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Chapter 116

The Mammary Gland in Mucosal and Regional Immunity

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INTRODUCTION

Milk Immunoglobulins and Passive Immunity: A Historical Perspective

The importance of the mammary gland (MG) in passive immune protection was known to herdsman centuries before the advent of science and immunology. It was well known that the foal that did not suckle the mare, the lamb that did not suckle the ewe, or the piglet that did not suckle the sow died in a few days. Whereas in 1892 Paul Ehrlich suspected antibodies in milk, it was not until 1946, when Emil Smith discovered “immune lactoglobulin” (now called immunoglobulin IgG1) in bovine colostrum, that a probable molecular basis for the passive protection was identified. However, effective systemic passive immunity also required that the intestine of the calf absorb these antibodies intact during a short window before protein degradation became the main role of the gastrointestinal (GI) tract. Thus, successful transfer of IgG1 required coordinated physiological regulation by both mother and offspring.

Although newborn foals, lambs, kids, and piglets die if they fail to suckle, human infants do not and can be raised on soy-based formula. This paradox was resolved when the placenta-tion of different mammals was studied. In primates, rabbits, and, to some extent, rodents and carnivores, IgG is actively transported from maternal serum across the placenta to the fetus, whereas this does not occur in horses, cattle, swine,

and other ungulates (Figure 1; Brambell, 1970). Survival of newborn primates and rabbits was not absolutely dependent on suckling, but it did not mean that suckling only provided nutrition. Roy (1956) recognized that suckling by calves had both long-term and immediate protective value. The long-term value persisted even when IgG1 levels dropped to 10% of protein in mature milk. The long-term value of suckling was reinforced by studies on infants in underdeveloped countries. One explanation for the long-term effect emerged a decade later, when various investigators showed that IgA (a minor serum protein), not IgG1 as in cattle, was the major immunoglobulin in rabbit and human colostrum (Figure 1). However, because the gut of the suckling ungulates becomes closed to the absorption of intact proteins within 12–24 h after birth and the infant gut has little capacity to absorb intact proteins even at birth (Section “The Role of Lacteal Secretions in Passive Immunity”), it was suspected that IgA antibodies in human colostrum and in mature milk of ungulates was acting in the gut lumen; this could explain the long-term effect reported by Roy. Because primates and rabbits have already transferred their passive IgG before birth, the passive antibody function of the MG is the transfer of IgA. The transition from the immediate short-term role of IgG to the long-term role of IgA is best demonstrated in swine, in which the immunoglobulin content of lacteal secretions change to favor IgA and hence become similar to that of human colostrum and milk (Table 1; Figure 2). Unlike IgM, IgG and IgA are both products of

FIGURE 1 Transmission of immunity from mother to young. The size of the type used for the various immunoglobulin isotypes depicts their relative concentration in colostrum. Revised from Butler (1974).

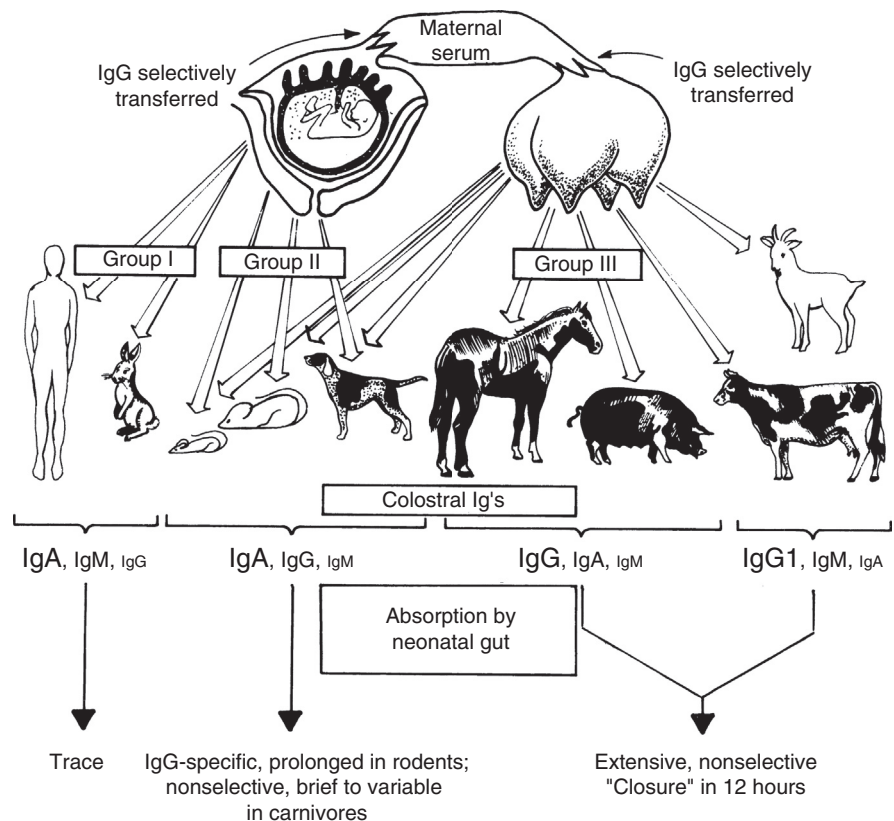


TABLE 1 The Immunoglobulins in Lacteal Secretions of Representative Species^a

| Species | Immunoglobulin | Conc. (mg/mL) ^b | | |
|---------------------|----------------|----------------------------|---------------------------|---------|
| | | Colostrum | Mature Milk | Serum |
| Human ^c | IgG (total) | 0.85 0.21 | 0.04 | 12.4 |
| | IgA | 86.8 13.6 | 1.8 (D4–D18) ^d | 2.91 |
| Rat ^e | IgM | 2.64 0.92 | 0.1 | 1.17 |
| | IgG (total) | 2.6 | 1.06 | 24.6 |
| | IgG2a | 0.67 | 0.8 | 6.91 |
| | IgG2b | 0.05 | 0.26 | 0.89 |
| | IgA | 1.15 | 1.02 | 0.18 |
| Mouse ^f | IgM | 0 | >0.002 | 0.95 |
| | IgG (total) | N.D. | 0.12 | 2.8–4.8 |
| | IgG2a | | | 0.4–1.5 |
| | IgG2b | | | 0.2–0.6 |
| | IgA | 0.05 | 0.3 | 0.6 |
| Rabbit ^g | IgM | 0.2 | 0.59 | 0.2 |
| | IgG | 1.5 | 0.1 | 5→10 |
| | IgA | ~30.0 | ~5.0 | 0.01 |
| Dog ^h | IgM | 0.01 | Trace | 0.01 |
| | IgG (total) | 12.1 | 0.15 | 15.6 |
| | IgA | 3.6 | 1.75 | 0.94 |
| Swine ⁱ | IgM | 0.6 | 0.13 | 1.6 |
| | IgG (total) | 61.8 | 1.6 | 24.0 |
| | IgG1 | ? | ? | ? |
| | IgG2 | ? | ? | ? |
| | IgG3 | ? | ? | ? |
| | IgG4 | ? | ? | ? |
| | IgG5 | ? | ? | ? |
| | IgG6 | ? | ? | ? |
| | IgA | 11.3 | 4.1 | 2.0 |
| | IgM | 3.8 | 1.5 | 2.5 |
| Cattle ^j | IgG1 | 46.4 | 0.58 | 11.2 |
| | IgG2 | 2.87 | 0.05 | 9.2 |
| | IgG3 | N.A. | N.A. | N.A. |
| | IgA | 5.36 | 0.10 | 0.37 |
| | IgM | 6.77 | 0.09 | 3.1 |
| Sheep ^k | IgG1 | 94→162 | 1.0 | 18.1 |
| | IgG2 | 2.0 | 0.1 | 7.9 |
| | IgA | 3.5 | 0.2 | 0.2 |
| | IgM | 1.3→21.2 | 0.2 | 3.6 |

Continued

TABLE 1 The Immunoglobulins in Lacteal Secretions of Representative Species^a—cont'd

| Species | Immunoglobulin | Conc. (mg/mL) ^b | | |
|--------------------|------------------|----------------------------|-------------|------------------|
| | | Colostrum | Mature Milk | Serum |
| Horse ^l | IgG1/IgG2 (IgGa) | 82 | 0.2 | 3.4 ^m |
| | IgG4/IgG5 (IgGb) | 183 | 0.3 | 19.6 |
| | ? (IgGc) | 0.3 | N.D. | 0.2 |
| | IgG3 (IgG(T)) | 44 | 0.1 | 4.0 |
| | IgA | 9 | 0.7 | 0.4 |
| Cat ⁿ | IgM | 2.3 | 0.07 | 1.6 |
| | IgG | 26.5 | N.D. | 14.7 |
| | IgA | 0.64 | N.D. | 2.2 |
| | IgM | 0.48 | N.D. | 5.3 |

^aData are based on samples collected from nonmastitic MGs. Data are means of reported values, some of which are themselves means. Immunoglobulin E has been reported in the lacteal secretions of many species but no quantitative data are available. When large differences occur, ranges are given.

^bColostrum samples in animals were collected 1–4 h after parturition; Mature milk = mid-lactation; Serum = generally from late or nonlactating animals. Most data are based on concentrations in whey that disregard losses in fat and casein. Because time of collection can drastically effect concentration values, comparative data for human samples collected immediately at parturition are also given.

^cBrandtzaeg (1983), Ammann and Steihm (1966), McClelland et al. (1978).

^dHennart et al. (1991) D4 = day lactation 4.

^eMcChee et al. (1975).

^fAll data are for mouse Ig Defresne (1998), except IgA Halsey et al. (1982), de Groot et al. (2000).

^gKraehenbuhl et al. (1979).

^hHeddle and Rowley (1975), Reynolds and Johnson (1970), Ricks et al. (1970), Glickman et al. (1988), Poffenbarger et al. (1991).

ⁱButler et al. (2009), Klobasa et al. (1987), Vaerman (1973); concentration of IgG subclasses not known.

^jButler (1986) and Guidry et al. (1980).

^kKlobasa and Werhahn (1989) and Lascelles et al., 1981.

^lVaerman (1973), McGuire and Crawford (1973), Kohn et al. (1989), Townsend et al. (1983), Sheoran et al. (2000).

^mNot representative since concentration ranges from 6.5 to 14.3.

ⁿCasal et al. (1996).

switch recombination and maturation of the B cell response that: (1) depend on cognitive T–B cell interaction, (2) usually represent secondary immune responses, and (3) is associated with affinity maturation. Simplistically, antibodies of these two isotypes in all mammals transfer the mother's mucosal (IgA) and systemic (IgG) humoral immunological experience, respectively (Figure 1).

All systems evolved for passive transfer of antibodies via the placenta, MG, or blood vascular systems must resolve problems in crossing the endothelial and epithelial barrier. These have succeeded using special Fc γ receptors and the polyIg receptor (pIgR), and by altered epithelial function. These will be discussed in later sections.

The Cellular Elements within the MG and Its Secretions

Historical studies on the passive transfer of immunity initially focused on antibody-mediated immunity. However, after the observation that lymphocytes could be transferred by milk (Beer et al., 1975), attention shifted to the characterization of lymphocytes in milk. Before this time the dairy industry focused on somatic cell counts (SCC) (largely neutrophils)

that were indicators of mastitis and might cause flavor alterations for cheese curd formation and indicate zoonotic disease, e.g., tuberculosis. Thus, SCC above 500,000/mL (in the United States) indicated mastitis and the milk should not be sold.

The phenotypic characteristics and origin of leukocytes in milk and the MG are described in Sections “The MG and Its Secretions” and “The Level and Origin of Lacteal Igs”, their role in defense of the MG in Section “The Immune Response of the MG”, and their role in passive immunity in Section “The Role of Lacteal Secretions in Passive Immunity”.

