Dairy Split Session III

Selected Topics on Mastitis Control

Larry Hutchinson, *Presiding*

Inducing Natural Defense Mechanisms to Promote Mastitis Control

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Introduction

Mastitis costs the U.S. dairy industry over 2 billion dollars annually due to milk losses, animal losses, and treatment costs. Strategies to control mastitis now rely heavily upon antibiotics and topical germicides; but are coming under increasing scrutiny and regulation because of the potential for adulteration of the food product. The considerable therapeutic efficacy of antibiotics especially when administered during the dry period, has led to too great reliance on curing the disease rather than preventing it on the farm, and has discouraged efforts in research laboratories to develop alternative means of protection, such as enhancement of natural mammary defense mechanisms. Dry cow therapy does not cure all existing infections and its preventive value is open to question. Additionally, there are good reasons for wishing to minimize use of antibiotics. These reasons include: 1) development of resistant strains of bacteria, 2) the maintenance of susceptible cows in the genetic pool, 3) inhibitory effect of some antibiotics on mammary defense mechanisms, and 4) potential health hazards to man. Futhermore, the dairy cow lives in a sea of bacterial pathogens. Milking machines, good herd management, and rigorous milking sanitation all require close attention to prevent their breakdown. When these breakdowns occur, the dairy cow usually succumbs to infection. Because we cannot prevent all exposure to bacterial challenge, it is important to stimulate the cow's mammary defense mechanisms to deal with invading pathogens and to prevent establishment of intramammary infection.

Several defense mechanisms for prevention of infection by mastitis pathogens exist within the udder. The streak canal, a duct which averages 8.6 mm in length and located at the end of the teat , is the first line of defense. It serves as a valve to control milk flow and to prevent the entrance of bacteria. The latter is accomplished by bactericidal, bacteriostatic and mechanical barriers that are located in the canal.

If bacteria are successful in bypassing these barriers they enter the teat cistern and encounter the second line of defense, composed of mammary secretions. These include the enzyme lysozyme, the lactoperoxidase/thiocyanate/hydrogen peroxide system, the iron binding protein lactoferrin, complement components, the various classes of immunoglobulins and leukocytes. The monocytes and polymorphonuclear neutrophilic leukocytes (PMNL) function in the phagocytic defense. Among the components which comprise the intramammary defense mechanisms, the streak canal, lactoferrin, and phagocytic activity of PMNL seem the most accessible to enhancement. The methods used to enhance these defense mechanisms will be discussed in this paper.

First Line of Defense

The streak canal. The first line of defense against invading mastitis pathogens is the streak canal. The streak canal averages 8.6 mm in length and 1.2 mm in diameter, when distended by milk flow (1,2,3). At rest, the canal is substantially occluded over a distance of a few mm (4). The streak canal is occluded by an oily wax-like material that contains long chain fatty acids and basic proteins that in vitro are bactericidal and bacteriostatic to a variety of contagious pathogens (5).

Recent wax reconstructions of the epithelial linings of the streak canal revealed a tendency of the canal to form folds with a predominantly spiraling course (6). From this observation, the authors concluded that the traditional concept of a distinct circular sphincter of the streak canal may be considered obsolete. The streak canal terminates in an area identified as Furstenberg's rosette. This tissue has been said to contain a high concentration of a cationic

polypeptide called ubiquitin (7). Ubiquitin is bacteriocidal and causes the swelling and lysis of bacteria.

Numerous leukocytes are found in the epithelium and in the underlying connective tissue of the streak canal, Furstenberg's rosette and teat cistern (8). The highest concentrations of leukocytes were observed in the area of the Furstenberg's rosette and lowest concentrations in the streak canal. Infected mammary quarters contained more cells than noninfected quarters. Involuting mammary quarters contained the fewest cells. The presence of macrophages, of lymphocytes and of plasma cells within the epithelium suggests the existence of mechanisms for detecting antigenic material and initiating immune responses.

