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## Enhancing Mammary Immunity: New Opportunities

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The most critical period in the lactation cycle of a cow starts at dry off and continues until about 60 days of lactation. This is the 120 day period that includes the major proportion of fetal growth, marked endocrine dynamics in preparation for parturition, abrupt changes in diet and gastrointestinal physiology, the highest incidence of metabolic disease, dramatic changes in energy balance, and the formation of production performance for the entire lactation. It is generally accepted that this is the most critical four month period with respect to mammary health as well. The mammary gland undergoes marked biochemical, cellular and immunological changes to accommodate involution, the preparation for parturition, the transition of colostrum to milk, and the attainment of peak milk production. Additionally, the early dry period and periparturient period have been promoted as the times of highest incidence for new intramammary infections, and the first 30 days of lactation has been reported to be the time of highest incidence of clinical mastitis. This paper offers a brief review of mammary immunity during this period of the lactation cycle, research efforts to enhance immune function, and opportunities that may possibly lie ahead.

Although the efficiency of neutrophil phagocytosis and killing in the mammary gland is lower than other tissues, it remains the most critical defense once the teat end barrier is breached. Involution of the mammary parenchyma begins one to two days after the end of lactation and continues for ten to 14 days. During this time, the gland is particularly vulnerable to new IMI, and in conjunction with the periparturient period, constitutes a period of high risk for new IMI in the lactation cycle of the cow. In contrast, the completely involuted mammary gland offers the most hostile immune environment of the lactation cycle. The most important defense, as with lactating cows, remains the teat canal, which is enhanced by the formation of a keratin plug. Populations of macrophages and lymphocytes, and concentrations of complement and immunoglobulins, increase as well. This helps orchestrate more efficient phagocytosis. Lactoferrin, a potent iron chelating protein, also markedly increases in dry cow secretions, thus helping to inhibit growth of Gram-negative bacteria, particularly *E. coli*. Consequently, the dry period is an ideal time to attain synergy between antimicrobial therapy and immune function to eliminate pathogens from the gland, and not incur the extensive costs typical of therapy of lactating cows. Additionally, doses or formulations that allow extended drug presence in the gland are of minimal concern for milk residues or discarded milk costs because dry periods are usually 45 to 60 days in length.

### **Dry cow therapy**

Intramammary administration of antibacterials at the end of lactation has been a standard of mastitis management for 30 years. In addition to the important role that dry cow therapy has in eliminating existing IMI, it also helps prevent new IMI. This is the simplest form of immune enhancement, antibacterials serve to augment clearance of bacterial pathogens. However, most commercial dry cow products have little or no activity against Gram-negative pathogens, nor will these products be effective for new IMI which begin in association with the periparturient period. Although cure rates for all pathogens (those IMI that existed prior to the dry period, but were not detected following calving) were reported in initial studies as averaging 75%, true efficacy of conventional dry cow treatments in eliminating more chronic IMI such as caused by *S. aureus*, may be low. Undoubtedly, success may vary from herd to herd and cow to cow. However, many studies did not utilize consistent sampling methods to identify infected quarters before or after the dry period. Thus, it is possible that the discrepancies between studies may be accounted for in the differences of experimental method used to determine IMI.

As with lactational therapy, the use of systemic administration as an adjunct to intramammary administration has stimulated interest in potential alternative therapeutic regimens, particularly for chronic IMI caused by Gram-positive cocci. Subcutaneous norfloxacin nicotinate administered at the start of the dry period achieved a better cure rate and lower new infection rate over the dry period for *S. aureus* infections, as compared to untreated cows and cows administered intramammary cephapirin benzathine preparations (Soback *et al.*, 1990). However, in a Michigan study, cows administered intramuscular oxytetracycline and intramammary cephapirin did not have better cure rates for quarters infected with *S. aureus* than cows treated with cephapirin only (Erskine *et al.*, 1994), although oxytetracycline was beneficial in a Dutch study (Sol, 1990). The later study was performed in a herd with a prevalence of *S. aureus* of 50% of the cows in the herd, and IMI were of considerably long duration. An evaluation of dry cow therapy should not be based on the success of eliminating IMI caused by *S. aureus* only, and for many other Gram-positive cocci, therapeutic efficacy can be 80 to 90%. One possible limitation that we encounter with traditional antibacterial therapy of mastitis is the relatively short duration of therapy. Most antibacterial activity used for dairy cattle (with the exception of fluoroquinolones) is based on a time-dependent effect. Thus, efficacy is based on the continuous time spent above the MIC for the pathogen, not peak concentration of the drug in the gland. Many of our mastitis regimens only promote two to three days of therapeutic drug concentrations in the gland, thus enhancing the chances for failure.

