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INHIBITION OF BOVINE PERIPHERAL BLOOD MONONUCLEAR CELL BLASTOGENESIS BY MAMMARY SECRETIONS

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

Effects of bovine mammary secretions obtained at various stages of the lactation cycle on bovine peripheral blood mononuclear cell blastogenesis were evaluated in Part II using concanavalin A and phytohemagglutinin as mitogens. In Part III, response of blood mononuclear cells to mitogens throughout the dry period was determined at drying off, 14-16 and 28-30 d post drying off, 14-12 d prior to parturition and at parturition. Concanavalin A, phytohemagglutinin, and Escherichia coli O111:B4 lipopolysaccharide were used as mitogens. Effects of fractionated and whole mammary secretion whey on bovine peripheral blood mononuclear cell blastogenesis were determined in Part IV. Concanavalin A and phytohemagglutinin were used as mitogens. Mammary secretions were collected from four cows 16-2 d prior to parturition and fractionated by gel filtration chromatography. In all studies, mononuclear cells were isolated from bovine blood by density gradient centrifugation. Mononuclear cell blastogenesis was evaluated by methyl-^{3}H-thymidine incorporation.

In Part II, all secretions markedly inhibited mononuclear cell blastogenesis compared to stimulated controls. Normal milk caused the least inhibition of blastogenesis. Colostrum and secretions obtained 7 d post drying off were associated with greatest inhibition of mononuclear cell blastogenesis. Mammary secretion wheys caused greater inhibition than mammary secretion skims. Phytohemagglutinin-stimulated cell blastogenesis was less suppressed by mammary secretions than concanavalin A-stimulated blastogenesis.

In Part III, stimulation of mononuclear cells by concanavalin A increased slightly through 28 d post drying off then decreased to its lowest point at parturition. Phytohemagglutinin-induced blastogenesis decreased consistently throughout involution and was lowest at parturition. Lipopolysaccharide-induced blastogenesis increased from drying off to mid-dry period. However, due to lack of mononuclear cell response to lipopolysaccharide compared to concanavalin A and phytohemagglutinin, use of lipopolysaccharide was discontinued.

Mammary secretion whey collected 16-2 d prior to parturition markedly depressed the response of peripheral blood mononuclear cells to mitogens in Part IV. Mammary secretion whey eluted from column chromatography in four major groups designated protein peaks 1, 2, 3 and 4. Concanavalin A-stimulated mononuclear cell blastogenesis was most inhibited by concentrated preparations of protein peak 1. Phytohemagglutininstimulated mononuclear cell blastogenesis was most inhibited by concentrated preparations of protein peak 2. Unconcentrated protein peak 2 was associated with greatest inhibition of both concanavalin A- and phytohemagglutinin-stimulated mononuclear cell blastogenesis. Protein peaks 3 and 4 inhibited mononuclear cell blastogenesis to a lesser degree than protein peak 2. Phytohemagglutinin-stimulated cells were less inhibited by both whole and fractionated mammary secretion whey than concanavalin A stimulated cells.

Insensitivity of phytohemagglutinin-stimulated cells compared to concanavalin A-stimulated cells to blastogenic inhibition by mammary secretions in Part II and fractionated and whole mammary secretion

whey in Part IV may reflect different subpopulations of T-lymphocytes in bovine blood. Variation in response of mononuclear cells to concanavalin A and phytohemagglutinin throughout involution in Part III may also support this hypothesis. Results of Part II indicated that mammary secretions collected during physiological transitions of the mammary gland were most inhibitory to bovine peripheral blood mononuclear cell blastogenesis. Suppression of mitogen-induced mononuclear cell blastogenesis at parturition observed in Part III may have been caused by hormones and other factors associated with impending parturition and lactation. Variation in inhibition of concanavalin A- and phytohemagglutinin-induced mononuclear cell blastogenesis by whey fractions in Part IV may reflect different immunosuppressants in mammary secretions with different specificities for subpopulations of cells stimulated by concanavalin A and phytohemagglutinin. Bovine mammary gland mononuclear cells are derived from peripheral blood. Suppression of mononuclear cell blastogenesis by mammary secretions in the gland may contribute to observed variation in rates of new intramammary infection during involution, especially during physiological transitions of the gland.

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PART I

LITERATURE REVIEW

INTRODUCTION

Of all cattle diseases of importance to the dairy producer, bovine mastitis causes the greatest economic loss. Economic losses from mastitis include reduced milk production, discarded milk, cost of veterinary services and cost of drugs (Blosser, 1979). Reduced milk production accounts for the greatest percentage of loss due to prevalence of subclinical mastitis (Blosser, 1979). Control of mastitis is hampered by the wide range of causative organisms, limited understanding of mechanisms of establishment of intramammary infection, and inability to manipulate the immunological capabilities of the mammary gland (Smith, 1983).

Total elimination of mastitis is unlikely due to the wide variety of organisms capable of causing the disease. The most common organisms associated with mastitis include Streptococcus agalactiae, Strep. dysgalactiae, Staphylococcus aureus and environmental pathogens such as Strep. uberis and coliform bacteria (McDonald, 1979). Environmental pathogens are so named because they are derived from sources external to the cow. Possible methods for mastitis control include immunization, antibiotic dry cow therapy, breeding of resistant cows, or improved management procedures (Dodd, 1983). Researchers have been successful in increasing antibody (Ab) titres in the bovine mammary gland against specific antigens (Lascelles, 1979; Chang et al., 1981). However, efforts to immunize the mammary gland have been mostly unsuccessful in terms of preventing mastitis due to: a) wide variety of organisms capable of causing intramammary

infection (IMI) compared to specificity of antigen-antibody reactions, b) relative lack of components of immune defense in the mammary gland, c) vast surface area of mammary secretory epithelium requiring protection, and d) ability of milk to sustain bacterial growth (Colditz, 1985).

Improved management and dry cow therapy have been the most successful means of controlling mastitis to date. Implementation of postmilking teat dipping, improved sanitation procedures, use of antibiotic dry cow therapy, and advances in milking machine research have made possible the reduction or elimination of IMI by Staph. aureus, Strep. agalactiae and Strep. dysgalactiae (Philpot, 1979; Bramley and Dodd, 1984). Unfortunately, mastitis caused by environmental pathogens is not controlled by methods successful in reducing IMI caused by non-environmental pathogens (Bramley and Dodd, 1984).

Selection of resistant cows may be a promising field of research in the control of mastitis. Heritable factors such as teat canal patency, intramammary leukocyte count, and humoral immune response are important in resistance to IMI (Poutrel, 1982). However, improvement of herd resistance to mastitis by selection is a slow process and is further complicated by lack of knowledge about specific gene markers involved in resistance to infection (Poutrel, 1982).

Current methods of mastitis control are relatively ineffective against environmental pathogens. It is generally agreed that environmental streptococci are of economic significance (Dodd,

1983). However, coliform infections have been considered of little economic significance. Coliform infections tend to be of short duration in comparison to infections caused by Strep. agalactiae and Staph. aureus (Eberhart et al., 1979). In addition, small (<100) numbers of colony forming units (cfu)/ml appear sufficient to establish coliform infection (Smith, 1983). Standard procedures for diagnosis of bovine mastitis require a minimum of 100 cfu/ml of milk isolated from two consecutive milk samples (Brown et al., 1981). It is likely that occurrence of IMI by coliform bacteria is greatly underestimated, especially in light of the fact that the majority of cases of coliform mastitis do not become acute (Smith, 1983). Antibiotics approved for treatment of bovine mastitis are not effective against coliform infections (Eberhart et al., 1979). In addition, current trends in dairy management tend to increase exposure of teat ends to coliforms and other environmental pathogens, thereby increasing risk of infection.

Perhaps the most obvious approach to the control of bovine mastitis is to increase natural resistance of the mammary gland to infection. The mammary gland and its secretion contain several naturally occurring factors which contribute to defense of the gland against IMI. Ability to manipulate defense mechanisms of the gland may prove effective in reduction of IMI, especially those caused by environmental pathogens. During mammary gland involution, concentrations of protective factors increase markedly, making the dry period a logical time to enhance the natural resistance of the mammary gland.

NON-CELLULAR DEFENSES OF THE MAMMARY GLAND

Mammary gland defense is mediated by a wide variety of protective factors operating independently and in concert to provide protection against IMI. These defense mechanisms span a range of humoral and cellular factors, and constitute both specific and nonspecific defenses. Certain defense factors in the gland serve dual roles, protecting the mammary gland against infection and providing the neonate with temporary passive immunity.

Teat duct defenses

The teat end constitutes the first line of defense against IMI (Craven and Williams, 1985). A direct correlation between tightness of closure of teat end sphincter muscles and incidence of IMI has been demonstrated (Murphy, 1944). Increased teat duct patency may provide access to and transport through the teat duct for microorganisms (Cousins et al., 1980; Craven and Williams, 1985). Keratin in the teat duct may also play a role in preventing IMI. Teat ducts susceptible to IMI have been shown to have wider diameters and thinner keratin layers than resistant ducts (McDonald, 1970). Removal of keratin from the teat duct increased susceptibility to IMI (Murphy, 1959). Keratin may also possess bactericidal properties. Certain fatty acids and basic proteins which are constituents of keratin can inhibit microbial growth *in vitro* (Hibbitt and Cole, 1968; Hibbitt et al. 1969). In contrast, whole keratin has no antibacterial activity *in vitro* (Forbes,

1970a). Recent studies demonstrated the presence of intraepithelial lymphocytes and plasma cells in teat end tissue (Nickerson et al., 1984). Localized release of immunoglobulin (Ig) and lymphokines in response to antigen in the teat duct would greatly enhance the ability of the mammary gland to prevent bacterial penetration and subsequent colonization.

Lysozyme

Lysozyme is an enzyme which cleaves peptidoglycans from cell walls of Gram-positive organisms and outer membranes of Gram-negative organisms (Reiter, 1978). Wardlaw (1962) postulated that lysozyme interacts with complement and Ig to lyse Gram-negative organisms. Lysozyme may play a role in enhancing binding of lactoferrin to bacterial cell walls (Schanbacher and Smith, 1975). Intestinal and alveolar tissues, tears, saliva, milk, monocytes, macrophages and polymorphonuclear neutrophils (PMN) contain lysozyme. However, levels of lysozyme in bovine milk are about 300 times less than in human milk (Chandran et al., 1964).

Lactoperoxidase/thiocyanate/hydrogen peroxide system

Growth of certain species of streptococci which produce lactic acid is inhibited by the lactoperoxidase/ thiocyanate/hydrogen peroxide (LP/SCN-/H_2O_2) system (Reiter et al., 1963). Lactoperoxidase is always present in milk (Poutrel, 1982). However, the level of SCN- in mammary secretion depends upon plane of nutrition (Poutrel, 1982) and the source of H_2O_2 is variable.

Certain streptococci produce endogenous H_2O_2. In the case of Escherichia coli and other catalase negative organisms, exogenous H_2O_2 may be required, either cellular (neutrophilic) or enzymatic in origin (Poutrel, 1982). Low oxygen tension in the mammary gland may limit production of H_2O_2 in vivo, thus limiting effectiveness of the LP/SCN-/H_2O_2 system in the mammary gland.

Complement

Low levels of hemolytic complement have been detected in the mammary gland at all stages of lactation (Poutrel and Caffin, 1983). All components of complement have been detected in colostrum (Brock et al., 1975), though total complement activity seems variable in the lactating gland. Mueller et al. (1982) demonstrated a high correlation between serum albumin concentration and levels of the third component of complement (C3) during inflammation of the mammary gland. Components of complement possessing chemotactic properties may exist in high enough concentrations in the mammary gland during involution and inflammation to play a biological role in recruitment of PMN at these times (Craven and Williams, 1985).

Lactoferrin

Lactoferrin (Lf), an iron binding protein composed of a single chain glycoprotein with a molecular weight of approximately 78,000, is located in certain biological fluids and specific granules of PMN (Querinjean et al., 1971). Lactoferrin binds two molecules of iron per molecule of Lf and retains affinity for iron down to pH 2.0

(Castellino et al., 1970). Bicarbonate is required for binding of iron to Lf (Masson and Heremans, 1968). As Lf becomes iron-saturated it assumes a characteristic reddish-pink color, resulting in the original designation of Lf as the "red protein" of milk (Groves, 1960). Lactoferrin is bacteriostatic by virtue of its high iron affinity, causing greatest growth inhibition of microorganisms such as staphylococci and coliforms which have high iron requirements (Bishop et al., 1976; Bullen et al., 1978). The bacteriostatic effect of Lf can be reversed by citrate (Bishop et al., 1976; Law and Reiter, 1977) which chelates iron in a form available for uptake by microbes. Some coliform organisms are able to remove iron from Lf via secretion of high affinity iron chelators called enterochelins (Neilands, 1981). Lactoferrin may work in conjunction with specific antibody which blocks enterochelin production (Bullen et al., 1972), conferring a degree of specificity to the antibacterial effects of Lf.

Recent research indicated a possible role for Lf in cellular defenses against infection. Specific receptors for Lf have been demonstrated on human PMN (Maneva et al., 1983), mouse peritoneal (Van Snick and Masson, 1976) and alveolar macrophages (Markowetz et al., 1979), and human B-lymphocytes and adherent mononuclear cells (Bennett and Davis, 1981). Iron-saturated Lf may inhibit PMN colony formation <u>in vitro</u> and granulopoiesis <u>in vivo</u> by inhibiting granulocyte-macrophage colony stimulating activity (GM-CSA) production and release by human monocytes and macrophages (Broxmeyer et al., 1978). Lactoferrin was shown to have no effect on production

of B-lymphocyte CSA by macrophages (Broxmeyer et al., 1980). Bagby et al.(1981) demonstrated that Lf inhibited release of a soluble factor from human monocytes which stimulated in vitro production of GM-CSA by human T-lymphocytes. Broxmeyer and Platzer (1984) found that Lf inhibited release of GM-CSA by murine macrophages in the absence of T-lymphocytes.

Several possible roles exist for Lf in intracellular killing of bacteria by PMN. Incubation of rabbit PMN with a ferritin-antibody complex decreased ability of PMN to destroy phagocytosed bacteria (Bullen and Armstrong, 1979) possibly due to iron saturation of intracellular Lf. Similar in vivo experiments in which mice were injected with bacteria and ferritin-antiferritin complexes resulted in high mortality rates associated with ability of the bacteria to survive and multiply within PMN (Bullen and Joyce, 1982). Iron is important in production of hydroxyl radicals by PMN. Lactoferrin could serve in iron transport to PMN for hydroxyl radical production (Ambruso and Johnston, 1981) and may also function in transport of iron for use by macrophages (Van Snick et al., 1977).

Lactoferrin is possibly involved in the process of migration of phagocytes to sites of inflammation. Membrane-bound Lf may enhance PMN adherence to cell surfaces in response to chemotactic stimuli, thus aiding in cell to cell interactions which occur at sites of infection (Boxer et al., 1982). Increased PMN adherence could also aid in PMN clearance by macrophages following inflammation (Boxer et al., 1982). Kijlstra and Broersma (1984) reported that in in vitro

studies, low concentrations of Lf stimulated mononuclear leukocytes to produce granulocyte migration inhibition factors (indirect inhibition of granulocyte migration at sites of inflammation by Lf), whereas higher Lf concentrations directly inhibited granulocyte migration. Lactoferrin induced suppression of murine in vitro primary Ab responses (Duncan and McArthur, 1981) and mitogenic response of bovine peripheral blood lymphocytes (Smith et al., 1979) have been reported.

Lactoferrin may be an integral part of the cellular response to infection and inflammation. Iron is required for the existence of all living cells and tends to accumulate at sites of infection (Weinberg, 1978). Iron salts may affect lymphocyte circulation (de Sousa, 1978), implying that lymphocytes tend to migrate to iron-rich areas such as sites of inflammation. Lactoferrin could serve as a mediator for direction of cellular traffic to sites of inflammation via high iron affinity and presence of specific receptors for Lf on various cell types associated with immune and phagocytic defense (de Sousa, 1978; de Sousa et al., 1978). Lactoferrin was shown to be taken up by the murine reticuloendothelial system (RES; Van Snick et al., 1974). As iron saturation of Lf increased, time required for Lf to be taken up by the RES decreased. Van Snick et al. (1974) suggested that Lf may serve in clearance of exogenous iron from sites of infection or inflammation. Presence of Lf, and all cell types known to interact with Lf, has been demonstrated in the bovine mammary gland, and mechanisms similar to those mentioned previously may exist in the bovine mammary gland.

Immunoglobulins

Immunoglobulins in mammary secretion serve a dual role, providing defense against intramammary infection and passive immunity to the neonate (Watson, 1980). The type of Ig predominating in mammary secretion depends upon route of maternal transfer of IgG to the neonate. In rodent, human and other primate species, selective transplacental IgG transfer occurs and IgA is the primary Ig transferred to the neonate via colostrum (Beer et al., 1974). In ruminant, equine, and porcine species transplacental transfer of Ig is absent and IgG predominates in colostrum (Lascelles et al., 1981). Major classes of Ig detected in mammary secretion of most species are IgG, IgA, IgM, and possibly IgE (Watson, 1980; Butler, 1983; Eigel et al., 1984). In bovine mammary secretions, IgG1 and IgG2 are the only subclasses of IgG found (Lascelles, 1979). Immunoglobulin G1 is distinguished from IgG2 by its more rapid electrophoretic mobility (Beer et al., 1974). In mammary secretions, IgA is found primarily as secretory IgA (sIgA) which is composed of two IgA monomers, a J chain, and secretory component (SC; Head, 1977).

Most IgG in the ruminant mammary gland is of serum origin (Lascelles, 1979). Increased ratios of IgG1:IgG2 in mammary secretion compared to blood serum indicate either local synthesis or selective transport of IgG1 into the mammary gland (Lascelles, 1979). Lack of gamma globulin-bearing plasma cells (Beer et al., 1974) and lack of IgG1 response to intramammary infusion of antigen (Lascelles et al., 1981) in normal bovine mammary secretions

indicate that selective transport of serum IgG1 into mammary secretion is the most plausible explanation. Brandon et al. (1971) postulated that transport of humoral IgG1 into mammary secretions is mediated by specific receptors for IgG1 on basal membranes of secretory epithelial cells. Following receptor-IgG1 binding, a pinocytotic vesicle forms and IgG1 is transported across the secretory epithelial cell and discharged into the alveolar lumen.

Normally IgG2 is scarce in bovine mammary secretions (Guidry et al, 1980). Immunoglobulin G2 may gain entrance to the mammary gland nonspecifically through ruptured tight junctions between secretory epithelial cells or trapped in IgG1-containing pinocytotic vesicles (Watson, 1976). Immunoglobulin G2 may also enter the gland bound specifically to migrating PMN (Watson, 1976). Opsonization of antigen for subsequent PMN phagocytosis may be the primary function of IgG2 in mammary secretions (Craven and Williams, 1985).

Low concentrations of sIgA and IgM are present in normal bovine mammary secretions (Watson, 1980). Whereas most IgG in the ruminant mammary gland is of serum origin, evidence favors local synthesis of sIgA and IgM (Lascelles, 1979). Antigen infused into the bovine mammary gland resulted in increased concentrations of sIgA and IgM in mammary secretion without significant changes in serum levels of these Igs (Lascelles et al., 1966). In the ruminant mammary gland, sIgA and IgM may function in preventing bacterial adherence to mammary secretory epithelium (Poutrel, 1982).

CELLULAR DEFENSES OF THE MAMMARY GLAND

The majority of cells in mammary secretion were once considered to be sloughed epithelial cells (Targowski, 1983). Cells in mammary secretion are known now to be leukocytic in origin (Lee et al., 1980; Jensen and Eberhart, 1981; McDonald and Anderson, 1981a; McDonald and Anderson, 1981b) and have been identified as PMN, macrophages, monocytes and lymphocytes. In normal milk approximately 98% of cells are leukocytes with fewer than 2% epithelial cells (Lee et al., 1980).

Phagocytic defenses

Phagocytosis involves recognition, ingestion, and digestion of foreign bodies such as bacterial cells by phagocytic cells. Following contact and adherence of phagocyte and bacterium, the phagocyte envelops the bacterium with pseudopods, drawing the cell in and forming a phagosome. Intracellular digestion of foreign particles is effected by fusion of cytoplasmic granules of the phagocyte with the phagosome forming a phagolysosome. Contents of cytoplasmic granules (lactoferrin, oxygen radicals, and hydrolytic enzymes) are released into the phagolysosome, lysing the foreign cell (Paape et al., 1979; Hohn, 1977).

Polymorphonuclear neutrophils and macrophages are the primary phagocytes of the bovine mammary gland, constituting 80-90% of cells in normal bovine milk (Lee et al., 1980). Phagocytosis is the primary function of each cell type, both random and in conjunction with specific opsonins. Immunoglobulin G2 may be a primary opsonin

for PMN (Lascelles, 1979) while both IgG1 and IgG2 may be opsonic for macrophages (Desiderio and Campbell, 1980). Possible opsonic roles for IgM and sIgA have not been established (Craven, 1985). Polymorphonuclear neutrophils and macrophages are attracted to the mammary gland by chemotactic agents, with PMN being the first cell type entering the gland in response to tissue injury (Targowski, 1983). The bovine mammary gland is able to rapidly mobilize PMN from stores of circulating mature blood PMN and bone marrow-derived immature PMN during inflammation (Paape et al., 1974). The importance of leukocyte populations in the bovine mammary gland was demonstrated by Jain et al. (1968). As a result of experimentally induced neutropenia utilizing equine anti-bovine leukocyte serum, growth of Enterobacter aerogenes in the bovine mammary gland was unrestricted. In spite of the mammary glands ability to mobilize PMN, the gland remains susceptible to IMI, possibly due to the lag time between initiation of infection and peak PMN response (Paape et al., 1981b). The ability of certain bacteria to survive intracellular killing by phagocytes may reduce effectiveness of phagocytes in coping with established infection (Craven and Anderson, 1981).

Phagocytic efficiency of milk PMN and macrophages is reduced compared to blood PMN and macrophages, possibly due to nonspecific ingestion of fat and casein (Paape et al., 1979; Lee et al., 1969) which may reduce cell surface membrane area, thereby inhibiting phagocytosis. Scanning electron microscopy showed that 37% of milk PMN had pseudopods over 50% of their surface area (Wergin and Paape, 1977). Ninety-eight percent of blood PMN isolated from the same

donors had pseudopods over 50% of their surface area. Nonspecific ingestion of fat and casein may interfere with intracellular killing of phagocytosed bacteria (Paape and Guidry, 1977) by exhausting available supplies of hydrolytic enzymes in specific granules of phagocytes. Russell and Reiter (1975) determined that milk PMN had significantly depressed ability to kill phagocytosed Staph. aureus compared to blood PMN. Other possible explanations for reduced PMN effectiveness in the mammary gland include depressed PMN intracellular glycogen stores (Naidu and Newbould, 1973), relative absence of specific opsonins in milk (Wisnioski et al., 1965) and blockage of Fc receptors on PMN by components of mammary secretion (Pickering et al.,1983; Targowski and Niemialtowski, 1986). Corticosteroids have been shown to inhibit in vitro phagocytosis and intracellular killing of bacteria by PMN (Paape et al., 1979). Injections of 250 IU adrenocorticotrophic hormone (ACTH) increased corticosteroid levels and subsequently depressed PMN adhesiveness and phagocytic ability. Results of these experiments may parallel effects of ACTH-induced corticosteroid release observed during the periparturient period and during mastitis (Paape et al., 1981a).

Mammary macrophages and monocytes may have functions in the mammary gland other than phagocytosis. While PMN constitute the primary cellular response to initiation of infection in the gland, macrophages predominate during chronic mastitis (Nickerson and Heald, 1982). Interleukin 1(IL-1) and certain cleavage products of complement are potent PMN chemotaxins which may be synthesized by macrophages (Craven and Williams, 1985). Weissman (1983) implicated

macrophages in the synthesis and release of prostaglandins (PG) which are potent vasoactive substances that may aid in PMN migration to sites of inflammation. Ovine mammary macrophages enhanced phytohemagglutinin (PHA)-stimulated incorporation of ^{3}H-thymidine by lymphocytes (Outteridge and Lee, 1981). Macrophages and monocytes appear to be necessary accessory cells in the induction of suppressor cell activity in bovine peripheral blood lymphocytes (Ohmann et al., 1983). In humans, macrophages may be required for T-cell activation (DeVries et al., 1979) while lymphokines produced by T-cells may enhance bacteriocidal activity of macrophages (Craven, 1985). Macrophages are implicated also in the removal of fat from the mammary gland during involution (Lee et al., 1969).

A preexisting leukocytosis has been suggested as a method of enhancing effectiveness of phagocytosis in the mammary gland. Schalm et al. (1964) indicated that protection of the mammary gland was greatest when PMN were present in the gland at concentrations of $\geq$500,000 cells/ml of milk prior to infection. Higher PMN concentrations may serve as an improved phagocytic barrier and stimulate more rapid PMN infiltration into the gland, reducing lag time between establishment of infection and PMN response (Jain et al., 1968). Attempts at establishing leukocytosis have centered primarily on the use of intramammary devices (IMD) and increasing somatic cell counts (SCC) via experimental infection with minor pathogens. Paape et al. (1981c) demonstrated that implantation of a polyethylene IMD in the mammary gland cistern increased SCC and subsequent leukocyte response to challenge with *E. coli* endotoxin

and Staph. aureus. Infection of the bovine mammary gland with minor pathogens may also protect the gland against subsequent infection by major pathogens (Bramley, 1978; Poutrel and Lerondelle, 1980). Increases in SCC in Corynebacterium bovis-infected quarters have been reported (Forbes, 1970b; Brooks and Barnum, 1984) indicating that leukocytosis following the establishment of minor pathogen infection is responsible for subsequent protection against IMI by major pathogens. However, increases in SCC following natural infection by C. bovis are marginal (Brooks and Barnum, 1984; Honkanen-Buzalski and Bramley, 1984). Protection of the mammary gland by minor pathogens may be due to competitive inhibition of establishment of major pathogen IMI by an as yet unidentified mechanism.