Schultz *et al* (4) reported that the likelihood of endotoxin passage through the canal varied according to how deeply it was deposited, and over about the same range of depths as in studies with bacteria (9). In a late study (10) Schultze reported that there was the strong tendency for endotoxin to pass through certain streak canals, and failure to pass through others. Penetration of endotoxin through the duct results in an increase in milk somatic cells in subsequent quarter milk samples. Analysis of results from 75 cows indicated the existence of three populations of cows: those that failed to respond to the endotoxin in any teat (extremely closed ducts), those that responded to the endotoxin in all teats (extremely open ducts), and an intermediate group with mixed responses. Furthermore, responses among cows of the first two populations were very repeatable while among the third group individual teat responses were not repeatable. These results offer the possibility of selecting cows on the basis of extremes of streak canal penetrability and could be a useful tool as an index for mastitis resistance, providing it measures resistance to passage of live bacteria.

The penetrability assay has also been used to show a progressive reduction in penetrability to endotoxin during the first 2 hours after milking (11). This suggests that streak canals remain open for approximately 2 hours after milking and contamination of the teat during this time could lead to infection. Lefcourt (12) reported peristaltic contractions of the teat sphincter and suggested that the contractions may serve as an expulsion mechanism for getting rid of debris that becomes lodged in the canal. Importantly, the author also reported that the teat sphincter was refractory for 4 hours after milking suggesting, as in Schultze's study (11), that bacteria may pass through the canal more easily during the first 2 to 4 hours after milking.

The Second Line of Defense

Lysozyme The enzyme lysozyme catalyzes the hydrolysis of the B(1-4) glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine, biochemical components found in the cell walls of all bacteria. Several Gram positive and Gram negative bacteria were reported to be susceptible to lysozyme purified from bovine milk (13) and bovine leukocytes (14). Unfortunately, concentrations of lysozyme are either very low or absent in tissues, leukocytes and fluids of cattle (15, 16, 17). For example, the concentration of lysozyme in human milk is 3000 times greater than the concentration found in cow's milk (18). The low concentration of lysozyme in cattle milk may represent a weakness in the second line of defense againt bacteria.

Lactoperoxidase/thiocyanate/hydrogen peroxide system This system is inhibitory to the growth of *Streptococcus agalactiae* and *Streptococcus uberis* (19). Lactoperoxidase is always present in milk but the concentration of thiocyanate in milk is variable and is dependent upon the composition of the animal's diet (20). The third component of this system, hydrogen peroxide (H_2O_2), is produced metabolically by streptococci. *E. coli* and *S. aureus* do not produce H_2O_2 and are not controlled by this system unless exogenous H_2O_2 or glucose plus glucose oxidase (20) is supplied. The system is inhibited by cystine which is found in milk (21). Although this system has been suggested as being accessible to enhancement (20), little has been done to develop this system into an effective defense against microbial invasion. This system is considered to play an important role in extending the shelf life of raw milk, especially in underdeveloped countries.

Lactoferrin The iron binding protein lactoferrin is found in mammary secretions (22) and within the secondary granules of PMNL (23). Lactoferrin has been shown (24) to inhibit growth of *E. coli* which requires iron for growth. Citrate, which is also found in mammary secretions, competes with lactoferrin for iron and makes it available to *E. coli* for growth (24). Therefore, the interaction of lactoferrin and citrate is important in controlling growth of *E. coli* in mammary secretions. Concentrations of citrate are high in milk and in mammary secretions around the time of parturition and early dry period (24). Concentrations decrease with involution of the mammary gland or with the decrease in secretory activity of mammary epithelial cells. Concentrations of lactoferrin are low in milk and in mammary secretions around the time of parturition and the early dry period. Concentrations increase with involution of the mammary gland. Thus, citrate and lactoferrin appear to be reciprocally related. High concentrations of citrate prevent the inhibitory effects of lactoferrin on microbial growth.

The importance of this reciprocal relationship was recently established in a study by Eberhardt (25). The number of persistent new intramammary infections established at different times in the dry period was monitored in 212 untreated quarters. During the first four weeks of the dry period 18 out of 26 new intramammary infections occurred during the first week after drying off. Ten out of the 18 infections were caused by coliforms. During the two week period before calving, 9 out of 11 infections occurred during the week before calving. Five out of the 9 infections were caused by coliforms.

Increasing the concentration of lactoferrin and reducing

the concentration of citrate during the first week of the dry period was accomplished by intramammary injection of colchicine and endotoxin (26). The injections produced the desired effect, causing a 50% reduction in the isolation of mastitis pathogens when compared to control quarters. The study established the importance of early mammary involution in controlling new intramammary infections at drying off.