Innate Immunity, Regulatory Factors, and Biotechnology

Proteomics provide insight into protein synthesis in the MG and transgenic and knockout animals are helping to identify various immune mechanisms while leading to transgenic cattle, swine, and sheep for the production of important human factors and human antibodies in blood and milk (Kuroiwa et al., 2002; Mendicino et al., 2011; Ramsoondar et al., 2011). Thus, the MG of large animals of veterinary importance can be expected to be a part of the future in immunotherapy and immunomodulation in both veterinary and human medicine.

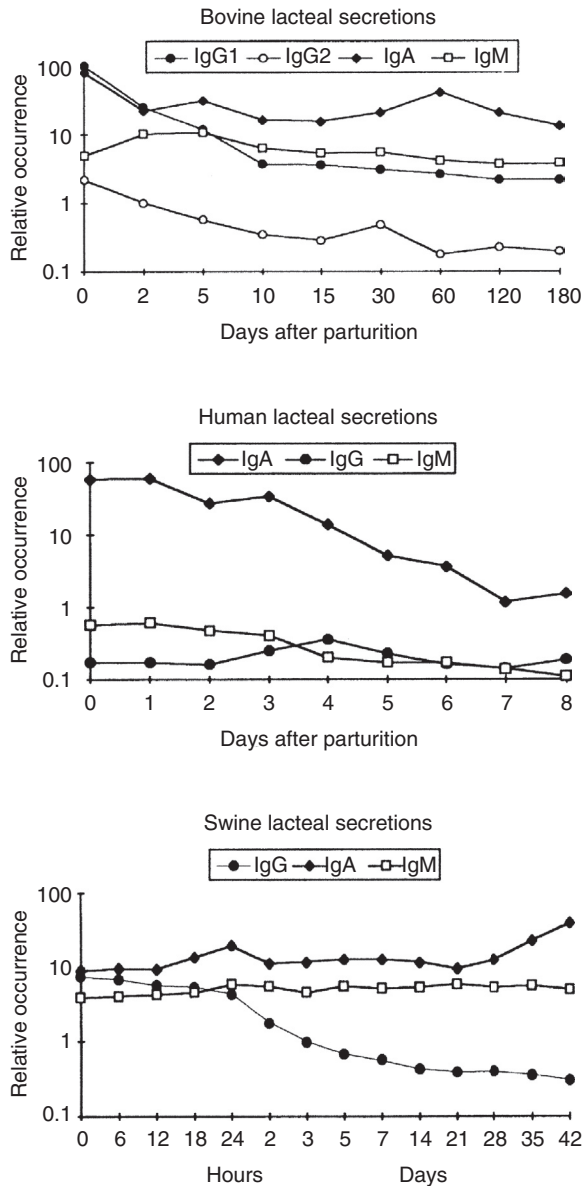


FIGURE 2 The relative occurrence of major immunoglobulins in lacteal secretions of cows, women, and sows during lactation. Immunoglobulin and albumin concentrations in milk and serum are measured at each time point.

$$\text{Relative occurrence} = \frac{\text{Lacteal Ig conc.} \times \text{serum albumin conc.}}{\text{Lacteal albumin conc.} \times \text{serum Ig conc.}}$$

THE MG AND ITS SECRETIONS

Architecture, Mammogenesis, and Cellular Constituency

The architecture of the MG is similar among species. Under the influence of the hormones of pregnancy and lactation and local growth factors, the narrow ducts bud alveoli and parenchyma tissue successively replaces the fat pads of the undeveloped MG. Vascularization occurs mostly in the

fatty tissue, with some capillary vessels around the epithelial cells. Epithelial cells differentiate into a columnar layer separated from the stroma by a thin myoepithelium. Before lactation, the duct lumens remain narrow while the alveoli blossom into clusters of chambers. Mononuclear and small numbers of granulocytes can be found in the stroma along with capillaries and lymphatics. A few weeks before parturition, the lumen of the ducts and alveoli expand, forcing the myoepithelium of adjacent alveoli nearly against each other and resulting in many large adjacent chambers. At this time, secretory products within the alveolar epithelial cells enter the distended lumens and fat appears as droplets surrounded by milk fat globule membranes (MFGMs). The functions of the alveolar epithelial cells may also change during lactation. Tactile stimulation, e.g., suckling, causes contraction of the myoepithelium and thus the release or injection of milk from the alveolar epithelial cells into the lumen. Cessation of suckling causes stasis that may last 2–3 days, after which the gland begins irreversible involution. Involution results in loss of the spherical alveoli and necrosis of the alveolar cells. Expanded cells and cell debris are ejected into the lumen by contraction of the myoepithelium. The dying cells are heavily vacuolated and their nuclei are forced to one side by the vacuoles. These are accompanied by debris- and fat-laden phagocytic cells, sometimes called foamy macrophages. In swine, cell types potentially involved in a local immune response are present in early stages of gestation and lactation, and move closer to the alveolar epithelium as gestation proceeds. These include CD4+ and CD8+ cells, B cells, and other class II-bearing cells, especially T helper cells that accumulate early in pregnancy (Chabaudie et al., 1993; Salmon and Delouis, 1982; Salmon, 1987). The local accumulation of immune cells and the increase in CD8+ intraepithelial lymphocytes (IELs) suggest the potential for a local immune defense (Chabaudie et al., 1993; Salmon, 1987).

In rats, leukocytes are confined to clusters between distended alveoli and between the epithelial cells of acini. At the onset of lactation, there is a two- to threefold increase in these cells (Seelig, 1980), 70% of which are macrophages (Kumar et al., 1991). However, macrophages constitute only 20% of the cells intimately associated with the epithelium. The highest cellularity in rats is seen in late pregnancy, where T cells predominate and CD4/CD8 ratios are about 0.5 (Kumar et al., 1991). The level of immunoglobulin-containing cells (mostly IgA cells) increases toward the end of lactation in mice and rats (Boumahrou et al., 2012; Parmely and Manning, 1983; Tanneau et al., 1999; Weisz-Carrington et al., 1977) to a maximum level at day 18 of lactation, whereas T cells decrease to a third of their numbers at the end of pregnancy (Tanneau et al., 1999). Whereas B cells and monocytes typically reside in the parenchyma, T cells (predominately CD8+ IELs) occur within the glandular alveolar epithelium, in keeping with the membrane expression of $\alpha\text{E}\beta 7$ integrins (Tanneau et al., 1999). These IELs are also CD45R(+) whereas T cells in

periductal clusters and interalveolar spaces are predominantly CD4 cells (Tatarczuch et al., 2000). At variance with most investigators, Parmely and Manning (1983) reported that most IELs were CD4+ whereas others reported at least equal numbers of CD8+ and CD4+ cells (Kumar et al., 1991; Steven et al., 1991).

In cattle, 17–25% of cells in the MG parenchyma have been reported to be B cells or plasma cells that were located mainly in the connective tissues (Nickerson et al., 1984). The highest percentage of B cells was found in the lactating MG (Shafer-Weaver and Sordillo, 1996; Sordillo and Nickerson, 1988) but twice as many T cells were present. The CD4/CD8 ratio in cattle is ~0.5 in the whole MG (Shafer-Weaver and Sordillo, 1996) and 0.7 in MG connective tissue (parenchyma) (Yamaguchi et al., 1999); it may be as low as 0.28 in MG epithelium (Yamaguchi et al., 1999). This is similar to the ratio for IELs in swine (Chabaudie et al., 1993), in rats (Kumar et al., 1991) and ewes (Lee et al., 1989). Unless the MG is infected, neutrophil levels in the MG stroma (parenchyma) or glandular epithelium are low (circa 1%). Macrophages are the major cell type in stromal regions in both cows and sows but plasma cells are also found in stromal areas.

The Biochemistry of Colostrum and Milk

Fractionation of Lactal Secretions

Dairy chemists recognize three major fractions of lacteal secretions: fat, whey, and casein. These terms evolved from the cheese-making trade. However, instead of rennin to coagulate casein, dairy chemists now employ acid (pH to 4.6) to precipitate casein from milk to avoid unwanted proteolysis caused by rennin. In the past 4 decades of experimental research, ultracentrifugation at 149,000×g for 1 h has been used for rapid, simultaneous separation of fat, whey, and casein (Frenyo et al., 1986). The relative contribution of fat, casein, and whey proteins to lacteal secretions depends on (1) the species under investigation (Table 2) and (2) the stage of lactation. Colostrum and especially preparturient secretions contain relatively small amounts of casein and are whey protein concentrates. These can be extremely viscous, and protein concentrations can exceed 200 mg/mL.

Traditional dairy chemical fractionation procedures ignored the nature and diversity of milk cells. As interest developed in the nature of these cells, they have typically been pelleted at 550×g before separation of the major milk fractions. Further fractionation is done using Percoll or Ficoll density gradients or using antibody-coated magnetic beads followed by identification using flow cytometry (FCM).

Non-Immunoglobulin Whey Proteins

Proteomics has allowed investigators to identify hundreds of proteins in whey (D'Amato et al., 2009). Before the emergence of proteomics, Igs, albumin, β-lactoglobulin, α-lactalbumin, lactoferrin, reactive oxygen generators,

cathelicins, defensins, various complement proteins, major histocompatibility class (MHC) components, fragments of β-casein, and >43 enzymes had to be identified immunologically or biochemically. Information on the constituency and nomenclature of bovine milk proteins have been regularly updated (Farrell et al., 2004). Although proteomics provide information on gene expression, information on concentrations and histological localization still depends on classical biochemistry and immunochemistry.

Among the major whey proteins, α-lactalbumin interacts with galactosyltransferase to produce lactose, the signature disaccharide of milk. The role of β-lactoglobulin remains unknown although it may transport small hydrophobic molecules. β-Lactoglobulin is characteristic of ruminant milks (2–4 mg/mL) and swine may have a related protein. There is apparently no β-lactoglobulin in human or rodent milks, and data for other species are insufficient (Jenness, 1974). The relative concentration of the various whey proteins is also highly species-dependent. Lactoferrin levels can be 10-fold higher in human lacteal secretions than in cattle (Goodman and Schanbacher, 1991). Secretory components (SC) are abundant in bovine and human colostrum but nearly absent from swine milk and colostrum (Section “The Level and Origin of Lacteal Igs”). Differences between colostrum and mature milk are common and supported by proteomic data showing that over 30 proteins are significantly up- or down-regulated in abundance in colostrum compared with mature bovine milk (Le et al., 2011). Changes in gene expression indicate a change in MG function, which is correlated with changes in the transport of Igs and their role in passive immunity (Sections “The Level and Origin of Lacteal Igs” and “The Immune Response of the MG”). Some of these are Igs, peptidoglycan recognition proteins, and complement components. Because Igs are a major category of whey proteins, and this chapter concerns mucosal immunology, Section “The Level and Origin of Lacteal Igs” is devoted to these Igs and their origin.

Immunological Significance of Fat and Casein

Milk fat does not exist as free lipids but is encapsulated in MFGM. These membranes are derived in part from internal cells membranes as well as fragments of the plasma membrane of MG epithelial cells (Wooding, 1971; Keenan, 2001). Thus, it is not surprising that MFGM contain or are associated with membrane proteins of the alveolar epithelial cells or proteins secreted across these membranes (Mather, 2000), some of which may be of immunological significance. The MFGM are labile and proteins associated with the MFGM are proteolytically released in the first 30 min after secretion (Pringnitz et al., 1985).