Based on the premise that no antibiotic can eliminate an infection without a functional immune system, and our limited ability to manipulate immune function at the time of treatment, mastitis therapy should be initiated on an assessment, even on a crude scale, of immune competence of the treated cow. This assessment is especially important when deciding on augmenting traditional dry cow therapy with systemic drug use. Intramammary infections from older cows, particularly of longer duration, that more consistently shed pathogens over time, and from cows that have multiple infected quarters are a poorer therapeutic risk. These are somewhat crude predictors of therapeutic success, but suggest that we should explore genetic markers in dairy

cattle that may allow us to identify potentially immune impaired breeding lines as well as target our therapeutic efforts and expectations of treated animals.

### **Immune modulation**

Attempts to enhance immune function and clearance with immune modulators, either solely, or synergistically with antimicrobials is also an area of potential future importance for mastitis therapy. Numerous cytokines, which regulate cell function during immune and inflammatory processes, have recently been identified and purified from cattle. With subsequent identification of the genes responsible for cytokine synthesis, recombinant production has been made possible.

Cytokines that have demonstrated promise for use in the prevention and or treatment of bovine mastitis are interleukin-1 (IL-1) and interleukin-2 (IL-2), interferons, and colony stimulating factor. Intramammary IL-1 $\beta$  and IL-2 increase mammary gland polymorphonuclear leukocyte diapedesis, and IL-2 activates inducible superoxide production. However, field trials using intramammary IL-2 as an adjunct therapy with intramammary cephalosporin determined little or no benefit of IL-2 in curing or preventing IMI in the dry period, and has been associated with abortions in treated cattle three to five days after infusion (Hogan *et al.*, 1995; Erskine *et al.*, 1998). Future research may elucidate potential uses of immune modulation, but as this class of bioagents are extremely potent and have complex actions, care will be necessary to attain the desired antimicrobial effect without other side effects. Other attempts at nonspecific immunostimulation to prevent mastitis have been described for levamisole (Anderson, 1984); concanavalin A (Oliver, 1989), interleukin-2 (Nickerson, 1989a), and thymosine (Kehrli, 1989). Most methods of non-specific immunostimulation have only been tested under experimental conditions.

### **Parturition**

Hill *et al.* (1979) reported twenty years ago that newly calved dairy cows had more severe cases of coliform mastitis than cows later in lactation. It was implicated that a slower migration of neutrophils into the gland was responsible for this effect. During calving, a complex symphony of endocrine expression takes place. The critical hormone that may be related to mammary immunity is cortisol, which markedly rises during calving. Cortisol is critical for expulsion of the fetus during parturition, however as with all corticosteroids, it may have a deleterious effect on mammary immunity near calving. Previous research has demonstrated that leukocyte function is significantly altered in cattle challenged with dexamethasone (a synthetic glucocorticoid), and when dexamethasone was administered to lactating cows with subclinical infections, increased bacterial shedding led to the development of clinical mastitis (Burton, 1995a; Lohuis, 1988). Further research demonstrated that important phagocyte adhesion molecules that are essential for trafficking into inflammatory sites are down-regulated by dexamethasone (Burton, 1995b). Leukocytes isolated from blood of cows at parturition have been determined to have lower adhesion molecule expression than cows later in lactation or in the dry period (Figure 1). Cortisol functions by binding to cytoplasmic glucocorticoid receptors in target cells. Once bound to the receptor, the hormone-receptor complex is activated and translocates into the nucleus to bind to regulatory regions of glucocorticoid responsive genes. One of the genes that are bound and apparently down-regulated is the L-selectin group, which would explain the

lowered ability of calving cows, or those treated with steroids, to resist infection. Thus, we must accept the reality that calving cows cannot migrate neutrophils into the gland as well as at other times of the lactation cycle because of cortisol associated with calving, and will thus be more prone to infection (Figure 2). Pharmacologic use of steroids must be used judiciously at this time. It should also be noted serum immunoglobulin responses to vaccination are lower when vaccination is administered at the time of calving as compared to other times of lactation.