Lactoferrin, also, exerts several important regulatory effects on PMNL. During phagocytosis the secondary granules, which contain lactoferrin, migrate towards the plasma membrane that is forming the phagosome around the particle being phagocytosed and begin to extrude their contents into the incompletely formed phagosome. As a result, it has been estimated that approximately 90% of the lactoferrin is released to the outside of the cell (27). Some of the released lactoferrin is bound to specific receptors for lactoferrin located on the surface of PMNL (28). The lactoferrin together with its bound iron is internalized where lactoferrin and iron enhances bactericidal hydroxyl (-OH) radical formation from superoxide (O_2) and H_2O_2 (29, 30). The lactoferrin released from specific granules of PMNL also has a negative feedback effect on granulopoiesis in bone marrow (31). Lactoferrin also controls adhesiveness of PMNL and keeps PMNL in the area of the inflammatory response, thereby intensifying the inflammatory reaction (32).

Leukocyte phagocytosis Because milk leukocyte phagocytosis is affected by multiple factors they will be discussed separately.

Phagocytic cell types. Macrophages and PMNL comprise the majority of the phagocytic cells of the mammary gland (33). Macrophages predominate in the noninfected gland and constitute approximately 70% of the cells in milk. PMNL predominate in inflamed glands. Depending on the degree of inflammation they constitute 50 to 100% of the cells in milk. Of the phagocytic cells in milk the PMNL have been the most studied. This can be attributed to the important role that they play in inflammation (34) and the ease in obtaining large numbers for study (35).

The PMNL are characterized primarily by a multilobed nucleus. Isles of glycogen and the bactericidal granules that are used by the cell in killing bacteria are found throughout the cytoplasm. Ruminant PMNL have been reported to contain three distinct populations of granules while other species contain two (27). Like other species, ruminant PMNL contain azurophilic and specific granules. The third novel granule is larger, denser and more numerous than the other two granules. It contains lactoferrin, which is also found in secondary granules, but does not contain constituents common to azurophil granules. Instead, it contains a group of highly cationic proteins and is the exclusive store of powerful oxygen-independent bactericidal agents (27).

The PMNL are formed in bone marrow, a process requiring 10 to 14 days. Mature PMNL leave the hematopoietic compartment of the bone marrow and enter the vascular sinus by traveling in migration channels through the endothelial cell (36). The PMNL circulate in the bloodstream briefly (half-life of 8.9 hours) (37), leave the bloodstream by diapedesis between endothelial cells, and enter tissues where they function as phagocytes.

Chemotaxis. The complex nature of the PMNL is evident from the specific way the cell moves (chemotaxis) towards chemicals emitted at sites of inflammation (chemotactic agents) and from the phagocytic and bactericidal activities that this cell is capable of performing.

Chemotaxis and phagocytosis are controlled by specific receptors located on the plasma membrane surface of PMNL. Chemotactic receptors on human PMNL include those for the complement component C_5a (38) and leukotrienes (39). Leukotrienes are a newly discovered family of bioactive substances derived from polyunsaturated fatty acids (arachidonic acid) (40).

Recent studies (41, 42) with bovine PMNL, using the agarose chemotaxis assay, indicated that the chemoattractants for bovine PMNL, in contrast to PMNL from other species, are not derived from bacteria and lack receptors for the formyl-methionyl-leucyl-phenylalanine (FMLP) chemotactics peptides. The FMLP peptides are similar to the chemotactic peptides derived from *E. coli* (43). Lipopolysacharide has been shown, however, to inhibit random migration of bovine PMNL but to act through lymphocytes causing the synthesis and the release of leukocyte inhibitory factor (44).

The macrophage, the predominant cell type in milk from noninfected quarters, plays a key role in recruitment of PMNL. As a result of the activation of the arachidonic acid cascade, $PGF_2\infty$ and leukotriene B_4 are released which are chemotactic (45, 46). Also released is PGE_2 and together with leukotriene B_4 increase capillary permeability, allowing for a greater influx of PMNL (47, 48). Complement components are also released which are chemotactic (49). Proteases are also released which can causing formation of C_5a and can also increase capillary permeability (50). Endotoxin can also activate complement causing release of C_5a (51). As a consequence of becoming activated, macrophages also release toxic oxygen metabolites such as O_2, H_2O_2, and -OH radicals which increase capillary permeability (52).

