Role of Vitamin D in the Immune System

ABSTRACT
Classically, the only roles attributed to vitamin D have been regulation of intestinal calcium absorption and maintenance of skeletal homeostasis. This review examines the new evidence describing a regulatory role for vitamin D (1,25-dihydroxyvitamin D) in immune cell functions along with research that has linked immune cell functions with skeletal homeostasis. The possible significance of this evidence with respect to the parturient dairy cow is discussed.

INTRODUCTION
The primary recognized role for the steroid hormone 1,25-dihydroxyvitamin D [1,25-(OH)₂ D] is its regulation of calcium transport in its classical target tissues, which include intestine, bone, and kidney (18, 19, 38). It is well-established that the cellular actions of 1,25-(OH)₂ D in these tissues are mediated through a specific intracellular receptor protein (39). It was recently discovered that many tissues not thought to participate in calcium homeostasis possess specific receptors for 1,25-(OH)₂ D; this finding has required reconsideration of the role that 1,25-(OH)₂ D plays in the biology of the animal (39). In this review we will discuss evidence that suggests a role for 1,25-(OH)₂ D in the regulation of immune cell functions, thus expanding the close association that exists between the endocrine system and immune cell function to include vitamin D.

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Vitamin D Metabolism and Mechanisms of Action
Vitamin D₃ is a prohormone that becomes a required nutrient only with inadequate exposure to sunlight. Ultraviolet irradiation of skin converts 7-dehydrocholesterol to previtamin D₃. At body temperature, previtamin D₃ rapidly isomerizes to vitamin D₃, which then enters the blood (17). Dietary vitamins D₂ and D₃ are absorbed in the small intestine where they enter the lymphatic circulation. Vitamin D is then taken up by the liver and hydroxylated to form 25-hydroxyvitamin D (25-OHD). This is the major circulating form of vitamin D, and it is transported in the blood primarily associated with vitamin-D-binding protein. 25-Hydroxyvitamin D is then taken up by the kidney where it is further hydroxylated to form the steroid hormone 1,25-(OH)₂ D. Production of 1,25-(OH)₂ D is elevated with increasing demands for calcium and declines during calcium excess. During calcium excess, 25-OHD is converted predominantly to 24,25-(OH)₂ D, whose biological functions, if any, remain controversial. 1,25-Dihydroxyvitamin D is the active form of vitamin D and appears to be the sole mediator of the known biological actions attributed to vitamin D (18, 19, 38, 39).

The mechanism of action of 1,25-(OH)₂ D is virtually identical to that described for other steroid hormones (Figure 1). 1,25-Dihydroxyvitamin D circulates bound to plasma proteins, primarily vitamin-D-binding protein. A small portion of the hormone circulates in the free state and readily enters cells due to its lipophilic nature. Binding of 1,25-(OH)₂ D to its intracellular receptor results in the “transformation” of the receptor-hormone complex to a form that has increased affinity for nuclear components over that of the unoccupied

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receptor (66, 67). Specific DNA domain binding or acceptor site binding by the receptor-hormone complex results in the induction or repression of mRNA transcription. These events lead to specific alterations in protein synthesis, which modulate cell function. Specific examples of 1,25-(OH)2D-mediated changes in the synthesis of biologically important cell proteins are shown in Figure 1.

Receptors for 1,25-Dihydroxyvitamin D in the Immune System

Because all known biological effects of 1,25-(OH)2D are receptor-mediated, it is clear that cells of the immune system must possess receptors for 1,25-(OH)2D in order for 1,25-(OH)2D to modulate immune cell function. This possibility was first raised by the finding that calf thymus glands and lymph nodes possess 1,25-(OH)2D receptors indistinguishable from those described for classical target tissues of 1,25-(OH)2D (50). The concentration of 1,25-(OH)2D receptors in thymus and lymphatic tissue was about 10 to 20% of that found in intestine (Table 1), suggesting only a small percentage of cells in these tissues possess specific receptors for 1,25-(OH)2D. Provvedini et al. (43) and Ravid et al. (49) showed that 1,25-(OH)2D receptors were only present in large, mitotically active medullary thymocytes and that 1,25-(OH)2D receptors were absent in the small, immature cortical thymocytes.