### **Vaccination**

Development of vaccines for streptococcal mastitis has been slow to develop. However, there has been considerable effort to develop a vaccine against *S. aureus*. Previous attempts to develop vaccines against cell wall components (protein A) and bacterial adhesion proteins (fibronectin-binding) have met with mixed results. However, new technology has developed a vaccine against the pseudocapsule that surrounds the bacterial cell wall when *S. aureus* grows in milk (Watson, 1990). The vaccine enhances anti-pseudocapsular antibodies that aid in opsonization of bacteria for phagocytosis by neutrophils. This approach, combined with alpha- and beta-toxin antigens has demonstrated positive results in the prevention of subclinical IMI and clinical mastitis cases caused by this organism (Watson, 1996; Sears, 1990; Nickerson, 1993). Commercial development has not as yet been provided, and eventual large scale efficacy trials would be needed to demonstrate long-term efficacy as well as economic feasibility. However, this concept is deserving of being pursued as a sound scientific method to prevent new *S. aureus* IMI.

One of the more promising methods of enhancing mammary resistance for coliform (and all Gram-negative) IMI is by the use of core antigen vaccination. Historically, attempts to immunize cows against coliform infections had met with little success. Much of this failure resulted from the diversity of surface (AO@ or capsular) antigens presented on invading pathogens. Among herds and cows within herds, a variety of species and serotypes may cause mastitis. Serologically identical strains are rarely found in intramammary infections of different cows within a herd (Eberhart, 1979). However, development of a bacterin derived from the J5 mutant of *E. coli*, a strain that lacks the capsular antigens but retains the lipopolysaccharide core of the cell wall, has proven effective in reducing the severity of coliform mastitis. The lipopolysaccharide core is highly conserved genetically and in phenotypical expression throughout most Gram-negative bacteria. Thus, exposure and presentation of the endotoxin antigens in the bacterin to the immune system should result in nearly universal recognition, and hopefully a response to any Gram-negative bacterial pathogen. California studies demonstrated that cows receiving two vaccine doses during the dry period and one dose after calving had an incidence of clinical coliform mastitis of 2.6%, as compared to 12.8% in unvaccinated controls (Gonzalez, 1989). A second field trial determined that vaccinated cows had a threefold decrease in clinical coliform mastitis (Cullor, 1991). Subsequent field studies from Ohio have confirmed the benefits of this technology (Hogan, 1992a). Titers among cows for naturally occurring serum antibody specific for the J5 strain of *E. coli* were negatively correlated to risk of clinical coliform mastitis in a California dairy herd (Tyler, 1988). Similarly, titers in response to immunization were negatively correlated with severity of clinical outcome following experimental challenge of

*E. coli* (Hogan, 1992b). Experimental challenge models of *E. coli* mastitis have indicated that vaccination with J5 bacterins: 1) decreases clinical severity, but does not prevent infection, 2) reduces the concentration of bacteria in milk, and 3) is beneficial for multiparous cows as well as primigravid heifers. (Hogan, 1992b, Hogan,1999). Commercial availability of this vaccine technology has allowed widespread use in many herd mastitis control programs. Despite the potential benefits, these vaccines cannot overcome poor environmental conditions for housing, or be expected to affect clinical mastitis caused by non-Gram negative pathogens. However, development of this vaccine has been the single biggest advance in active transfer of immunity against mastitis for dairy cattle, and depending on the incidence of clinical mastitis caused by Gram-negative bacteria in a herd, should be considered for a complete mastitis control program.

### **Beyond the dry period?**

Should we consider enhancing immunity beyond the dry and periparturient period? We have recently completed a clinical trial comparing therapy of severe clinical mastitis in six Michigan dairies. One-hundred cases were enrolled in the trial over 18 months, of which 55 were identified as coliforms cases from culture of milk from affected quarters. Unlike previous studies of clinical mastitis caused by environmental pathogens, the distribution of cases with respect to days in milk from the Michigan study revealed that the time of highest risk was approximately 120 days in milk (Figure 3). This suggested a potential lack of resistance at a time other than the dry/periparturient period. Interestingly, cows that were vaccinated with J-5 bacterin for four additional vaccinations at 30 day intervals beyond the standard three dose regimen (extending 120 days in milk) demonstrated increased serum concentrations of anti-coliform immunoglobulins as compared to three vaccinations. In another Michigan study, steers administered multiple vaccinations of J-5 bacterin at three week intervals had a consistent increase in plasma IgG1, IgG2, and IgM (Figure 4). The most profound elevation occurred after 6 immunizations. This raises questions as to the need for more extensive vaccination regimens for coliform mastitis in herds that have coliform mastitis problems in cows that are beyond the periparturient period. However, no clinical trials demonstrating any clinical or economic benefits to this strategy have been published.