The idea that Igs are found exclusively in milk whey is inconsistent with the evidence that IgM (Euber and Brunner, 1984), IgA (Lavens et al., 1981; Honkanen-Buzalski and Sandholm, 1981) and other Igs are associated with fat globules and that casein depletion concentrates the Igs in the whey fraction (Fleener and Stott, 1981). For example, bovine SIgA

TABLE 2 Composition of Milk from Different Species

| Species | Fat % | Protein % | Lactose % | Ash % | Total Solids % |
|---------------------|-------|-----------|-----------|-------|----------------|
| Antelope | 1.3 | 6.9 | 4.0 | 1.30 | 25.2 |
| Ass (donkey) | 1.2 | 1.7 | 6.9 | 0.45 | 10.2 |
| Bear, polar | 31.0 | 10.2 | 0.5 | 1.2 | 42.9 |
| Bear, black | 24.5 | 5.7 | 0.4 | 1.8 | 44.5 |
| Bison | 1.7 | 4.8 | 5.7 | 0.96 | 13.2 |
| Buffalo, Philippine | 10.4 | 5.9 | 4.3 | 0.8 | 21.5 |
| Camel | 4.9 | 3.7 | 5.1 | 0.7 | 14.4 |
| Cat | 10.9 | 11.1 | 3.4 | – | – |
| Cow | | | | | |
| Ayrshire | 4.1 | 3.6 | 4.7 | 0.7 | 13.1 |
| Brown Swiss | 4.0 | 3.6 | 5.0 | 0.7 | 13.3 |
| Guernsey | 5.0 | 3.8 | 4.9 | 0.7 | 14.4 |
| Holstein | 3.5 | 3.1 | 4.9 | 0.7 | 12.2 |
| Jersey | 5.5 | 3.9 | 4.9 | 0.7 | 12.2 |
| Zebu | 4.9 | 3.9 | 5.1 | 0.8 | 14.7 |
| Deer | 19.7 | 10.4 | 2.6 | 1.4 | 34.1 |
| Dog | 8.3 | 9.5 | 3.7 | 1.20 | 20.7 |
| Dolphin | 14.1 | 10.4 | 5.9 | – | – |
| Elephant | 15.1 | 4.9 | 3.4 | 0.76 | 26.9 |
| Goat | 3.5 | 3.1 | 4.6 | 0.79 | 12.0 |
| Guinea pig | 3.9 | 8.1 | 3.0 | 0.82 | 15.8 |
| Horse | 1.6 | 2.7 | 6.1 | 0.51 | 11.0 |
| Human | 3.8 | 0.6 | 7.0 | 0.2 | 12.4 |
| Kangaroo | 2.1 | 6.2 | Trace | 1.20 | 9.5 |
| Mink | 8.0 | 7.0 | 6.9 | 0.7 | 22.6 |
| Monkey | 3.9 | 2.1 | 5.9 | 2.60 | 14.5 |
| Opossum | 6.1 | 9.2 | 3.2 | 1.60 | 24.5 |
| Pig | 8.2 | 5.8 | 4.8 | 0.63 | 19.9 |
| Rabbit | 12.2 | 10.4 | 1.8 | 2.0 | 26.4 |
| Rat | 14.8 | 11.3 | 2.9 | 1.5 | 31.7 |
| Reindeer | 22.5 | 10.3 | 2.5 | 1.40 | 36.7 |
| Seal, grey | 53.2 | 11.2 | 2.6 | 0.70 | 67.7 |
| Sheep | 5.3 | 5.5 | 4.6 | 0.90 | 16.3 |
| Whale | 34.8 | 13.6 | 1.8 | 1.60 | 5.12 |

Data compiled by [Jenness \(1974\)](#).

and IgM were fivefold more prevalent in milk fat than IgG1 and IgG2 and the concentration of IgM and SIgA was three- and twofold higher, respectively, in fat than whey ([Frenyo et al., 1986](#)). Homogenization of the fat layer followed by

centrifugation resulted in a 30% release of SIgA and IgM, which indicated that these immunoglobulins were associated with the MFGM. Because milk-laden macrophages are well-described ([Outteridge and Lee, 1981](#)) and IgA has been

found in milk macrophages (Pittard et al., 1977), the IgM and SIgA in the fat layer may simply be associated with fat-laden macrophages. Because casein exists in a complicated micellar form (the reason why milk is white), entrapment of other proteins is expected. Frenyo et al. found that 20% of IgM was associated with the casein pellet, even in cell-free skim milk. At least in cattle, it is unlikely that the IgA in fat is associated with SC on MFGM because most SC is free in milk and not associated with fat globules (Pringnitz et al., 1985). The biological significance of the association of IgM and IgA with milk fat or IgM with casein remains unresolved. Therefore, these observations mainly affect antibody and Ig determinations in milk. Measuring Igs or antibodies in whey underestimates their total levels in lacteal secretions.

The MFGM have also been shown to contain antimicrobial properties. Milk fat globule membranes could inhibit *Helicobacter pylori* infection in mice by inhibiting attachment of the bacteria to the gastric mucosa (Wang et al., 2001). A major protein of the MFGM is xanthine oxidase (XO), which is held responsible for the antibacterial activity of the MFGM against several bacterial species including *Escherichia coli* and *Salmonella typhimurium* (Clare et al., 2008). Proteomics show that antimicrobial proteins such as cathelicidins and defensins are also found in the MFGM (T. Reinhardt, pers. comm.).

Species Variations in Lacteal Secretions

Table 2 compares the composition of milk from 28 species in 10 taxonomic orders. Phylogenetic similarities are apparent by comparing cattle with goats and dogs with cats. Differences between species in the composition of their milks are reflected in differences in total solids, fat, and lactose content. The milks of aquatic mammals (seal and whale) are highly concentrated, whereas those of carnivores, rabbits, some rodents, some marsupials, and artiodactyls such as antelope, deer, and reindeer are intermediate and those of primates, domesticated ruminants, and horses are nearly fivefold less concentrated than those of aquatic mammals. The high fat content of aquatic mammals and certain carnivores is particularly noteworthy whereas horses, bison, and antelope are at the low end. The ratio of casein to whey protein is also highly species-dependent; it is ~5 for ruminants and mice, <1.0 for humans and rabbits, and nearly equal for swine and horse. Whereas the lactose level is high in human milk, it is low in bears, aquatic mammals, and rabbits. Some differences follow along taxonomic lines but others do not and suggest special environmental adaptations.

Leukocytes in Lacteal Secretions

Leukocyte Differentials in Lacteal Secretions

The consistency of leukocytes within the MG (Section “Architecture, Mammogenesis, and Cellular Constituency”) does not necessarily reflect their consistency in lacteal

secretions or blood. Studies on milk leukocytes in humans and rodents are usually limited to lactation, whereas these cells in cows, ewes, and even swine can be studied throughout the reproductive cycle. In cattle, the SCC of noninfected glands is typically between 2×10^6 and 5×10^6 /mL during the nonlactating period but can be much higher during infection (Jensen and Eberhart, 1981; Sordillo et al., 1987). In cattle, SCC (mostly macrophages) increase at drying off to a peak of $>10^7$ 3 weeks before parturition. A precipitous, dilution-dependent decline in SCC begins as the gland becomes active in secretion. This further declines during the transition from colostrum to mature milk. In humans, levels are 2 logs lower in mature milk than in colostrum (Ho et al., 1979, Table 3) whereas blood leukocyte levels remain relatively constant (Ogra and Ogra, 1978). Lymphocyte immigration to the MG may change (Tanneau et al., 1999) but blood neutrophils do not because their level in blood is highest at this time (Guidry et al., 1976). Stress neutrophilia associated with the periparturient surge in cortisol is attributed to a transient reduction in CD62L expression on circulating neutrophils (Lee and Kehrl, 1998). Changes in SCCs also fluctuate daily (Ogra et al., 1977) and diurnally (Cullen, 1967), and especially during inflammation of the gland (Section “The Immune Response of the MG”).

In most studies in rodents, humans, and cattle, 40–50% of the SCC recovered in colostrum from healthy glands are monocyte/macrophages (Table 3; Figure 3). As many as two thirds of the mononuclear cells in human colostrum and in secretions from the dry bovine MG are monocytes (Concha et al., 1996; Hurley et al., 1990; Saito et al., 1991; Wilson et al., 1986). Exceptionally high neutrophil levels have also been reported in swine colostrum, although their concentration decreases during lactation to equal those seen in mature milks of other species (Table 3). A decline in the proportion of neutrophils as lactation progresses is also seen in humans (Ho and Lawton, 1978). Discrepancies in leukocyte differentials among SCC can be an identification problem. Ficoll and Percoll gradients used for blood do not perform well with milk cells and most early studies were done without FCM. Studies based on rosetting techniques, anti-immunoglobulin reagents, and lectin affinity may have overestimated B cell levels and underestimated T cell levels (Concha et al., 1978; Diaz-Jouanen and Williams, 1974; Schore et al., 1981). Many milk cells are associated with Igs (Berning et al., 1991; Crago et al., 1979; Pittard et al., 1977), including T cells (Duhamel et al., 1987), thus rendering unreliable the use of anti-Igs reagents for B cell identification. Most likely, much of the surface Ig expression on milk cells reflects Igs bound to Fc receptors, which decorate neutrophils and monocytes in all species. However, those binding IgG2 on sheep neutrophils appear to be necessary for the phagocytosis of *Staphylococcus aureus*, a causative agent of mastitis (Watson, 1976). Even using FCM, cell types are not resolved as discrete populations

TABLE 3 The Distribution of Leukocytes in Lacteal Secretions^a

| Parameter Measured | Species | | | |
|---|----------------------------------|------------------------|----------------------------------|------------------|
| | Human ^b | Cattle ^c | Swine ^d | Rat ^e |
| Total cells/mL | | | | |
| Colostrum | 10 ⁶ –10 ⁷ | 5 × 10 ⁵ | 10 ⁶ –10 ⁷ | |
| Mature milk | 10 ⁴ –10 ⁵ | <4.5 × 10 ⁵ | 1–5 × 10 ⁶ | |
| Monocyte/macrophage (%) | | | | |
| Colostrum | 47–66 | 21–46 | 1–11 | |
| Mature milk | 44 | 25–45 | 2–22 | |
| Neutrophils (%) | | | | |
| Colostrum | 21–60 | 37–40 | 45–92 | |
| Mature milk | 16 | 5–40 | 14–33 | |
| Lymphocytes (%) | | | | |
| Colostrum | 5–11 | 17–23 | 10–26 | |
| Mature milk | 2 | <20; 6–50 | 0.5–13 | |
| Lymphocyte subsets (% of total lymphocytes) | | | | |
| B cells (total) | 7–35 | 3.5 | 10–30 | 55 |
| T cells (total) | 50–88 | 88 | 70–90 | 45 |
| CD4(+) | 43 | 21 | 25–33 | 35 |
| CD8(+) | 48 | 51 | 45–57 | 34 |
| NK | 9 | 15 | NR | 21 |
| Epithelial cells (%) | | | | |
| Colostrum | <1 | <1 | 0.4–20 | NR |
| Mature milk | 10–90 | <25 | 30–99 | NR |

NR = not reported.

^aData for four species are mean values from the publications identified with corresponding footnotes b–e.^bDiaz-Jouanen & Williams (1974), Ho et al. (1979), Ogra and Ogra (1978), Wirt et al. (1992).^cDuhamel et al. (1987), Taylor et al. (1994, 1997), Paape et al. (1981b).^dLeJan (1994), Magnusson et al. (1991).^eHuang et al. (1992).

because of forward scatter drift that results from degradation of neutrophil and side scatter drift resulting from fat ingestion by macrophages. For example, phagocytic cells of milk and colostrum are typically vacuolated, having engulfed fat globules, casein, and SC debris (Outteridge and Lee, 1981; Paape et al., 1981a; Tatarczuch et al., 2000), which could also explain why their in vitro phagocytic activity and level of activation are generally lower than those of blood phagocytes taken at the same time from the same animal (Cai et al., 1994; Evans et al., 1982; France et al., 1980; Gilbert et al., 1993; Nonnecke and Kehrl, 1985; Riollet et al., 2001). Specifically, fat globules, casein, and colostrum are known to inhibit phagocytosis

(Paape et al., 1981a). Neutrophils that have most recently entered from blood show the highest activity (Berning et al., 1991, Section IV).