Lymphocytes

The presence of viable populations of B- and T-lymphocytes in mammary secretion at all stages of lactation has been documented. Mitogen and antigen responsive lymphocytes have been identified in human (Diaz-Jouanen and Williams, 1974), bovine (Smith and Schultz, 1977; Schore et al., 1981; Concha et al., 1980), ovine (Lee and Outteridge, 1981) and canine (Smith and Schultz, 1977) mammary secretions. Bovine mammary B-cells have been characterized by fluorescent staining of surface Ig (Concha et al., 1978) and formation of erythrocyte- antibody-complement (EAC) rosettes (Schore et al., 1981). Bovine mammary T-cells have been characterized by ability to form erythrocyte (E) rosettes (Schore et al., 1981) and

binding of Helix pomatia (HP) agglutinin (Concha et al., 1978), a marker of human (Hammarstrom et al., 1973) and bovine (Morein et al., 1979) T-lymphocytes. Reactivity of mammary lymphocytes from various species to a range of mitogens and antigens has been characterized.

Since the serendipitous discovery by Nowell (1960) that the plant lectin PHA stimulated mitosis in human lymphocytes, a wide variety of agents have been shown to possess mitogenic properties. Agents discovered to be mitogenic include plant and animal lectins, Ab to lymphocyte surface components, calcium ionophores, bacterial lipolysaccharide (LPS) and allogeneic lymphocytes (Schechter, 1980). In vitro exposure to mitogen stimulates lymphocyte proliferation, B-lymphocyte antibody production and release of soluble factors (lymphokines) from T-lymphocytes (Cogoli and Pearson, 1976). Therefore, mitogen stimulation has been proposed to be a good in vitro model of in vivo lymphocyte response to antigen.

The sequence of events leading to mitogenic stimulation of lymphocytes is not well defined. Initial binding of mitogen to cell surface receptors and cross-linking of receptors appears to be the first step in mitogenic stimulation of lymphocytes (Cogoli and Pearson, 1976). Mitogens appear to interact specifically with single sugar structures on cell membranes (Schechter, 1980). Cross-linking of receptors may involve the microtubule structure of lymphocytes. Albertini and Clark (1975) noted polar accumulation of receptors (capping) following incubation of rabbit ovarian granulosa cells with concanavalin A (con A). Capping of receptors was accompanied by

reorganization of cellular microtubules and association of microtubules with capped areas of cellular membranes. Identity of the "second messenger" of mitogenic stimulation of lymphocytes is less clear. Increased calcium (Ca^{+2}) influx, increased production of certain cyclic nucleotides, and association of mitogen receptors with cytoplasmic proteins capable of initiating mitosis have been implicated (reviewed by Cogoli and Pearson, 1976). Calcium may also be involved in mitogenic stimulation of bovine splenic and peripheral blood lymphocytes (Atluru et al., 1980). Binding of con A increased Ca^{+2} influx into rat splenic cells (Wolff and Akerman, 1982). Although influx of Ca^{+2} into cells is known to be complete within 1 minute of mitogenic stimulation, removal of con A during the first 18 hr of incubation abolished mitogenic stimulation (Cogoli and Pearson, 1976). These data indicate that Ca^{+2} is not the second messenger of lymphocyte stimulation. Increased Ca^{+2} influx may be a result of increased cell membrane permeability (Cogoli and Pearson, 1976). A combination of interaction of cell surface receptors and cytoplasmic protein complexes followed by increased production of cyclic nucleotides (especially cyclic GMP) may serve as the second messenger (Cogoli and Pearson, 1976). The lymphocyte undergoes a change from a resting state to an activated state of DNA synthesis followed by proliferation and immunoglobulin or lymphokine synthesis (Cogoli and Pearson, 1976; Schechter, 1980).

Use of mitogens has made possible <u>in vitro</u> studies of interactions between B- and T-lymphocytes. Finesilver and Braley-Mullen (1983) demonstrated the presence in murine spleen cell populations

of con A-inducible helper T-cells which augmented Ab response to a T-cell independent antigen. The murine thymus gland contains cells which amplify response of peripheral blood B- lymphocytes to lipopolysaccharide (Norcross and Smith, 1977). T-cells may also function in stimulation of B-cells by T-cell mitogens via release of lymphocyte activation factors (MacDermott et al., 1978) although stimulation of ostensibly pure B-cell populations by T-cell mitogens has been reported (Chess et al., 1974). B- and T-lymphocyte interactions in the mammary gland may play a role in defense of the gland against IMI.

Mammary gland lymphocytes (MGL) from various species have been characterized as to response to certain mitogens and antigens. Diaz-Jouanen and Williams (1974) noted that human colostral lymphocytes respond to in vitro stimulation by PHA and pokeweed mitogen (PWM), though to a lesser degree than peripheral blood lymphocytes (PBL). Bovine and canine MGL responded in reduced fashion to con A, PHA and PWM (Smith and Schultz, 1977). In contrast, response to tuberculin was often higher in MGL than PBL of sensitized cattle (Smith and Schultz, 1977; Nonnecke et al., 1986). Depressed response of bovine MGL to con A, PHA, PWM and Salmonella typhimurium LPS was reported by Concha et al. (1980). Kohl et al. (1980) indicated that human colostral lymphocytes were capable of mediating antibody-dependent cell-mediated cytotoxicity (ADCC), but to a lesser degree than PBL from the same donor. Parmely et al. (1976) demonstrated that milk lymphocytes were able to respond to allogeneic blood and milk lymphocytes in mixed lymphocyte reactions

(MLR). Again, responses of MGL were depressed compared to PBL from the same donor. Further investigations showed that whereas blood lymphocytes of Candida albicans-sensitized donors responded in vitro to C. albicans, MGL did not (Parmely et al., 1976). Milk lymphocytes of sensitized donors responded to E. coli K1 antigen while PBL from the same donor did not (Parmely et al., 1976). It is interesting to note that C. albicans is a component of normal human gastrointestinal flora while E. coli is considered an enteropathogen. In further studies, Parmely et al. (1977) examined responses of MGL and PBL to various antigenic stimuli. Tetanus toxoid, C. albicans and streptokinase were more stimulatory to PBL than MGL. Mumps, measles, and tuberculin Ags were equally effective in stimulation of PBL and MGL. Escherichia coli K1 antigen stimulated only MGL. These data, coupled with the discovery that maternal MGL show reduced capacity to respond to paternal alloantigens (Parmely and Williams, 1979) may indicate that mammary glands select for certain clones of T- and B-lymphocytes while excluding others.

A possible source of mammary lymphocytes has been postulated to be gut-associated lymphoid tissue (GALT). The observation that MGL with specific reactivity to organisms commonly encountered in the gastrointestinal (GI) tract appear in human mammary glands (Parmely et al., 1977) would appear to support this hypothesis. Kortbeek-Jacobs et al., (1984) proposed that porcine lymphocytes migrate from intestinal Peyers Patches to mesenteric lymph nodes to mammary lymph nodes following oral administration of antigen. Primary responses to

K88 antigen in swine were found to be of the IgA class, but IgG- and IgM-positive cells were also observed depending upon vaccination schedule. Specific plaque-forming cells were demonstrated in human colostrum following oral immunization with live nonpathogenic E. coli (Goldblum et al., 1975). The work of Parmely and Beer (1977) indicated that in addition to GALT the mammary gland may be able to draw upon bronchial- associated lymphoid tissue (BALT) as a source of specifically reactive T-cell clones. In patients sensitized to mumps, measles or tuberculin antigen (to which sensitization is often acquired via the respiratory tract), colostral lymphocytes exhibited in vitro reactivity to these antigens on the same order of magnitude as PBL. The observation of Manning and Parmely (1980) that radiolabeled peripheral (cervical lymph node) and intestinal (mesenteric lymph node) T-cell blasts migrate equally well to rat mammary glands support this hypothesis. Limited data exist to support the existence of GALT-mammary gland and BALT-mammary gland cell transfer (Lascelles, 1979; Chang et al., 1981; Nonnecke et al., 1986) in the bovine, and further work is required to establish clearly the existence of specific selection of lymphoid clones by the bovine mammary gland.

Hyporesponsiveness of mammary lymphocytes to mitogen may be due to absence of certain clones of cells, lack of accessory cells or presence of suppressor cells, but none of these hypotheses have been proven. Various hormones and proteins associated with pregnancy and lactogenesis appear to be immunosuppressive (reviewed by Lloyd, 1983) and pregnancy-associated factors may contribute to depressed

mitogenic response of mammary lymphocytes. During involution, barriers to entry of serum proteins into the mammary gland are less effective (Fleet and Peaker, 1978), and blood-borne immunosuppressants could gain entrance into the gland. Immunosuppressive substances native to mammary secretion also provide a likely explanation for observed hyporesponsiveness of mammary lymphocytes. Suppression of PBL response to PHA, con A and PWM by human colostrum and milk has been reported (Diaz-Jouanen and Williams, 1974; Ogra and Ogra, 1978; Crago et al., 1981; Drew et al., 1982). Collins and Oldham (1986) reported that bovine colostrum and dry secretion inhibit bovine PBL response to PHA and con A. Interleukin-2 (IL-2) production was unaffected. Clonal expansion of cytotoxic T-cells may be associated with IL-2, which is itself produced by a subpopulation of T-cells (Smith, 1980). Kohl et al. (1980) demonstrated colostral inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC) by human PBL. Colostrum was shown to inhibit effector rather than target cells. Bovine Lf, the major whey protein of the involuted bovine mammary gland, was shown to suppress in vitro bovine PBL response to con A (Smith et al., 1979). Free secretory component has been shown to suppress PWM-stimulated expression of cytoplasmic Ig by human PBL (Crago et al., 1981).

Mammary lymphocyte hyporesponsiveness may serve a function in transfer of immunity to the neonate. When rats recieved skin grafts from their own offspring, the nursing offspring developed symptoms of graft-vs-host disease (Beer et al., 1974). Fischer strain rats nursing Lewis strain mothers tended to develop immunological

tolerance to skin grafts from Lewis strain rats (Head and Beer, 1979). It is possible that maternal transfer of immunity to the neonate takes place via the cellular component as well as Ig content of colostrum. Trypan blue-labelled bovine leukocytes were observed invading neonatal rabbit gastrointestinal epithelium when adoptively transferred (Kmetz et al., 1970). Conditions in the neonatal intestine may favor survival of ingested maternal lymphocytes (reviewed by Head, 1977) due to higher pH, inadequate production of digestive enzymes and high buffering capacity of colostrum and milk. Depressed reactivity of mammary gland lymphocytes could provide a means of transferring immunologically competent lymphocytes that will not react against paternal alloantigens, but reduced immunocompetence may also contribute to increased susceptibility of the mammary gland to infection.

INTRAMAMMARY INFECTIONS DURING PHYSIOLOGICAL TRANSITIONS OF THE MAMMARY GLAND

The nonlactating or dry period of the dairy cow serves a physiological as well as nutritional purpose. In order to maximize milk production during the subsequent lactation, the dairy cow requires a dry period of approximately 60 days (Swanson, 1965). The dry period is related to etiology of mastitis within the herd in that new IMI during the dry period contributes to increased numbers of infected quarters in subsequent lactations (Dodd et al., 1977). Antibiotics used for dry cow therapy are not equally effective, and

susceptibility of the mammary gland to particular pathogens varies according to organism and stage of involution (Smith and Todhunter, 1982).

The bovine mammary gland appears to be most susceptible to new IMI during early involution and just prior to parturition. Neave et al. (1950) showed that highest rates of new IMI occur during the first three weeks of the dry period. Ward and Schultz (1974) noted highest rates of IMI during the second and third weeks of the dry period. Infection rates rose again near calving. Most new streptococcal IMI were observed during early involution while most new staphylococcal IMI were seen at parturition (Ward and Schultz, 1974). In a study of 61 clinical cases of mastitis, 40 were shown to have begun in the dry period and all but 14 became clinical after parturition (Eberhart, 1982). Number of new IMI was highest during the first week post-drying off, decreased to 0 by 4 weeks post-drying off and increased again during the week prior to calving. Higher producing cows (Oliver et al., 1956) and older cows (Oliver and Mitchell, 1983a) appear to be more susceptible to new IMI during the dry period. However, in a study by Oliver and Mitchell (1983b) approximately 27% of quarters of primigravid heifers were infected at parturition, indicating that cow age is not the most important factor in susceptibility to new IMI in the dry period.

During the dry period, environmental pathogens may be a major cause of mastitis. Streptococcus agalactiae and Staph. aureus are transmitted from cow to cow during the milking procedure and can be

reduced or eliminated by postmilking teat disinfection and dry cow therapy, whereas the teat end is in constant contact with sources of environmental pathogens (Eberhart, 1977). In one study, approximately 50% of new IMI isolated during the dry period were caused by "green" streptococci of which 86% were identified as Strep. uberis (Oliver et al., 1956). Oliver and Mitchell (1983a) found in a study of the dry period that 98.2% of new IMI were caused by coliform bacteria and streptococci other than Strep. agalactiae. Over 50% of new coliform IMI detected early in involution were eliminated without treatment prior to calving. At parturition, 22 infected quarters were detected of which only 2 shed coagulase-positive staphylococci. All other infections were caused by coliforms and streptococci other than Strep. agalactiae (Oliver and Mitchell, 1983a). Increased susceptibility of the mammary gland to Strep. uberis during the prepartum period was reported by McDonald and Anderson (1981c). In a separate study, 88% of glands inoculated with E. coli during the latter portion of the dry period became infected compared to 32% of glands inoculated during the first half of the dry period (McDonald and Anderson, 1981d). All infections detected in the first half of the dry period were eliminated without treatment whereas 14 infections that occurred near parturition persisted into the subsequent lactation and developed into peracute mastitis.

Several possible explanations exist for the variation in susceptibility of the bovine mammary gland to infection. Neave and Oliver (1962) observed that Staph. aureus was frequently isolated on

teat ends following the final milking of lactation whereas Strep. uberis was rarely isolated. By 28 days of involution, Strep. uberis was most frequently isolated. Apparently, changes in the bacterial population to which teat ends are routinely exposed affect susceptibility to infection. During physiological transitions of the mammary gland (initiation of involution and colostrogenesis) increased intramammary (imam) pressure due to fluid accumulation may increase teat duct patency, thus increasing susceptibility to IMI (Cousins et al., 1980). Alterations in defense mechanisms of the mammary gland may also affect susceptibility of the gland to infection, especially during physiological transitions. Experiments designed to accelerate the rate of involution were shown to reduce number of infections detected during early involution, apparently by reducing fluid accumulation and increasing concentrations of natural protective factors of the mammary gland (Oliver and Smith, 1982; Breau and Oliver, 1985). Further efforts directed at reducing rate of infection during physiological transitions of the mammary gland by manipulation of natural protective factors may reduce number of infections carrying over into subsequent lactations.

CHANGES IN NATURAL PROTECTIVE MECHANISMS OF THE MAMMARY GLAND THROUGHOUT THE DRY PERIOD

During periods of functional transition the bovine mammary gland undergoes a series of changes associated with natural protective factors in the gland. Synthesis and secretion of normal

milk constituents is reduced or eliminated. Fluid levels decrease and levels of natural protective factors increase. It is paradoxical that the mammary gland appears to show increased susceptibility to new IMI during the dry period when levels of protective factors such as Lf, Igs and phagocytic cells have been demonstrated to be highest (Welty et al., 1976; Breau and Oliver, 1985; Jensen and Eberhart, 1981).

Humoral defenses

Alterations in humoral defenses of the bovine mammary gland are associated primarily with Lf and Ig concentrations. Marshall et al. (1986) stated that levels of LP increase during involution and reach a maximum at 7 days post-drying off. Minor fluctuations in SCN- concentrations were recorded. Levels of complement in mammary secretion increased five-fold by 21 days of involution, possibly reflecting increased passive transfer of complement from serum to mammary secretion through leaky "tight junctions" between secretory epithelial cells as involution progressed (Poutrel and Rainard, 1986). However, the roles of complement and the LP/SCN-/H_2O_2 system in defense of the bovine mammary gland against infection are not fully understood. Levels of lysozyme are low in bovine milk compared to human milk (Chandran et al., 1964) and levels of lysozyme in secretions of the nonlactating mammary gland have not been reported (Schanbacher and Smith, 1975).

Lactoferrin is a minor whey protein in bovine milk compared to human milk (Bezkorovainy, 1977). In bovine milk, Lf is found at

concentrations of .35 mg/ml (Schanbacher and Smith, 1975). As involution progresses, Lf concentration increases, reaching levels of 25-35 mg/ml in the fully involuted gland (Welty et al., 1976). The molar ratio of citrate to Lf (cit:Lf) decreases significantly during this period (Smith and Oliver, 1981). Lactoferrin levels subsequently decrease as parturition approaches (Welty et al., 1976). During acute mastitis, milk Lf levels increase to as much as 30 times normal (Harmon et al., 1976). Increased Lf concentration was not due solely to decreased fluid volume and greatest Lf production was observed in infected quarters. As severity of infection subsided, Lf levels in infected glands returned towards normal values.

Concentrations of Ig are relatively low during early involution and reach maximum levels at parturition (Watson, 1980). Levels of IgG range from .5-1 mg/ml at drying off to 50-100 mg/ml at parturition (Lascelles, 1979; Lascelles et al., 1981). Transitory increases in levels of IgG1 have been reported 2-3 days post-drying off (Lascelles, 1979). At parturition, IgG1 predominates over IgG2 (52.3 mg/ml and 2 mg/ml, respectively; Schanbacher and Smith, 1975). Levels of sIgA and IgM remain elevated throughout involution (Schanbacher and Smith, 1975) but decrease markedly following initiation of lactation. Levels of sIgA in dry secretion and colostrum were 3.31 mg/ml and 2.8 mg/ml, respectively. Concentration of IgM in dry secretion and colostrum was 8.6 and 8.92 mg/ml, respectively. Immunoglobulin G increased to 15.5 mg/ml during the acute phase of coliform mastitis and gradually decreased as

infection subsided (Harmon et al., 1976). Levels of sIgA and IgM increased following intramammary infusion of antigen, indicating local production in the bovine mammary gland (Chang et al., 1981).

Cellular defenses

Numbers of bovine peripheral blood leukocytes remain fairly stable throughout involution (Wilson et al., 1986). In contrast, marked variations in mammary leukocyte populations occur. Total cell counts in mammary secretion increase following initiation of involution, reaching peak numbers in the fully involuted gland and declining thereafter to postpartum levels similar to those observed prior to drying off (Jensen and Eberhart, 1981; McDonald and Anderson, 1981a; McDonald and Anderson, 1981b; Wilson et al., 1986). It is interesting to note that concentrations of Lf and somatic cell counts in the bovine mammary gland follow similar patterns during the dry period, especially given the premise that Lf may serve as a mediator for direction of cell traffic (de Sousa, 1978).

Minor discrepancies exist between reports of mammary gland leukocyte population dynamics and concentrations, possibly reflecting differences in cell collection techniques, infection status of glands sampled, counting procedures and individual cow variation. However, certain trends can be identified. Phagocytic cells predominate in the gland during early involution. Polymorphonuclear neutrophils were the major cell type identified in uninfected mammary glands between 5 and 8 days of involution (Jensen and Eberhart,1981; McDonald and Anderson, 1981a) though differences

between macrophage and PMN numbers in these studies were minimal. In contrast, Wilson et al. (1986) reported that macrophages were the predominant cell type throughout involution.

Predominance of phagocytes in the mammary gland during early involution may be due to the role of these cells in removal of fat, casein and other residual milk constituents from the gland following cessation of milking (Lee et al., 1969; Lee et al., 1980). Numbers of macrophages and PMN appear to peak at 7-8 days of involution and decline thereafter until parturition, with number of PMN declining more rapidly than number of macrophages (Jensen and Eberhart, 1981; McDonald and Anderson, 1981a). Transient increases in macrophage and PMN concentrations during the peripartum period have been reported (Jensen and Eberhart, 1981). Colostrum contains increased amounts of cellular debris, and increased numbers of phagocytes in the gland during colostrogenesis may serve to remove this debris (Lee et al., 1980). At parturition, macrophages and PMN again predominate in the mammary gland in numbers similar to those observed at drying off (Jensen and Eberhart, 1981; McDonald and Anderson, 1981b; Wilson et al., 1986).

The dynamics of lymphocyte populations appear to follow a different pattern than phagocytes of the mammary gland. Whereas phagocyte concentrations peak at approximately one week of involution, lymphocyte numbers continue to increase for approximately 30 days (Jensen and Eberhart, 1981; McDonald and Anderson, 1981a; Wilson et al., 1986). Numbers of lymphocytes exceed those of PMN (Wilson et al., 1986; McDonald and Anderson, 1981a) and

possibly of macrophages (Jensen and Eberhart, 1981) in the fully involuted gland. Lymphocytes constitute approx- imately 40% of total number of cells (Jensen and Eberhart, 1981). Mammary lymphocyte concentrations remain relatively stable during the latter portion of the dry period up to two weeks prepartum, then begin to decline (Jensen and Eberhart, 1981; McDonald and Anderson, 1981b). Drastic reductions in numbers of mammary lymphocytes have been observed during the final 3-4 days prior to parturition (Jensen and Eberhart, 1981; McDonald and Anderson, 1981b).

Limited data are available regarding sequential changes in proportions of T- and B-cells in mammary secretions throughout the nonlactating period. Results of studies quantifying bovine peripheral blood and mammary secretion lymphocytes have varied and this is most likely due to variation in techniques of isolation and quantification of B- and T-cells. Bovine blood B-cells ranged from approximately 20% of the total lymphocyte population (Concha et al., 1978; Johansson and Morein, 1983; Morein et al., 1979) to about 30% (Schore et al., 1981; Canning et al., 1983). Blood T-cell percentages fluctuated from approximately 45% (Concha et al., 1978; Wilson et al., 1986) to more than 60% (Canning et al., 1983; Johansson and Morein, 1983; Morein et al., 1979). Mammary B-cells have been reported to constitute 19.9% of total lymphocyte populations in milk (Concha et al., 1978) and 28% of total lymphocyte populations in dry mammary gland secretion (Concha et al., 1980) indicating a possible influx of B-cells into the mammary gland as the gland goes from lactation to involution. T-cells have

been reported to constitute 60-70% of total lymphocytes in the involuted mammary gland (Concha et al., 1980; Wilson et al., 1986; Schore et al., 1981). Data from some studies (Wilson et al., 1986; Schore et al., 1981) show higher proportions of T-cells in mammary secretions compared to peripheral blood. Other studies suggest that the percentage of T-cells in bovine blood and mammary secretions is relatively similar (Concha et al., 1978; Concha et al., 1980; Johannson and Morein, 1983). Possibly, identity and functional characteristics of lymphocyte subpopulations in blood and mammary secretions may be of greater importance than absolute percentages of B- and T-cells.

Recently, Wilson et al. (1986) reported the relative proportions of mammary T-cell subsets at intervals throughout involution. T-cell subsets were separated by differential rosetting using untreated, neuraminidase (N)- treated and aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC). T-cell subsets were classified as $E_{N+}E_{AET+}$, $E_{N+}E_{AET-}$, $E_{N-}E_{AET+}$ and $E_{N-}E_{AET-}$ based on ability to form rosettes with N (E_N)- and AET (E_{AET})-treated SRBC. $E_{N-}E_{AET-}$ cells probably contained mostly B-cells and "null cells" (Wilson et al., 1986). Ignoring changes in relative proportions of T-cell subsets as involution progressed, Wilson et al. (1986) found that only percentages of $E_{N+}E_{AET-}$ and $E_{N-}E_{AET+}$ cells were similar in blood and mammary secretions. Mammary secretions contained a higher percentage of $E_{N+}E_{AET+}$ cells than blood (39% and 9%, respectively). In contrast, blood contained 42% $E_{N-}E_{AET-}$ cells compared to 16% $E_{N-}E_{AET-}$ cells in mammary secretions.

$E_{N+}E_{AET+}$ and $E_{N-}E_{AET+}$ cells were the most responsive in MLR and mitogenic assays (Belden et al., 1981). Predominance of $E_{N+}E_{AET+}$ and $E_{N-}E_{AET+}$ phenotypes in mammary secretions may indicate specific selection of active T-cell populations by the bovine mammary gland (Wilson et al., 1986).

Wilson et al. (1986) also quantified relative changes in percentages of T-cell subsets as involution progressed. Little change was observed in blood T-lymphocyte subpopulations. In mammary secretions, proportions of $E_{N+}E_{AET+}$ and $E_{N+}E_{AET-}$ cells decreased significantly during the dry period. In contrast, $E_{N-}E_{AET+}$ and $E_{N-}E_{AET-}$ cells increased up to and including the week prior to parturition. $E_{N-}E_{AET+}$ cells may represent a functional maturation of $E_{N+}E_{AET+}$ cells (Hurley and Mastro, 1983), indicating that concentrations of an active T-cell subset and of B- and null cells increase in the bovine mammary gland as parturition approaches. However, studies of functional capabilities of mammary T-cell subsets are required before any conclusions can be drawn about the relationship between changes in T-lymphocyte sub-populations and changes in mammary gland susceptibility to infection throughout involution.