Peripheral blood monocytes also possess 1,25-(OH)2D receptors, but peripheral blood T and B lymphocytes do not (9, 32, 45). These authors showed, however, that in vitro activation of peripheral lymphocytes with phytohemagglutinin (PHA), Concanavalin A (Con A), or pokeweed mitogen (PWM) resulted in rapid expression of the 1,25-(OH)2D receptor by the lymphocytes, which peaked by d 2 poststimulation and declined thereafter (46). However, in
TABLE 1. Tissue concentration of specific 1,25-dihydroxyvitamin D binding.

<table>
<thead>
<tr>
<th>Tissue (mucosa)</th>
<th>(mg/g tissue)</th>
<th>Specific 1,25-(OH)(_2)D binding</th>
<th>(pmol/mg DNA)</th>
<th>(pmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>9.3 .4</td>
<td>(\bar{X}) .68 .12 .6.2 .4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>24.1 1.2</td>
<td>(\bar{X}) .05 .01 1.2 .1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>11.3 1.4</td>
<td>(\bar{X}) .05 .01 .5 .1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

cows, peak 1,25-(OH)\(_2\)D receptor expression is delayed to d 3 or 4 poststimulation and declines much more slowly (Figure 2). The peak concentration of 1,25-(OH)\(_2\)D receptor expressed by mitogen-stimulated lymphocytes approaches the concentration of receptor we found in classical target tissues such as intestine (unpublished observations).

The expression of 1,25-(OH)\(_2\)D receptors in B cells also occurs in vitro following transformation by Epstein Barr virus as well as in normal lymphocytes activated by alloantigens in the mixed lymphocyte reaction (32, 45, 47). It is now clear that the expression of receptor in activated lymphocyte is not just an in vitro phenomenon. 1,25-Dihydroxyvitamin D receptors are present in thymus, lymph nodes, and tonsils (32, 43, 49, 50). In fact, the lymphocytes activated in vivo that circulate in people with rheumatoid arthritis possess the 1,25-(OH)\(_2\)D receptor (32). Many established promonocytic, monocytic, and macrophage precursor cell lines, as well as normal peripheral monocytes, express the 1,25-(OH)\(_2\)D receptor and respond to 1,25-(OH)\(_2\)D stimulation via differentiation or activation (1, 2, 35, 61).

Expression of 1,25-(OH)\(_2\)D receptor in lymphocytes, monocytes, and malignant cell lines of lymphoid origin suggests that the 1,25-(OH)\(_2\)D may be of general importance to these cells for activation, mitosis, and differentiation.

Vitamin D and Immune Function

Clinical observations on the effect of childhood rickets (vitamin D deficiency) suggested a possible role for vitamin D in immune cell function several years prior to the explosive growth of this research area. Rickets from vitamin D deficiency is often accompanied by increased rates and severity of infections (58). The reasons for this have not been studied in detail, and conclusions about the effects of human vitamin D deficiency effects on immune functions are clouded primarily because childhood rickets is a disease of families of lower socioeconomic status who already have increased susceptibility to infectious disease. However, monocytes from ricketic children have reduced ability (30 to 40%) to phagocytize Escherichia coli when compared with age-matched controls (59). Neutrophils from ricketic children had defective motility (30). Anemia and decreased bone marrow cellularity have been reported in humans and rats with severe vitamin D deficiencies. Administration of vitamin D leads to rapid improvement of these

Figure 2. Expression of 1,25-dihydroxyvitamin D receptors in bovine peripheral blood mononuclear leukocytes as a function of days in culture and mitogen. Con A = Concanavalin A, PWM = pokeweed mitogen.

defects (68, 69). Studies in rats and mice have shown that vitamin D deficiency resulted in impaired macrophage function, which can be reversed by 1,25-(OH)₂D₃ treatment (7). Also, vitamin D deficiency in rats results in decreased bone marrow cellularity and a time-dependent reduction in spleen colony-forming units (68).

It is clear that these in vivo data are limited and represent extremes in vitamin D status. Thus, to understand better the possible functions of 1,25-(OH)₂D in the cell, we will examine the in vitro data relating 1,25-(OH)₂D to specific effects on immune cell function.