The SCC does not consist exclusively of leukocytes. Epithelial cells are known to be shed into the gland lumen, especially during involution (Section “Architecture, Mammatogenesis, and Cellular Constituency”) and fat-laden macrophages called colostrum corpuscles or foamy macrophages are present (Outteridge and Lee, 1981). Epithelial cells may comprise 30–90% of the SCC in mature milk from healthy glands (Evans et al., 1982; Leitner et al., 2000; Le Jan, 1994; Magnusson et al., 1991, Table 3). Epithelial cells can be identified by the presence of both adventitious

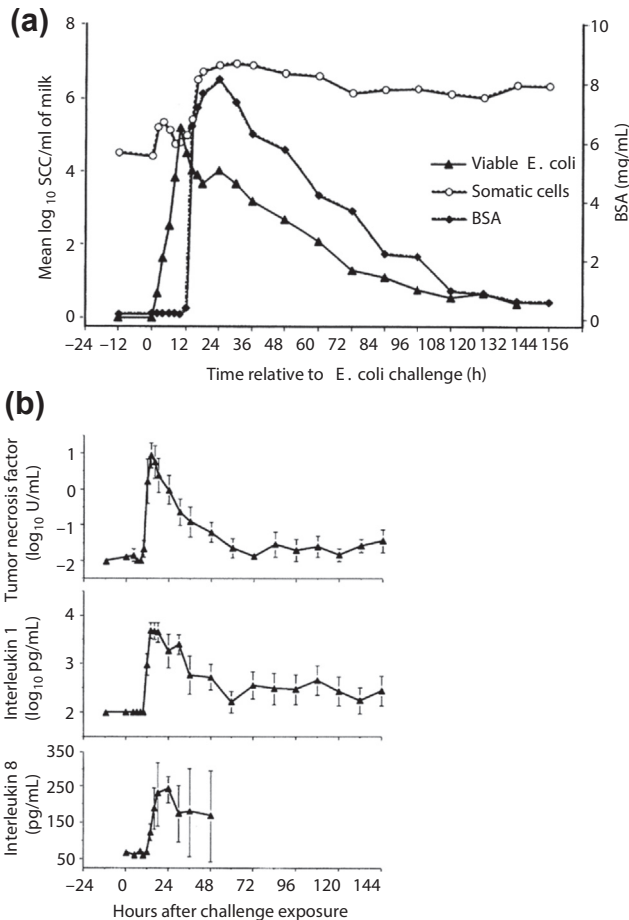


FIGURE 3 Changes in the bovine mammary gland during inflammation. (a) Changes in total leukocytes levels (SSC) and serum albumin in response to the infusion of the mammary gland with *E. coli*. (b) Changes in milk cytokines levels after infusion of bacteria.

immunoglobulin and SC on their membranes (Le Jan, 1994) and some have reported they express CD11a/CD18 (Leitner et al., 2000). The release of epithelial cells into milk in late lactation may reflect an apoptotic event triggered by CD8(+) cells (Tatarczuch et al., 2000). Identification of such cells as either epithelial or foamy macrophages has been historically controversial. In the case of sheep and cattle, this issue appears to have been resolved in favor of the macrophage (Outteridge and Lee, 1981; Paape et al., 1981a).

Lymphocyte in the MG and Lacteal Secretions Versus in Blood

The lymphocytes in milk are not representative of peripheral blood leukocytes (PBLs) because there is a higher frequency of T cells (Duhamel et al., 1987; Le Jan, 1994; Taylor et al., 1994; Wei et al., 1986; Wirt et al., 1992, Table 2) and a lower CD4/CD8 ratio among α/β T cells (Asai et al., 1998; Eglinton et al., 1994; Gibson et al., 1991; Hyung et al., 1992; Le Jan, 1994; Wirt et al., 1992, Section

II A3–5). Nevertheless, cells from blood enter the MG, can transit the alveolar epithelium, and enter the milk (Kumar et al., 1985). The ratio of γ/δ to α/β T cells is twice that in blood (Bertotto et al., 1991; Gibson et al., 1991). The higher proportion of γ/δ cells is not surprising because up to 50% of the circulating T cells in young ruminants and swine bear γ/δ T cell receptors and their greater representation in mucosal and secretory tissue is common to all mammals studied so far (Machugh et al., 1997). Actual reported numbers may vary because some γ/δ -specific mAb recognize only the noncovalent form (Bertotto et al., 1991). Milk lymphocytes contain a higher proportion of natural killer (NK) cells (Duhamel et al., 1987; Hyung et al., 1992; Na et al., 1992).

There is a fivefold higher level of CD45R(+) and CD8(+) cells in bovine milk compared with blood (Taylor et al., 1994; Asai et al., 1998), which suggests that they may represent resident IELs or IELs derived from the gut. Gibson et al. (1991) suggested that because of the two- to fourfold higher frequency of γ/δ cells, the lower CD4/CD8 ratio, and the 12-fold higher expression of HML-1 on human milk T cells, these cells have a mucosal phenotype similar to intestinal IELs. Human colostrum T cells preferentially express noncovalent γ/δ chains, a characteristic of many intestinal IELs (Bertotto et al., 1991), and thus display a memory cell phenotype (CD45R, LFA-1, and UCHL1) and secrete high levels of interferon (IFN)- γ (Eglinton et al., 1994; Wirt et al., 1992). The higher expression of CD45RO on both milk CD4 and CD8 cells also resembles that of T cells in the gut and buccal mucosa (Wirt et al., 1992).

Some differences between blood and milk lymphocytes could be microenvironmental. Collins and Oldham (1986) reported that the poor mitogenic response of colostrum mononuclear cells could be improved by removal of macrophages, and although colostrum appeared suppressive, mature milk was not. Others have also reported that milk lymphocytes were less responsive to mitogens (Harp and Nonnecke, 1986; Kohl et al., 1980; Nonnecke and Kehrl, 1985; Torre et al., 1992) and that colostrum can inhibit the mitogenic response of PBLs (Diaz-Jouanen and Williams, 1974; Kohl et al., 1980; Ogra and Ogra, 1978). However, Park et al. (1993) suggested that the poor response of CD4(+) cells in bovine milk resulted from suppression by a CD8(+) population bearing an activation phenotype (ACT2; Asai et al., 1998). The suppression of neutrophil activity in milk is discussed in Section “The Immune Response of the MG”.

Trafficking of Lymphocytes to the MG

Trafficking appeared to be controlled by the expression of particular structures on the surface of endothelial cells (vascular addressin) and complementary structures on

the membranes of lymphocytes (homing receptors). The endothelial venules within the lamina propria of the gut and of the capillaries around MG acina both express a common mucosal addressin cell adhesion molecule (MAdCAM-1) (Streeter et al., 1988). In mice, the expression of MAdCAM-1 on MG blood vessels increases during pregnancy and is correlated with the increase in the number of T cells expressing the $\alpha 4\beta 7$ integrin (the corresponding homing receptor for MAdCAM-1) (Tanneau et al., 1999). Thus, $\alpha E\beta 7$ T lymphocytes (mostly CD8 T cells) are localized as IELs (Tanneau et al., 1999). These accumulate in the MG during pregnancy in relationship to endothelial cells expressing MAdCAM-1. Because CCL25 was the only epithelial chemokine found to be increased during pregnancy (Bourges et al., 2008), CCL25 may be responsible for T cell recruitment in the MG during pregnancy, as was demonstrated for T cells in the gut. Swine, human, and rat milk T cells carry a phenotype resembling that of intestinal IELs, so their gut origin has been suggested (Asai et al., 1998; Gibson et al., 1991; Le Jan, 1994; Parmely and Manning, 1983; Taylor et al., 1994). Based on their memory or mucosal phenotype, they do not appear to be generated in a secondary lymphoid organ such as Peyer patches or tonsil. In cattle and sheep, a gut origin for MG lymphocytes seems unlikely because cells from the mammary lymph node home back to the mammary lymph node whereas those from the ileal mesenteric lymph node do not (Harp and Moon, 1987, 1988). Furthermore, the L-selectin phenotype of bovine milk lymphocytes resembles that of peripheral lymphocytes, not intestinal lymphocytes (Bosworth et al., 1993). Lymphocyte trafficking may change during inflammation. For example, 14 days after *S. aureus* antigen infusion in sheep MG, there was an accumulation of T and B cells originating from draining lymph nodes. The pattern of migration of these two major lymphocyte populations was clearly different, indicating differential regulation of adhesion molecules on B and T cells and/or their ligands on the endothelium during acute inflammatory reactions (Meeusen et al., 1991).

Regulatory Factors in the Mammary Gland

Cytokines and Chemokines

The vast spectrum of proteins and more than 43 different enzymes found in lacteal secretions include immunomodulatory factors such as cytokines, chemokines, and growth factors. The list increases on a regular basis as these factors are identified by proteomics, cloned by reverse transcriptase–polymerase chain reaction from an ever-increasing number of mammals, identified by intracellular FCM and quantified by sandwich enzyme-linked immunosorbent assay. Table 4 summarizes the occurrence of these

substances in three mammals. More information on cytokines in human milk is provided in the review by Garofalo and Goldman (1998). The immunomodulatory agents fall into two groups: those reflecting the inflammatory response of the gland and those that are the products of a healthy gland and therefore may be a part of passive immunity from mother to offspring. The latter group will be discussed in Section “The Role of Lacteal Secretions in Passive Immunity”, and the former in Section “The Immune Response of the MG”.

Innate Antimicrobial Properties of Milk

The antimicrobial properties of milk are well known (Wheeler et al., 2007; Stelwagen et al., 2008) and comprise the innate immune system, a system without memory. Some elements have a direct antimicrobial function; others can modulate immune responses or can sequester needed nutrients away from microbes. One of the first discovered was lysozyme (Salton, 1957). Lysozyme is thought to have evolved through gene duplication into α -lactalbumin, a constituent of the lactose synthetase complex in the MG. Lysozyme limits the growth of microbes that can hydrolyze lactose. This inhibition is not limited to bacteria and lactose intolerance is a common human trait. Lactoferrin is abundant in milk and sequesters iron away from bacteria; it can also destabilize the bacterial membranes (Legrand et al., 2005) and can complex with Igs (Butler, 1973). Xanthine oxidase and lactoperoxidase generate hydrogen peroxide and nitric oxide that can (1) inhibit adherence of pathogens, (2) enhance phagocytosis and killing, and (3) modulate cytokine production during infection (Vorbach et al., 2006). Conglutinin, a bovine collectin, has been shown to bind bacteria and can activate complement (Lachmann, 1962). Interestingly, serum levels of conglutinin decline at parturition and this coincides with increased susceptibility to infections such as mastitis (Kehrli et al., 1990). C3 (a component of the complement pathway) has been found in milk whey (Mueller et al., 1982), on MFGM (Reinhardt and Lippolis, 2008), and in milk exosomes (Reinhardt et al., 2011). Milk contains cathelicidins and defensins that have a direct antimicrobial activity against a range of different microbes. These can modulate a wide range of functions that affect infections and inflammation (Brown and Hancock, 2006). These factors also aid the nursing neonate by clearing microbes during a time when its immune system is not fully functional. These factors and many others work in tandem with macrophages, neutrophils, and MG epithelial cells (MEC) to form the first line of immune defense of the MG. Section “The Immune Response of the MG” describes the innate immune defense of the MG and how its stimulation can lead to adaptive immunity.