Various factors may influence the ability of bovine mammary lymphocytes to respond to antigenic challenge. Bovine PBL response to mitogen was lowest in February and highest in August (Soper et al.,1978). Yamamoto et al. (1980) reported a depressed response of human maternal PBL to PHA and conA during pregnancy. A similar suppression was reported for bovine PBL immediately prepartum (Soper

et al., 1978). Plasma from pregnant ewes suppressed response of ovine efferent lymph lymphocytes to allogeneic cells and con A (Miyasaka and McCullagh, 1981). Suppression of the maternal immune response during gestation has been proposed as a mechanism to prevent rejection of the fetus. Prepartum immunosuppression has been linked to: a) fetal and maternal immunoregulatory proteins produced during pregnancy, b) generation of suppressor cells and c) steroid and protein hormones that suppress the immune response (reviewed by Lloyd, 1983). Care must be taken in extrapolating these data to form hypotheses about observed hyporesponsiveness of bovine mammary lymphocytes. However, bovine MGL are derived from peripheral blood and immunosuppressive substances in blood would be expected to contribute to mammary lymphocyte hyporesponsiveness prior to lymphocyte entry into the gland. During involution, barriers to large molecular weight plasma proteins are more permeable (Fleet and Peaker, 1978), and pregnancy-associated immunosuppressants may enter the mammary gland and contribute to observed hyporesponsiveness of bovine MGL to mitogens.

Infection status of the mammary gland influences relative numbers and concentrations of mammary gland leukocytes. Lymphocytes increase in number during infection, but comprise a lesser percentage of total somatic cell counts than in the uninfected gland (Jensen and Eberhart, 1981). Mammary gland infection may also influence functional capacity of MGL. Virulence of certain bacterial and viral pathogens is mediated by their ability to suppress the immune response of the host (Kristensen et al., 1982). Bovine blood

mononuclear cells had impaired ability to respond to mitogens in cattle infected persistently with bovine viral diarrhea (Roth et al., 1986). Nonnecke and Harp (1985) noted that MGL from quarters experimentally infected with Staph. aureus showed reduced in vitro response to mitogens compared to MGL from uninfected quarters. Influx of PMN observed during infection may also affect MGL function. Polymorphonuclear neutrophils are the major cell type present in the mammary gland during infection (Jensen and Eberhart, 1981). Activated PMN release substances which may suppress human lymphocyte mitogenesis (Zosche and Messner, 1984), and may have a similar effect on bovine MGL.

SUMMARY

Susceptibility of the bovine mammary gland to IMI during the dry period is greatest during physiological transitions of the gland from lactation to involution and from involution to colostrogenesis. Increased susceptibility to new IMI is associated with changes in bacterial populations to which the teat end is exposed, increased fluid accumulation during physiological transitions of the gland, and changes in the composition of mammary secretion. One component of the defensive armament of the bovine mammary gland is a population of functionally competent, viable lymphocytes. Mammary lymphocytes are derived from blood and may function in defense of the mammary gland in a manner similar to PBL. Mammary gland lymphocytes are hyporesponsive to mitogens compared to PBL, and may,

therefore, be hyporesponsive to antigenic stimulation. Components of mammary secretion may be responsible for suppression of MGL activity in the gland. Factors associated with pregnancy have been shown to be immunosuppressive, and these may gain entry to the mammary gland and contribute to the hyporesponsiveness of mammary lymphocytes. Variation in MGL activity during involution may contribute to observed variation in susceptibility of the bovine mammary gland to new IMI in the dry period, especially during the latter portion of involution when numbers of lymphocytes are high. Therefore the primary focus of these studies was to determine possible causes of fluctuation in mammary lymphocyte activity. In addition, studies were conducted to provide further evidence about the portion of mammary secretion that inhibits the response of mononuclear cells to mitogens. Peripheral blood lymphocytes were used in all studies in order to obtain maximum cell responses to mitogens and avoid masking of variation of cell activity due to hyporesponsiveness of MGL.

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PART II

SUPPRESSION OF MITOGENIC RESPONSE OF PERIPHERAL BLOOD MONONUCLEAR CELLS BY BOVINE MAMMARY SECRETIONS

ABSTRACT

Effects of bovine colostrum, normal milk and dry secretions obtained at 7 and 28 d of involution on bovine blood mononuclear cell blastogenesis were evaluated. Mononuclear cells were isolated by density gradient centrifugation. Concanavalin A and phytohemagglutinin were used to induce blastogenesis, which was evaluated by methyl-^{3}H-thymidine incorporation. All mammary secretions inhibited mononuclear cell blastogenesis compared with stimulated controls. Normal milk was associated with the least inhibition of blastogenesis while colostrum and secretions obtained at 7 d post drying off caused the greatest inhibition. Dilution of secretions reduced blastogenic inhibition in a dose-dependent manner. Whey preparations of mammary secretions caused greater inhibition than mammary secretion skims. Mononuclear cells stimulated by PHA were less inhibited by mammary secretions than con A-stimulated cells. These data suggest that mammary secretions inhibit mononuclear cell blastogenesis, particularly during early involution and at parturition. Suppressed mononuclear cell activity may be related to increased susceptibility to new intramammary infection during physiological transitions of the bovine mammary gland.

INTRODUCTION

Bovine mammary glands are highly susceptible to new intramammary infection (IMI) during early involution (Neave et al., 1950; Ward and Schultz, 1974; Eberhart, 1982; Oliver and Mitchell, 1983) and

near parturition (Eberhart, 1982; Oliver and Mitchell, 1983; Ward and Schultz, 1974; Oliver, 1987). In contrast to transitional periods, the fully involuted mammary gland appears to be resistant to new IMI (Eberhart, 1982; Ward and Schultz, 1974). Increased susceptibility to new IMI during functional transitions has been attributed to several factors such as increased intramammary pressure caused by fluid accumulation (Cousins et al., 1980), absence of regular "flushing out" of pathogens during the milking process (Thomas et al., 1972), and changes in the biochemical composition of mammary secretions (Schanbacher and Smith, 1975). The cellular component of mammary secretions is another factor that is in all probability related to susceptibility or resistance of the mammary gland to new IMI during the dry period.

Total cell concentrations increase during the first week of involution, remain elevated during the dry period, and decrease following parturition (Jensen and Eberhart, 1981; Wilson et al., 1986). Polymorphonuclear neutrophils (PMN) and macrophages predominate during early involution and decrease in concentration as involution progresses (Jensen and Eberhart, 1981; McDonald and Anderson, 1981a; McDonald and Anderson, 1981b). On the other hand, the concentration of lymphocytes is highest in the fully involuted gland (Jensen and Eberhart, 1981; Wilson et al., 1986; McDonald and Anderson, 1981a) and lymphocytes may be the predominant cell type at that time (Jensen and Eberhart, 1981). Proportions of T- and B-lymphocytes vary according to stage of lactation (Concha et al., 1978; Concha et al., 1980;

Schore et al., 1981; Wilson et al., 1986). Wilson et al. (1986) reported that subpopulations of mammary gland lymphocytes (MGL) varied in concentration throughout involution. B-lymphocytes, null cells and a subset of T-lymphocytes increased in concentration up to and including the week prior to parturition.

An important factor in resistance to infection is the ability of lymphocytes to initiate and maintain an immune response. Human and bovine peripheral blood lymphocytes (PBL) are more responsive to *in vitro* mitogenic stimulation than MGL (Diaz-Jouanen and Williams, 1974; Smith and Schultz, 1977; Concha et al., 1980; Schore et al., 1981). Reasons for this remain unclear. However, hyporesponsiveness of MGL may be associated with components of mammary secretion. In support of this hypothesis, bovine and human mammary secretions have been shown to suppress response of PBL to mitogens (Collins and Oldham, 1986; Diaz-Jouanen and Williams, 1974; Ogra and Ogra; 1978; Crago et al., 1981; Drew et al., 1982). Lactoferrin (Lf) inhibited mitogenic stimulation of PBL (Smith et al., 1979) and concentration of this protein increases during involution of the mammary gland (Oliver and Smith, 1982; Welty et al., 1976). Immunosuppressants of serum origin (Lloyd, 1983) associated with pregnancy and lactation may also be involved.

Immunosuppression of bovine lymphocytes could affect the dynamics of IMI during involution. Depressed ability of mononuclear cells to mount a specific immunoglobulin or cytotoxic response to bacterial invasion may contribute to observed susceptibility of the mammary

gland to new IMI during involution, especially during physiological transitions. The purpose of this study was to determine if mammary secretions obtained at various stages of the lactation cycle varied in their ability to influence bovine blood mononuclear cell blastogenesis.

MATERIALS AND METHODS

Animals

Five multiparous lactating Holstein dairy cows served as blood donors. All animals had been lactating between 2 and 14 wk prior to initiation of experiments, and were producing approximately 31 kg of milk (range 24-40 kg)/day. Animal age ranged from 38-58 mo.

Secretions

Colostrum, normal milk, and mammary secretion obtained 7 (D+7) and 28 d (D+28) post-drying off were collected. Only secretions from mammary glands free of major mastitis pathogens based on microbiological analysis were used. Skim samples were prepared by centrifugation (37,000 x g for 30 min) to remove fat and cellular debris. Wheys were prepared by lowering the pH of skim samples to 4.5 with glacial acetic acid followed by centrifugation at 37,000 x g for 45 min. Skim and whey samples were adjusted to pH 7.0 with 1 N NaOH, filter sterilized (0.45 μm filter; Gelman Sciences Inc., Ann Arbor, MI) and stored frozen until required. Total protein concentration of mammary secretion skims and wheys was determined by Lowry analysis

(Lowry et al., 1951). Lactoferrin (Lf) and bovine serum albumin (BSA) concentrations were determined by electroimmunodiffusion (EID) on cellulose acetate (Schanbacher and Smith, 1974). Rabbit anti-bovine BSA was obtained commercially (Miles Scientific, Naperville, IL). Anti-bovine Lf was prepared by intramuscular injection of purified bovine Lf (Smith et al., 1971) into rabbits.

Mammary secretion skims and wheys were also characterized by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using the method of O'Farrell (1975) as modified by Horst and Roberts (1979). Dialyzed, lyophilized mammary secretions were solubilized in 5 mM K_2CO_3 containing 9.3 M urea, 2% (v/v) Nonidet-P40 and 0.5% (w/v) dithiothreitol. Isoelectric focusing in the first dimension used a disc gel electrophoretic procedure (Laemmli, 1970) which separated mammary secretion proteins by isoelectric point (pI). An average of 423 µg of total protein were loaded onto each gel (range 400-448 µg). The pH gradient of disc gels was established by addition of ampholines (pH ranges 3.5-10, 5-7 and 9-11) to the unpolymerized gel. Gels were electrophoresed under denaturing conditions in the presence of urea. The pH gradient of gels was determined by running a blank disc gel. The gel was cut into 1 cm lengths. Each piece was minced in deionized water, and pH was measured on a pH meter. The pH of slab gels ranged from 3.84 to 8.09. Following electrophoresis, disc gels were equilibrated briefly in .065 M Tris-HCl containing 1% (w/v) sodium dodecyl sulfate (SDS) and 1% (v/v) 2-mercaptoethanol. Disc gels were then placed on 10% polyacrylamide

slab gels and electrophoresed in the presence of SDS to separate mammary secretion proteins by molecular weight. Transferrin (M_r 78,000), ovalbumin (M_r 43,000), chymotrypsinogen (M_r 25,000), and ribonuclease A (M_r 13,899) were used as molecular weight markers. Slab gels were fixed in a solution of 40% ethanol and 7% acetic acid in deionized H_2O, stained with 0.125% Coomassie Brilliant Blue in ethanol, and destained in a solution of 10% acetic acid and 7% ethanol in deionized H_2O.

Mitogens

Concanavalin A (con A) and phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO) were utilized to induce mononuclear cell blastogenesis. Optimal concentrations of con A and PHA were determined in separate experiments (Part III). Concanavalin A (5 µg/ml) and PHA (25 µg/ml) were reconstituted in sterile ultrapure water and diluted in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO).

Isolation of mononuclear cells

Bovine peripheral blood mononuclear cells were isolated by the method of Boyum (1968) with modifications described by Nonnecke and Kehrli (1985). Bovine blood (80 ml) was collected by jugular venipuncture into 17 ml of modified Alsever's solution and diluted with two parts phosphate-buffered saline (PBS; pH 7.2, 0.15M). Diluted blood (12 ml) was overlaid carefully on 3 ml Ficoll-sodium diatrizoate with specific gravity 1.083 g/ml (Histopaque 1083; Sigma Chemical Co., St. Louis, MO). Following centrifugation (400 x g at 18°C)

for 50 min the mononuclear cell band was recovered and washed twice (300 x g for 15 min/wash at 18°C) in Ca^{+2}- and Mg^{+2}-free Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY). Residual red blood cells (RBC) were lysed by hypotonic shock treatment using sterile phosphate-buffered water (pH 7.2). Following a single wash in HBSS, cells were resuspended in RPMI 1640 medium containing 10 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin G and 100 µg streptomycin sulfate/ml. Mononuclear cell recovery and viability were determined by hemacytometer count using the trypan blue exclusion method (Patterson, 1979). No attempt was made to further purify mononuclear cell populations by removal of monocytes due to prior reports that ovine (Outteridge and Lee, 1981) and bovine (Ohmann et al., 1983) monocytes enhance response of lymphocytes to mitogens.

Cell quantification

Purity of isolated mononuclear cell populations was evaluated by differential microscopic count of cytocentrifuge (Cytospin 2; Shandon Southern Inc., Sewickley, PA) smears stained with Wright-Giemsa (Camco Quik Stain II: Cambridge Chemical Prod., Ft. Lauderdale, FL). Aliquots of cells from Ficoll gradients were also processed for light and electron microscopy using the method of Sordillo and Nickerson (1986). Briefly, cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0 at 37°C) for 2 hr and postfixed in 0.1 M cacodylate-buffered osmium tetroxide (pH 7.0 at 5°C) for 1.5 hr. Following dehydration in a graded series of ethanol washes, cell pellets were embedded in epoxy resin. Thick sections (0.5-1

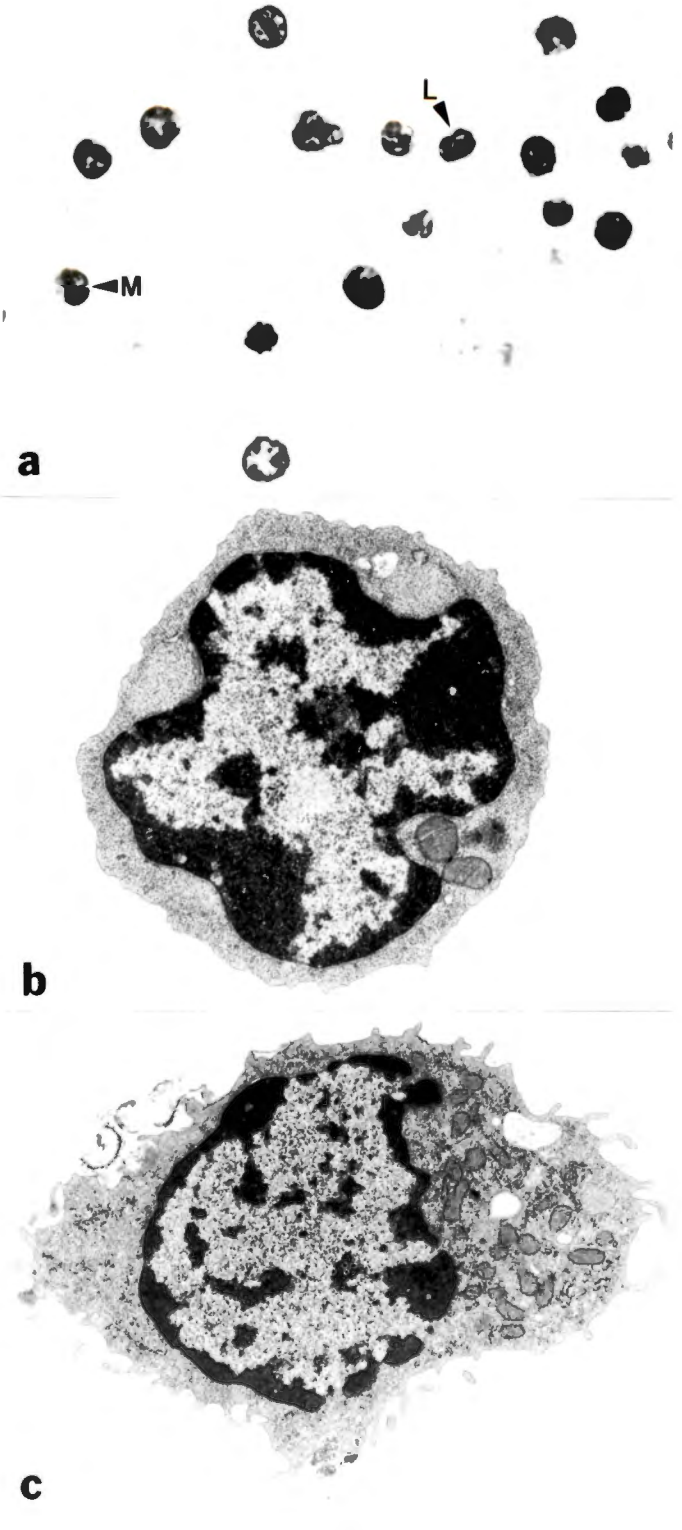
μm) were prepared and stained with toluidine blue for light microscopy. Ultrathin sections (60 nm) were prepared and stained for 20 min with 5% uranyl acetate in 50% methanol for electron microscopy. Representative lymphocytes and monocytes isolated by density gradient centrifugation are presented in Figure 1.

Cell culture procedures

Cells were cultured in 96 well flat-bottom microtitre plates (Corning Cell Wells; Corning Glass Works, Corning, NY). Fifty μl of RPMI 1640 containing $2x10^5$ viable mononuclear cells were added to each well. Additional components added to each well were: 100 μl of mitogen, 50 μl of fetal calf serum (heat inactivated and diluted in RPMI 1640 at a final concentration of 10%) and 50 μl of appropriately diluted bovine mammary secretion. Control wells received 150 μl of RPMI 1640 medium instead of mitogen and mammary secretion. Stimulated control wells received 50 μl of RPMI 1640 instead of mammary secretion.

Microtitre plates were incubated for 72 hr at 37°C in an atmosphere of 95% air and 5% CO_2. After 72 hr, 1 μCi of methyl-^{3}H-thymidine (ICN Radiochemicals, Irvine, CA) diluted in 50 μl of RPMI 1640 medium was added to each well. Following 18 hr incubation, mononuclear cells were harvested onto glass fiber filters using a multiple automated sample harvester (Mini-MASH II; MA Bioproducts, Walkersville, MD). Filters were placed in plastic scintillation vials and 2 ml of scintillation cocktail (Scintiverse II; Fisher Scientific

Figure 1. Electron micrographs of bovine peripheral blood mononuclear cells isolated by density gradient centrifugation. (a) Bovine mononuclear cells (x 600), L = lymphocyte, M = monocyte; (6) Representative bovine lymphocyte (x 15000); (c) Representative bovine monocyte (x 15000).


L
M
a
b
c

Co., Fairlawn, NJ) were added to each vial. Retained radioactivity was counted for 10 min/vial in a liquid scintillation counter (Isocap 300 Liquid Scintillation System; Searle Analytic Inc., Des Plaines, IL). Data were expressed as mean counts per min (cpm) x 10^3 of three replicate cultures. Data were expressed also as an index of inhibition where Index = cpm experimental/cpm stimulated control.

Sequential viability counts were performed by the trypan blue exclusion method on individual cultures at 24, 48 and 90 hr of culture. Viability counts were performed on duplicate wells for control, con A-stimulated control, PHA-stimulated control and D+28 skim and whey experimental cultures.

Statistical analysis

Count data were transformed to $\log_e$ for statistical analysis. However, actual count data are presented. Experimental design was split block and data were analyzed by least squares analysis of variance (Steel and Torrie, 1980) using the following model:

$$Y_{ijk} = \mu + C_i + D_j + S_k + DS_{jk} + \varepsilon_{ijk}$$

where: μ = overall mean cpm

C_i = cow

D_j = dilution of secretion

S_k = secretion

In order to further characterize differences in effects of individual secretions on bovine blood mononuclear cell blastogenesis, a series of contrasts were made between effects of different secretions using the same statistical model.

RESULTS

Mammary secretion composition

Representative 2D-PAGE gels of bovine mammary secretion skims are presented in Figures 2 and 3. Gels presented are of mammary secretions collected 7 and 28 d post drying off (Figure 2) and of colostrum and normal milk (Figure 3). The protein band at pH of approximately 8 and with M_r similar to that of transferrin (78,000) was presumed to be Lf. Light (IgG_L) and heavy (IgG_H) chains of IgG were identified based upon molecular weights and protein map patterns reported by Anderson and Anderson (1977) for human IgG_L and IgG_H. Serum albumin was identified based upon reported pI and molecular weight (Eigel et al., 1984) and upon comparison with electrophoretic patterns of serum albumin from human plasma (Anderson and Anderson, 1977), murine milk skim (Lee et al., 1984) and bovine milk whey (Shimazaki et al., 1983). Caseins are contained in the acidic region of the slab gel near the 25,000 M_r marker based upon comparison with migration of murine skim milk caseins (Lee et al., 1984), reported pI and mw of bovine milk caseins (Eigel et al., 1984) and relative absence of these spots in whey preparations. Identification of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) was based upon pI reported by Eigel et al. (1984), approximate M_r reported by Hurley and Rejman (1986) and comparison with 2D-PAGE protein maps of bovine milk whey presented by Shimazaki et al. (1983). Other protein bands located in the region of the 78000 M_r marker (Figures 2 and 3) may

Figure 2. Two-dimensional polyacrylamide gel electrophoresis of bovine mammary secretions collected at 7 and 28 d of involution. Gels were stained with Coomassie Blue. (a) PAGE of bovine mammary secretion skim obtained 7 d post-drying off; (b) PAGE of bovine mammary secretion skim obtained 28 d post-drying off; Lf, lactoferrin; BSA; bovine serum albumin IgG_H, heavy chain IgG; IgG_L, light chain IgG; $\underline{\alpha}$-LA, $\underline{\alpha}$-lactalbumin;β-LG, $\underline{\beta}$-lactoglobulin; c, caseins.