Phagocytosis. A number of receptors which mediate phagocytosis are located on the plasma membrane surface of PMNL—receptors for IgG_2, IgM and complement have been identified on ruminant PMNL (53, 54, 55, 56). Bovine IgM has also been shown to be highly opsonic for *E. coli* and *S. aureus* (57). A receptor for IgG_1 has also been identified on bovine macrophages (58). However, the binding of IgG_1 to its receptor has been shown not to mediate phagocytosis (58). Receptors for IgA have been identified on human PMNL and are located in close proximity to the Fc receptors for IgG_2 (59). Because IgA is a dimer, the binding of this immunoglobulin to its receptor prevents binding of

IgG_2 to its Fc receptor, thus inhibiting phagocytosis (59). IgG_2 and IgA immune complexes bind to Fc receptors on PMNL and also inhibit phagocytosis (60, 61, 62). Receptors for fibronectins, a closely related family of major glycoproteins, have been identified on PMNL (63). The fibronectins exist in two forms. The soluble form, also known as opsonic α-2, is found in plasma and in lymph and initiates clearance of particles (64). An insoluble form is associated with native collagens in the extracellular matrix and promotes adherence of PMNL (65). Receptors for leukotrienes are found on PMNL that mediate phagocytosis (66, 67). Casein binds nonspecifically with PMNL and blocks receptors that mediate phagocytosis (68).

Enhancers of phagocytic function. A number of factors have been shown to regulate phagocytic and bactericidal properties of bovine PMNL. Specific opsonins of the IgG_2 and IgM classes of immunoglobulins and the complement component C_3b enhance phagocytosis. Addition in vitro of immune serum and whey has been shown to increase phagocytosis (69). Multiple regression of percent phagocytosis simultaneously on concentration of IgG_2, IgG_1, IgA, and IgM (in skim milk samples collected from 48 cows throughout lactation over a three year period) indicated that IgM was most important to phagocytosis, followed by IgG_2 (70).

Naidu and Newbould (71) found that PMNL isolated from milk contained 38% less glycogen than PMNL from blood. This finding conflicts with what has been reported in other species that PMNL accumulate glycogen at inflammatory sites (72). However, the addition of glucose to media containing *S. aureus* and milk-derived PMNL increased phagocytosis (73).

Selenium has been reported to promote both phagocytosis and killing of ruminant PMNL (74, 75, 76). Selenium is associated with glutathione peroxidase, a selenium containing enzyme, that removes excess intracellular H_2O_2, thereby preventing PMNL from self destruction. Lactating dairy cows fed diets deficient in selenium have been reported (77) to have a higher incidence of new intramammary infections when compared to supplemented cows.

Vitamin A deficiency impairs the functional integrity of the reticuloendothelial system and the phagocytic function of PMNL (78). Dairy cows, fed diets supplemented with vitamin A and B-carotene, have been reported (79, 80) to have reduced milk somatic cell counts (MSCC) and reduced incidence of new intramammary infections in the early dry period when compared to unsupplemented cows.

Enhancement of the phagocytic and bactericidal capacities of PMNL was also reported with interferon (81), and the divalent cations Ca++, Mg++, Mn++ and Co++ (82). The anthelmintic drug, Levamisole, was reported to increase phagocytic activity of human PMNL (83) but not of bovine PMNL (84).

Iron (30) zinc (85) and copper (86) also increases the bactericidal properties of PMNL. Lactating dairy cows receiving diets supplemented with zinc have been reported (87) to have reduced incidence of mastitis and lower MSCC than nonsupplemented cows.

Inhibitors of phagocytic function. Inhibitory effects on phagocytosis of bovine PMNL have been reported for antibiotics (88) and corticosteroids (89). The antibiotics tiamulin, nitrofurantoin, rifampin, chloramphenicol, amikacin, gentamicin, tetracycline and novobiocin-penicillin reduced phagocytosis. Interestingly, one can select antibiotics within the same family that will not depress phagocytosis and still maintain the same degree of efficacy. For example, gentamicin and amikacin sulfate belong to the family of aminoglycosides. Both are equally efficacious (90) but amikacin sulfate will not suppress phagocytosis (91). Thus, knowledge of the effects of drugs on host defense, together with sensitivity of the pathogen to the drug, will allow for a more intelligent selection of a drug for future intramammary antibiotic therapy.