TABLE 4 The Occurrence of Cytokines, Chemokines and Growth Factors in Lactal Secretions

| Cytokine | Human | | | Bovine | | | Porcine | | |
|---------------------|-------|-----------|-------|--------|-----------|---------|-----------|-----------|-------|
| | Whey | Leukocyte | ME | Whey | Leukocyte | ME | Whey | Leukocyte | ME |
| IL-1 β | + | + | – | + | \pm^a | | | | |
| IL-2 | | | | | $+^b$ | | | | |
| IL-4 | | | | | $+^b$ | | c | | |
| IL-6 | + | + | + | | \pm | | c | | |
| IL-8 (CXCL8) | $+^d$ | $+^d$ | $+^d$ | + | | | + | | + |
| CXCL2 | $+^d$ | $+^d$ | $+^d$ | | | | | | |
| CXCL3 | $+^d$ | $+^d$ | $+^d$ | | | | | | |
| CXCL5 | $+^d$ | $+^d$ | $+^d$ | | | | | | |
| CXCL9 | $+^d$ | $+^d$ | $+^d$ | | | | | | |
| IL-10 | + | + | \pm | | \pm | | c | | |
| IL-12 | | | | | \pm | | c | | |
| IL-18 | + | | | | | | | | |
| INF- α | – | – | – | + | \pm | | | | |
| INF- γ | + | + | – | + | \pm | | c | | |
| TGF- β 1 | + | | | $+^e$ | | | $+^{c,f}$ | | |
| TGF- β 2 | | | | $+^e$ | | | | | |
| GM-CSF | + | + | – | | \pm | | | | |
| M-CSF | + | – | + | | | | | | |
| G-CSF | + | – | – | | | | | | |
| IGF | | | | + | | | + | | |
| EGF | + | | | + | | | $+^g$ | | |
| TNF- α | + | + | \pm | + | \pm | | c | | |
| RANTES | + | – | – | | | | | | |
| MCP-1 | + | \pm | – | | | | | | |
| CCL28 ^h | | | | h | | \pm^i | + | | $+^h$ |
| CCL25 | | | | h | | \pm^i | | | $+^h$ |
| CXCL14 ⁱ | | | | | | | | | $+^j$ |
| CCL21 ^j | | | | | | | | | $+^j$ |
| CCL19 ^j | | | | | | | | | $+^j$ |
| CXCL12 ^j | | | | | | | | | $+^j$ |

ME=Mammary gland epithelium.

^a \pm indicates that a transcript has been identified but the actual protein has not yet been reported.^bOnly in the involuted MG when T cell ratio resembles those in blood (Asai et al., 1998). Data are as cited in text or from Garofalo and Goldman (1998), Hagiwara et al. (2000), Riollot et al. (2001), Alluwarma and Culla (2002), Bianco et al. (2001).^cNguyen et al. (2007).^dMaheshwari et al. (2003).^eJin et al. (1991).^fXu et al. (1999).^gJaeger et al. (1987).^hBerri et al. (2008) and Meurens et al. (2006).ⁱDistelhorst et al. (2010).^jBourges et al. (2008) and Meurens et al. (2006).

THE LEVEL AND ORIGIN OF LACTEAL IGS

Relative Igs Levels Reflect the Mode of Passive Immunity

Table 1 summarizes data on the concentration of Igs of different isotypes in colostrum and mature milk of various mammals. The contrast between group I and group III mammals is noteworthy (Figure 1). In the latter group, the IgG/IgA ratio ranges from 5 to 40, whereas in humans it is ~0.01; group II mammals display intermediate ratios that can approach unity. These differences are correlated with the different pathways used to transfer IgG to their offspring (Figure 1). Group I mammals actively transport IgG to the fetus via the placenta to the extent that fetal IgG levels equal or exceed those of their mother. Although group III mammals have several different types of placentation, transport of IgG to the fetus in utero has not been shown. However, fetuses have trace amounts of de novo synthesized Igs. Fetal pig sera contains more IgG than other Ig (Butler et al., 2001), of which IgG3 is dominantly transcribed (Butler and Wertz, 2006; Kloep et al., 2012). Because the IgG level is only 1/1000 of that seen in maternal serum or newborn piglets after suckling, it is too low to protect the newborn. Hence, colostrum-deprived conventional group III offspring succumb to infection. Through suckling, group III neonates normally achieve higher serum IgG levels than their mothers in 12–24 h. A diverse group of mammals comprise group II but share the common feature of transmitting IgG both in utero and via colostrum (Figure 1), although the latter is the major pathway (Appleby and Catty, 1983; Heddle and Rowley, 1975; Poffenbarger et al., 1991; Suffin et al., 1979a; Yamada et al., 1991). Absorption of Igs by the gut of group II mammals shows considerable diversity (Section “The Role of Lacteal Secretions in Passive Immunity”).

Changes in absolute and relative Ig concentration during the transition from colostrum to milk is also related to the mode of passive immunity, which indicates a change in MG function. Among group III mammals, this decrease in Ig concentration is associated with a significant change in IgG/IgA ratios. In swine it changes from 6 to 0.3, in horses from 9 to 0.5, but in cattle only from 5 to 3. Although IgA levels in human and rabbit lacteal secretions (group I) also drop precipitously, there is only a slight change in IgG/IgA ratios. The precipitous decline in IgG in the lacteal secretions of farm animals immediately after parturition is paralleled by a rebound in serum IgG levels that again steadily decline during the last month of gestation (Guidry et al., 1980; Figure 4). This decline in serum IgG before parturition results from the well-known active transport of IgG (IgG1 in cattle) from blood into the colostrum-forming MG at the end of gestation. Serum level of bovine IgG2 do not change during the reproductive cycle and relative occurrence in lacteal secretions remains low (Figure 2), consistent with the view that active IgG transport in cattle is restricted to IgG1. Whereas IgA constitutes 80–95% of Ig in the mature milk

of humans, rabbits, and swine, its high relative occurrence in mature milk of all three species (Figure 2) indicates that it is available for defense of the MG and to provide long-term passive antibodies to the suckling neonate.

Information on relative Ig levels in group II mammals throughout lactation is limited. However, modest changes in IgG/IgA ratios are seen in rodents, whereas carnivores show changes similar to those in swine (Table 1: Heddle and Rowley, 1975). In rats, in which enterocytes actively transport IgG for 3 weeks, IgG2a levels exceed IgA levels 1 week after parturition and a ratio of 1:5 is maintained during suckling (McGhee et al., 1975). The pattern seen in eutherian mammals has also been observed in the brush tail possum (Adamski and Demmer, 2000).

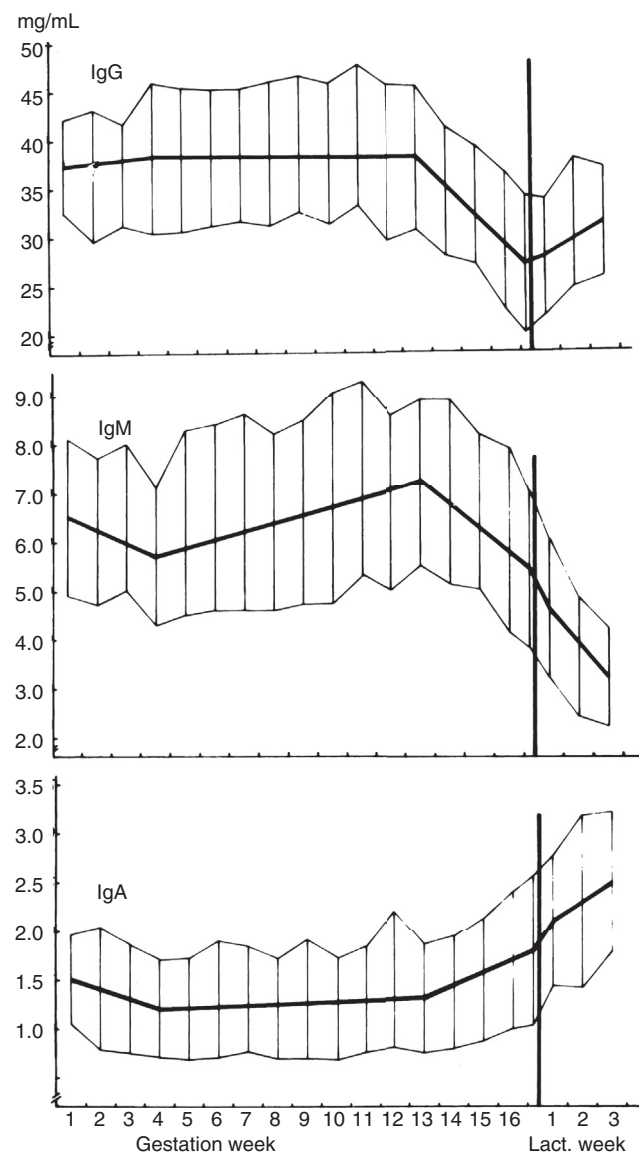


FIGURE 4 The concentration of IgG, IgM, and IgA in serum of more than 1000 sows throughout the reproductive cycle. Mean values are depicted by heavy solid lines and variations represent standard deviations. From Klobasa et al. (1985).

In addition to changes in concentration and isotype ratios, secretion rates also change during lactation indicating that MG function changes during lactation. In humans, ~100 mL/day is secreted on day 1, 500 mL/day on day 5, and >1 L/day after 4 weeks (McClelland et al., 1978). Also, day 5 milk contains 40–50% casein, whereas in colostrum casein accounts for ~10% of total proteins in humans. This pattern is seen in all mammals studied to date and defines colostrum as an Ig concentrate. Changes in secretion rates translate into differences in output. The daily output after 4 weeks in women is about <1 g IgA, in swine is >30 g IgA, and in cattle is ~3 g IgA and 6 g IgG1 (Beyer et al., 1986; Guidry et al., 1980; Lawrence, 1994; Sinclair et al., 1996).

Predicting the Origin of Igs in Lactal Secretions

Lactal Igs in exocrine secretions can arise as serum transudates, the result of active transport from serum, or the result of local synthesis. Serum transudation of Ig can be distinguished from active transport by normalizing the concentration of secreted Igs to those of albumin, which enters secretions by inflammation-induced transudation (Figure 3(a)). Using this yardstick, relative occurrence values >1.0 indicate that a particular Ig is either locally synthesized or actively transported from serum to the secretion in question (Figure 2). Indices of < 1.0 indicate that the Ig is derived by transudation from serum, which can result from loss of security at tight junctions owing to inflammation or physiological distention of the colostrum-forming MG (Figure 3(a)). This measurement predicts that colostrum IgA in cattle, humans, and swine most likely arises from active transport from blood or from local synthesis, whereas IgM and IgG in human milk and IgM and IgG2 in cattle can be largely explained by transudation (Figure 2).

Ig Synthesis in the Mammary Gland

Antibody-Containing Cells in the MG

In humans, 70–85% of all antibody-containing cells (ACCs) within the gland contain IgA; this is similar to the pattern seen in the parotid and lacrimal glands and in the colon (Brandtzaeg, 1983). Although a few IgG and IgM cells are found in the human MG (Brandtzaeg, 1983), relative occurrence values (Figure 2) and organ culture studies (Hochwald et al., 1961) suggest that IgA is the only lactal immunoglobulin synthesized in more than trace amounts in the human MG. Cell frequencies (Weisz-Carrington et al., 1977) and organ culture studies (Lawton et al., 1970) indicate this is true for mice and rabbits but not true for cattle (Butler et al., 1972). Local synthesis may also explain the increase in the relative occurrence of IgA in mature swine milk (Figure 2). Because IgA synthesized in the MG and in gut is believed

to enter the serum pool during lactation (Fisher et al., 1979), this may explain the high absolute postpartum serum IgA levels in sows (Klobasa et al., 1985; Figure 4).