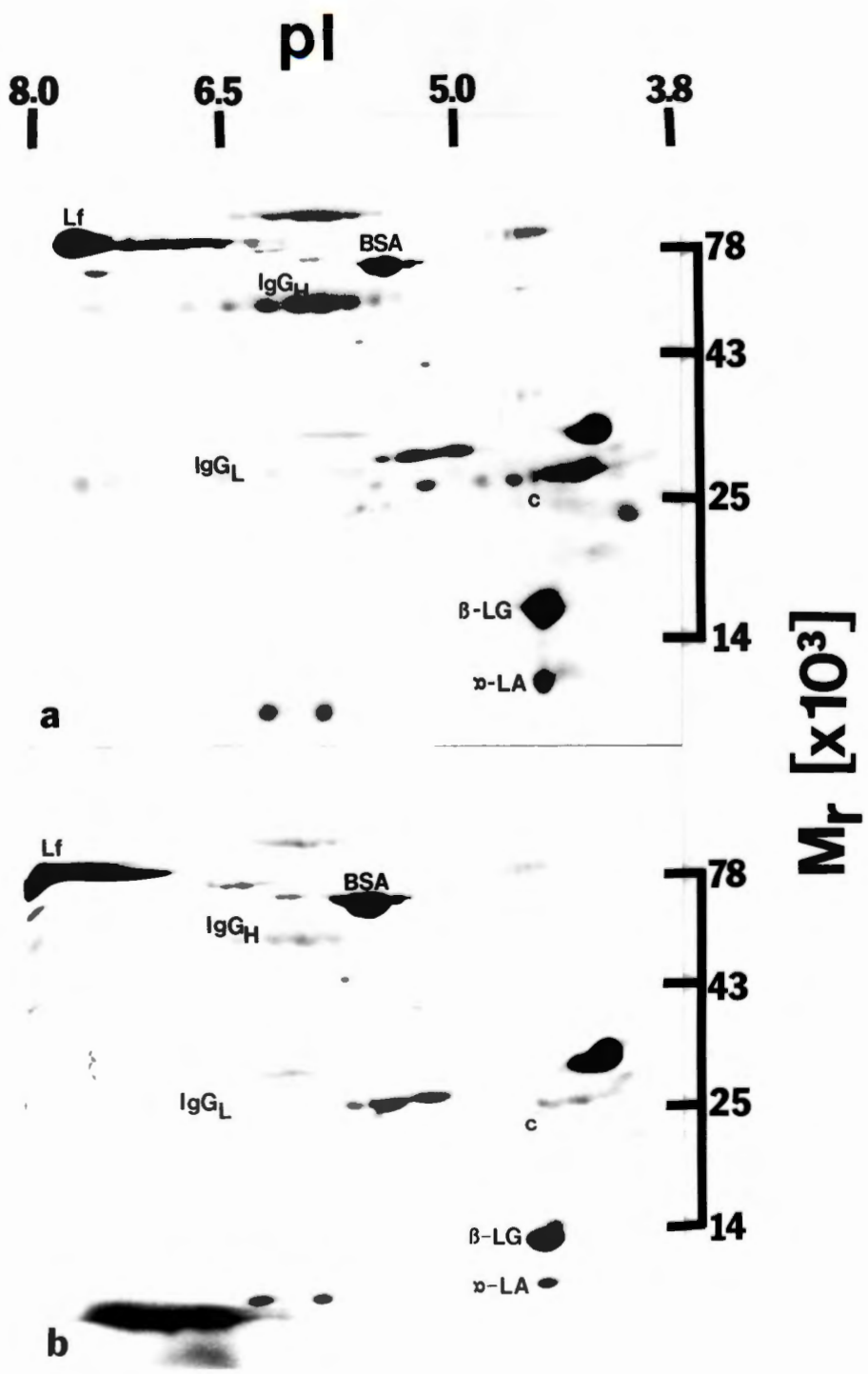
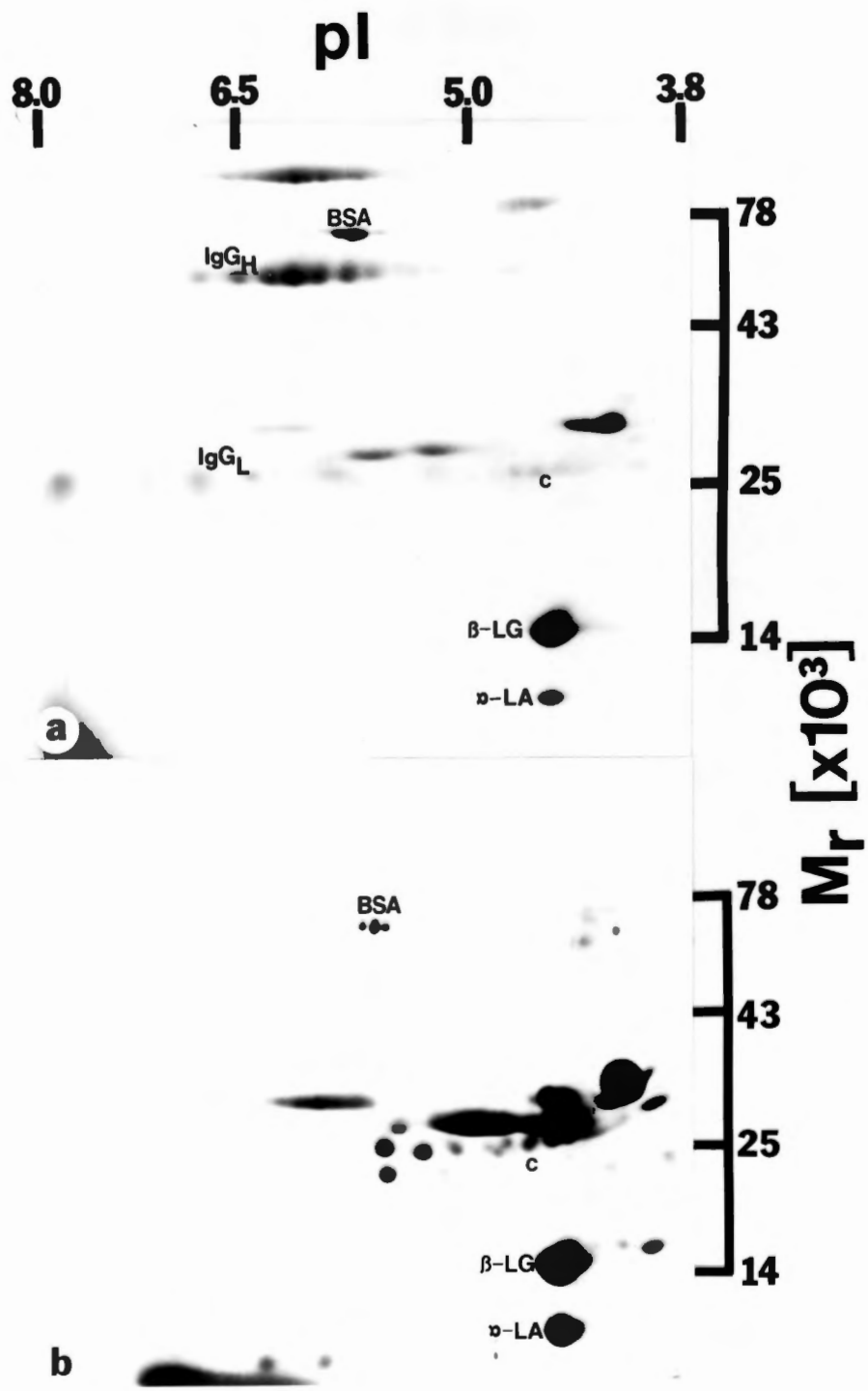

pI
8.0
6.5
5.0
3.8
Lf
BSA
IgG$_H$
IgG$_L$
c
ß-LG
α-LA
a
78
43
25
14
M$_r$ [x10^3]
Lf
BSA
IgG$_H$
IgG$_L$
c
ß-LG
α-LA
b
78
43
25
14

Figure 3. Two-dimensional polyacrylamide gel electrophoresis of bovine colostral and normal milk skims. Gels were stained with Coomassie Blue. (a) PAGE of bovine colostral skim; (b) PAGE of bovine normal milk skim; Lf, lactoferrin; BSA, bovine serum albumin; IgG_H, heavy chain IgG; IgG_L, light chain IgG; $\underline{\alpha}$-LA, $\underline{\alpha}$-lactalbumin; $\underline{\beta}$-LG, $\underline{\beta}$-lactoglobulin; c, caseins.


pI
8.0
6.5
5.0
3.8
BSA
IgG$_H$
IgG$_L$
c
β-LG
α-LA
a
78
43
25
14
M_r [x10^3]
BSA
c
β-LG
α-LA
b
78
43
25
14

represent fragments of higher molecular weight immunoglobulins.

Based upon relative size and intensity of staining of protein bands and spots, Lf, BSA, IgG_H and IgG_L appeared to be relatively similar between D+7 and D+28 (Figure 2). Casein, α-LA and β-LG remained relatively constant. In contrast, colostrum (Figure 3) had greater IgG_H, IgG_L, α-LA, and β-LG compared to D+28. Greater amounts of high M_r proteins were also observed in colostrum than in dry secretions. Lactoferrin and BSA decreased in colostrum. The majority of proteins contained in normal milk (Figure 3) were caseins, α-LA and β-LG. Only minor amounts of Lf, BSA, IgG_H, and IgG_L were detected. Determination of Lf and BSA concentrations in mammary secretions by EID corroborated 2D-PAGE results (Table 1).

Cell cultures

Bovine blood mononuclear cell isolation procedures resulted in a population of >99% pure mononuclear cells comprised of approximately 75% lymphocytes and 25% monocytes based upon differential microscopic cell counts of cytocentrifuge smears. Light microscope counts on thick sections from cell pellets and corroborated by EM preparations on mononuclear cells isolated from two cows showed that approximately 70% of cells were lymphocytes and 30% were monocytes with <1% granulocytic contamination.

Mean viability ± standard error of mononuclear cells recovered from Ficoll gradients prior to initiation of cultures was 81.3 ± 0.8%. Viabilities of various cell preparations at intervals throughout the 90 hr culture period are presented in Table 2. Only in the

Table 1. Concentration of lactoferrin and bovine serum albumin in mammary secretion wheys.

	Protein concentration (mg/ml)	
Secretion	Lf	BSA
Early involution	15.0	2.3
Mid-involution	17.2	3.1
Colostrum	.5	1.0
Normal milk	.2	.1

Table 2. Mean viability of selected cell preparations throughout 90 hr of culture.[1,2]

Hours of culture	Cell viability				
	Control	Con A	PHA	Con A + D+28 skim	Con A + D+28 whey
24	76.7 ± 2.1	74.3 ± 1.4	68.4 ± 2.1	82.2 ± .8	78.4 ± 1.7
48	77.4 ± 2.3	66.8 ± 2.5	61.4 ± 1.8	74.4 ± 2.7	67.2 ± 2.3
90	80.3 ± 2.0	64.2 ± 3.9	38.4 ± 4.8	79.6 ± 4.4	65.6 ± 2.6

[1]Data expressed as mean % viability ± standard error of the mean.

[2]Viability obtained by hemacytometer count using trypan blue exclusion method of duplicate wells of a culture plate.

case of PHA-stimulated cells did cell viability decrease to below 60% at 90 hr of culture. Viability of PHA-stimulated cells was consistently lower than con A-stimulated cells. However, mean cpm of PHA-stimulated cells was 8.5 times higher than control values.

Results of incubation of bovine blood mononuclear cells with undiluted bovine mammary secretions are presented in Figures 4 and 5. Total protein concentration of mammary secretion skims and wheys are presented in Table 3. Individual cow variation in magnitude of inhibition of mononuclear cell response to mitogens by mammary secretions was significant ($P< .05$). Mitogenic stimulation of bovine mononuclear cells incubated with PHA appeared to be less inhibited by mammary secretions than cells incubated with con A ($P<.05$). This was especially noticeable with skim preparations (Figure 4). Mammary secretion whey (Figure 5) caused consistently greater inhibition ($P<.05$) of mononuclear cell blastogenesis than skim preparations.

Normal milk skim and whey caused the least inhibition of mononuclear cell response to mitogens (Figures 4 and 5). In contrast, greatest inhibition of both con A- and PHA-stimulated cells was caused by secretions obtained 7 d post-drying off and at parturition. Inhibition of PHA-stimulated cells by secretions collected from fully involuted mammary glands was significantly less ($P<.05$) than for secretions obtained during early involution. Similarly, colostral skim and whey caused greater ($P<.05$) inhibition of mononuclear cell response to PHA than did normal milk skim and whey. Of the skim preparations used, colostral skim was associated with the greatest

Figure 4. Effect of undiluted bovine mammary gland secretion skims on bovine peripheral blood mononuclear cell blastogenesis. Data are expressed as counts per minute ± standard error of the mean x 10^3.

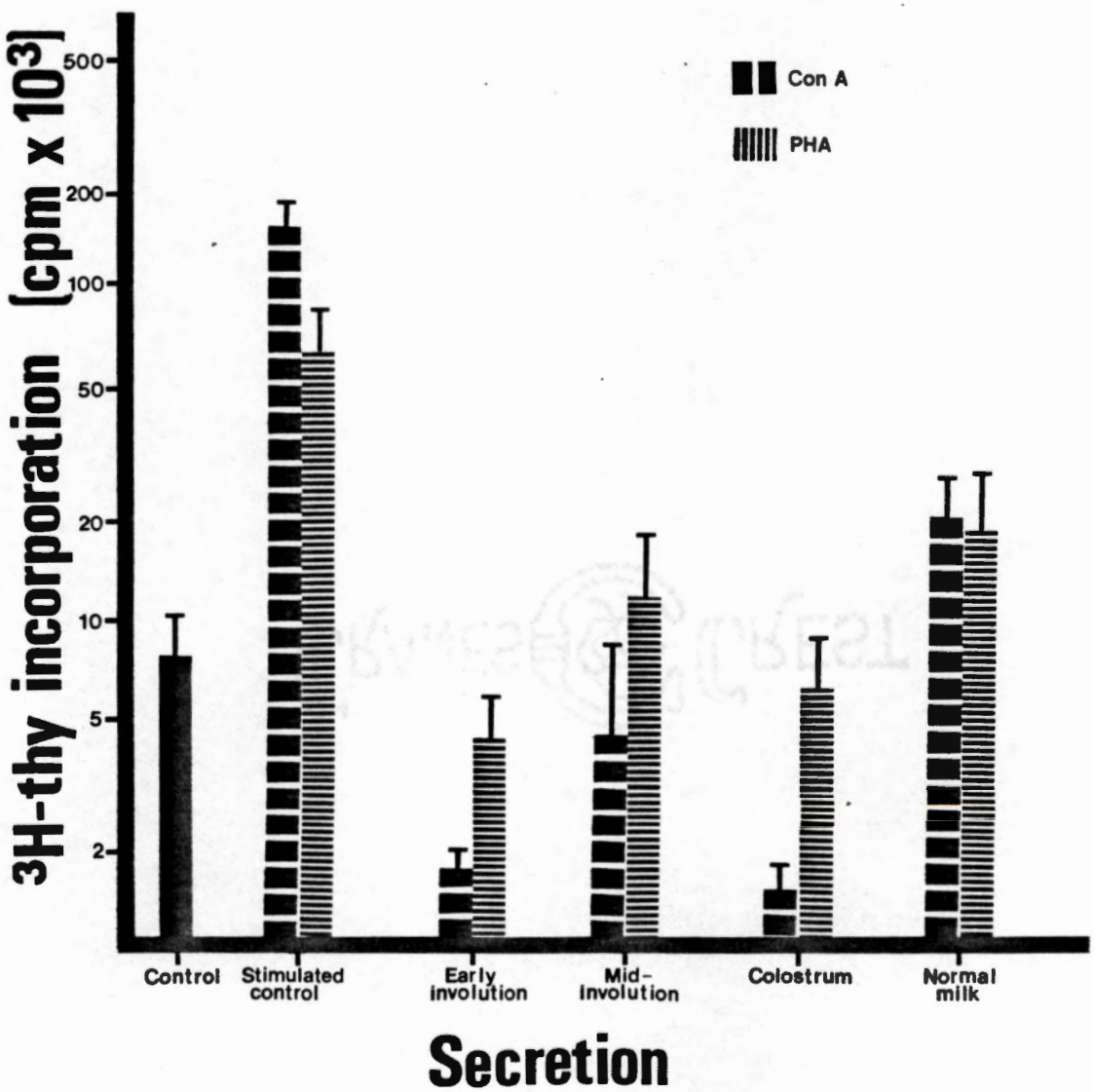

3H-thy incorporation (cpm x 10³)
500
200
100
50
20
10
5
2
Con A
PHA
Control
Stimulated control
Early involution
Mid-involution
Colostrum
Normal milk
Secretion

Figure 4

Figure 5. Effect of undiluted bovine mammary gland secretion wheys on bovine peripheral blood mononuclear cell blastogenesis. Data are expressed as counts per minute ± standard error of the mean x 10^3.

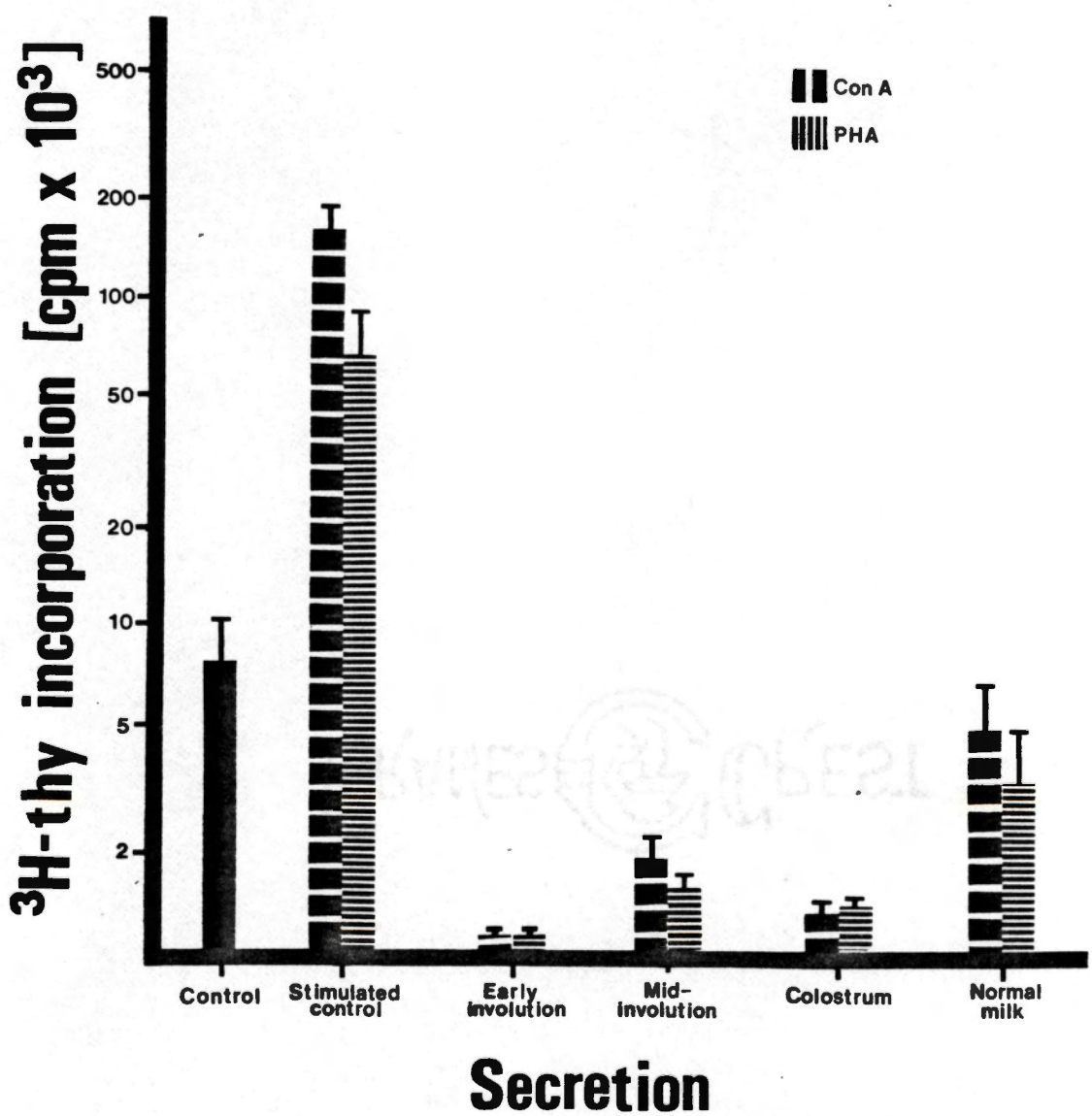

^{3}H-thy incorporation [cpm x 10^3]
500
200
100
50
20
10
5
2
Con A
PHA
Control
Stimulated control
Early involution
Mid-involution
Colostrum
Normal milk
Secretion

Figure 5

Table 3. Total protein concentration of bovine mammary secretions collected at various stages of the lactation cycle.[1]

Secretion		Protein concentration (mg/ml)
Early involution	skim	84.1
	whey	64.4
Mid-involution	skim	74.2
	whey	60.8
Colostrum	skim	87.6
	whey	69.4
Normal milk	skim	22.7
	whey	5.5

[1]Total protein concentration in secretions was determined by Lowry analysis.

inhibition of con A-stimulated mononuclear cell blastogenesis (Figure 4). In contrast, early involution whey caused the greatest inhibition of con A-stimulated cells (Figure 5). However, differences in inhibition of con A-stimulated cells by early involution secretion and colostrum were not statistically significant for both skim and whey preparations.

Effects of serial four-fold dilutions of mammary secretions on mononuclear cell blastogenesis are presented in Table 4. Dilution of secretions significantly ($P<.05$) reduced inhibition of mononuclear cell blastogenesis by both con A and PHA in a dose-dependent manner. However, results of dilution of mammary secretions did not follow identical patterns. The index of inhibition of mammary secretion whey was consistently lower than for skim preparations. Dilution of whey preparations generally affected PHA-stimulated cells to a greater degree than con A-stimulated cells. Effects of mammary secretion skims obtained 7 and 28 d post drying off were less completely removed by a 1:256 dilution for con A-stimulated cells compared to PHA-stimulated cells. In contrast, effects of colostrum and normal milk skims on PHA-stimulated cells were less completely removed by a 1:256 dilution.

DISCUSSION

Human colostrum and milk (Diaz-Jouanen and Williams, 1974; Ogra and Ogra, 1978; Crago et al., 1981; Drew et al., 1982) and bovine colostrum and dry secretion (Collins and Oldham, 1986) have been

Table 4. Effects of dilution of mammary secretions on bovine peripheral blood mononuclear cell blastogenesis.[1]

		Skim					Whey				
		Dilution of secretion					Dilution of secretion				
Secretion	Mitogen	0	1:4	1:16	1:64	1:256	0	1:4	1:16	1:64	1:256
Early involution	Con A	.009	.086	.125	.412	.633	.001	.030	.150	.439	.608
	PHA	.067	.546	.832	1.000	.936	.002	.055	.504	.792	.714
Mid-involution	Con A	.029	.068	.159	.348	.707	.012	.030	.086	.340	.746
	PHA	.190	.598	.989	1.096	1.034	.016	.127	.525	.758	.748
Colostrum	Con A	.006	.195	.508	.899	.950	.004	.091	.427	.677	.690
	PHA	.095	.324	.419	.773	.779	.011	.071	.312	.683	.861
Normal milk	Con A	.129	.567	.880	.986	.987	.037	.373	.639	.781	.861
	PHA	.288	.556	.821	.873	.844	.050	.419	.807	.813	.896

[1]Data expressed as an index of inhibition where index = cpm experimental/cpm stimulated control.

shown to suppress peripheral blood lymphocyte responsiveness to both T- and B-cell mitogens. Of the secretions used in our study, colostrum and early involution mammary secretion caused greatest inhibition of bovine peripheral blood mononuclear cells for both con A and PHA. Proteinaceous components of bovine mammary secretions which have been shown to suppress in vitro response of lymphocytes to mitogens include Lf (Smith et al., 1979) and secretory component (Crago et al., 1981). If Lf alone was responsible for variation in lymphocyte activity, then inhibition of PBL response to mitogens should have been similar for mammary secretions collected at 7 and 28 d of involution, since the concentration of Lf was similar. However, such was not the case. Significantly greater inhibition of mononuclear cell blastogenesis by mammary secretions collected 7 d post-drying off may be indicative of the presence of an as yet unidentified immunosuppressant present in greater concentration in mammary secretions from early rather than fully involuted bovine mammary glands.

Crago et al. (1981) reported that of the components of human colostrum tested, only free secretory component (FSC) significantly inhibited human PBL response to PWM via suppression of B-cell cytoplasmic Ig. Bovine colostral secretory IgA concentrations are elevated compared to dry secretion and milk (Butler, 1983) and therefore FSC concentration might also be assumed to be elevated. However, no data are available concerning inhibition of T-cell mitogen-induced bovine lymphocyte blastogenesis by FSC and thus no conclusions can

be made about the role of FSC as an immunosuppressant in bovine colostrum. The concentration of Lf in colostrum was low. Therefore, it is also unlikely that the immunosuppressive effects of colostrum were due solely to Lf.

A possible explanation for increased immunosuppression of colostrum compared to normal milk may be related to factors of serum origin. Pregnancy-associated immunosuppression of ovine PBL has been reported (Yamamoto et al., 1980; Miyasaka and McCullagh, 1981). Similar maternal immunosuppression was observed in humans and mice (Lloyd, 1983) which may be related to prevention of fetal rejection. Suppression of bovine PBL response to mitogens during the peripartum period has been reported (Soper et al., 1978) and could be associated with the transfer of maternal immunity to the neonate via lymphocytes which do not react against paternal alloantigens in the neonates tissues (Parmely and Williams, 1979). Hormones such as prolactin, estrogens, adrenal glucocorticoids, prostaglandins and placental lactogen are involved critically in lactogenesis (Tucker, 1981), and may possess immunosuppressive properties (Clemens et al., 1979; Kelly and Dineen, 1973; Morse, 1976). Increased concentrations of hormones in colostrum which may be both lactogenic and immunosuppressive may account for increased suppression of PBL by colostrum compared to normal milk and secretions from fully involuted mammary glands.

Marked suppression of bovine blood mononuclear cell response to PHA compared to con A was observed in this study. Lower viability of PHA-stimulated cells at 90 hr of culture may have contributed

to depressed count values. Inhibitory effects of colostral and normal milk skim on PHA-stimulated cells were more difficult to remove by dilution compared to con A-stimulated cells. Conversely, inhibitory effects of secretions collected 7 and 28 d post drying-off were more difficult to remove for con A-stimulated cells. Concanavalin A and PHA preferentially stimulate T-lymphocytes (Schechter, 1980). Differences in stimulation of mononuclear cells may reflect differences in effectiveness of con A and PHA as T-cell mitogens. On the other hand, Stobo and Paul (1973) proposed the existence of two distinct subsets of murine T-cells. One subset of T-cells responded equally well to con A and PHA while the other subset responded best to con A. Using differential erythrocyte rosetting techniques, Belden and Strelkauskas (1981) demonstrated the presence of subsets of bovine blood T-cells which responded differently to mitogens and in the mixed lymphocyte reaction. Wilson et al. (1986), using similar rosetting techniques, demonstrated the presence of similar subsets of bovine mammary gland T-cells. Evidence is unclear as to whether different subsets of bovine T-cells are entirely different populations of T-cells. Each subset may be a different maturational stage of bovine T-lymphocyte ontogeny. However, it is possible that differences in response of bovine blood mononuclear cells to con A and PHA in our study may reflect the presence of subpopulations of T-cells found in bovine blood. Lessened ability of mammary secretions to inhibit PHA stimulation of mononuclear cells may indicate relative

insensitivity of a PHA-responsive subpopulation of bovine T-lymphocytes to immunosuppressive factors in mammary secretions. The inhibitory effect of normal milk on bovine mononuclear cell blastogenesis was more easily removed by dilution, possibly due to lower concentration of immunosuppressive substances in milk compared to secretions collected from involuted mammary glands. Difficulty in removing inhibition of blastogenesis of PHA-stimulated cells compared to con A-stimulated cells by dilution of colostrum and milk skim could indicate the presence of immunosuppressants in those secretions which exert more lasting effects on the subset of T-cells stimulated by PHA. Similarly, secretions collected during the first 30 d of involution may exert more permanent immunosuppressive effects upon the subset of T-cells stimulated by con A. However, a further understanding of the nature of immunosuppressive substances in mammary secretions and the mechanisms by which these substances inhibit mitogenic stimulation of lymphocytes are required to substantiate these hypotheses.

