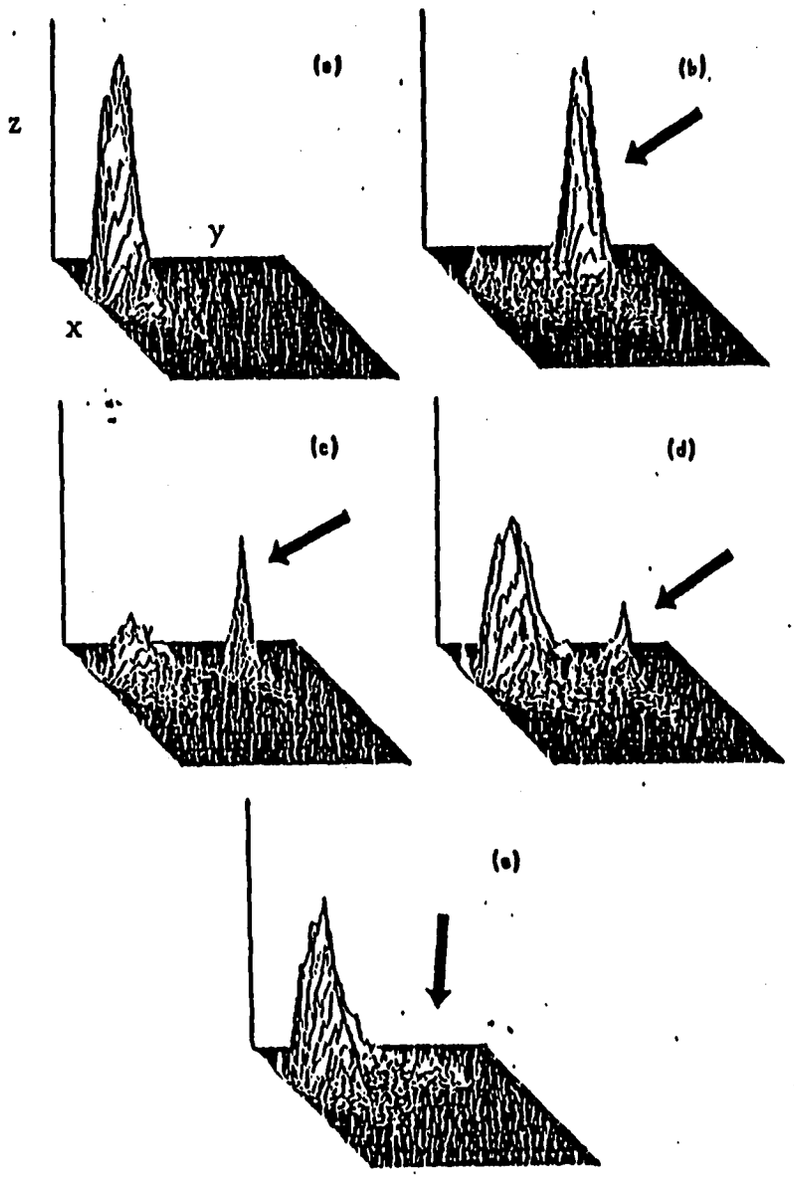

Figure 2. Three dimensional flow cytometric analysis of sheep peripheral blood lymphocytes labelled with the monoclonal antibodies SBU-T1, SBU-T4, SBU-T8 and SBU-T19. Sheep lymphocytes were reacted with monoclonal antibody supernatant and then with FITC-rabbit anti-mouse IgG. Forward angle light scatter as a relation to cell size is plotted on the X axis in arbitary units, relative fluorescence is plotted on the Y axis and relative cell number on the Z axis. The controls for each cell type consists of PBS/Az followed by FITC-conjugated second antibody and in each case shows only negligible fluorescence. Profiles are (a) control; (b) SBU-T1; (c) SBU-T4; (d) SBU-T8, and (e) SBU-T19.

APPENDIX F

Outline of β-Hydroxy-β-MethylButyrate Analysis by Gas Chromatograph/Mass Spectrometry (GC/MS) and Data Collected

1. Add sample (1 ml plasma or 1 ml standard) + 50 ul internal standard to a 50 ml glass culture tube.

Internal Standard

5 ul D^9 β-hydroxy-β-methylbutyrate

75 ml triple distilled water

2. Adjust pH < 2.0 with 3N HCl using pH paper. Vortex.
3. Add 30 ml diethyl ether (Fisher Scientific Co., Fair Lawn, NJ).
4. Vortex and centrifuge at 425g for 10 minutes.
5. Remove as much water layer as possible by aspirating.
6. Place culture tube into liquid nitrogen , placing tube at a depth just beyond the water layer, submerge until water layer and plasma proteins are frozen.
7. Transfer diethyl ether layer into a clean culture tube.
8. Add 1 ml 0.01M phosphate buffer, adjust pH ~ 7.0.

0.01M Phosphate Buffer

0.68 ml phosphoric acid/1 distilled water

Adjust pH ~ 7.0 with sodium hydroxide

9. Vortex and centrifuge at 425g for 10 min.
10. Transfer buffer layer at the bottom of the tube into a dram vial, allowing no ether to contaminate sample in vial.
11. Dry sample under nitrogen gas at 60 ^{o} C.
12. Add 100 ul of methylene chloride (Fisher Scientific Co., Fair Lawn,

NJ) to vial, dry under nitrogen gas at 60 ^{o} C. Repeat two more times.