The virgin MG of mice contains few plasma cells, but their numbers rapidly increase during the first week of lactation and most produce IgA (Tanneau et al., 1999; Weisz-Carrington et al., 1977). The resting MG of sows contains few lymphocytes, but helper T cells accumulate rapidly during gestation and are followed by an influx of IgA ACCs (Chabaudie et al., 1993). Immunoglobulin A cells predominate throughout gestation and lactation in rats, although IgG and IgM cells are also present (Lee et al., 1978). The increase in the number of IgA ACCs in rat, mouse, and sow MG during lactation (Bourges et al., 2008; Chabaudie et al., 1993; Parmely and Manning, 1983; Tanneau et al., 1999; Weisz-Carrington et al., 1977) is consistent with the increase in relative occurrence of lactal IgA as lactation progresses, especially in swine (Figure 2). Immunoglobulin A-containing cells also predominate in the lactating rabbit mammary gland (Hurlimann and Lichaa, 1976) and may preferentially express certain IgA subclasses (Spieker-Polet et al., 1993).

Organ culture studies reveal modest immunoglobulin synthesis in the lactating bovine MG and IgG1 synthesis equals or exceeds IgA synthesis; by comparison, IgA synthesis is dominant in the bovine intestinal tract and salivary and lacrimal glands (Butler et al., 1972). Leary et al. (1982) found few IgG-containing cells whereas Sordillo and Nickerson (1988) reported them to be numerous and showed that they increased during lactation. Consistent with organ cultures (Butler et al., 1972) IgG1 ACC were more frequent than IgA ACC. Enumeration of ACCs in human (Brandtzaeg, 1983), mouse (Weisz-Carrington et al., 1977), and pig (Bourges et al., 2008; Brown et al., 1975; Chabaudie et al., 1993) showed a predominance of IgA synthesis, so the MG of domesticated ruminants is an exception. Thus, the immune response of the ruminant MG may be of a peripheral nature in contrast to the MG of monogastric mammals and evolved the well-described serum to MG transport of IgG1. However, serum transport of Igs does not apply carte blanche to the transport of IgA or IgM (Section “Trans epithelial Transport of Igs into Colostrum and Milk”).

B-cell Trafficking and the Gut–MG Axis

General lymphocyte trafficking was discussed in Section “The MG and Its Secretions”. Here we focus on the accumulation of B cells in the MG especially of monogastrics, which correlates with the increase in plasma cells synthesizing IgA and the development and proliferation of glandular epithelium local Ig synthesis (Weisz-Carrington et al., 1977). During the first week of lactation, there is a marked increase in the accumulation of B cells in the mouse MG. The suggestion by Bohl and Saif (1975), that milk IgA in

sows originated from IgA ACC that were derived from cells stimulated in the GI tract has been confirmed by experiments in mice (Roux et al., 1977) and swine (Salmon, 1987) showing that plasmablasts from the gut home to the lactating MG. Homing receptors and chemotactic factors in the MG may be responsible for their selective accumulation (Bourges et al., 2008; Czinn and Lamm, 1986; Salmon, 1999). The predominance of IgA antibodies in human milk to oral and intestinal microorganisms suggests that a similar axis occurs in primates (Ahlstedt et al., 1975; Allardyce et al., 1974; Eggert and Gurner, 1984; Holmgren et al., 1976).

The cellular axis between gut and MG does not appear to be universal among species. Sheldrake and Husband (1985) found little evidence for a well-developed gut-MG axis in sheep; this finding was confirmed by Harp and Moon (1987). Moon and McDonald (1983) failed to find antibodies in milk when cattle were orally vaccinated with *E. coli* K99 and Harp and Moon (1988) found that most ⁵¹Cr-labeled lymphocytes from the intestinal lymph nodes of cattle and sheep returned to the mesenteric-lymph node of intestine, whereas in parallel studies homing of such cells to the lymph node of the MG of sows was observed (Harp and Moon, 1988). However, some cell or antigen traffic must occur because Chang et al. (1981) recovered IgA and IgG1 plaque-forming cells specific to T₄-phage from the bovine MG after intestinal infusion of T₄. Thus, the substantial cellular gut–mammary gland axis seen in rodents, primates, and swine appears to be lacking in ruminants.

A cellular link between the MG and upper respiratory tract (URT) was suggested by the presence of IgA in sow milk when animals were infected late in gestation with the porcine respiratory coronavirus, a deletion mutant of TGEV, that replicates in the respiratory tract (Lanza et al., 1995). Thus, $\alpha 4\beta 1+$ immigrant cells (Bourges et al., 2007) may arrive from the URT in pigs (Bourges et al., 2008) and in mice (Xu et al., 2003) via the vascular cell-adhesion molecule (VCAM-1)/ $\alpha 4\beta 1$ interaction. This adds support to the concept of compartmentalization of mucosal immune system (see Chapter 4). Recently, in a model of intestinal lengthening there was an increase in milk IgA that correlated with both pIgR expression and IgA plasma cell accumulation in the lactating mammary gland (Boumahrou et al., 2012); these findings strengthen the notion of an entero-mammary gland link, whereby the neonatal gut is protected by the maternal gut through the immune function of the mammary gland. Whereas these examples prove the widespread protection among maternal and neonatal mucosal tissues, a variation on this theme is seen in ruminants.

The Mechanism of Recruitment of B Cells to the Lactating MG

In mice and in sows, MadCAM-1 is expressed in endothelial cells of MG during pregnancy and lactation (Bourges

et al., 2008; Tanneau et al., 1999). However, an increase in $\alpha 4\beta 7$ during lactation at a time when MAdCAM-1 levels are beginning to decline in both sows and mice suggests that additional factors might be involved (Czarneski et al., 2002; Tanneau et al., 1999). This was reminiscent of work by Czinn and Lamm, who in 1986 searched for chemoattractants in milk that might explain B cell recruitment of B cells from the MLN to the exclusion of cells from other peripheral LN. Candidates included a peptide derived from bovine β -casein, which is a chemoattractant for pig lymphoblasts, that is as effective as lactoserum (Fronteau et al., 1998). A small peptide of mammary-associated amyloid SAA3 (McDonald et al., 2001) was isolated from a sow milk ultrafiltrate (10kDa) and appears to be a chemoattractant for an entire population of Ig-bearing B lymphoblasts (Rodriguez et al., 2009).

CCL28 is a chemokine expressed by many different types of epithelial cells, including those of the gut and MG (Hieshima et al., 2003; Lazarus et al., 2003; Meurens et al., 2006; Mickanin et al., 2001). It is up-regulated during lactation (Berri et al., 2008) and was speculated to have a major role in the accumulation of IgA plasma cells in the lactating MG. This was demonstrated by blocking plasma cell immigration into MG with anti-CCL28 antibodies (Wilson and Butcher, 2004) or by knocking out the CCR10 receptor (Morteau et al., 2008). These treatments resulted in an absence of MG plasma cells and also an absence of IgA in milk.

In other studies in mice, VCAM-1 is present in the large blood vessels of the mouse MG to the exclusion of small vessels, which represent the sites of lymphocytes extravasation (Tanneau et al., 1999). However, in sows, VCAM-1 is also expressed in endothelial cells of small blood vessels as well as in connective cells of the lactating MG, where it may contribute to IgA-cell retention, particularly for $\alpha 4\beta 1+$ originating from the URT (Bourges et al., 2007). Vascular cell-adhesion molecule was found on the large blood vessels as in lactating mice and IgA plasma cell recruitment could be blocked in mice. However, MAdCAM-1 was not detected in the bovine MG at any of four different physiological stages of MG development, in agreement with the absence of $\beta 7$ cells in MG (Hodgkinson et al., 2007).

Hormonal Regulation of IgA ACC Recruitment into the MG

The density of prolactin receptors in mammary tissue has been shown to be correlated with the accumulation of lymphocytes in this organ (Salmon, 1987). In virgin mice, treatment with a combination of progesterone, estrogen, and prolactin induces MG development and leads to an increase in the number of IgA plasma cells in this organ and intraepithelial IgA (Weisz-Carrington et al., 1977). This increase in IgA plasma cells could be a consequence of mammarygenesis

resulting in entrapment of circulating lymphoblasts. The presence of an estrogen-responsive element in the promoter of MAdCAM-1 gene (Sampaio et al., 1995) is in agreement with the higher expression of MAdCAM-1 in pregnancy than in lactation. Recently, it was shown that supplemental beta-carotene increases IgA-secreting cells in mammary gland and IgA transfer from milk to neonatal mice, probably by an increase in immigration of IgA cells from the ileum to the MG (Nishiyama et al., 2010). Shark-liver oil supplied to sows during gestation and lactation (Mitre et al., 2005) may have a similar effect.

Transepithelial Transport of Igs into Colostrum and Milk

Basic Principles

Serum proteins that gain access to the ductal lumen must navigate the endothelial barrier and the acinar epithelial barrier to be secreted into milk or colostrum. The latter normally results from selective secretion through the acinar epithelial or a breakdown of tight junctions between these cells. The former is also believed to be receptor-mediated or due to non-receptor mediated transudation that occurs in inflammation (Figure 3; Gitlin et al., 1976). Therefore, Igs available at the basal region of the acinar epithelial cells in the healthy MG could arrive there by selective transport from the blood across the capillary endothelial barrier or be secreted from Ig-producing cells in the parenchyma. Therefore, the appearance of Igs in lacteal secretions depends on the efficacy of endothelial and epithelial transport, the Ig concentration gradient, the diffusion coefficient of the Ig, the level of receptor expression, and the degree of inflammation. The ruminant model is perhaps the best example of transporting an IgG subclass protein from blood to lacteal secretions that involves crossing both the capillary endothelium and the acinar epithelium. In the case of dIgA, transport across the acinar epithelium is mediated by the pIgR (Chapter 12 in third edition) but not across the capillary endothelium because no receptor has been described that can perform this function. When there are no specific receptors to control transport, passage relies on transudation. In contrast to rats and mice, absorption of Igs by the gut of newborn ungulates is not receptor mediated, but relies on aggressive pinocytosis by enterocytes (Section “The Role of Lacteal Secretions in Passive Immunity”).

Selective Transport of IgA into Lacteal Secretions

Immunoglobulin A is the major Ig synthesized by plasma cells in the MG in species studied to date, except for ruminants. Mammary gland epithelial cells in all species synthesize the pIgR, the N terminal domains of which are shed into the lacteal and other secretions as free SC (Butler,

1971; Mach et al., 1969; Mostov and Bloebel, 1982; O’Daly and Cebra, 1971; Ricks et al., 1970; van Munster et al., 1971; Chapter 12 of third edition). The expression of pIgR in conventional animals may be influenced by many factors including hormones (Weisz-Carrington et al., 1984) and cytokines such as interleukin (IL-4) and IFN- γ (Kaetzel, 2005). Expression may result from metabolic products such as from E-box elements (Martin et al., 1998). Nearly all IgA recovered in the lacteal secretions is polymeric and is bound to SC to form SIgA. Thus, active transport by pIgR-mediated transcytosis almost certainly accounts for most lacteal IgA. However, selection for transport of dIgA across the capillary endothelium of the MG (which lacks the pIgR) is less convincing. Dimeric IgA (dIgA) in the serum of rats, mice, and rabbits is readily transported into bile (Fisher et al., 1979; Koertge and Butler, 1986a) and evidence that IgA can be transported from serum into the MG has been offered by three studies: Halsey et al. (1980), Sheldrake et al. (1984), and Newby and Bourne (1977). If transport of serum dIgA into milk is universal among mammals, one might expect its depletion from serum during colostrum formation in the same manner as seen for IgG in group III mammals and that IgA levels should rebound after this period of active transport. The opposite is seen in swine (Fig. 4), which raises questions about the three studies described. Examination of these studies revealed there was minimal control for the quality of the recovered ^{125}I product that was reported as IgA. When the study was repeated, Koertge and Butler (1986a) recovered only degraded fragments of dIgA in milk. However, they found extremely efficient transport of intact dIgA into bile in rats and somewhat less efficient biliary transport in mice (Koertge and Butler, 1986b). Although Halsey et al. recovered IgA in milk, they also recovered as much albumin, which, when the relative occurrence formula was applied (Figure 2), suggested that the IgA arrived by transudation from serum. Newby and Bourne injected colostrum SIgA and their results conflicted with the concept that only dIgA can be transported across epithelial membranes (Fisher et al., 1979). Newby and Bourne found that the amount of radioactivity recovered in milk was similar to that reported by Sheldrake et al. (1984). When their experiment was repeated, most of the transported IgA was of low molecular weight (although still trichloroacetic acid precipitable) and 50% was dialyzable, which suggested a molecular weight of <20kD (Butler et al., 1986). When corrected for degradation, only 1.47% and 0.54% of intravenously administered SIgA was recovered intact from milk and bile, respectively, whereas SIgA in serum remained intact throughout the study. Oestensson and Lun (2008), and others showed that in experimental mastitis, serum transudation into the MG was common and it is known that udder distention causes similar effects (Campbell, 1971). The studies of Koertge and Butler (1986a,b) and Butler et al. (1986) were conducted in animals during normal lactation and with no

evidence of MG inflammation. The best data at this point indicate that SIgA in milk comes from IgA-producing cells in the MG and is not derived from serum, except for a minor portion derived by serum transudation.