The bovine mammary gland is particularly susceptible to infection during physiological transitions from lactation to involution and from involution to colostrogenesis. Highest rates of new IMI occur during the first three weeks following drying off (Neave et al., 1950; Ward and Schultz, 1974) and during the peripartum period (Eberhart, 1982; Oliver and Mitchell, 1983; Ward and Schultz, 1974). Increased susceptibility of the mammary gland to new IMI has been related to fluid accumulation (Cousins et al., 1980), relative paucity of certain protective factors such as Lf (Smith and Todhunter,

1982) and reduced capacity of mammary phagocytes to effect intracellular killing of invading bacteria (Paape et al., 1981; Paape and Guidry, 1977). Limited data are available concerning the role of lymphocytes in the defense of the mammary gland against IMI during physiological transitions. However, our study suggests that mammary secretions collected during the times of highest susceptibility to new IMI are most inhibitory to bovine blood mononuclear cell blastogenesis. Increased suppression of mammary mononuclear cell blastogenesis during early involution and the peripartum period could contribute to increased susceptibility of the mammary gland to infection. However, further studies of mononuclear cell function throughout involution will be required before definitive conclusions can be formed about the role of mononuclear cells in variation in rates of IMI during involution.

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PART III

CHANGES IN BLASTOGENIC ACTIVITY OF BOVINE BLOOD MONONUCLEAR CELLS THROUGHOUT THE NONLACTATING PERIOD

ABSTRACT

Mononuclear cells were isolated from bovine blood by density gradient centrifugation. Optimum concentrations of concanavalin A, phytohemagglutinin, Salmonella typhimurium lipopolysaccharide and three Escherichia coli lipopolysaccharides were determined using six cows as blood donors. Cellular response to mitogens was evaluated by methyl-^{3}H-thymidine incorporation. Concanavalin A, phytohemagglutinin and Escherichia coli 0111:B4 lipopolysaccharide were selected for further experiments. Mitogenic responses of blood mononuclear cells from five cows were evaluated at drying off, 14-16 and 28-30 d post drying off, 12-14 d prepartum and at parturition. Concanavalin A-treated cells exhibited greater blastogenic activity than phytohemagglutinin-treated cells. Response of cells to concanavalin A increased slightly through 28-30 d post drying off and decreased markedly at parturition. Blastogenic activity of cells treated with phytohemagglutinin decreased throughout the nonlactating period and was lowest at parturition. Activity of lipopolysaccharide-treated mononuclear cells increased through 28-30 d post drying off. However, response of mononuclear cells to lipopolysaccharide was minimal compared to concanavalin A and phytohemagglutinin. Variation in peripheral blood mononuclear cell activity throughout involution may parallel mammary gland mononuclear cell activity, affecting mammary gland susceptibility to new intramammary infections.

INTRODUCTION

The dry period is an important time in the etiology of mastitis. Rates of new intramammary infection (IMI) increase dramatically during the dry period (Neave et al., 1950). However, the rate of new IMI is not constant throughout the dry period. The mammary gland is increasingly susceptible to new IMI during the first three weeks after drying off (Neave et al., 1950; Ward and Schultz, 1974; Oliver and Mitchell, 1983) and susceptibility to new IMI increases also during the peripartum period (Eberhart, 1982; Smith et al., 1985; Oliver and Mitchell, 1983; Oliver, 1987). On the other hand, the fully involuted gland appears to be resistant to new IMI (Smith and Todhunter, 1982).

Little attention has been focused on the role of cellular defenses of the mammary gland, in particular lymphocytes, in the dynamics of IMI throughout the nonlactating period. The bovine mammary gland possesses a population of viable, functionally competent lymphocytes of peripheral blood origin which may contribute to the defense of the gland against IMI (Smith and Schultz, 1977; Concha et al., 1978; Concha et al., 1980; Schore et al., 1981). A factor which contributes to dynamics of infection is variation in lymphocyte number and activity. Bovine peripheral blood lymphocyte activity varies seasonally, with age, and by individual cow (Soper et al., 1978). The objective of this study was to determine variation in mitogen-induced bovine peripheral blood mononuclear cell activity throughout the nonlactating period.

MATERIALS AND METHODS

Animals

Four multiparous and two primiparous Holstein cows from the University of Tennessee dairy research herd were selected as blood donors to establish optimum concentrations of mitogens for further experiments. Cow age ranged from 33-55 mo ($\bar{x}$ 44 mo). Cows had been lactating 26 to 42 weeks and were producing an average of 20 kg of milk/day (range 18-24 kg/day).

Five mature Holstein cows were selected as blood donors for determination of changes in bovine blood mononuclear cell blastogenic activity throughout involution. The experiment was conducted from July-September. Cow age ranged from 34-77 mo ($\bar{x}$ 51 mo). Cows were producing an average of 15 kg of milk/day at drying off (range 10-21 kg/day) and had gone through 1-4 lactations prior to cessation of milking. Blood samples were collected from each cow on the day of drying off (D-0), 2 wk after drying off (D+14-16), 4 wk after drying off (D+2830), two weeks prior to expected parturition (C-14-12), and within 24 hr of parturition (C-0).

Mitogens

T-cell mitogens used were concanavalin A (con A) and phytohemagglutinin (PHA; Sigma Chemical Co, St. Louis, MO). B-cell mitogens selected were lipopolysaccharides (LPS) from Salmonella typhimurium and Escherichia coli serotypes 0111:B4, 026:B6, and 0127:B8 (Difco

Laboratories, Detroit, MI). Concanavalin A was added to cultures at initial concentrations of 1, 5, 10, 25, 50 and 100 µg/ml. Phytohemagglutinin was added at concentrations of .2, 1, 5, 10, 25 and 50 µg/ml. Lipopolysaccharides were added at concentrations of .2, 1, 5, 10 20 and 40 µg/ml. Concanavalin A (5 µg/ml), PHA (25 µg/ml) and E. coli 0111:B4 LPS (20 µg/ml) were selected as mitogens for studies of bovine blood mononuclear cell blastogenic activity throughout involution.

Isolation of mononuclear cells

Bovine blood mononuclear cells were isolated by the method of Boyum (1968) with modifications described by Nonnecke and Kehrli (1985). Blood (40 ml) was collected by jugular venipuncture using modified Alsever's solution as the anticoagulant. Blood was diluted in two parts of phosphate-buffered saline (PBS; 0.15 M, pH 7.2). Diluted blood (12 ml) was layered over 3 ml of Ficoll-sodium diatrizoate with specific gravity of 1.083 g/ml (Histopaque 1083; Sigma Chemical Co., St Louis, MO) and centrifuged at 400 x g at 18°C for 50 min. Following aspiration of plasma, the mononuclear cell band was recovered and washed two times in Hanks balanced salt solution (HBSS; Sigma Chemical Co., St Louis, MO) at 300 x g for 15 min/wash. Residual erythrocytes were lysed by hypotonic shock treatment using sterile phosphate-buffered water. Cells were reconstituted in HBSS, washed, and resuspended in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO) containing 10 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin-G and 100 µg streptomycin sulfate/ml. Mononuclear cell

recovery and viability were determined by hemacytometer count using the trypan blue exclusion method (Patterson, 1979). Mononuclear cell purity was evaluated by differential microscopic cell counts of cytocentrifuge (Cytospin 2; Shandon Southern Inc., Sewickley, PA) smears stained with Wright-Giemsa (Camco Quik Stain II; Cambridge Chemical Prod., Ft Lauderdale, FL). Isolation procedures resulted in a population of mononuclear cells consisting of approximately 75% lymphocytes and 25% monocytes with <1% granulocytic contamination. No attempt was made to further purify mononuclear cell populations based on reports that ovine (Outteridge and Lee, 1981) and bovine (Ohmann et al., 1983) monocytes enhance response of lymphocytes to mitogens. Mean viability of mononuclear cells prior to initiation of cultures was 79.3 ± 1.4%.

Cell cultures

Cells were cultured in 96-well flat bottom microtitre plates (Corning Cell Wells; Corning Glass Works, Corning, NY). For determination of optimum concentrations of mitogens, each well contained $2x10^5$ viable cells in 50 µl of RPMI 1640 medium and 50 µl of heat-inactivated (56°C for 30 minutes) fetal calf serum diluted in RPMI 1640 medium to give a final concentration of 10%. Experimental wells received 100 µl of the appropriate mitogen. Control wells received 100 µl of RPMI 1640 instead of mitogen. For studies of mononuclear cell blastogenic activity throughout involution, each well contained $2x10^5$ viable cells in 100 µl of RPMI 1640. All other parameters

were the same as described for determination of optimal mitogen concentrations.

Cultures were incubated for 72 hr at 37°C in an atmosphere of 95% air and 5% CO_2. After 72 hr, 1 µCi of methyl-^{3}H-thymidine (ICN Radiochemicals, Irvine, CA) diluted in 50 µl of RPMI 1640 was added to each well. Following 18 hr incubation, cultures were terminated and harvested onto glass fiber filters using a multiple automated sample harvester (Mini-MASH II; MA Bioproducts, Walkersville, MD). Filters were placed in plastic scintillation vials. Two ml of scintillation cocktail (Scintiverse II; Fisher Scientific Co., Fairlawn, NJ) were added to each vial and retained radioactivity was counted for 10 min/vial in a liquid scintillation counter (Isocap 300 Liquid Scintillation System, Searle Analytics Inc., Des Plaines, IL). Data were expressed as mean counts per min (cpm) ± standard error of the mean x 10^3 of four replicate counts and as a stimulation index (SI) where SI = cpm experimental/cpm control.

Statistical analysis

Data were analyzed as a split block experiment by least squares analysis of variance (Steel and Torrie, 1980) using the model:

$$Y_{ijk} = \mu + M_i + C_j + MC_{ij} + D_k + MD_{ik} + \varepsilon_{ijk}$$

Where: μ = mean cpm

M = mitogen

C = cow

D = day of dry period

Count data were transformed to $\log_e$ values for statistical analysis, but actual count data are presented. In order to determine significant differences in effects of individual mitogens between sampling days, a series of contrasts were made using the model:

$$Y_{ij} = \mu + C_i + D_j + \varepsilon_{ij}$$

Where: μ = mean cpm

C = cow

D = day of dry period

RESULTS

Mitogenic stimulation of bovine peripheral blood mononuclear cells by con A, PHA, S. typhimurium LPS and various E. coli LPS is presented in Figures 6 and 7. Greatest stimulation of cells was elicited by con A and PHA. The optimal concentration of con A (5 µg/ml) caused greater stimulation of mononuclear cells than the optimal concentration of PHA (25 µg/ml; Figure 6). Lipopolysaccharide preparations caused marginal stimulation of cells at all concentrations (Figure 7). Escherichia coli 0111:B4 LPS caused greatest stimulation of cells at a concentration of 20 µg/ml due mainly to the effects of the LPS on mononuclear cells of two cows.

Effects of con A, PHA and E. coli 0111:B4 LPS on bovine peripheral blood mononuclear cells obtained throughout involution are presented in Figures 8 and 9. Use of LPS was discontinued following the third sampling period (D+28-30) due to lack of response of LPS-

Figure 6. Dose response curve of stimulation of bovine peripheral blood mononuclear cells by concanavalin A and phytohemagglutinin. Data are expressed as mean counts per minute ± standard error of the mean x 10^3.

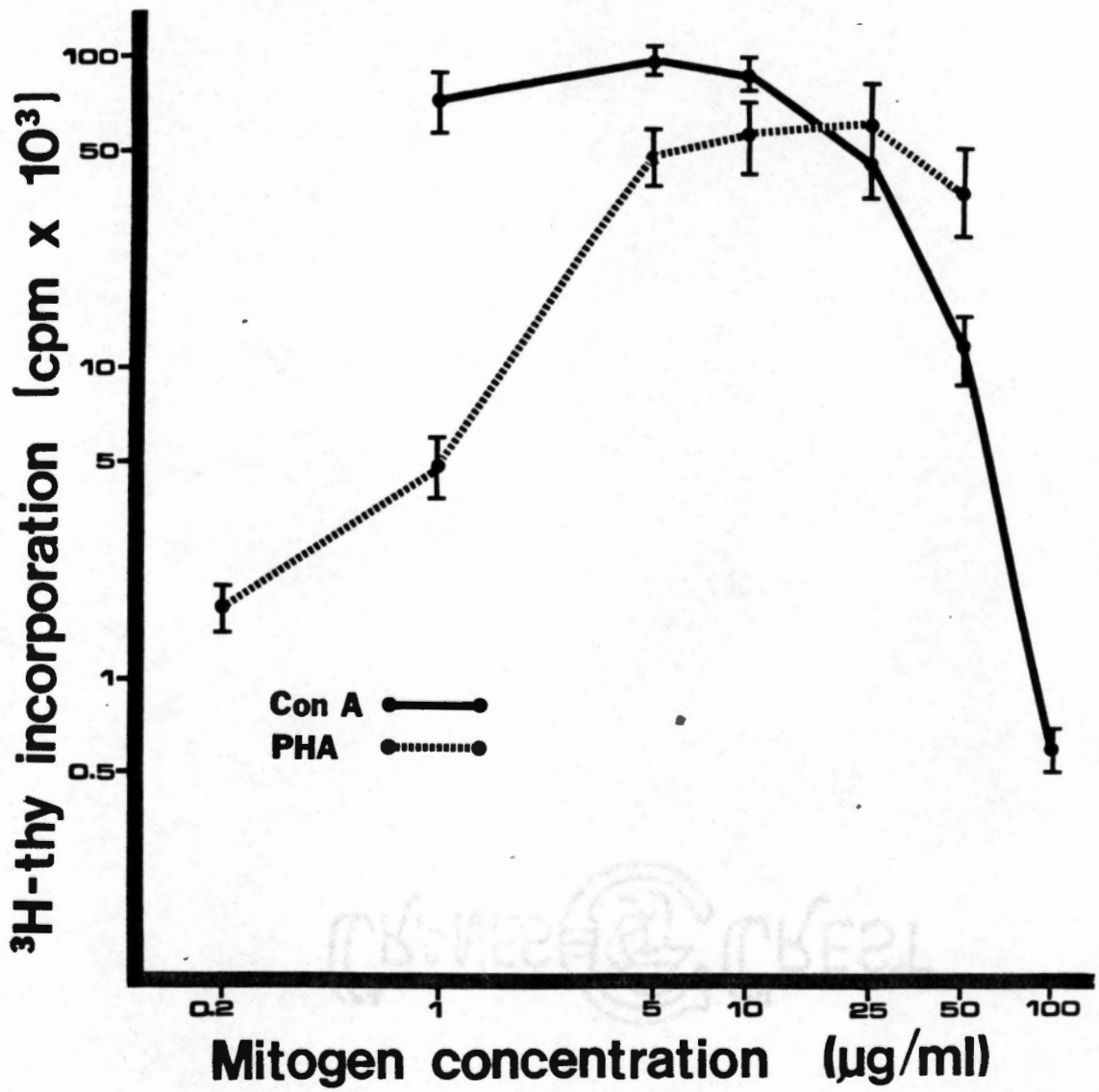

100
50
10
5
1
0.5
^{3}H-thy incorporation (cpm x 10^3)
Con A
PHA
0.2
1
5
10
25
50
100
Mitogen concentration (µg/ml)

Figure 6

Figure 7. Dose response curve of stimulation of bovine peripheral blood mononuclear cells by various lipopolysaccharides. Data are expressed as mean counts per minute ± standard error of the mean x 10^3.

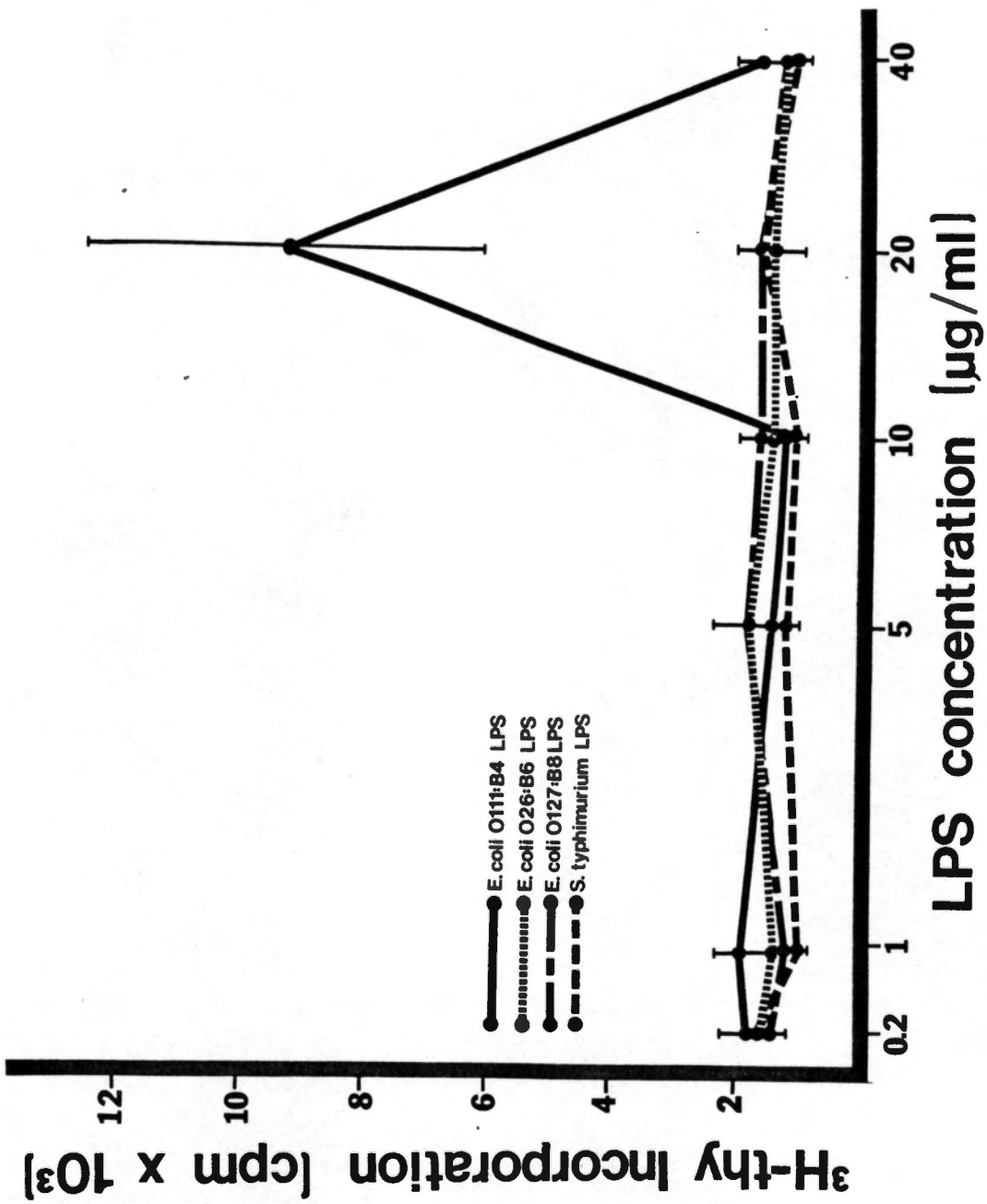

3H-thy Incorporation (cpm x 10³)
12
10
8
6
4
2
0.2
1
5
10
20
40
LPS concentration (µg/ml)
E.coli O111:B4 LPS
E.coli O26:B6 LPS
E.coli O127:B8 LPS
S. typhimurium LPS

Figure 7

Figure 8. Mitogenic stimulation of bovine peripheral blood mononuclear cells throughout the nonlactating period. Data are expressed as mean counts per minute ± standard error of the mean x 10^3.  , control;  , con A;  , PHA;  , LPS.

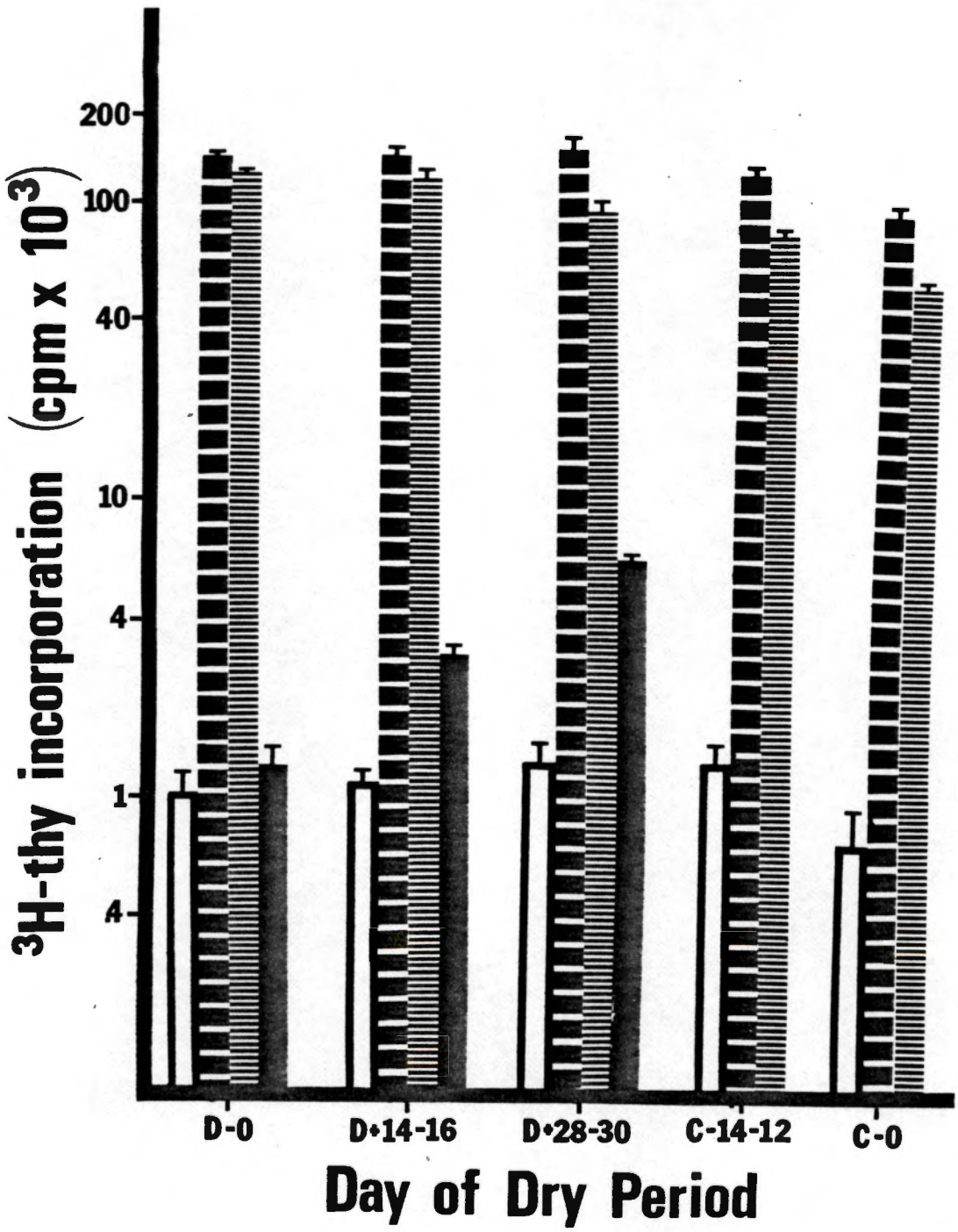

200
100
40
10
4
1
.4
^{3}H-thy incorporation (cpm x 10^3)
D-0
D+14-16
D+28-30
C-14-12
C-0
Day of Dry Period

Figure 8

Figure 9. Mitogenic stimulation of bovine peripheral blood mononuclear cells throughout the nonlactating period. Data are expressed as a stimulation index where SI = cpm experimental/cpm control.