13. Derivatize with 100 ul acetonitile (Regis Chemical Co., Morton Grove, IL) and 100 ul N-Methyl-N-(t-Butyldimethylsilyl) trifluoroacetamide (Regis Chemical Co., Morton Grove, IL).
14. Sonicate for 1 minute, then vortex thoroughly.
15. Heat for 1 hr at 60^{o} C.
16. Transfer into GC/MS vials and inject into gas chromatograph/mass spectrometer (Hewlett-Packard 5890/5790a using DATA:HMBSIM.M, which monitors 175, 178 and 183 AMU with injector @ 230 ^{o} C and detector @ 285^{o} C, and oven initially at 50^{o} C. Oven temperature initial temperature at 50^{o} C, ramp 1 at 20^{o} C/min to 170^{o}, hold for 0 min., and ramp 2 at 50^{o}/min to 270^{o} C, hold for 0 min. Retention time is roughly 5.5 min. Column is 25m x .22 mm i.d. x .11 um film thickness, crosslinked silicone gum phase capillary column (HP-1, Hewlett-Packard, Avondale, PN).

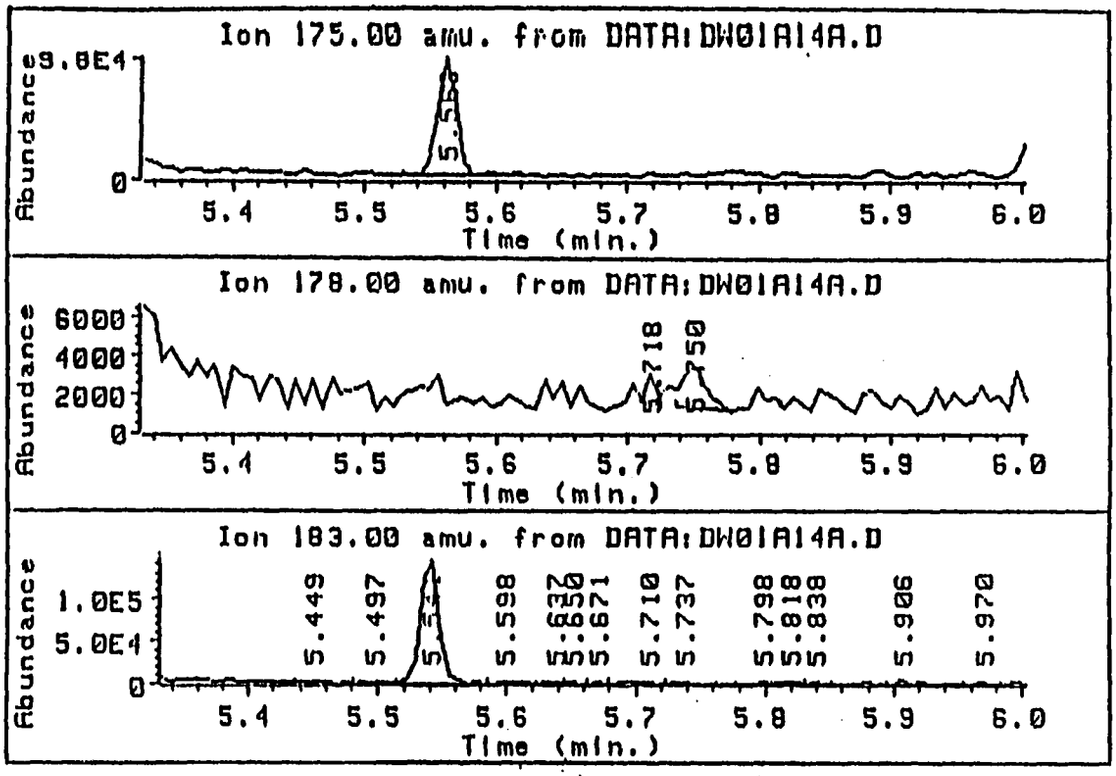

Figure 1. Selected ion monitoring (SIM) chromatographs of β-hydroxy-β-methylbutyrate with a Hewlett-Packard 5790a mass selective detector monitoring for masses 175, 178 and 183 AMU as described in the outline above

APPENDIX G

Plasma β-Hydroxy-β-MethylButyrate Concentrations Following Oral Supplementation of α-Ketoisocaproate in Sheep

Plasma samples were collected from sheep fed control, KIC or Leu supplemented diets for approximately 60 days in Experiments 1 and 2. GC/MS analysis was performed (see appendix F) to determine HMB concentrations.

β-Hydroxy-β-methylbutyrate

(Mean of 2 experiments)

(uM) ± SEM

Control	18.91 ± 4.49
KIC	31.86 ± 4.64
Leu	22.89 ± 4.64

KIC fed animals had higher plasma HMB concentrations than Leu fed or control animals. This difference was significant only in the first experiment ($P < 0.05$). A large difference existed in the values collected for HMB concentrations of the animals between the 2 experiments. This difference may be partially explained by the different genders of the animals or by the different environmental temperatures during the 2 experiments. However, the oral administration of KIC to sheep for 60 days results in elevated plasma HMB concentrations.