Because IgM is also able to bind the polyIgR, we wondered whether the IgM found in cattle and swine milk was serum-derived (Frenyo et al., 1987). We found that most IgM recovered in bovine milk and bile was in degraded form and there was also no transport of IgM into saliva. It is believed that the barrier for IgM transport (and dIgA) is not due to their affinity for the pIgR, but rather the restrictions of the capillary endothelial barrier regarding mass transfer and the diffusion coefficients of the molecule in question (Natvig et al., 1997). In the absence of inflammation, vascular basement membranes are relatively impermeable to molecules the size of IgM and dIgA. Whereas IgM and dIgA may be efficiently transported across the acinar epithelium with the aid of the pIgR, a mechanism to explain their transport across a healthy capillary endothelial barrier is lacking. This would suggest that some portion of SIgA and IgM (as SIgM) may be derived from serum during colostrum formation when the gland is distended or under conditions of inflammation.

Control of dIgA Transport across the Epithelial Barrier

Because one pIgR molecule transports only one dIgA molecule and because the polyIgR is not recycled after each round of transport, this implies that the amount of available receptor or ligand (dIgA) could be a rate-limiting factor for transport of dIgA into the milk. The use of overexpressing transgenic mice (60- to 270-fold) showed that SIgA levels could be increased twofold, indicating that the level of the polyIgR can influence the rate of transport (de Groot et al., 2000). In cattle, the concentration of free SC is high (Butler, 1971; Mach et al., 1969; Pringnitz et al., 1985). Thus, the relatively low level of SIgA in bovine milk and colostrum cannot be explained as a deficiency of the pIgR. By contrast, SC is difficult to detect in swine colostrum and not at all in milk, yet swine milk is rich in SIgA (Table 1). Therefore, the IgA deficiency in ruminant milks (Butler et al., 1972; Lascelles et al., 1981) appears not to be a deficiency of the pIgR, but rather is the amount of dIgA available for transport. PolyIg receptor production in humans and rats must be robust because their colostrum has normal IgA levels as well as free SC in excess.

Receptor-Mediated Transport of IgG into the Mammary Gland

The selective accumulation of serum IgG1 into colostrum in ruminants and IgG (subclass unknown) in other group III mammals (Figure 1) suggested the presence of receptor-mediated epithelial transport. In cattle, as much as 500 g

of IgG1 is transported from blood to colostrum in each of the 3 weeks preceding parturition (Brandon et al., 1971); similar amounts are transported in swine and horses. This transport is accompanied by a corresponding depression of serum IgG levels as seen in swine (Figure 4) and by the shorter serum half-life of IgG1 in cattle during the transport period (Sasaki et al., 1976). No such depression of serum IgA levels occur that might support selective transendothelial transport (Figure 4).

The serum to milk transport of IgG1 appears to be mediated by Fc receptors on alveolar epithelial cells (Kemler et al., 1975; Leary et al., 1982) that could be induced by the administration of estrogens and progesterone to nonpregnant heifers (Smith, 1971). However, such a receptor must also be present in the capillary endothelium. Leary et al. noted that the presumed IgG1 transport receptor is down-regulated by prolactin, which simultaneously up-regulates α -lactalbumin (Barrington et al., 1997). This signals the changeover from the high rate of IgG1 transport by alveolar epithelial cells to the prominent role for the MG in the synthesis and secretion of milk proteins, e.g., casein. Sasaki et al. (1977) identified a high-affinity receptor for IgG1 in the colostrum-forming gland and a low-affinity receptor that was present during the remainder of lactation. Transport of serum IgG into the MG in mice, cattle, and sheep appears to be regulated by the neonatal Fc receptor (FcRn), an MHC I-related receptor composed of an α -chain and β 2-microglobulin (β 2m; Simister and Mostov, 1989; Jacobowitz et al., 1995). This was originally identified as the protein that mediates the transport of IgG ingested by suckling rat pups into their circulation through enterocytes of the small intestine (Jones and Waldmann, 1972). Fc receptor has also proven to be a key player in regulating the transport of IgG in other venues while serving to rescue IgG and albumin from degradation, thereby prolonging their serum half-lives (Ward and Ober, 2009). It also has a major role in antigen-IgG immune complex phagocytosis and in antigen presentation by professional APCs (Baker et al., 2011; Liu et al., 2011; Mi et al., 2008; Qiao et al., 2008; Vegh et al., 2012).

In mice, Cianga and colleagues localized the FcRn to the epithelial cells of the mammary gland acini and found that the transport of the IgG subclasses into milk showed an inverse correlation with their affinity to the FcRn, which suggests that the FcRn in the lactating mammary gland has a role in recycling rather than secreting selected IgG subclasses from the milk gland back into the circulation. Thus, the authors suggested that IgG subclasses that bind poorly to FcRn are preferentially secreted into milk (Cianga et al., 1999). Therefore, the differential affinity of IgG subclasses for FcRn could explain selective transport of IgG1 into lacteal secretions of ruminants and other group III mammals. Fc receptor has been identified in the MG of cattle and sheep (Kacsokovics et al., 2000; Mayer et al., 2002) and its localization in the mammary epithelial cells was

determined by immunohistochemistry. Those investigators found a remarkable difference in MG expression patterns of this receptor around parturition. The cytoplasm of the epithelial cells of the acini and ducts stained homogeneously before parturition; however, only the apical side of the cells was positive after parturition. This striking change in distribution before and after parturition suggested that the cellular position of FcRn may have a role in the transport of IgG during colostrum formation in ruminants (Mayer et al., 2002; Mayer et al., 2005). This seems correlated with the low- and high-affinity IgG1 receptor described by Sasaki et al., 1977.

To clarify the role of the bovine FcRn (bFcRn) in colostrum or milk production, transgenic mice overexpressing the bFcRn in their lactating mammary glands were generated. The IgG level was surprisingly increased both in serum and milk in these animals, owing to better serum IgG protection that consequently resulted in a greater amount of IgG to be transported into the milk. Nevertheless, FcRn overexpression did not facilitate IgG secretion into milk; rather, it blocked transport of IgG isotypes into the milk that bind strongly to it (Lu et al., 2007). In agreement with this observation, a recent study indicated that the bFcRn binds better to bovine IgG2 than bovine IgG1 (six- to sevenfold difference in Kd) and thus it was concluded that the role of bFcRn is to recycle IgG2 from the udder to blood instead of secreting it from blood to colostrum/milk, as in mice and humans (Takimori et al., 2011). This may explain the preferential transport of IgG1 by the MG of cattle and sheep and the exclusion of IgG2 (Figure 2; Table 1), as well as the longer serum half-life of IgG2; bovine IgG2 has twice the half-life of IgG1 (Butler, 1983).

These studies still leave partially unresolved whether the robust transport of IgG1 in ruminants and rodent IgG2b is solely the result of their poor affinity for FcRn. This begs the question of whether yet another IgG transport receptor is operative because receptor-less transport has not been described in any eukaryotic system and when it occurs it is called serum transudation. Another question concerns the control of transport, because FcRn expression is constitutive in the bovine MG even when not forming colostrum (Kacskovics et al., 2000). The apparent logistical difficulty might be explained by immunohistochemical evidence suggesting that FcRn is differently localized in the colostrum-forming alveolar epithelial cells but not in the inactive gland, and that different localization mean different function (Mayer et al., 2002, 2005).

The study that analyzed the bovine IgG1 and IgG2 interaction with bFcRn also showed that IgGs secreted into bovine colostrum are more highly sialylated than in milk samples obtained at later time points. This is curious because the interaction between IgG and FcRn was not affected by the structure of the N-glycans attached to IgG (Takimori et al., 2011). Recently, Fc sialylation of IgG

received increased attention, because it was reported that increased sialylation makes IgGs better anti-inflammatory agents (Anthony and Ravetch, 2010; Kaneko et al., 2006). Considering that such a high amount of immunoglobulin goes directly into the circulation of the newborn calves, the most plausible explanation for the high level of sialylated colostrum IgG is its potential function as an anti-inflammatory agent or immune modulator. Of course, these IgGs may influence the immune system of the cow. The dramatic change of the IgG N-glycosylation around parturition in the cow is similar to that seen in human pregnancy (van de Geijn et al., 2009; Einarsdottir et al., 2012).

Fc receptor was also located in the human mammary gland (Cianga et al., 2003); however, its role has not been functionally evaluated because of ethical considerations. In humans, IgG comprises only 1–5% of total Ig in colostrum and milk (Table 1) although the subclass distribution is not the same as in serum. Mehta et al. (1989) showed that whereas the proportion of IgG3 and IgG4 in milk and colostrum was similar to that in blood, IgG1 levels were increased and IgG2 levels decreased. However, these results have not been substantiated by others (Kim et al., 1992; Thom et al., 1994). Differences may be methodological and in any case do not allow conclusions regarding selective subclass transport into the human MG. Nevertheless, that human milk contains a limited amount of IgG suggests that the role of human FcRn must be similar to its mice ortholog, in that the human FcRn functions very efficiently in recycling IgG from milk into serum (Cianga et al., 1999). A more global role of FcRn in passive immunity and immune homeostasis may emerge as comparative studies on FcRn expression and function in, e.g., marsupials (Adamski and Demmer, 2000) and wallabies (Daly et al., 2007) are undertaken.

Besides ruminants, the presence of the FcRn has been detected in the porcine mammary gland 3 days prepartum and on the day of farrowing (Schnulle and Hurley, 2003). Serum transport of IgG to the swine MG is robust and is accompanied by a depression in serum IgG levels (Figure 4). Early studies by Curtis and Bourne (1971) suggested selective subclass transport; Huang et al. (1992) reported that certain allotypes (presumably expressed on certain subclasses) accumulate in the swine MG. Studies on the specificity of subclass transport in this species is complicated by the lack of purified forms of the six different subclasses in this species (Butler et al., 2009; Kloep et al., 2012). Fortunately, these have now been synthesized in vitro (Butler et al., 2012), so this can potentially be resolved.