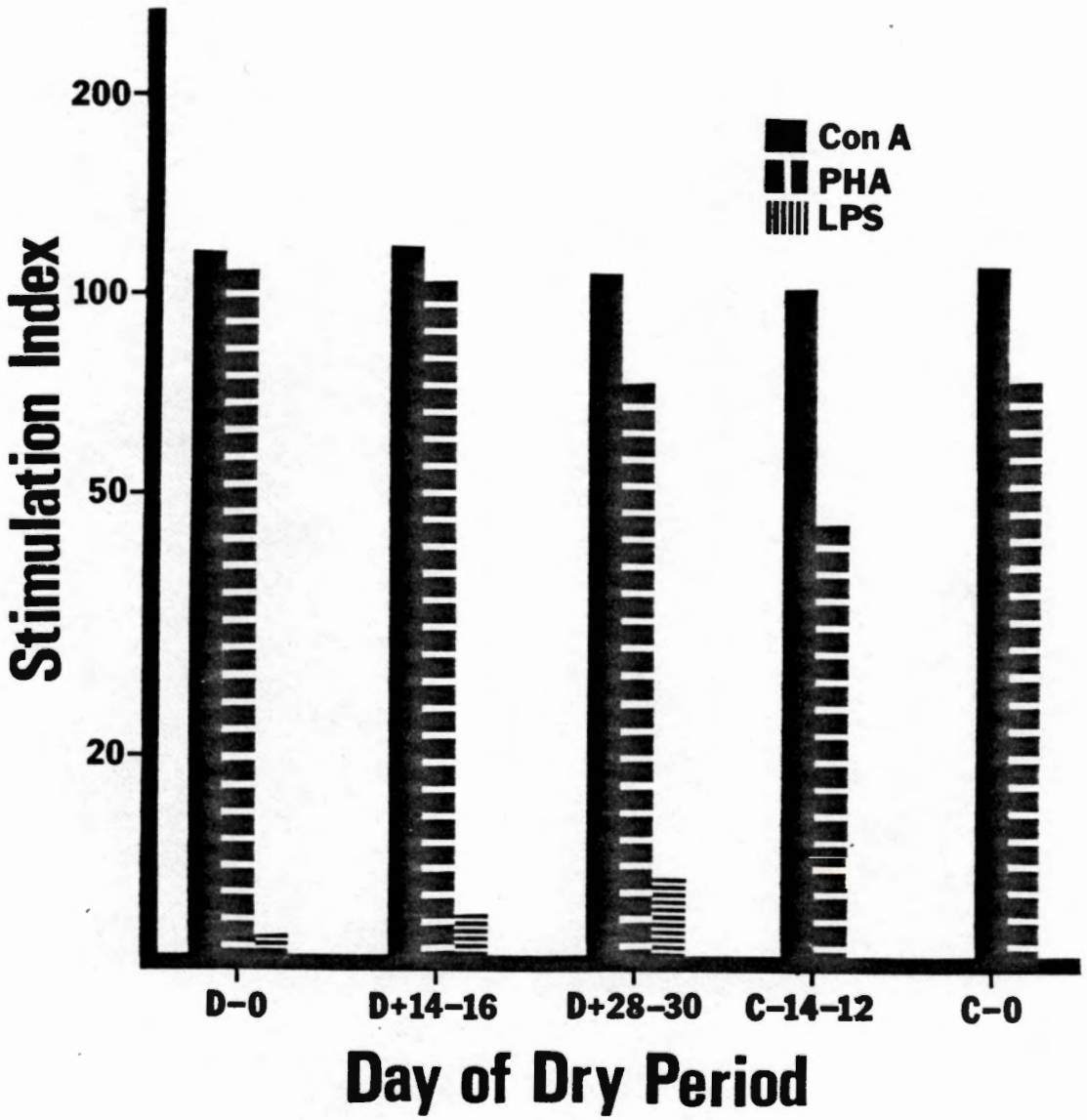

200
100
50
20
Stimulation Index
Con A
PHA
LPS
D-0
D+14-16
D+28-30
C-14-12
C-0
Day of Dry Period

Figure 9

stimulated cells compared to con A- and PHA-stimulated cells. Individual cow variation was significant ($P<.05$). Concanavalin A caused the greatest stimulation of bovine mononuclear cells throughout involution. However, differences between con A- and PHA-induced mononuclear cell blastogenesis were not significant ($P<.05$) until mid-involution (D+28-30).

Control values for ^{3}H-thymidine incorporation did not vary significantly from 1×10^{3} throughout involution. A slight but insignificant increase was observed in the response of mononuclear cells to con A between D-0 and D+28-30. Stimulation of cells by con A was significantly lower ($P<.05$) at C-0 than at D-0 or D+28-30. When data were expressed as SI to eliminate the possible contribution of background radiation to overall cpm, the minimum response to con A was observed at C-14-12 (Figure 9). Phytohemagglutinin-induced stimulation of mononuclear cells was less ($P<.1$) at D+28-30 than at D-0 and significantly lower ($P<.05$) at C-0 when compared to D-0 and D+28-30. Stimulation indices indicated that the minimum response of bovine mononuclear cells to PHA occurred at C-14-12 (Figure 9). Stimulation of bovine mononuclear cells by *E. coli* 0111:B4 LPS increased ($P<.05$) between D-0 and D+28-30. However, the largest value for stimulation of mononuclear cells by LPS was fourteen- and ninefold less than the smallest values for con A and PHA, respectively.

DISCUSSION

Marked variation was observed in response of bovine blood mononuclear cells to mitogens. In blood, T-lymphocytes are responsible for cell-mediated immunity (Lawman, 1982), whereas B-lymphocytes are responsible for the humoral immune response (Jerne, 1973). Concanavalin A and PHA, which preferentially stimulate T-lymphocytes (Schechter, 1980), caused the greatest stimulation of bovine mononuclear cells. Response of bovine mononuclear cells to E. coli LPS, a B-cell mitogen (Schechter, 1980), was significantly lower than responses elicited by con A and PHA. Concanavalin A was associated with the greatest stimulation of mononuclear cells. Variation in T-cell response to con A and PHA may reflect differences in the ability of these mitogens to stimulate the same cell population. However, con A and PHA could preferentially stimulate different subpopulations of T-cells. Stobo and Paul (1973) described the presence of subpopulations of murine T-cells which differed in response to T-cell mitogens and in density of Θ determinants, which are surface antigens that distinguish T-cells from B-cells (Reif and Allen, 1964). One subpopulation of T-cells bore a high density of Θ determinants and responded equally well to con A and PHA. The other subpopulation had a reduced density of Θ determinants and responded primarily to con A. Wilson et al. (1986), using differential erythrocyte rosetting assays, reported the presence of four subsets of lymphocytes in bovine blood and mammary secretions during involution, of which three appeared

to be T-cells. Functional analysis of bovine blood lymphocyte subsets isolated by differential erythrocyte rosetting indicated that subsets of T-cells exist which respond differently to mitogens and in the mixed lymphocyte reaction (Belden and Strelkauskas, 1981). Each subset of bovine T-cells may represent a different stage in the functional maturation of bovine T-cells. However, it is equally possible that subsets of bovine T-cells with different functional capacities exist. Differential responses of bovine mononuclear cells to con A and PHA observed in our study may reflect different subsets of functional T-lymphocytes present in bovine blocd.

Lack of response of bovine lymphocytes to E. coli 0111:B4 LPS might imply that low numbers of B-lymphocytes are present in bovine blood. However, the presence of bovine peripheral blood B-cells in concentrations similar to those of human blood has been documented (Schore et al., 1981; Concha et al., 1978; Concha et al., 1980). The inability of LPS to stimulate bovine B-cells may reflect the absence of certain accessory cell populations necessary for B-cell stimulation, or conversely the presence of other cell populations or factors that are inhibitory to B-cell stimulation. Evidence for the presence of T-cell populations which regulate B-cell response to LPS has been documented in the murine (Finesilver and Braley-Mullen, 1983; Norcross and Smith, 1977) and human (MacDermott et al., 1978) immune systems.

As involution progressed, variation in response of bovine mononuclear cells to individual mitogens was observed. Incorporation

of ^{3}H-thymidine by cells incubated without mitogen did not fluctuate significantly throughout involution. A minor increase in stimulation of mononuclear cells by con A was observed between D-0 and D+28-30. This difference might have approached significance if more cows had been used. In contrast, stimulation of cells by PHA decreased consistently throughout involution. Response of cells to con A and PHA was significantly depressed during the prepartum period compared to early involution. Suppression of mitogenic stimulation of bovine mononuclear cells during the prepartum period may be related to blocd-borne factors associated with impending parturition and lactation. Using whole blocd cultures, Yamamoto et al. (1980) reported suppression of con A- and PHA-induced lymphccyte transformation in pregnant compared to nonpregnant women. Pregnant ewe plasma collected during the final trimester of pregnancy was shown to suppress blastogenic responses of ovine maternal and allogeneic lymphocytes (Miyasaka and McCullagh, 1981).

A variety of pregnancy associated serum factors, both fetal and maternal in origin, appear to possess immunosuppressive properties. Maternal factors implicated in suppression of the maternal immune response include progesterone (Clemens et al., 1979; Szekeres et al., 1981), estrogen (Clemens et al., 1979), prolactin (Kelly and Dineen, 1973), cortisol (Clemens et al., 1979; Murray and Chenault, 1982) and placental lactogen (Morse, 1976) which are also associated with lactogenesis (Tucker, 1981). Bovine prolactin and 17-β estradiol, in particular, reach peak serum concentrations immediately prior to parturition (Tucker, 1985) and therefore may contribute

to peripartum maternal immunosuppression. Bovine pregnancy associated α_2-glycoprotein has been shown to suppress *in vitro* proliferation of human lymphocytes (Milton, 1971). Fetuin (α-fetoprotein), a major fetal plasma protein, has been shown to be immunosuppressive in humans and mice (Yachnin, 1975).

Similar variation in lymphocyte activity may occur in the mammary gland. Mammary lymphocytes migrate into the gland from a peripheral blood source, reach peak concentrations in the fully involuted mammary gland and remain elevated until parturition (Jensen and Eberhart, 1981). Increased lymphocyte concentrations in the bovine mammary gland may imply a greater contribution of lymphocytes to mammary gland defenses during the latter portion of the dry period. During involution, mammary epithelial cell permeability barriers are less resistant to influx of large molecular weight serum proteins into the mammary gland (Fleet and Peaker, 1978). Immunosuppressive proteins of fetal and maternal origin could pass into the mammary gland and exert immunosuppressive effects on mammary lymphocytes similar to those observed in peripheral blood lymphocytes. Thus, immunosuppression of lymphocytes destined for the mammary gland both prior to and following entry into the gland by pregnancy-associated serum proteins, hormones, or other factors might contribute to increased susceptibility of the bovine mammary gland to new IMI, especially during the prepartum period. Phytohemagglutinin-induced stimulation of bovine blood mononuclear cells obtained at parturition in this study was consistent with PHA-induced stimulation of bovine blood mononuclear cells obtained from lactating cows, whereas con A-induced

stimulation was lower (Part II). However, incorporation of ^{3}H-thymidine by con A-stimulated bovine mononuclear cells obtained at parturition was 90 times greater than con A-stimulated bovine blood mononuclear cells incubated with colostrum (Part II). Stimulation of blood mononuclear cells with PHA was 8.6 times greater than PHA-stimulated mononuclear cells incubated with colostrum (Part II). Therefore, it is likely that inhibition of mammary mononuclear cell blastogenesis is not due entirely to immunosuppressive substances of serum origin. Studies on mammary lymphocyte activity throughout involution and further research on factors associated with fluctuation in lymphocyte activity are required, however, to determine the contribution of mammary lymphocytes to changes in susceptibility of the bovine mammary gland to infection throughout involution.

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PART IV

SUPPRESSION OF BOVINE PERIPHERAL BLOOD MONONUCLEAR CELL BLASTOGENESIS BY FRACTIONATED AND UNFRACTIONATED BOVINE MAMMARY SECRETION WHEY

ABSTRACT

Effects of fractionated and unfractionated bovine dry secretion whey on bovine mononuclear cell blastogenesis were evaluated. Dry secretion whey from four dairy cows in late involution was fractionated by gel filtration chromatography. Blood was collected from cows at drying off, 14-16 and 28-30 d post drying off, 14-12 d prepartum and at parturition. Mononuclear cells were isolated by density gradient centrifugation. Concanavalin A and phytohemagglutinin were used to evaluate blastogenesis which was measured by methyl-^{3}H-thymidine incorporation. Unfractionated dry secretion whey markedly inhibited mononuclear cell blastogenesis compared to stimulated controls. Concentrated preparations of the first and second protein peaks that eluted from column chromatography also inhibited blastogenesis. Concentrated protein peak 1 caused greatest inhibition of concanavalin A-stimulated cells, while concentrated protein peak 2 was associated with greatest inhibition of phytohemagglutinin-stimulated cells. Unconcentrated protein peak 2 caused greatest blastogenic inhibition of both concanavalin A- and phytohemagglutinin-stimulated cells. Unconcentrated protein peaks 3 and 4 inhibited mononuclear cell blastogenesis to a lesser degree than protein peaks 1 and 2. Phytohemagglutinin-treated mononuclear cells were less inhibited by fractionated and unfractionated mammary secretion whey than concanavalin A-treated cells.

INTRODUCTION

Susceptibility of the bovine mammary gland to intramammary infection (IMI) varies as involution progresses. In a classic study, Neave et al. (1950) demonstrated increased susceptibility of the bovine mammary gland to IMI during the first three weeks of the dry period. Subsequent studies indicated that the bovine mammary gland is also increasingly susceptible to IMI during the peripartum period (Eberhart, 1982; Oliver and Mitchell, 1983; Oliver, 1987). Various studies concerning changes in susceptibility to IMI of the bovine mammary gland during involution have concentrated on effects of changes in volume and biochemical composition of mammary secretions on mastitis-causing bacteria (Cousins et al., 1980; Breau and Oliver, 1985; Smith and Oliver, 1981; Harmon et al., 1976; Schanbacher and Smith, 1975). However, mononuclear cells may also play a role in defense of the mammary gland against IMI, and interactions between mammary secretions and mammary gland mononuclear cells, while not fully understood, cannot be ignored.

Mammary gland lymphocyte concentrations peak in the fully involuted gland and do not return to pre-involution concentrations until the peripartum period (Jensen and Eberhart, 1981; McDonald and Anderson, 1981). Dynamics of bovine MGL populations may imply a greater role of MGL in the defense of the gland against IMI during the latter portion of involution. However, rates of IMI are elevated during this period (Eberhart, 1982; Oliver and Mitchell, 1983; Oliver, 1987).

Hyporesponsiveness of MGL to *in vitro* mitogenic and antigenic stimulation has been reported for human (Diaz-Jouanen and Williams, 1974) and bovine (Smith and Schultz, 1977; Concha et al., 1978; Concha et al., 1980; Schore et al., 1981) lymphocytes. Human MGL have also been shown to be hyporesponsive in the mixed lymphocyte reaction (Parmely et al., 1976) and in mediating antibody-dependent cell-mediated cytotoxicity (Kohl et al., 1980), implying a similar *in vivo* hyporesponsiveness.

Suppression of mammary mononuclear cell activity may be associated with components of mammary secretions. Human colostrum (Diaz-Jouanen and Williams, 1974; Ogra and Ogra, 1978) and bovine dry secretion and colostrum (Collins and Oldham, 1986) have been shown to suppress *in vitro* blastogenic response of human and bovine peripheral blood lymphocytes (PBL). Immunosuppression of human MGL has been related to maternal transfer of cell-mediated immunity to the neonate (Beer et al., 1974; Head and Beer, 1979; Parmely and Williams, 1979), and this may also be true of bovine mononuclear cells. However, suppression of bovine mononuclear cell activity may also affect dynamics of IMI during involution. Studies concerning the nature of immunosuppressive substances in bovine dry secretion may help to further elucidate the mechanism of blastogenic suppression of mammary mononuclear cells. The objective of this study was to determine which fraction of bovine dry secretion whey was associated with greatest inhibition of mitogen-induced bovine mononuclear cell blastogenesis. Bovine peripheral blood mononuclear cells were utilized to

obtain maximum response to mitogens and avoid possible masking of inhibitory properties of bovine mammary secretion whey fractions due to native hyporesponsiveness of bovine mammary gland mononuclear cells.

MATERIALS AND METHODS

Animals

Five cows from the University of Tennessee dairy research herd were selected as blood donors. Average cow age was 51 mo (range 34-77 mo). Cows were producing 10-21 kg of milk/day ($\bar{x}$ 15 kg/day) and had gone through 1-4 lactations prior to drying off. Cows were bled at drying off (D-0), 14-16 (D+14-16) and 28-30 (D+28-30) d post drying off, 14-12 d prior to parturition (C-14-12) and within 24 hr of parturition (C-0).

Mammary secretion

Mammary secretions were collected from four dairy cows between 16 and 2 d prior to parturition and pooled. Fat and cellular debris were removed by centrifugation at 37,000 x g for 30 min. Whey preparations were made by lowering the pH of skims to 4.5 using glacial acetic acid and centrifuging at 37,000 x g for 45 min. Whey was adjusted to pH 7.0 with 1 N NaOH and filter sterilized (0.45 μm filter; Gelman Sciences Inc., Ann Arbor, MI). Concentrations of lactoferrin (Lf) and bovine serum albumin (BSA) in secretion whey were determined by electroimmunodiffusion (EID) on cellulose acetate (Schanbacher

and Smith, 1974). Rabbit anti-BSA was obtained commercially (Miles Scientific, Naperville, IL). Anti-bovine Lf was prepared by repeated intramuscular injections into rabbits of bovine Lf purified by the method of Smith et al. (1971). Total protein content of mammary secretion whey was determined using the Lowry (Folin-Ciocalteau) method (Lowry et al., 1951). Protein content of mammary secretion whey was mapped by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of denatured secretion whey proteins as in Part II. The method of O'Farrell (1975) was used in which isoelectric focusing of mammary secretion proteins was carried out in tube gels prior to electrophoresis of proteins over 10% polyacrylamide slab gels. Gels were stained with Coomassie Blue. Transferrin (M_r 78,000), ovalbumin (M_r 43,000), chymotrypsinogen (M_r 25,000) and ribonuclease A (M_r 13,899) were used as molecular weight markers. The pH gradient of slab gels ranged from 3.84 to 8.09.

Column chromatography

A 2.6 x 100 cm column packed with Sephacryl S-200 (Pharmacia, Piscataway, NY) with a buffer system of 10 mM Tris in 0.3 M NaCl (pH 8.0) was prepared. Ten ml samples of mammary secretion whey were loaded onto the column and allowed to elute by gravity. Elution volume was 130 ml. Five ml fractions (120) were collected and read at A_{280} on a spectrophotometer (Bausch and Lomb Spectronic 2000; Bausch and Lomb Analytical Systems Div., Rochester, NY). Proteins eluted from the column in 4 major groups which were referred to as

protein peaks 1, 2, 3, and 4 (Figure 10). Individual peaks were pooled and dialyzed against ultrapure water. Preparations of peaks 1 and 2 were concentrated by ultrafiltration in order to more closely approximate actual secretion concentrations of proteins present in those peaks. Concentrations of Lf and BSA in each peak were determined by EID (Schanbacher and Smith, 1974). Total protein content of each peak was determined by the Lowry method (Lowry et al., 1951). Proteins contained in each peak were mapped by 2D-PAGE (O'Farrell, 1975) as described previously.

Mitogens

Concanavalin A (con A) and phytohemagglutinin (PHA; Sigma Chemical Co., St. Louis, MO) were used to induce mononuclear cell blastogenesis. Optimum concentrations of con A (5 µg/ml) and PHA (25 µg/ml) were determined prior to initiation of the experiment (Part III).

Isolation of mononuclear cells

Bovine mononuclear cells were isolated by the method of Boyum (1968) with modifications described by Nonnecke and Kehrli (1985). Blood (40 ml) was collected by jugular venipuncture using modified Alsever's solution as the anticoagulant and diluted in two parts phosphate-buffered saline (0.15 M, pH 7.2). Diluted blood (12 ml) was layered over 3 ml of Ficoll-sodium diatrizoate with specific gravity of 1.083 g/ml (Histopaque 1083, Sigma Chemical Co., St. Louis, MO) and centrifuged at 400 x g (18°C) for 50 min. Following aspiration of plasma, the mononuclear cell band was recovered and washed

Figure 10. S-200 chromatogram of bovine mammary secretion whey collected 2-16 d prior to parturition from 4 cows.

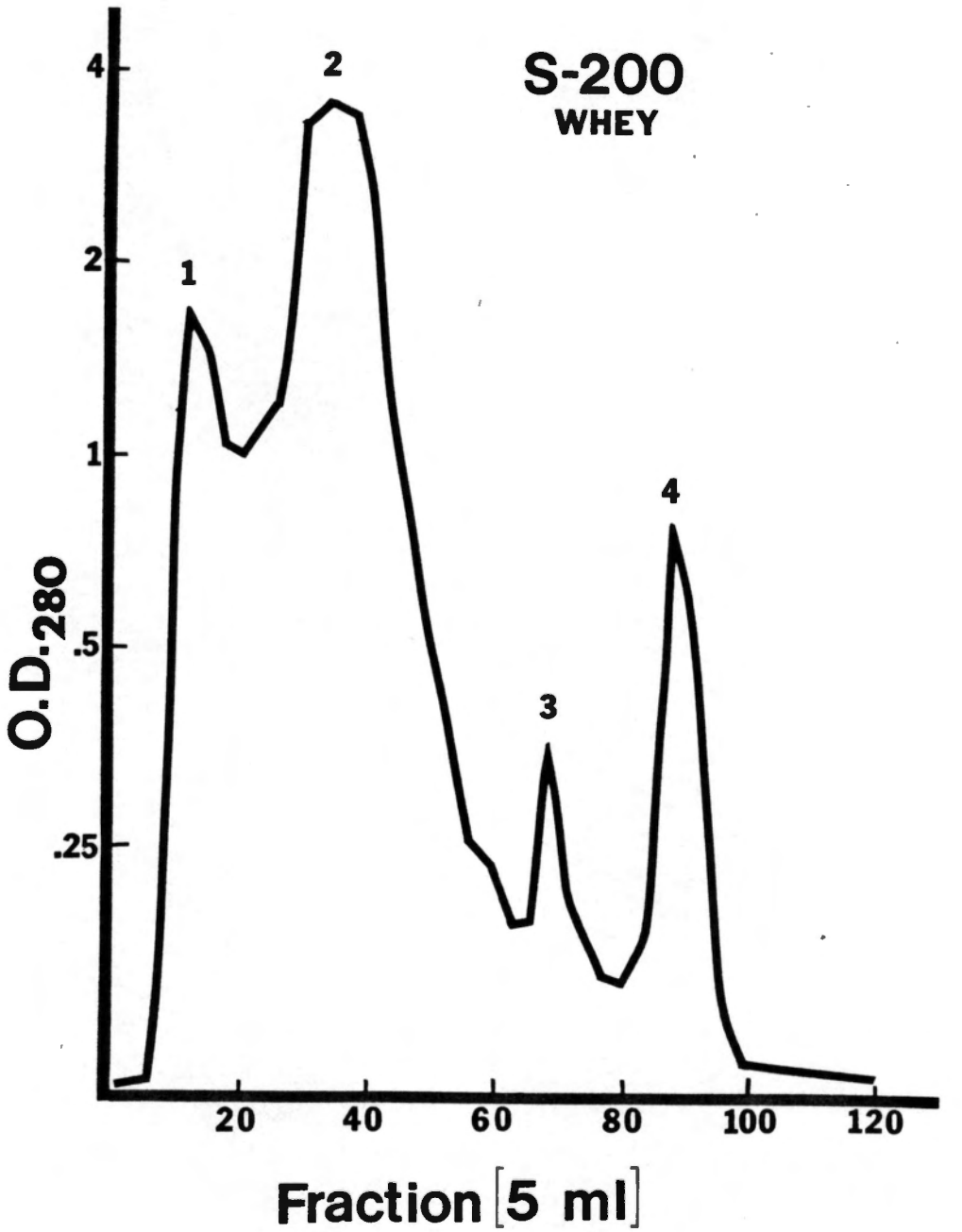


Figure 10

twice in Ca^{+2}- and Mg^{+2}-free Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) at 300 x g for 15 min/wash. Residual erythrocytes were lysed by hypotonic shock treatment using phosphate buffered water (pH 7.2). Following a single wash in HBSS, cells were resuspended in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10 mM HEPES buffer, 2 mM L-glutamine, 100 U penicillin G and 100 µg streptomycin/ml. No attempt was made to further purify mononuclear cell populations by removal of monocytes based on reports that ovine (Outteridge and Lee, 1981) and bovine (Ohmann et al., 1983) monocytes enhance response of lymphocytes to mitogens. Mononuclear cell recovery and viability were determined by hemacytometer count using trypan blue exclusion (Patterson, 1979). Cytocentrifuge (Cytospin 2; Shandon Southern Inc., Sewickley, PA) smears of purified mononuclear cells were prepared and stained with Wright-Giemsa (Camco Quik Stain II; Cambridge Chemical Prod., Ft. Lauderdale, FL) for mononuclear cell quantification.

Cell cultures

Cells were cultured in 96 well flat-bottom microtitre plates (Corning Cell Wells; Corning Glass Works, Corning, NY). Components added to each well were: $2x10^5$ viable mononuclear cells in 50 µl of RPMI 1640, 50 µl of heat inactivated (56°C for 30 min) fetal calf serum diluted in RPMI 1640 to a final concentration of 10%, 100 µl of mitogen, and 50 µl of dilutions of fractionated or unfractionated

mammary secretion whey. Control wells received 150 µl of RPMI 1640 in place of mitogen and whey. Fifty µl of RPMI 1640 were added to stimulated control wells in place of secretion whey or protein peaks.