Results of in vitro phagocytosis studies (68, 92, 93, 94) show that fat globules and casein inhibit phagocytic and bacteriocidal properties of PMNL. The losses in activities were associated with the binding of casein to the surface of the PMNL (68), the loss of PMNL plasma membrane (95) and the degranulation of the cells after ingestion of fat and casein (96).

Artificial enhancement of phagocytic function by man. Enhancement of the phagocytic defense mechanisms of the udder was recently accomplished by inserting an abraded polyethylene intramammary device (AIMD) into the mammary gland cistern of cows (97). The principle involved was to induce a leukocytosis in that fraction of milk (foremilk or strippings) closest to a pathogen's point of entry. To avoid any change in the composition of the milk from the entire mammary gland, any cellular or humoral response to the AIMD must be confined to the area of the mammary gland cistern. The device had the desired effect of increasing the MSCC in stripping milk to 900,000/ml and caused only modest increases in the milk from the entire gland. Results from experimental challenge studies (98) indicate that the AIMD was 60 to 70% successful in the prevention of infection after challenge with *E. coli, Strep. uberis* and *S. aureus*.

The AIMD is currently being field tested in Israel (99). Thirteen dairy herds totaling 3660 Friesian dairy cows were selected for study. Half of the cows received AIMD in all four quarters. There were 164 reported cases of clinical mastitis among AIMD cows and 366 cases among controls. Of these clinical cases, 41 of the AIMD cows and 197 of the control cows had systemic involvement that required treatment. Seventy percent of the isolates from clinical cases with systemic involvement were coliforms. Significantly, these studies indicate that an intramammary defense mechanism can be successfully enhanced and developed into a practical deterrent against mastitis. An update on the AIMD will be presented at the 25th Annual Meeting of the National Mastitis Council (NMC), February 9-12, 1986 in

Columbus, Ohio and will be published in the NMC Proceedings.

Artificial enhancement of the bactericidal properties of phagocytic cells can be accomplished with liposomes. Liposomes are lipid coated vesicles that contain antibiotics. When internalized by phagocytic cells, the liposomes enhance the bacteriocidal properties by emptying their contents into the phagolysosomes (100). Liposomes have been shown to enhance intracellular killing of *Brucella abortus* (100) and *S. aureus* (101) and are currently being studied in the treatment and prevention of bovine mastitis (102).

Significant variation has been reported (35, 103) among cows in the ability of PMNL to infiltrate milk after irritation of the mammary gland, in the ability of milk to support phagocytosis and in the ability of PMNL to phagocytose. Knowledge of the variability among cows might help explain some of the differences in susceptibility of the mammary gland to infection. The extent to which this variation was due to different ratios of PMNL subpopulations among cows was recently examined (104). In that study, 16 monoclonal antibodies were produced against bovine PMNL, indicating the existence of subpopulations of PMNL. The phagocytic and chemotactic properties of these subpopulations are currently under investigation by scientists in the Milk Secretion and Mastitis Laboratory at Beltsville. The identification of subpopulations of PMNL that are functionally superior to others may provide a marker for the genetic selection of mastitis resistant animals. The identification of a subpopulation of PMNL chemotactically superior to other subpopulations was recently made in the human (105).

Conclusions

A far-sighted mastitis research thrust would have as its ultimate objective a dairy cow population with heightened natural resistance to the disease. The intramammary defense mechanisms that were discussed in this report that appear to be accessible to enhancement by man include the streak canal, lactoferrin, and phagocytic leukocytes.

The presence of macrophages, of lymphocytes and of plasma cells within the epithelium of the teat offer possibilities of presenting antigenic material to these cells to initiate immune responses. The discovery that significant variation exists among cows in the ease in which substances can penetrate the streak canal, offers the possibility of selecting cows with heightened genetic resistance to mastitis.

The successful manipulation of the lactoferrin to citrate ratio in mammary secretions offers the possibility of enhancing the control of coliforms.

The phagocytic and bactericidal properties of leukocytes appear to be regulated by a number of control mechanims that are accessible to man. The provision of proper dietary levels of selenium, vitamin A, and zinc appears to reduce intramammary infection levels by increasing efficiency of phagocytic cells. Increasing optimal concentrations of specific antibodies in milk has been shown to increase phagocytic function. Procedures for increasing these antibodies have yet to be devised.

Localized recruitment of PMNL was shown to be possible through the insertion of a polyethylene intramammary device into the gland cistern of cows. Although much still needs to be known about the device, the successes obtained in experimental and field trials in reducing mastitis appear promising.