In summary, the best way to take advantage of the enhanced immune ability of the dry period remains dry cow therapy with antibacterials, possibly enhanced with systemic therapy. Sound pharmacokinetic and pharmacologic principles must be applied. Maternal cortisol associated with parturition reduces the expression of adhesion molecules on the surface of phagocytes, thus decreasing their ability to migrate into the gland during inflammation. This is a physiologic phenomenon that we are not likely to be able to alter. Making prudent choices regarding steroid and vaccine use should reflect this reality. We also may have to think outside the usual boundaries of when in lactation mastitis can occur, and the number of immunizations that may be necessary to most effectively and economically enhance immune resistance.

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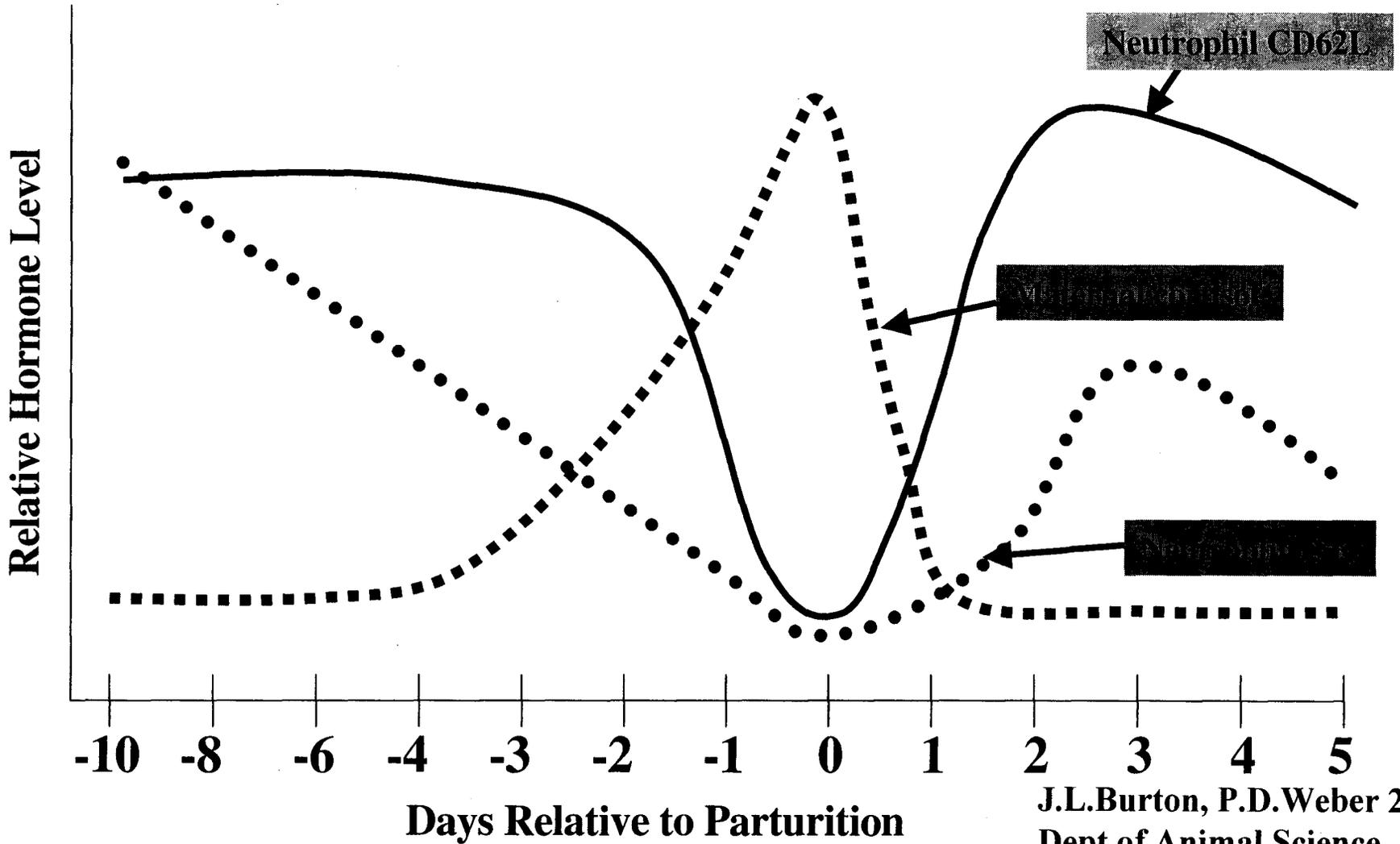
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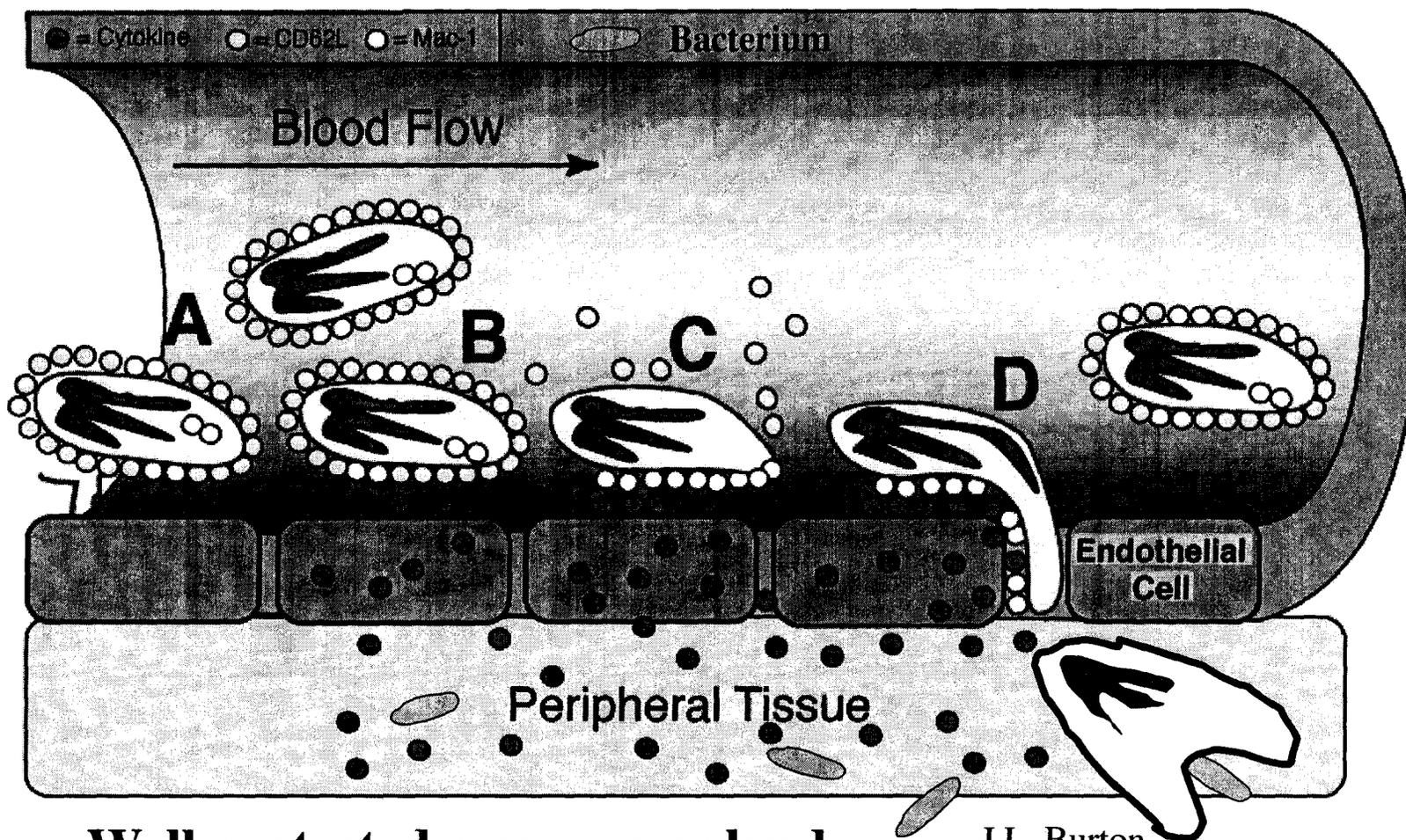
**Figure 1. Relative Concentrations of Cortisol, L-selectin, and PMN GR at Calving**