Monocyte and Macrophage Functions and 1,25-Dihydroxyvitamin D

Physiologic doses of 1,25-(OH)₂D promote differentiation of several mono- and promonocyte cell lines toward a macrophage phenotype while decreasing the rate of cell proliferation (Figure 3) (1, 2, 35, 61). The criteria for differentiation and specialization utilized in these studies were morphologic changes, increased adherence to plastic plates, decreased growth rates, induction of phagocytosis, increased lysosome enzyme activity, appearance of nonspecific esterase, and expression of cell

Figure 3. General model depicting the regulatory role of 1,25-dihydroxyvitamin D in immune cell function.

membrane surface receptors for immunoglobulin (Fc) and complement (C3) (5, 14, 40). The 1,25-(OH)2 D-mediated phenotypic changes seen in these cells were preceded by decreased expression of the c-myc oncogene (51, 63).

Evidence for similar in vivo effects of 1,25-(OH)2 D on cell differentiation and growth have been obtained from study of the survival of syngenic SL mice and nude mice inoculated with M-1 cells (2). Animals treated with either 1,25-(OH)2 D3 or 1α-hydroxyvitamin D3 (1α-OHD3) survived an average 50% longer than vehicle-treated controls. These results are attributed to a 1,25-(OH)2D3-mediated increased rate of M-1 cell differentiation to a nondividing cell with a macrophage phenotype.

Important to the demonstration that 1,25-(OH)2 D affects monocyte function is the demonstration that these effects are receptor-mediated. Cells in the model systems used in the studies mentioned expressed specific receptors for 1,25-(OH)2 D. More important, the ability of different vitamin D analogs to promote cell differentiation correlates positively with their affinity for the 1,25-(OH)2 D receptor. Additional evidence that 1,25-(OH)2 D-mediated cell differentiation is receptor-dependent comes from studies utilizing clones of the promonocyte cell line HL-60, which express different amounts of 1,25-(OH)2 D receptor (27, 33). Cells that contain less than 400 receptors per cell are resistant to the actions of 1,25-(OH)2 at physiological doses of hormone, whereas those with 4000 receptors per cell are fully responsive to the actions of 1,25-(OH)2 D.

1,25-Dihydroxyvitamin D also increases both specific and nonspecific functions of macrophages associated with the defense of the host in addition to phagocytosis already discussed. 1,25-Dihydroxyvitamin D3 enhances the cytotoxicity of pulmonary macrophages (2). It also enhances the competence for H2O2 secretion in monocytes isolated from normal humans and from the cell line U937 (12, 55). The 1,25-(OH)2 D3 effect on H2O2 secretion at 10^-8 M was equivalent to that of cells exposed to 1000 U/ml γ-interferon. The authors suggested that the concentrations of 1,25-(OH)2 D that circulate in vivo may permit maintenance of normal competence for H2O2 secretion, which is thought to be important in microbicidal and tumoricidal activity of these cells.

Finally, recent evidence suggests that 1,25-(OH)2 D3 enhances the expression of Class II major histocompatibility antigens (Ia antigens) (36). Because these molecules mediate antigen presentation to lymphocytes, 1,25-(OH)2 D might promote monocytes to function more efficiently as antigen-presenting cells.

Much of the evidence for a 1,25-(OH)2 D3 effect on monocyteic function has come from data obtained in transformed cell lines. Recent evidence suggests that normal monocytes respond in vitro to 1,25-(OH)2 D in a manner similar to that of cell lines (44). In normal monocytes, 1,25-(OH)2 D induces morphological changes as well as accelerated differentiation, increases in lysosomal enzymes, enhances adherence, accelerates cell fusion, increases synthesis of heat shock proteins, and controls the proliferation of intracellular Mycobacterium tuberculosis in monocytes (2, 42, 44, 54, 60).

Taken together, these data suggest that 1,25-(OH)2 D may play both a permissive and active role in monocyte differentiation and function; however, detailed in vivo studies will be required to address this hypothesis.