Microtitre plates were incubated for 72 hr at 37°C in an atmosphere of 95% air and 5% CO_2. After 72 hr, 1 µCi of methyl-^{3}H-thymidine (ICN Radiochemicals, Irvine, CA) diluted in 50 µl of RPMI 1640 was added to each well. Following 18 hr of incubation, cultures were harvested onto glass fiber filters using a multiple automated sample harvester (Mini-MASH II; MA Bioproducts, Walkersville, MD). Filters were placed in plastic scintillation vials and 2 ml of scintillation cocktail (Scintiverse II, Fisher Sci. Co., Fairlawn, NJ) were added to each vial. Retained radioactivity was counted for 10 min/vial in a liquid scintillation counter (Isocap 300 Liquid Scintillation System; Searle Analytic Inc., Des Plaines, IL). Data were expressed as mean counts per min (cpm) ± standard error of the mean x 10^3 of four replicate cultures. Additionally, data were expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control.

Statistical analysis

Count data were transformed to $\log_e$ for statistical analysis. However, actual count data are presented. Due to lack of uniformity in cow numbers and dilutions of protein peaks between experiments, no attempt was made to compare data between days of involution. Experimental design was split block and data were analyzed by least squares analysis of variance (Steel and Torrie, 1980) using the following model:

$$Y_{ijkl} = \mu + C_i + M_j + P_k + MP_{jk} + DP_{kl} + MDP_{jkl} + CMDP_{ijkl} + \varepsilon_{ijkl}$$

Where: μ = overall mean cpm

C = cow

M = mitogen

D = dilution of protein peak

P = protein peak

Significance of each variable and interaction was tested using cow x mitogen x dilution x protein interaction as the error term. To further characterize differences in effects of individual protein peaks on bovine blood mononuclear cell blastogenesis, a series of contrasts were made using the following model:

$$Y_{ijk} = \mu + C_i + D_j + P_k + DP_{jk} + \varepsilon_{ijk}$$

Where: μ = overall mean cpm

C = cow

D = dilution of protein peak

P = protein peak

In certain analyses, degrees of freedom (df) for individual variables equalled 1. In those cases, variables with df = 1 and all interactions involving those variables were removed from the model prior to statistical analysis.

RESULTS

Mammary secretion composition

Results of 2D-PAGE of whole and fractionated mammary secretion whey are presented in Figures 11 and 12. Identification of BSA,

Figure 11. Two-dimensional polyacrylamide gel electrophoresis of fractionated and unfractionated bovine mammary secretion whey. Gels were stained with Coomassie Blue. (a) PAGE of bovine mammary secretion whey collected from 4 cows 2-16 d prior to parturition; (b) PAGE of protein peak 1; (c) PAGE of protein peak 2; Lf, lactoferrin; BSA, bovine serum albumin; IgG_H, heavy chain IgG; IgG_L, light chain IgG; α-LA, α-lactalbumin; β-LG, β-lactoglobulin.

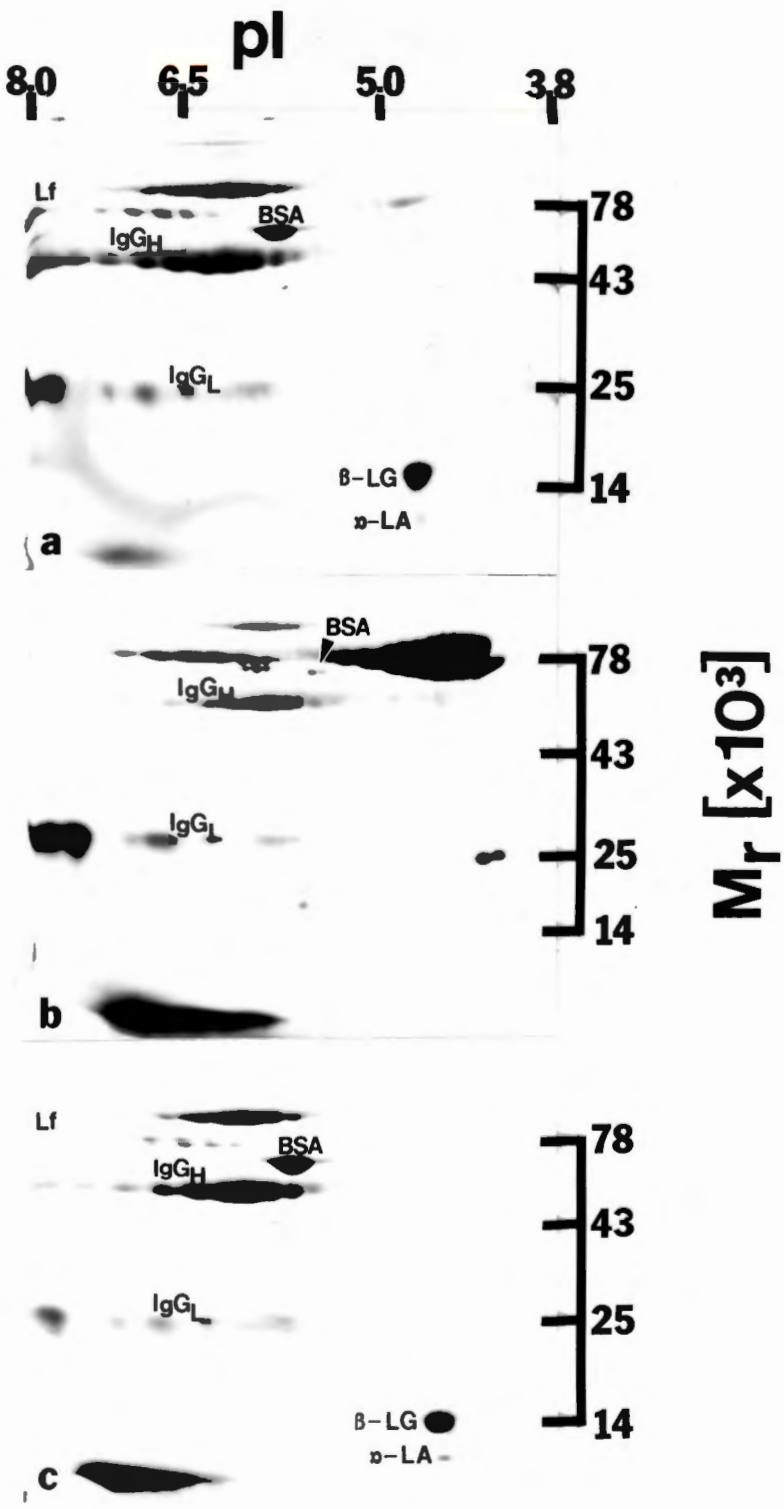

pI
8.0
6.5
5.0
3.8
Lf
BSA
IgG$_H$
IgG$_L$
β-LG
α-LA
a
78
43
25
14
BSA
IgG$_H$
IgG$_L$
b
78
43
25
14
Lf
BSA
IgG$_H$
IgG$_L$
β-LG
α-LA
c
78
43
25
14
M$_r$ [x10^3]

Figure 11
141
Torre, 1987

Figure 12. Two-dimensional polyacrylamide gel electrophoresis of fractionated bovine mammary secretion whey. Gels were stained with Coomassie Blue. (a) PAGE of protein peak 3; (b) PAGE of protein peak 4.

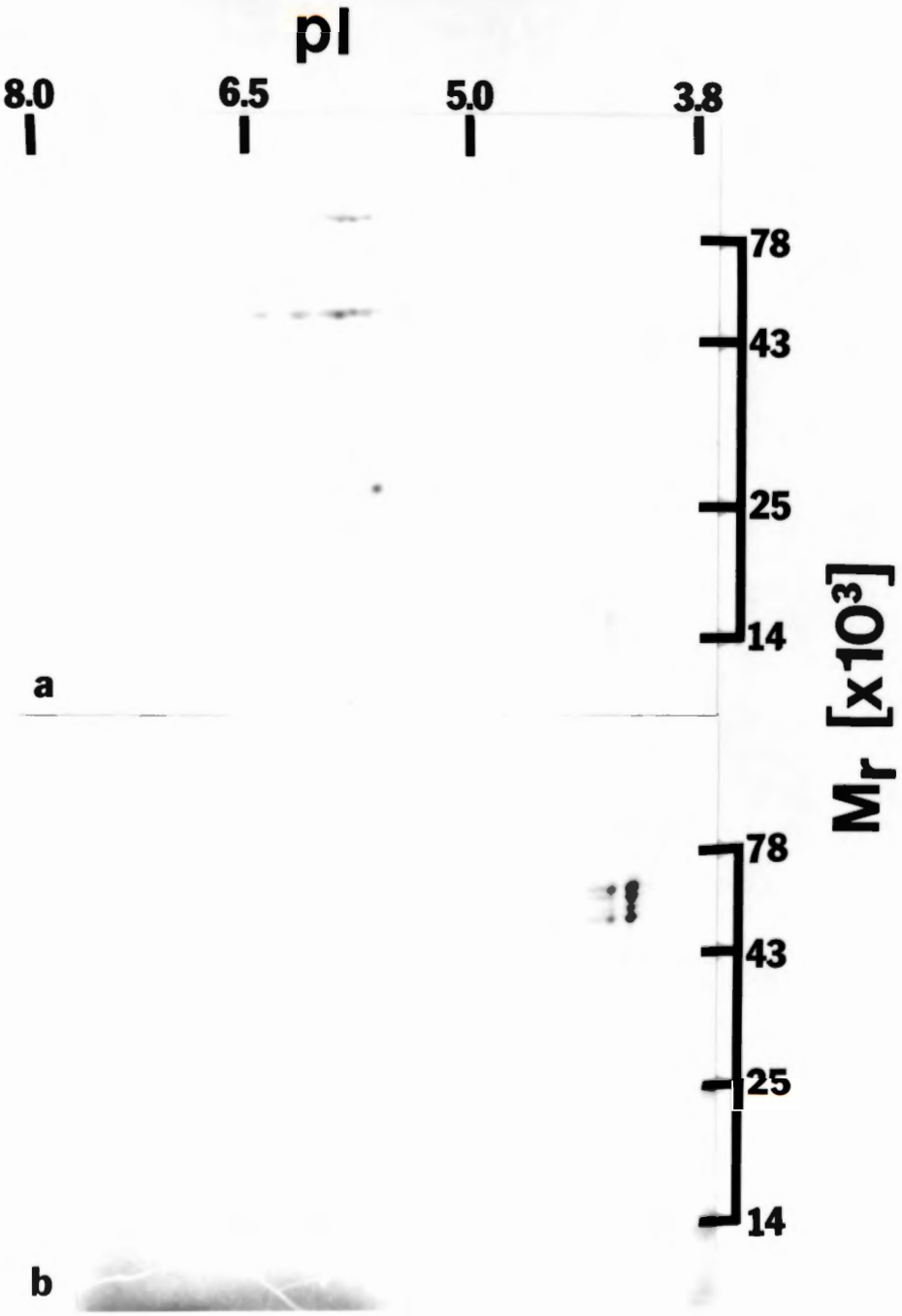

pI
8.0
6.5
5.0
3.8
78
43
25
14
a
78
43
25
14
b
Mr [x10³]

Figure 12
143
Torre, 1987

heavy (IgG_H) and light (IgG_L) chain IgG, α-lactalbumin (α-LA), and β-lactoglobulin (β-LG) in 2D-PAGE gels of fractionated and unfractionated secretion whey was made based upon known isoelectric points (pI) and M_r (Eigel et al., 1984). Additionally, gels were compared with protein maps formed by similar PAGE of serum (Anderson and Anderson, 1977), murine milk skim (Lee et al., 1984), and bovine milk whey (Shimazaki et al., 1983). The protein band with pI at pH of approximately 8 and M_r similar to that of transferrin (78,000) was presumed to be Lf. Other protein bands located near the 78,000 M_r marker (Figure 11) may be fragments of higher M_r Igs. Electroimmunodiffusion analysis of mammary secretion whey indicated that concentrations of Lf and BSA were typical of bovine mammary secretions collected during late involution (Table 5). The majority of secretion whey proteins eluted from S-200 column chromatography in peaks 1 and 2 (Figure 10). Protein peak 1 contained greater amounts of high M_r proteins. Most Lf, BSA, IgG_H, and IgG_L eluted in protein peak 2 (Table 5, Figure 11). The majority of α-LA and β-LG also eluted from the column in peak 2 (Figure 11). Minimal amounts of protein were detected in peaks 3 and 4 by Lowry analysis (Table 6), EID (Table 5) and 2D-PAGE (Figure 12).

Cell cultures

Bovine blood mononuclear cell isolation procedures resulted in a population of >99% pure mononuclear cells comprised of approximately 75% lymphocytes and 25% monocytes. Representative lymphocytes

Table 5. Concentration of lactoferrin and bovine serum albumin in pooled mammary secretion whey collected during late involution.[1]

Whey fraction	Protein concentration (mg/ml) Lf	BSA
Whole whey	3.4	5.4
Concentrated protein peak 1	.30	.28
Concentrated protein peak 2	1.2	.89
Unconcentrated protein peak 1	<.046	<.048
Unconcentrated protein peak 2	.14	.14
Unconcentrated protein peak 3	ND[2]	ND
Unconcentrated protein peak 4	ND	ND

[1]Mammary secretions collected from 4 cows 2-16 d prior to parturition.

[2]ND - not detectable.

Table 6. Total protein concentration of fractionated and unfractionated bovine mammary secretion whey by Lowry analysis.[1]

Whey fraction	Protein concentration (mg/ml)
Concentrated protein peak 1	8.9
Concentrated protein peak 2	9.7
Whole whey	147.9
Unconcentrated protein peak 1	.2
Unconcentrated protein peak 2	2.0
Unconcentrated protein peak 3	<.01
Unconcentrated protein peak 4	<.01

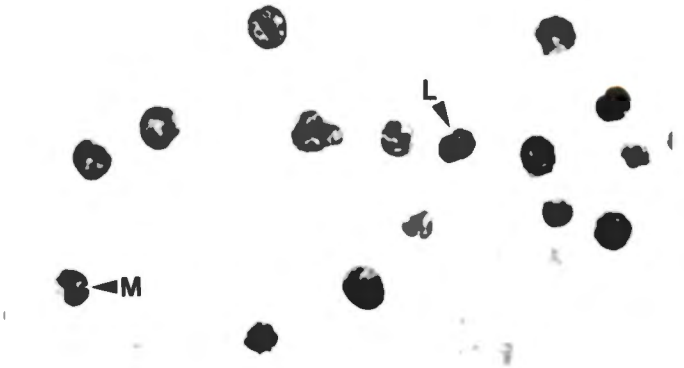
[1]Mammary secretions collected from 4 cows 2-16 d prior to parturition.

and monocytes isolated by density gradient centrifugation are in Figure 13. Mean viability ± standard error of the mean of mononuclear cells recovered from Ficoll gradients prior to initiation of cultures was 79.3 ± 1.4%.

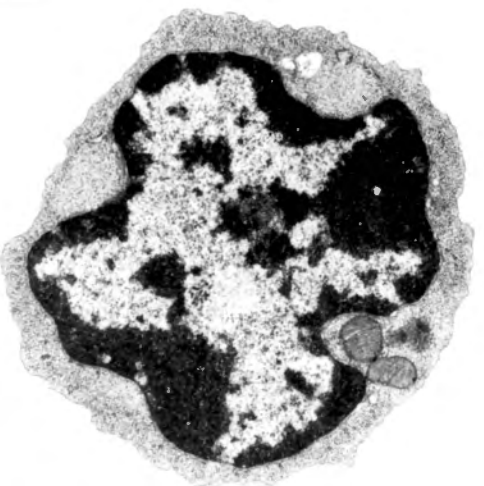
Effects of mammary secretion whey and protein peaks 1 and 2 concentrated by ultrafiltration on mitogen-induced blastogenesis of bovine mononuclear cells collected at D-0 are presented in Figure 14. Secretion whey and protein peaks were added to cultures at an initial dilution of 1:2. Mammary secretion whey and protein peaks 1 and 2 significantly ($P<.05$) inhibited mononuclear cell blastogenesis compared to stimulated controls. However, PHA-stimulated cell blastogenesis was less inhibited than con A-stimulated blastogenesis ($P<.05$). Of the concentrated protein peaks, peak 2 caused greatest inhibition of PHA-treated mononuclear cells. In contrast, greatest inhibition of con A-treated mononuclear cells was associated with concentrated protein peak 1. Differences between inhibition of mononuclear cell blastogenesis by concentrated protein peak 2 and mammary secretion whey were not significant at a 1:2 dilution ($P<.05$). However, concentrated protein peak 1 caused significantly ($P<.05$) greater inhibition of con A-induced mononuclear cell blastogenesis and significantly less ($P<.05$) inhibition of PHA-induced mononuclear cell blastogenesis than whole mammary secretion whey.

Mammary secretion whey and concentrated proteins from peaks 1 and 2 were added to cells collected at D+14-16, C-14-12 and C-0 at increasingly higher dilutions (Tables 7, 8, and 9). Dilution

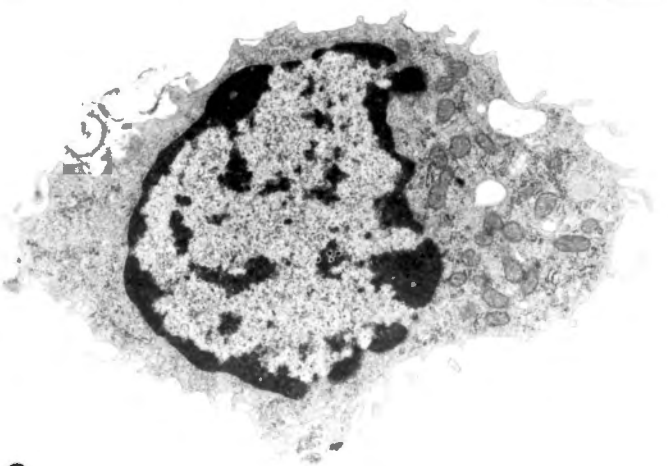
Figure 13. Electron micrographs of bovine peripheral blood mononuclear cells isolated by density gradient centrifugation. (a) Bovine mononuclear cells (x 600), L = lymphocyte, M = monocyte; (b) Representative bovine lymphocyte (x 15000); (c) Representative bovine monocyte (x 15000).



a



b



c

Figure 14. Inhibition of bovine peripheral blood mononuclear cell blastogenesis by fractionated and unfractionated mammary secretion whey. Mononuclear cells were collected at D-0. All protein preparations were added to cultures at a 1:2 dilution. Data are expressed as mean counts per minute ± standard error of the mean x 10^3.

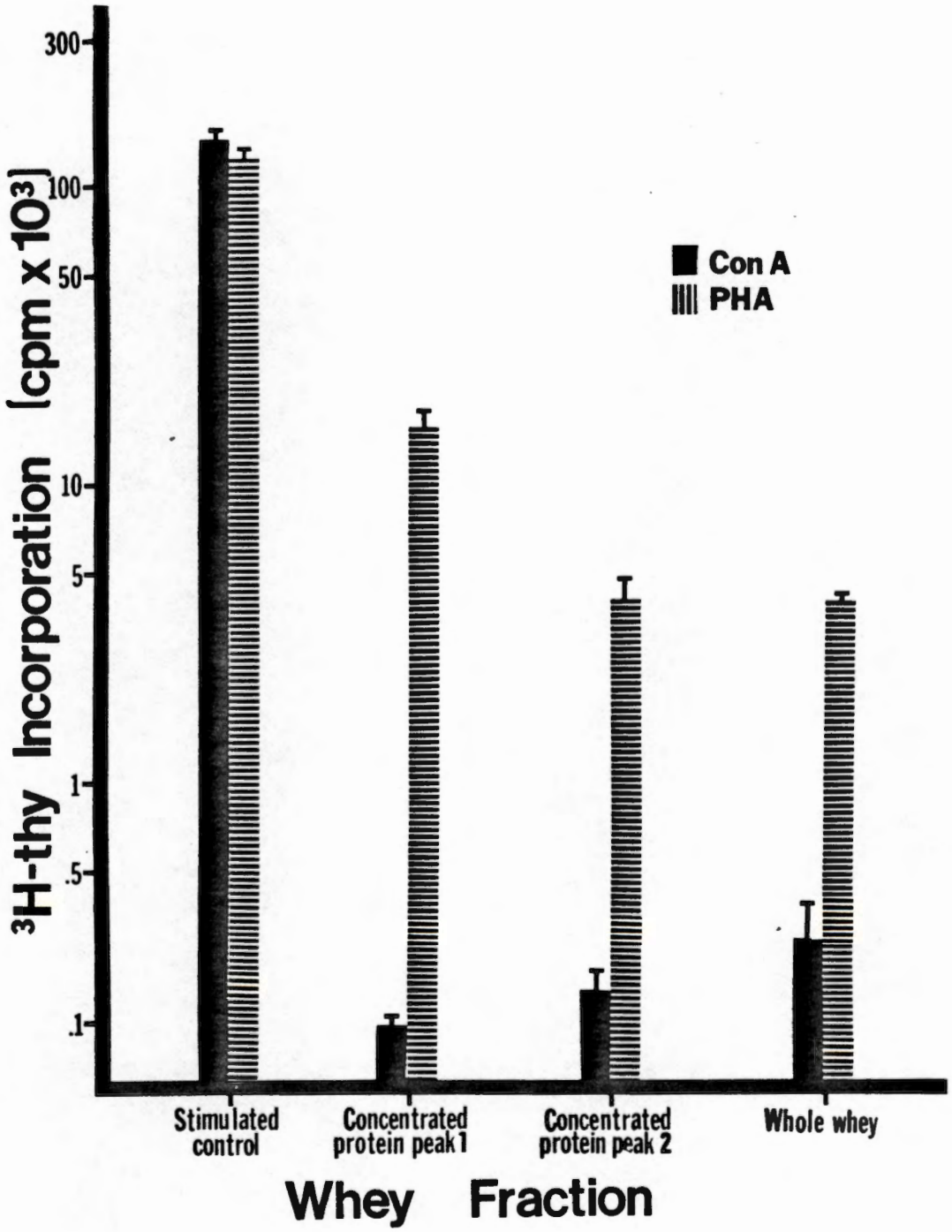

300
100
50
10
5
1
.5
.1
³H-thy Incorporation (cpm x 10³)
Con A
PHA
Stimulated control
Concentrated protein peak 1
Concentrated protein peak 2
Whole whey
Whey Fraction

Figure 14

Table 7. Inhibition of bovine peripheral blood mononuclear cell blastogenesis by fractionated and whole mammary secretion whey during late involution.[1]

Whey fraction	Dilution of whey fraction	Mitogen: Concanavalin A $\bar{x}$ ± SEM[2]	Concanavalin A Index[3]	Mitogen: Phytohemagglutinin $\bar{x}$ ± SEM	Phytohemagglutinin Index
Concentrated protein peak 1	1:4	.6 ± .1	.004	19.3 ± 6.3	.25
	1:32	88.3 ± 8.4	.54	59.2 ± 10.0	.77
	1:512	129.4 ± 4.4	.79	81.4 ± 6.1	1.06
Concentrated protein peak 2	1:4	1.7 ± .3	.01	11.7 ± 3.0	.15
	1:32	94.7 ± 4.5	.58	45.0 ± 3.1	.59
	1:512	141.4 ± 8.8	.86	66.4 ± 3.8	.86
Whole whey[4]	1:4	.7 ± .1	.004	6.1 ± 1.7	.08
	1:32	1.1 ± .1	.007	46.4 ± 1.7	.60
	1:512	120.5 ± 5.0	.73	71.4 ± 4.4	.93

[1]Blood collected at C-14-12 from 2 cows.

[2]Mean counts per minute ± standard error of the mean x 10^3.

[3]Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control. Concanavalin A stimulated control = 164.4 ± 16.4. Phytohemagglutinin stimulated control = 76.8 ± 5.7.

[4]Secretions collected from 4 cows 2-16 d prior to parturition.

Table 8. Inhibition of bovine peripheral blood mononuclear cell blastogenesis by fractionated and whole mammary secretion whey at parturition.[1]

Whey fraction	Dilution of whey fraction	Mitogen: Concanavalin A $\bar{x}$ ± SEM[2]	Concanavalin A Index[3]	Mitogen: Phytohemagglutinin $\bar{x}$ ± SEM	Phytohemagglutinin Index
Concentrated protein peak 1	1:4	2.0 .9	.02	15.1 2.0	.28
	1:32	77.5 6.3	.86	48.4 3.1	.89
	1:512	96.6 6.3	1.07	52.9 4.6	.98
Concentrated protein peak 2	1:4	6.7 3.3	.07	16.8 1.5	.31
	1:32	85.6 7.0	.95	41.8 3.8	.77
	1:512	79.6 8.6	.88	53.5 4.2	.99
Whole whey[4]	1:4	2.3 .7	.02	2.4 .3	.04
	1:32	2.3 .8	.02	26.4 3.4	.49
	1:512	86.7 6.9	.96	58.1 3.8	1.07

[1]Blood collected at C-0 from 5 cows.

[2]Mean counts per minute ± standard error of the mean x 10^3.

[3]Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control. Concanavalin A stimulated control = 90.1 ± 9.6. Phytohemagglutinin stimulated control = 54.2 ± 3.7.

[4]Secretions collected from 4 cows 2-16 d prior to parturition.