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**Figure 2. Neutrophil margination is mediated by CD62L and migration by CD11b/CD18 (Mac-1)**

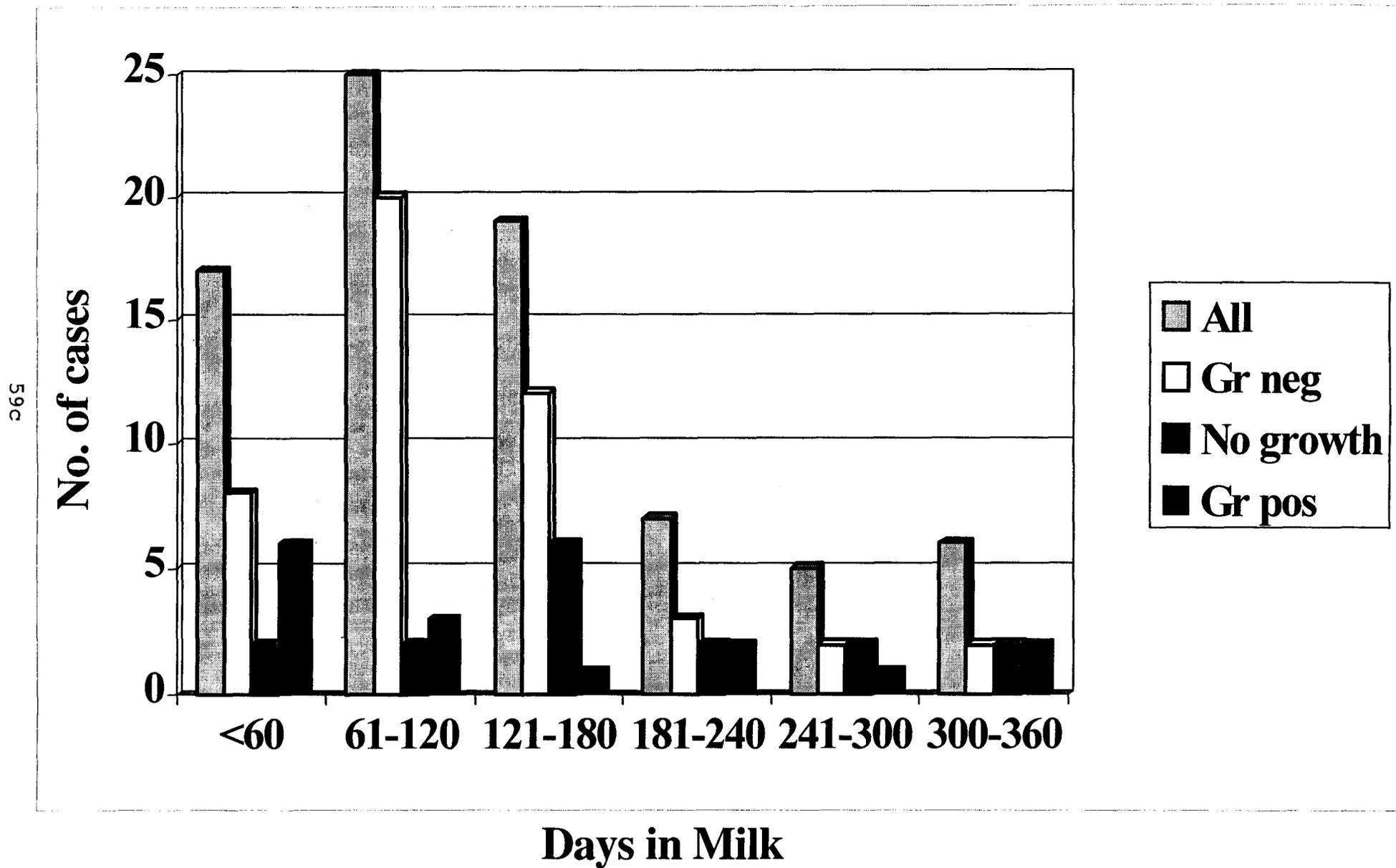


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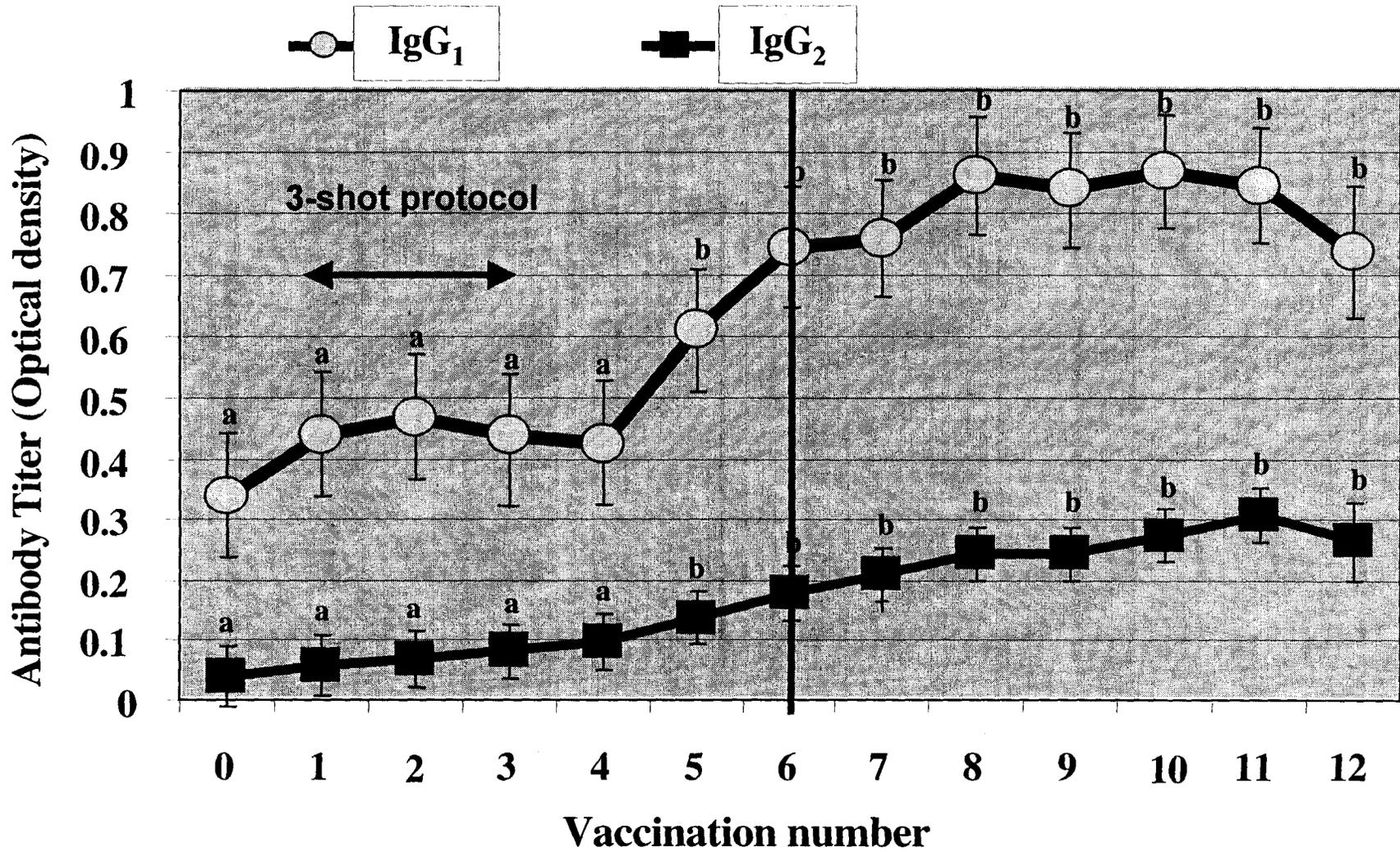
**Well protected mammary gland**

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**Fig 3. Severe clinical mastitis in six Michigan dairies**



**Fig 4. Mean anti-coliform antibody titers in serum of six Holstein steers hyperimmunized by J5 vaccine**



[Smith et al. 1999. CRWAD]