Effects of 1,25-Dihydroxyvitamin D on Thymocyte Function

There is little information available regarding the effect of 1,25-(OH)2 D on thymic function, although the distribution of 1,25-(OH)2 D receptor in thymic tissue suggests any effects will be cell specific and limited to medullary thymocytes (43, 49). The most interesting phenomenon yet described is that 1,25-(OH)2 D slows the spontaneous lytic involution seen normally in cultured medullary thymocytes (32). Ravid et al. (49) reported that 1,25-(OH)2 D inhibits the mitogenic response of cultured thymocytes to PHA, interleukin-2 (IL-2), and interleukin-1 (IL-1) (25, 43, 49). They also showed that 1,25-(OH)2 D had no effect on the receptor negative cortical thymocytes, as would be expected. Although the physiological significance of these observations is unknown, research is needed to examine possible roles for 1,25-(OH)2 D in thymic involution and T-cell differentiation.

Effects of 1,25-Dihydroxyvitamin D on Lymphocyte Function

The activation of lymphocytes by either polyclonal mitogens or specific antigens results
in the production and release of lymphokines, such as IL-2, along with the expression of IL-2 receptors on the T-lymphocytes. These activated lymphocytes proliferate and differentiate into effector T-cells whose functions include cytotoxicity and help or suppression of B-cell antibody production.

The discovery that activated lymphocytes express large amounts of the 1,25-(OH)₂D receptor lead to a series of studies examining the effects of 1,25-(OH)₂D₃ on lymphocyte lymphokine production, proliferation, and differentiation (10, 11, 52, 53, 64). These studies demonstrated that 1,25-(OH)₂D is a potent inhibitor of IL-2 production by PHA-activated human and murine peripheral lymphocytes. Order of potency of various vitamin D metabolites on inhibition of IL-2 production correlated with their affinity for the 1,25-(OH)₂D receptor, thus indicating that 1,25-(OH)₂D inhibition of IL-2 synthesis is a receptor-mediated event (32).

The IL-2 is important in lymphocyte proliferation; therefore, it was expected that 1,25-(OH)₂D₃ would also affect lymphocyte proliferation. In fact, several laboratories have shown that 1,25-(OH)₂D at 10⁻¹¹ to 10⁻⁹ M markedly inhibits mitogen-stimulated lymphocyte proliferation. The inhibitory effect of 1,25-(OH)₂D on lymphocyte proliferation is due to a marked inhibition of IL-2 production 24 h earlier (32). We have shown an antiproliferative effect of 1,25-(OH)₂D on mitogen-activated bovine lymphocytes (Figure 4). Paradoxically, we have also shown that the 1,25-(OH)₂D₃ effect on Con A-stimulated bovine lymphocytes is biphasic. The hormone is antiproliferative at low doses and promotes proliferation at moderate doses. This suggests that a subpopulation of lymphocytes are specifically activated by Con A, which responds differently to 1,25-(OH)₂D, or that moderate doses of hormone have a secondary effect on monocytes, leading to increased IL-1 production. The significance of this observation is under study.

Cell cycle analysis has been utilized to characterize better the antiproliferative effect of 1,25-(OH)₂D₃ on lectin-activated lymphocytes. Rigby et al. (53) and Provvedini et al. (46) have shown that 1,25-(OH)₂D₃ blocks the transition of these cells from the early low RNA compartment G₁₀ to the high RNA compartment G₁₅ without affecting IL-2 receptor expression. Exogenous IL-1 was unable to overcome this block; however, both indomethacin and IL-2 slightly reversed the effects of 1,25-(OH)₂D₃. These results indicate a prostaglandin component to 1,25-(OH)₂D₃ effects and suggested that part of antiproliferative effect of 1,25-(OH)₂D₃ results from enhanced prostaglandin E₂ production by monocytes (53).

In addition to 1,25-(OH)₂D effects on IL-2 production and cell proliferation, evidence is accumulating for 1,25-(OH)₂D effects on B-cell and effector cell function (28, 29). Several reports suggest 1,25-(OH)₂D₃ inhibits lectin, T-cell-dependent antigens, and Epstein Barr virus (EBV)-induced immunoglobulin production (20, 26, 47, 56). Exogenous IL-2 could not reverse 1,25-(OH)₂D inhibition of immunoglobulin production by EBV-activated B-cell, suggesting again that 1,25-(OH)₂D inhibitory effects are mediated through complex interaction of cell types (47). Little information is available describing 1,25-(OH)₂D effects on effector cells. However, evidence has been presented demonstrating that 1,25-(OH)₂D₃ decreases both the development of cytotoxic and helper and inducer T-cells (29, 32). Whether 1,25-(OH)₂D directly affects B cell or effector cell function or is the result of the antiproliferative effects of 1,25-(OH)₂D remains to be determined.
Bone Resorption and Cells of Immune System