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Questions & Answers:

Question: Would you comment on the level of leukocytes?

Answer: I think the increase in leukocytes seems to be very modest during early to mid-lactation. You may get an increase on the order of maybe 20, 30, or 40,000, which probably isn't going to impact very much in terms of causing possible secretory damage and reduced milk production. During later lactation, towards the end of the lactation, near the dry period, it appears the leukocyte count in milk will increase, say from 200,000 in controlled quarters, to closer to 400,000 in the IMD quarters. So now we're sort of impacting on those secretory cells. However, we're at a point now where the gain is producing a lot less milk and so we're probably not impacting as much in terms of total milk production in the IMD cows. In addition it appears that the gain that you get from reduced clinical mastitis (clinical mastitis causes a lot of tissue damage) is going to more than offset the slight decrease in IMD quarters.

Question: What kind of results did you obtain?

Answer: The problem with such a large study like this involving 6 or 7,000 cows is that we have so much data being accumulated it is going to take us a couple of years to really sit down and look at all these things. For example, looking at milk production in a herd relative to the incidence of clinical mastitis, we know that we get a lot of variation. Well, not a lot

of variation, we get variation among herds where some herds don't show any difference between the IMD and the control cows in milk production. Probably due to the fact that they don't have a lot of clinical mastitis. We don't know why, for example, cows are responding differently to the IMD and this could be related, possibly, to just when the IMD is inserted. Certainly we are looking at the spontaneous cure rate. Interestingly enough the spontaneous cure rate is higher in the IMD quarters than in the control quarters. So it is affecting the spontaneous cure rate which could affect maybe some of these clinical cases where you don't have systemic signs.

Question: Have you had any bad effects?

Answer: In the Israeli study we have not had any bad effects. We know in studies at Beltsville, everytime you put an IMD into a cow, microscopically, at least, you can see some red blood cells in the milk. Visually you can't see them. We don't know, it appears they're coming from somewhere in the gland cistern, but we don't know what the long term effects of these red cells in the milk may be.

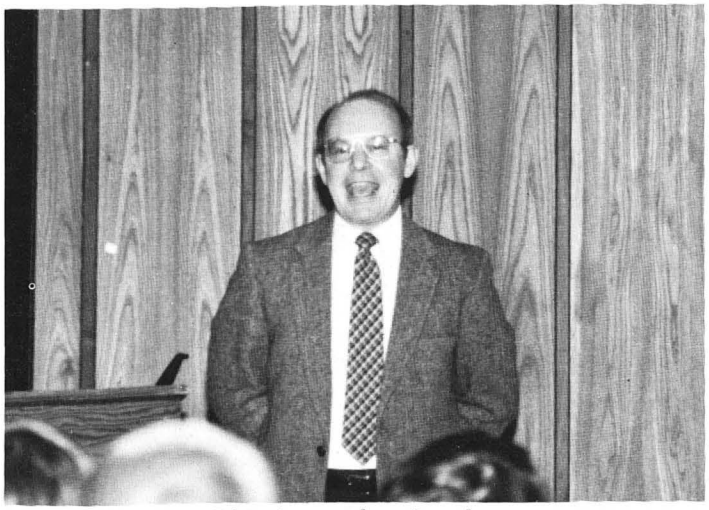
Question: Any changes in the milk?

Answer: No, they haven't noticed any blood in the milk. No abnormalities to speak of. And it appears to be very well tolerated in the mammary glands.

Question: What about somatic cell counts?

Answer: The important concept here is that the somatic cell count only increases in the stripping portion of the milk. So that means that when the cow comes into the milking parlor at the time of milking, that would be the foremilk fraction. And that fraction is just usually discarded in the strip cup. So that milk normally wouldn't enter the weigh jar. The only time it shows an increase in the somatic cell counts in the weigh jar is during late lactation. Interestingly enough in Israel, where they have half the herd with IMDs, they have not shown any change in bulk tank somatic cell counts. They are averaging 150,000 to 200,000 per ml. They have not shown any change. And the reason for that is you have such a dilution effect. If you have a cow producing 400,000 leukocytes per ml in 10 or 20 lbs. of milk, and that's diluted by 40,000 per ml in say 80 or 100 lbs. of milk, it just disappears.

Post Convention Tour



Dean Phemister welcoming the group.