Table 9. Inhibition of mitogen-induced bovine peripheral blood mononuclear cell blastogenesis by fractionated and unfractionated mammary secretion whey.[1]

Mitogen	Dilution of whey fraction	Whey fraction: Whole whey[2] $\bar{x}$ ± SEM[3]	Whole whey Index[4]	Concentrated protein peak 1 $\bar{x}$ ± SEM	Concentrated protein peak 1 Index	Concentrated protein peak 2 $\bar{x}$ ± SEM	Concentrated protein peak 2 Index
Con A	1:8	.7 .07	.005	40.4 4.8	.28	56.0 9.0	.39
	1:16	.7 .09	.005	107.4 10.2	.76	109.6 15.2	.77
	1:32	3.1 2.0	.02	129.0 10.8	.91	150.2 12.9	1.06
	1:64	43.6 13.4	.31	128.3 15.1	.90	155.1 13.6	1.09
	1:128	107.8 17.4	.76	157.5 11.9	1.11	167.7 12.5	1.18
PHA	1:8	56.6 17.1	.43	77.8 10.8	.59	76.5 7.3	.58
	1:16	55.3 7.0	.42	117.2 13.0	.89	81.0 7.5	.61
	1:32	102.7 13.5	.78	135.0 13.5	1.02	134.7 12.7	1.02
	1:64	122.1 11.1	.92	136.0 14.2	1.03	233.8 19.2	1.77
	1:128	131.6 11.1	1.00	144.6 13.7	1.10	111.7 8.4	.85

[1]Blood collected at D+14-16 from 3 cows.

[2]Mammary secretions collected from 4 cows 2-16 d prior to parturition.

[3]Mean counts per minute ± standard error of the mean x 10^3.

[4]Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control. Concanavalin A stimulated control = 155.8 ± 15.2. Phytohemagglutinin stimulated control = 95.6 ± 8.9.

significantly ($P<.05$) reduced inhibitory effects of protein preparations. Differences in level of inhibition of mononuclear cell blastogenesis between mammary secretion whey and concentrated protein peaks became more marked as dilution increased. Mammary secretion whey at a 1:4 dilution caused significantly ($P<.05$) greater inhibition of PHA-induced mononuclear cell blastogenesis than did concentrated protein peaks 1 and 2 (Tables 7 and 8). However, inhibition of con A-induced mononuclear cell blastogenesis by whole mammary secretion whey was not different ($P>.05$) than that associated with concentrated protein peak 1. Inhibition of PHA-induced mononuclear cell blastogenesis by concentrated protein peak 2 at a 1:4 dilution was not different ($P>.05$) than inhibition by concentrated protein peak 1.

Differences in level of inhibition of PHA-induced mononuclear cell blastogenesis by concentrated protein peaks 1 and 2 and whole whey were not significant following addition of protein peaks at an initial dilution of 1:8 (Table 9). Adding mammary secretion proteins to cultures at increasingly higher dilutions caused the index of inhibition to approach 1, indicating dose-related removal of inhibitory effects (Tables 7, 8, and 9).

Unconcentrated protein peaks 1 and 2 were added to mononuclear cells collected at D+28-30, C-14-12 and C-0 (Tables 10, 11, and 12). Additionally, preparations of the 3rd and 4th protein peaks eluting from S-200 column chromatography were added to cell cultures. Unconcentrated, undiluted protein peaks 1, 2, 3, and 4 inhibited mononuclear cell blastogenesis (Tables 10 and 11). However, significantly

Table 10. Inhibition of bovine peripheral blood mononuclear cell blastogenesis by fractionated mammary secretion whey during late involution.[1]

Whey fraction	Dilution of whey fraction	Mitogen: Concanavalin A $\bar{x}$ ± SEM[2]	Concanavalin A Index[3]	Phytohemagglutinin $\bar{x}$ ± SEM	Phytohemagglutinin Index
Unconcentrated protein peak 1	0	26.9 1.7	.16	29.0 2.5	.38
	1:2	92.5 8.8	.56	56.4 3.8	.73
	1:32	124.7 5.4	.76	72.6 7.4	.95
Unconcentrated protein peak 2	0	.6 .2	.004	13.0 2.6	.17
	1:2	40.9 5.3	.25	43.5 3.8	.57
	1:32	112.6 9.2	.68	64.4 5.4	.84
Unconcentrated protein peak 3	0	36.2 2.1	.22	20.5 2.1	.27
	1:2	84.0 4.1	.51	49.4 4.7	.64
	1:32	154.8 7.4	.94	86.6 7.2	1.13
Unconcentrated protein peak 4	0	43.3 2.6	.26	26.3 2.8	.34
	1:2	87.4 4.5	.53	60.5 6.8	.79
	1:32	148.7 5.1	.90	70.6 5.9	.92

[1]Blood collected at C-14-16 from 2 cows.

[2]Mean counts per minute ± standard error of the mean x 10^3.

[3]Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control. Concanavalin A stimulated control = 164.4 ± 16.4. Phytohemagglutinin stimulated control = 76.8 ± 5.7.

Table 11. Inhibition of bovine peripheral blood mononuclear cell blastogenesis by fractionated mammary secretion whey at parturition.[1]

Whey fraction	Dilution of whey fraction	Mitogen: Concanavalin A $\bar{x}$ ± SEM[2]	Concanavalin A Index[3]	Phytohemagglutinin $\bar{x}$ ± SEM	Phytohemagglutinin Index
Unconcentrated protein peak 1	0	30.9 3.4	.34	18.3 1.3	.34
	1:2	53.9 4.1	.60	34.8 2.9	.64
	1:32	79.0 5.8	.88	47.8 6.2	.88
Unconcentrated protein peak 2	0	.4 .1	.004	8.8 1.4	.16
	1:2	38.2 3.8	.42	31.2 3.3	.58
	1:32	72.6 7.8	.80	56.7 4.2	1.04
Unconcentrated protein peak 3	0	49.5 4.1	.55	21.8 1.5	.40
	1:2	58.1 3.7	.64	39.0 3.7	.72
	1:32	100.9 11.7	1.12	51.7 5.9	.95
Unconcentrated protein peak 4	0	43.0 3.5	.48	21.6 1.7	.40
	1:2	72.7 5.8	.81	34.4 3.5	.63
	1:32	88.8 10.3	.98	52.6 5.8	.97

[1]Blood collected at C-0 from 5 cows.

[2]Mean counts per minute ± standard error of the mean x 10^3.

[3]Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control. Concanavalin A stimulated control = 90.1 ± 9.6. Phytohemagglutinin stimulated control = 54.2 ± 3.7.

Table 12. Inhibition of concanavalin A-induced bovine peripheral blood mononuclear cell blastogenesis by fractionated and unfractionated mammary secretion whey.[1]

Dilution of whey fraction	Whey fraction														
	Whole whey[2]			Unconcentrated protein peak 1			Unconcentrated protein peak 2			Unconcentrated protein peak 3			Unconcentrated protein peak 4		
	$\bar{x}$ ± SEM[3]		Index[4]	$\bar{x}$ ± SEM		Index	$\bar{x}$ ± SEM		Index	$\bar{x}$ ± SEM		Index	$\bar{x}$ ± SEM		Index
1:2	.5	.06	.003	131.1	9.4	.84	46.9	5.3	.36	154.9	10.6	.99	148.1	13.5	.95
1:4	.6	.08	.004	125.9	11.2	.81	96.3	7.8	.62	154.7	13.1	.99	149.9	14.4	.96
1:8	.9	.2	.006	123.1	11.3	.79	129.0	9.2	.83	153.3	9.8	.98	147.8	13.6	.95
1:16	.8	.07	.005	132.8	10.9	.85	142.6	11.2	.92	154.4	14.1	.99	145.4	13.6	.94
1:32	.9	.04	.006	134.1	9.9	.86	139.3	10.0	.89	147.1	13.4	.94	145.6	13.8	.94
1:64	17.9	4.5	.12	142.2	11.9	.91	139.0	10.9	.89	151.9	13.8	.97	152.9	12.8	.98
1:128	48.9	10.8	.31	147.5	12.0	.95	132.7	13.5	.85	150.2	14.9	.96	148.0	12.7	.95
1:256	86.1	12.0	.55	149.3	11.5	.96	137.0	12.9	.87	141.0	13.9	.90	151.7	12.1	.97

[1]Blood collected at D+28-30 from 5 cows.

[2]Mammary secretions collected from 4 cows 2-16 d prior to parturition.

[3]Mean counts per minute ± standard error of the mean x 10^3.

[4]Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control. Concanavalin stimulated control = 155.8 ± 15.2. Phytohemagglutinin stimulated control = 95.6 ± 8.9.

greater ($P<.05$) inhibition of con A- and PHA-stimulated cells was associated with protein peak 2. At a 1:2 dilution, only unconcentrated protein peak 2 retained inhibitory effects on con A-induced mononuclear blastogenesis (Table 12).

Bovine serum albumin at various concentrations was added to con A-stimulated cells collected at D+14-16 and C-0 to determine if addition of a protein with no known immunosuppressive properties could affect mitogen-induced blastogenesis (Figures 15 and 16). Total protein in the BSA preparation containing 5 mg/ml was comparable to the total protein content of the 1:2 dilutions of concentrated peaks 1 and 2 (Table 6). Inhibition of mononuclear cell blastogenesis was also observed following addition of BSA to cell cultures.

Viability of mononuclear cells collected at C-0 and cultured with all protein preparations used was determined from duplicate wells at 48 and 90 hr of culture by the trypan blue exclusion method (Table 13). Mononuclear cell viability was lower at 90 hr of culture in wells containing protein peaks and BSA compared to whole mammary secretion whey (Table 13). However, only in the cases of concentrated protein peak 2 and BSA did cell viability decrease below 60%.

DISCUSSION

Concanavalin A and PHA preferentially stimulate T-lymphocytes (Schechter, 1980). Increased inhibition of cells stimulated with con A and relative insensitivity of PHA-stimulated cells to inhibition

Figure 15. Effect of bovine serum albumin on concanavalin A-induced bovine peripheral blood mononuclear cell blastogenesis. Mononuclear cells were collected from 4 cows at D+14-16. Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control.

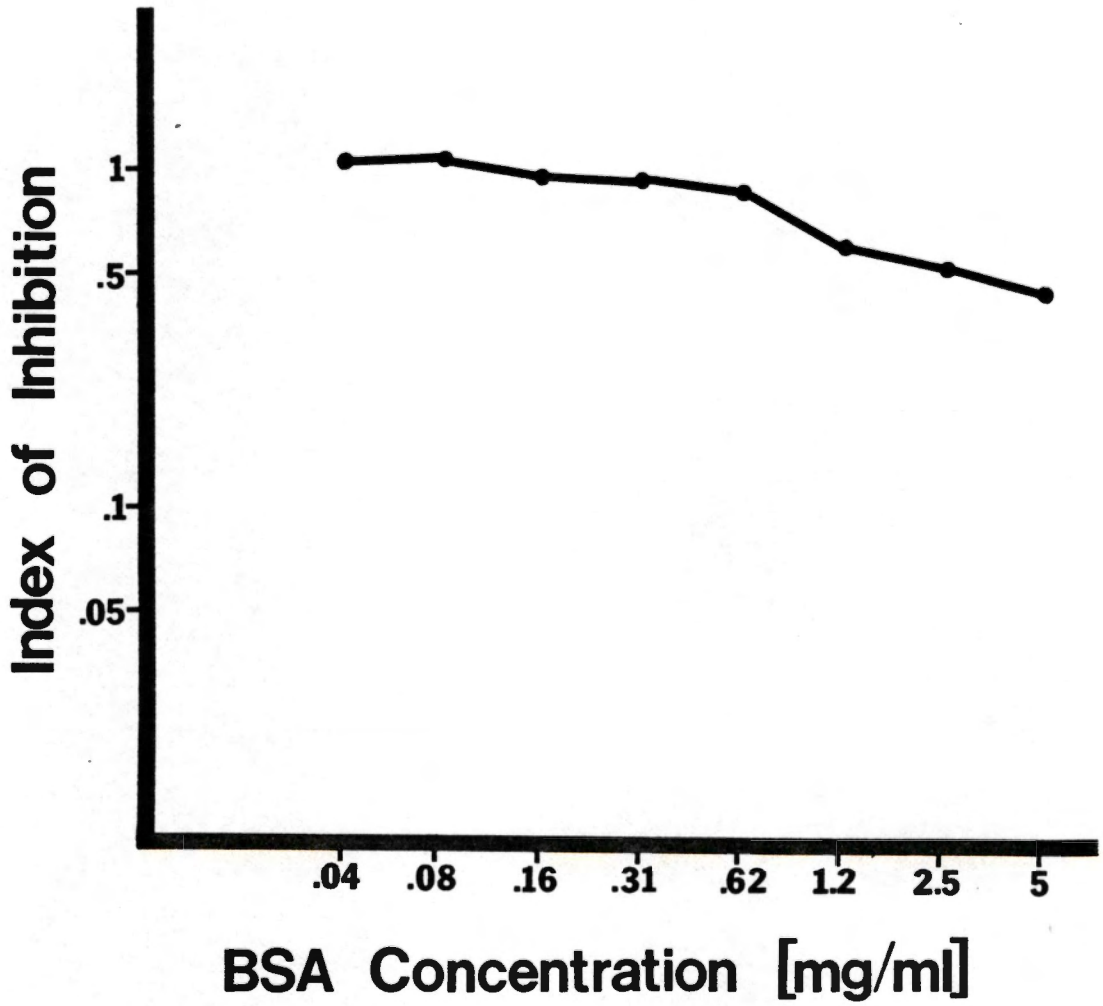

Index of Inhibition
1
.5
.1
.05
.04
.08
.16
.31
.62
1.2
2.5
5
BSA Concentration [mg/ml]

Figure 15

Figure 16. Effect of bovine serum albumin on concanavalin A-induced bovine peripheral blood mononuclear cell blastogenesis. Mononuclear cells were collected from 3 cows at C-0. Data are expressed as an index of inhibition where Index = cpm experimental/cpm stimulated control.

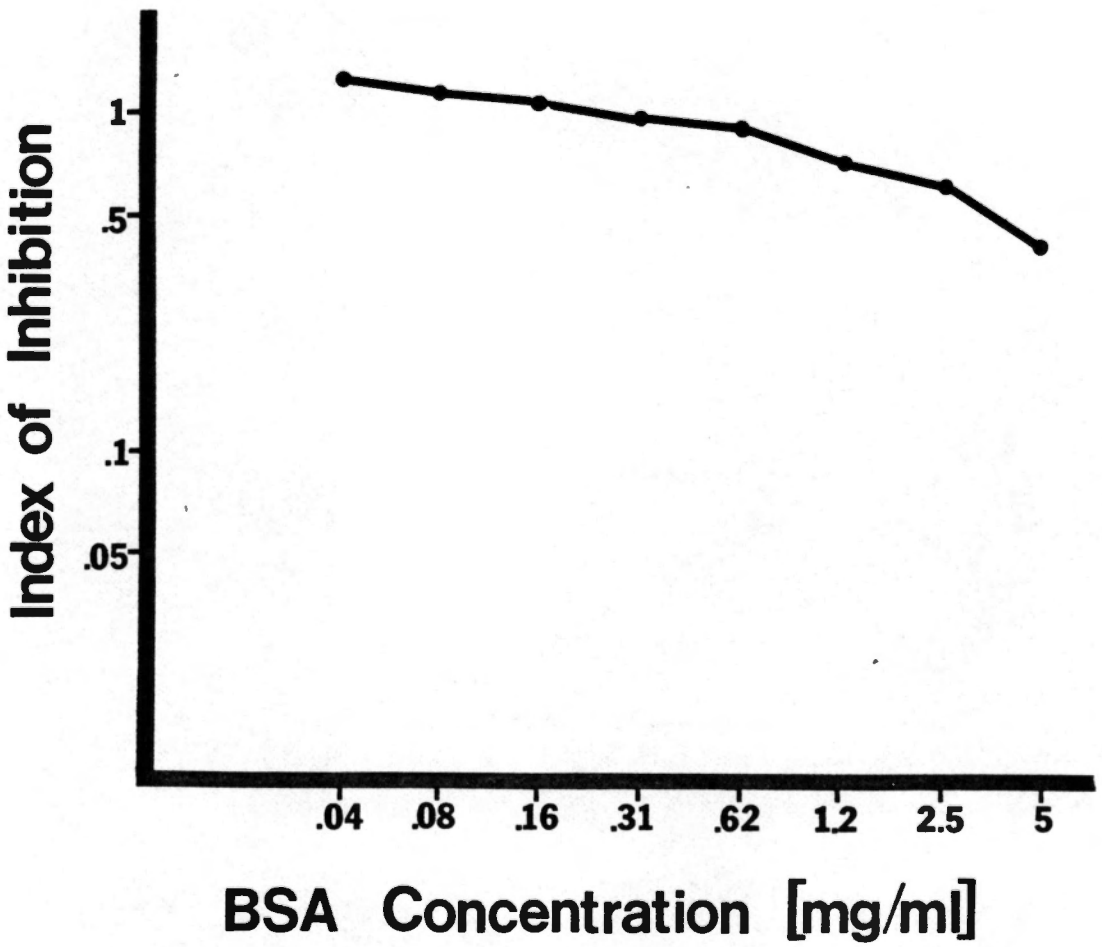

Index of Inhibition
1
.5
.1
.05
.04
.08
.16
.31
.62
1.2
2.5
5
BSA Concentration [mg/ml]

Figure 16

Table 13. Effect of fractionated and unfractionated bovine mammary secretion whey on bovine blood mononuclear cell viability.[1]

Cell preparation	Hours in culture	Cell viability[2] $\bar{x}$	SEM
Whole whey	48	81.0	3.1
	90	81.5	.9
Concentrated protein peak 1	48	71.5	2.7
	90	66.8	2.2
Concentrated protein peak 2	48	65.8	2.2
	90	55.8	4.2
Unconcentrated protein peak 1	48	61.8	4.5
	90	63.8	5.3
Unconcentrated protein peak 2	48	70.2	5.5
	90	66.0	2.7
Unconcentrated protein peak 3	48	59.2	2.0
	90	63.0	4.4
Unconcentrated protein peak 4	48	63.8	1.9
	90	66.0	3.3
BSA	48	72.2	3.1
	90	59.8	3.2

[1]Blood collected from 2 cows at parturition.

[2]Data expressed as x % viability ± standard error of the mean of 2 duplicate cultures.

of blastogenic activity by whey and whey fractions may reflect differences in effectiveness of con A and PHA as T-lymphocyte mitogens. However, Stobo and Paul (1973) suggested that subsets of murine blood T-lymphocytes existed which differed in their response to con A and PHA. Wilson et al., (1986), using differential erythrocyte rosetting techniques, demonstrated the presence of four subsets of bovine PBL and MGL, three of which appeared to be T-lymphocyte subsets. Functional analysis of subsets of bovine blood T-lymphocytes (Belden and Strelkauskas, 1981) indicated that blood T-lymphocyte subsets responded differently to mitogens and in the mixed lymphocyte reaction. Therefore, differential responses to mitogens and to inhibition of blastogenic activity of mammary secretion whey and whey fractions in our study may be due to the presence of subsets of T-lymphocytes in bovine PBL.

Lowry analysis detected only minor protein concentrations in protein peaks 3 and 4 (<10 µg/100 µl). Slight suppression of blastogenic activity associated with these peaks may have been caused by changes in osmolarity in culture wells following the addition of protein solutions suspended in unbuffered ultrapure water. Decreased viability of mononuclear cells co-cultured with mammary secretion whey fractions versus whole whey may also have been the result of a change in osmolarity. However, the <20% decrease in viability in all probability could not have accounted for the 75 to almost 100% suppression of blastogenic activity associated with concentrated and unconcentrated protein peaks 1 and 2.

Bovine serum albumin, a whey protein in mammary secretion with no known immunosuppressive properties, also caused inhibition of mononuclear cell blastogenic activity. Total protein content of the BSA preparation containing 5 mg/ml was similar to the total protein content of the 1:2 dilutions of concentrated peaks 1 and 2. However, inhibition by BSA of con A-induced blastogenic activity of mononuclear cells collected at C-0 was 18 and 5-fold less than that associated with concentrated peaks 1 and 2, respectively. Additionally, mononuclear cell blastogenic inhibition by unconcentrated, undiluted protein peak 2 was greater than that associated with BSA at a concentration of 5 mg/ml. Arthur (1978) noted a similar suppression of bovine mononuclear cell blastogenic activity by BSA and attributed it to possible cytotoxicity of the BSA preparation used. It is also possible that BSA nonspecifically prevented mononuclear cell blastogenesis by blocking cell surface mitogen receptors.

Concentrated preparations of the first two peaks eluting from S-200 column chromatography inhibited bovine mononuclear cell blastogenic activity. At lower dilutions, concentrated protein peak 1 caused greatest inhibition of con A-stimulated cells, while concentrated protein peak 2 caused greatest inhibition of PHA-stimulated mononuclear cells. In contrast, unconcentrated protein peak 2 was associated with greatest blastogenic inhibition of both con A- and PHA-stimulated mononuclear cells. In agreement with results of Smith et al. (1971), the majority of Lf and IgG contained in mammary secretion whey used in this study eluted from S-200 column chromatography in the second peak based on EID and 2D-PAGE analysis.

Lactoferrin, a major whey protein of the involuted bovine mammary gland (Schanbacher and Smith, 1975; Welty et al., 1976) has been shown to suppress in vitro response of bovine PBL to con A (Smith et al., 1979). It is possible that inhibitory effects of protein peak 2 may have been due to Lf. Concentrated and unconcentrated protein peak 1 contained Lf, although in lower concentrations compared to protein peak 2. Suppression of con A-induced mononuclear cell blastogenesis by concentrated protein peak 1 might be due to elevated Lf concentration caused by ultrafiltration. However, concentrated peak 1 caused greater inhibition of con A-induced mononuclear cell blastogenesis in spite of the fact that Lf concentration in concentrated protein peak 2 was greater than that in concentrated protein peak 1. Therefore, it is possible that other factors eluting from S-200 chromatography in peak 1 may have greater immunosuppressive effects when added at higher concentrations on the subset of T-lymphocytes preferentially stimulated by con A.

Immunoglobulin G, which increases in concentration in mammary secretion throughout involution (Smith and Todhunter, 1982) may also be involved in suppression of bovine mononuclear cell blastogenesis. Human IgG has been shown to inhibit lymphocyte response to PHA (Gatti et al., 1973; Faulk et al., 1974) and tuberculin (Faulk et al., 1974). Human IgG has also been shown to inhibit lymphocyte production of migration inhibitory factor (Rocklin et al., 1976), response of human lymphocytes in the mixed lymphocyte reaction (Gatti et al., 1973; Faulk et al., 1974), and spontaneous incorporation of ^{3}H-thymidine

by unstimulated lymphocytes (Faulk et al., 1974). Immunoglobulin G_1, IgG_2 and IgG_4 were the only subclasses of human IgG shown to possess inhibitory effects (Gatti et al., 1974). Immunosuppressive effects of human IgG may be related to blockage of maternal lymphocyte immune response to specific paternal human leukocyte antigens (HLA) to prevent rejection of the fetal allograft (Gatti et al., 1974; Gatti et al., 1975). However, IgG from non-paternal donors has also been shown to suppress maternal lymphocyte activity (Faulk et al., 1974). It is possible that inhibition of mononuclear cell blastogenic activity observed in this study may have been associated with IgG. Differential blastogenic inhibition of con A-and PHA-stimulated mononuclear cells by concentrated protein peaks 1 and 2 may be related to differential specificities of subclasses of IgG for subsets of bovine blood T-cells. However, further research is required to support this hypothesis.

Mammary gland secretions obtained at the end of involution contain elevated concentrations of certain hormones, in particular prolactin and 17-β-estradiol, which are involved in the initiation of lactation (Tucker, 1981). Prolactin (Kelly and Dineen, 1973) and 17-β-estradiol (Clemens et al., 1979) have been shown to possess immunosuppressive properties. Cortisol has also been shown to be immunosuppressive to bovine blood lymphocytes (Murray and Chenault, 1983). However, cortisol affected only con A-induced blastogenesis and not PHA-induced blastogenesis. Therefore, it is also possible

that immunosuppressive effects of mammary secretion whey and whey fractions may be due in part to hormonal or other influences associated with parturition and lactogenesis.

The bovine mammary gland is most susceptible to new IMI during the first few wk following drying off (Neave et al., 1950; Eberhart, 1982; Oliver and Mitchell, 1983) and near parturition (Eberhart, 1982; Oliver and Mitchell, 1983; Oliver, 1987). Changes in rate of IMI throughout involution have been related to fluid accumulation (Cousins et al., 1980) and resulting paucity of natural protective factors such as Lf (Smith and Todhunter, 1982). However, the bovine mammary gland contains a population of viable, functionally competent lymphocytes which may also contribute to defense of the gland against new IMI (Smith and Schultz, 1974; Concha et al., 1978; Concha et al., 1980; Schore et al., 1981). Bovine and human mammary secretions have been shown to suppress mitogenic response of peripheral blood lymphocytes (Drew et al., 1982; Collins and Oldham, 1986; Ogra and Ogra, 1978). Results of a previous study (Part II) indicated that bovine mammary secretions collected at various stages of the lactation cycle differed in their ability to inhibit peripheral blood mononuclear cell blastogenesis. Results of the present study suggest the presence of immunosuppressive factors with some specificity towards subsets of bovine blood T-lymphocytes in mammary secretion obtained during the latter stages of involution. However, further research concerning the exact nature of immunosuppressive substances in mammary secretion and their effect on mammary gland mononuclear cell

subsets is needed. In addition, research is required to determine the role of individual subsets of bovine mammary gland mononuclear cells, in particular lymphocytes, in the defense of the mammary gland against IMI especially during early involution and the periparturient period.

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