Another concept important to the role of vitamin D in immune cell function is the hypothesis that cells of the monocyte and macrophage lineage serve as precursors to bone-resorbing cells, the osteoclasts, and that lymphocytes or monocytes produce osteoclast-activating factors (OAF), which may be important either for normal bone resorption or in pathological conditions associated with high resorption rates (6, 22, 62, 63). Early theories on the origin of osteoclasts were based on the idea that bone cells (osteoblasts, osteocytes, and osteoclasts) were derived from a common progenitor cell (48). Recent studies suggest that osteoclasts are of hemopoietic origin (31, 34), as first suggested in 1925 (21), and are related to macrophages, as earlier suggested (15, 16). A large body of evidence suggests that osteoclasts originate from a bloodborne precursor cell and not from a local marrow-derived precursor. This hypothesis comes from a long series of experiments of osteoclast defects that could be corrected by transplantation of blood, thymus, or spleen cells from normal animals into affected animals (31, 34). Because macrophages readily fuse to form multinucleated cells and fusion can be accelerated by 1,25-(OH)2D3, Abe et al. (2) and Tanaka et al. (60) hypothesized that 1,25-(OH)2D3 may cause the fusion of osteoclast precursors to form osteoclasts in a similar fashion. Supporting evidence includes the finding that macrophages possess calcitonin receptors, as do osteoclasts. Monocytes and macrophages can resorb bone directly in culture, but macrophages from vitamin D-deficient rats lose their ability to resorb bone in vitro (8, 62). This defect cannot be corrected in vitro by adding 1,25-(OH)2D; however, macrophages regain their ability to resorb bone in vitro if that rat’s vitamin D deficiency is corrected (62). This parallels evidence suggesting 1,25-(OH)2D does not act directly on osteoclasts but acts through another mechanism. Furthermore, monocytes regulate the production of IL-1 and the lymphokine OAF, two potent mediators of bone resorption (37).

Evidence against osteoclasts being derived from cells of monocytic origin include 1) macrophages in contact with bone lack a ruffled border at the bone cell interface, as seen in osteoclasts; 2) macrophages do not show a morphological response to calcitonin as do osteoclasts; 3) osteoclasts lack C3 receptors found on macrophages; and 4) transplanted macrophages do not correct the resorption defect associated with osteopetrosis (65). However, macrophages are a heterogeneous population of cells in various stages of differentiation, so attempts to confirm or refute the relationship between macrophages and osteoclasts require experiments utilizing many phenotypic and functional markers. It should be pointed out that lack of certain common markers does not preclude the loss or capping of a large number of macrophage-like markers as cells differentiate to an osteoclast phenotype. Recent evidence demonstrates this problem. Until recently, lack of the Fc receptor on osteoclasts was used as evidence against the kinship of monocytes. However, reexamination of osteoclasts for the presence of the Fc receptor demonstrated this receptor on osteoclasts at the cell bone interface and that these receptors are capped (41).

The most compelling evidence for a monocyte and osteoclast precursor relationship was provided by the results of a clinical study by Key et al. (23). They gave an infant suffering from osteopetrosis (defective osteoclast function) high doses of 1,25-(OH)2D3 for 3 mo. Although no active osteoclasts were observed at biopsy prior to treatment, numerous active osteoclasts were present in biopsy taken at the end of treatment. Most importantly, this infant’s monocytes were incapable of resorbing bone in culture prior to treatment. However, following 1,25-(OH)2D therapy, in vitro monocyte bone resorption capacity increased to greater than three times control levels.

Horst (18) recently suggested that the osteoclastic response in milk fever is similar to that observed in congenital osteopetrosis. A component of the etiology of milk fever may represent an acute nutritional osteopetrosis similar to the chronic form seen in bulls fed high calcium diets. Thus, one might hypothesize that milk fever might be associated with decreased numbers or a malfunction of proposed osteoclast precursors, the monocytes. There have been published reports of decreased monocyte numbers in cows at parturition (57). However, monocyte function at parturition has not been described. Preliminary studies in our
laboratory have examined the ability of bovine monocytes to resorb bone in vitro during late pregnancy, parturition, and postpartum. Early findings show a marked decline in monocyte-mediated bone resorption at parturition in the Jersey cow, which is prone to milk fever. Further research will be required to determine if these data represent a real cellular defect that contributes to the development of milk fever.

The interested reader is referred to detailed reviews of the evidence linking cells of the immune system to skeletal homeostasis (6, 22).

1, 25-Dihydroxyvitamin D3 and Immunology in the Dairy Cow

The actual role that 1,25-(OH)2D plays in infectious disease states of the dairy cow is only a matter of conjecture at this point. However, it is clear from the available evidence utilizing in vitro and in vivo experimental models that 1,25-(OH)2D permits and perhaps stimulates the nonspecific components of the immune response, which are mediated by macrophages (27).

One problem in developing an understanding of any 1,25-(OH)2D regulatory role in immune cells is that 1,25-(OH)2D concentrations in the microenvironment of an immune response must be regulated or maintained independently of systemic (plasma) concentration of the hormone. This paradox may have begun to be resolved by recent findings that 1,25-(OH)2D can be produced from 25-(OH)2D by cells involved in inflammatory processes and cells associated with disorders such as sarcoidosis (3, 4). Specifically, 1,25-(OH)2D production can occur in monocytes and macrophages, and γ-interferon increases production of 1,25-(OH)2D (24). These data suggest that 1,25-(OH)2D may be a monokine and 1,25-(OH)2D concentrations might be regulated in the microenvironment of an immune response independently of the systemic regulation of its concentration. Rigby et al. (53) hypothesized that 1,25-(OH)2D3 may act in vivo in an inhibitory feedback loop to suppress T-cell proliferation once adequate γ-interferon is produced by activated lymphocytes in the presence of activated macrophages. If this hypothesis is correct, then factors that dramatically alter systemic 1,25-(OH)2D3 production (such as vitamin D deficiency, calcium deficiency, or the varying degrees of hypocalcemia associated with parturition in all dairy cows) could dramatically affect immune function by overwhelming microenvironmental changes in 1,25-(OH)2D3, which influence immune cell function.

The concept that local 1,25-(OH)2D production by monocytes is important for immune function has its greatest potential impact in the parturient dairy cow. Virtually all dairy cows suffer some degree of hypocalcemia postpartum, and hypocalcemia is associated with large increases in systemic concentrations of 1,25-(OH)2D (19). This increase in systemic concentration of 1,25-(OH)2D happens at a time when lymphocyte function in these animals has already been suppressed for unknown reasons for several days (Figure 5). The exposure of this animal to an infectious agent (antigens) during this time will result in rapid expression by lymphocytes of receptors for 1,25-(OH)2D. Thus, lymphocytes with receptors for 1,25-(OH)2D may be exposed to large amounts of 1,25-(OH)2D early in the immune response, possibly resulting in a decreased capacity to respond due to the well documented antiproliferative effects of 1,25-(OH)2D on lymphocytes. One might speculate that the immunosuppressive effects of 1,25-(OH)2D might explain the finding that cows with clinical milk fever are more susceptible to infectious diseases, such as mastitis (13). Concomitantly, cows that do not develop clinical milk fever, but experience hypocalcemia with increased systemic 1,25-(OH)2D concentrations, will also be

Figure 5. Peripheral blood bovine lymphocyte blastogenesis during late pregnancy and early parturition. Con A = Concanavalin A, PHA = phytohemagglutinin, PWM = pokeweed mitogen.
immunosuppressed but to a lesser extent. Research is in progress that examines the practical consequences of elevated systemic 1,25-(OH)₂D on immunocompetence in the hypocalcemic parturient cow.

Conclusion

The field of vitamin D metabolism and function has realized an explosion of new information in relation to nutrition and physiology. The recent addition of the immune system to the list of vitamin D functions adds to the list of nutrients and hormones whose functions are important to the immunology of the animal. Recognition of the importance of nutrition in the maintenance of normal immune functions is likely to increase in importance as our cows' nutrient requirements rapidly change with the large increases in milk production expected with the practical application of growth hormone technology and other biotechnology developments.

REFERENCES

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