Initial studies in horses provide no evidence for preferential subclass transport (Rouse and Ingram, 1970), which is supported by the studies of Sheoran et al. (2000). Carnivores (group II) also accumulate IgG in their colostrum at levels more than twofold greater than IgA. Because the IgG/IgA ratio dramatically shifts to favor IgA in mature

milk (Table 1), it suggests less robust transport of serum IgG during the remainder of lactation, reminiscent to that seen in ungulates. Although selective transport of a single subclass (IgG1) in ruminants has been recognized for 4 decades, data on ruminants are incomplete because there are no transport data for IgG3 (Rabbani et al., 1997). Whatever the case, one must be mindful that IgG diversified into subclasses after speciation (Butler et al., 2009), so the same name designation, e.g., IgG1, does not apply to other species except closely related ruminants. The subclass designations for horse IgG in Table 1 reflect the recent work of Wagner et al. (2002). Those for swine are from Butler et al., 2009; neither are intended to imply homology of same-name subclasses among species.

THE IMMUNE RESPONSE OF THE MG

The Innate Response to Infection

The innate immune system of the MG is composed of barriers, signaling molecules, antimicrobial molecules, and cells (Rainard and Riollot, 2006; Sordillo and Streicher, 2002). The first line of defense that is unique to the mammary gland is the teat canal barrier (Rainard and Rollet, 2006). The teat canals acts as a one-way valve allowing milk flow and preventing pathogens from gaining entrance. Hence, hypocalcemia near the teat sphincter can make the animal more susceptible to infection (Goff, 2008).

Sensing the Pathogens

If a pathogen breaches the physical barriers, the immune system is activated by a complex set of warning signals. Recognition of bacteria by host cells relies on a variety of molecular sensors called pattern recognition receptors (PRR) that belong to several families, including the Toll-like (TLR) and the nucleotide-binding oligomerization domain (NOD)-like (NLR) receptors. Toll-like receptors recognize MAMPs (Microbiol Associated Molecular Patterns), which are conserved motifs unique to microbes such as lipopeptides, lipopolysaccharides, double-stranded RNA, and bacterial DNA. Some of the NLR, known as inflammasomes, can trigger inflammation through caspase-1, which in turn matures and releases IL-1 β . The NLR NALP3 binds uric acid, which is a molecule released from injured or necrotic cells (Fritz et al., 2006). Thus, PRR are involved in not only detection of microorganisms, but also detection of any injury.

Sensing of biological aggressors in the MG through their MAMPs by PRR is well documented. Purified lipopolysaccharide (LPS) of *E. coli* or *Salmonella* (TLR4 agonist) has long been used to induce experimental mastitis (Shuster et al., 1996; Figure 3(a)). Other MAMPs, such as lipoteichoic acid from *S. aureus*, synthetic agonists of the heterodimers TLR1/TLR2 (Pam3CSK4) or TL2/TLR6

(Pam2CSK4), and agonists of the NLRs NOD1 (C12-iE-diamino-pimelic acid) and NOD2 (muramyl dipeptide MDP), trigger inflammation when infused into the lumen of the MG (Bougarn et al., 2010; Porcherie et al., 2012; Wellnitz et al., 2011). However, flagellin of *Salmonella* (agonist of TLR5) did not induce MG inflammation (Porcherie et al., 2012).

The sentry cells in healthy MG may be macrophages or mammary epithelial cells (MEC) that comprise the epithelium lining of the lumen of the MG. It has been shown using a mouse mastitis model that MG macrophages, but probably not dendritic cells, are necessary for the response of the MG to LPS, but not to living *E. coli* (Elazar et al., 2010a,b; Gonen et al., 2007). Mammary epithelial cells in culture react to the MAMPs that induce inflammation when infused into the MG (Strandberg et al., 2005; Wellnitz and Kerr, 2004) or to whole mastitis-causing bacteria (Günther et al., 2011; Lahouassa et al., 2007; Yang et al., 2008). Moreover, the expression of several PRR by MEC has been reported at the mRNA level (Bougarn et al., 2010; Goldammer et al., 2004; Petzl et al., 2008; Porcherie et al., 2012). At the protein level, surface expression on MEC has been shown for TLR2 (Petzl et al., 2008) and the MFGM has been shown to express TLRs, which indicates that these receptors are on the apical membrane of MEC (Reinhardt and Lippolis, 2006). An important molecule that acts as a co-receptor for TLR2 and particularly TLR4 in the recognition of LPS is the glycosyl-phosphatidyl-inositol anchored membrane CD14 (Wright et al., 1990). Mammary epithelial cells apparently do not express membrane CD14, but they could be the one source of the high concentration of soluble sCD14 found in human milk (Labeta et al., 2000). In the MG of lactating mice, CD14 is not detected on the alveolar epithelium by immunohistochemistry, but epithelial cells become positive 2 days after MG involution induced by removal of the pups (Stein et al., 2004). Soluble CD14 is also present in bovine milk and could help bovine MEC to respond to LPS (Wang et al., 2002). Another co-receptor of TLRs, the membrane scavenger CD36, has been found in the MFGM (Reinhardt and Lippolis, 2006). Information on the involvement of the inflammasomes in the MG is still lacking.

The Inflammatory Response

The result of TLR or NOD recognition of a pathogen is the activation innate immune cells that secrete cytokines and chemokines (Figure 3(b)). Expression levels, timing of expression, and the milieu of cytokines and chemokines expressed depend on the type of pathogen causing the infection (Bannerman, 2009). Transcriptomic studies have shown that shortly after exposure to MAMPs or bacteria, large numbers of genes are up-regulated in the mammary tissue and MEC, which suggests that MEC can exert sentinel as well as effector functions (Günther et al., 2009). Among these are genes

for beta-defensins, pentraxins, complement components, iNOS, lactoferrin, proteins of the S100 family, and acute phase proteins such as serum amyloid A3 and haptoglobin. Chemokines are also up-regulated, such as those targeting neutrophils (CXCL1, CXCL2, CXCL3, CXCL5/6, CXCL8, or IL-8) and those targeting mononuclear leukocytes (CCL2, CCL5, CCL20, or CXCL10). This is in line with the prompt influx of leukocyte in mammary tissue and milk after bacterial intrusion into the MG lumen (Figure 3(a)).

The most prevalent of the innate immune cells that have a role in MG defense are neutrophils and monocytes/macrophages. During acute mastitis, SCC increase and cell differentials change so that there are ~90% neutrophils and 5% macrophages per 10^7 SCC (Leitner et al., 2000). Experimental induction of neutropenia using anti-bovine leukocyte serum strongly aggravated *E. coli* mastitis and converted subclinical *S. aureus* mastitis into gangrenous mastitis (Jain et al., 1971; Schalm et al., 1976). In a mouse model of *E. coli* mastitis, specific neutrophil depletion before intramammary infection was associated with unrestricted bacterial growth, tissue damage, severe sepsis, and mortality (Elazar et al., 2010a). These data indicate a predominant role for neutrophils in the defense of the MG against bacterial infections.

Neutrophils have various killing mechanisms to destroy pathogens. Phagocytosis of bacteria stimulates neutrophils to produce oxidizing agents in a process referred to as the respiratory burst, in which oxygen radicals serve as precursors to various antimicrobial oxidants. Neutrophils also contain numerous antimicrobial granule proteins such as cathelicidins, hydrolases, proteases, lactoferrin, and lysozyme. These proteins are released into phagosomes to destroy ingested pathogens, or the granule contents are released out of the cell. Compared with neutrophils in blood, those isolated from milk have a decreased ability to kill bacteria in vitro (Paape and Guidry, 1977) and a decreased capacity to generate reactive oxygen species (Mehrhad et al., 2001). Interestingly, when blood neutrophils are incubated in milk in vitro, loss of neutrophil function is quickly manifest. Preincubation with casein results in reduced reactive oxygen species (Cooray, 1996) but not incubation in skim milk, whey, or media (Paape et al., 1975, 1977). Recently, an alternate killing mechanism for neutrophils was described, called neutrophil extracellular traps (NETs) (Brinkman et al., 2004). Unlike other neutrophil antimicrobial mechanisms, NETs are unaffected by incubation in milk and therefore may be an important neutrophil killing mechanism in milk (Lippolis et al., 2006).

Monocyte-derived macrophages and dendritic cells have the ability to kill pathogens and specifically stimulate adaptive immunity. These cells phagocytose pathogens and process antigenic peptides from the pathogens that are presented by MHC to T lymphocyte, which then initiates an adaptive response. Thus, monocytes are a bridge between the innate immune system and the adaptive immune system.

Lymphoid cells are primarily recruited as part of the passive immunity (Sections “The Level and Origin of Lactal Igs”) but also enter the gland during the inflammation. As in other inflammatory settings, they cooperate with other cell types such as neutrophils, monocytes/macrophages, and epithelial cells to modulate the responses to pathogens. Because most entering lymphocytes are of the activated/memory phenotype, they also contribute to the production of cytokines found in mastitic milk, such as tumor necrosis factor (TNF)- α , IFN- γ , IL-10, and transforming growth factor (TGF)- β (Figure 3(b)).

Because IL-6 levels are also elevated in blood and milk of mastitic cattle (Shuster et al., 1993), its role seems primarily related to defense of the gland rather than regulation of the neonatal immune system (Hagiwara et al., 2000). Infection and/or inflammation of the MG would be expected to result in a cytokine profile in colostrum or milk that differs from that of healthy glands. During inflammation, one anticipates an increase in the transcription and secretion of the endogenous pyrogens from macrophages, i.e., IL-6, TNF- α , IL-12, IL-8, and IL-1. The bovine MG responds in the anticipated manner. Figure 3(b) shows the abrupt rise in TNF- α , IL-1, and IL-8 after infusion of the bovine MG with *E. coli*. Rapid elevation of TNF- α , IL-8, and granulocyte macrophage-colony-stimulating factor was also observed in the sheep MG infused with LPS (Waller et al., 1997). This is also associated with elevation of IL-6 not only in the gland but in serum (Hagiwara et al., 2000), and by increased expression of IL-12 (Taylor et al., 1997). This suggests that the neutrophil influx that is the hallmark of mastitis (Figure 3(a)) results from the activation of macrophages/monocytes/DC within the MG. Accepting that the epithelia in contact with potentially colonizing microorganism exist in a mild inflammatory state, subtraction of the cytokine profile of the inflamed gland from that of the normal gland should provide insight into those cytokines/chemokines/growth factors that are important in healthy glands, may function in the neonatal GI tract under healthy gland conditions, and are a feature of the inflammatory response of the MG. Whereas the situation regarding the cytokine profile of the infected MG fits nicely with what is generally known about the inflammatory response, the cytokine profile of the healthy gland offers minimal insight into how the cytokine profile of colostrum and milk in healthy animals functions. As discussed above, TGF- β and IL-6 may be needed for the mucosal response of the neonate. This is discussed in Section “The Role of Lactal Secretions in Passive Immunity”.

The Adaptive Humoral Immune Responses

Adaptive Versus Innate Immune Response

Innate immunity provides the first line of defense in the MG as in all other regions of the body. Stimulation of the innate

immunity is necessary to initiate the events leading to an adaptive immune response, as illustrated by the role of gut colonization and MAMPs in germ-free piglets (Butler et al., 2002, 2005). Thus, the same principle applies to the MG.

The Antibody Response After Intramammary Infusion and Direct Injection

Efforts to stimulate antibodies in milk by some form of local immunization have been a focus of veterinary and agricultural scientists with the aim of protecting the MG (reviewed by Outteridge and Lee, 1988) or the suckling offspring (reviewed by Salmon, 1999). One approach has been infusion of antigens via the teat canal, which is perhaps the route by which the MG naturally encounters environmental antigen. Nashar et al. (1990) compared the response to ovalbumin (OVA) and a particulate antigen (*Streptococcus uberis*). The soluble antigen readily entered the circulation after infusion and probably by this pathway also gained access to adjacent MGs, i.e., the noninfused quarters in the case of cattle. Soluble OVA stimulated an influx of neutrophils and OVA was also taken up by acinar epithelial cells similar to the uptake of soluble antigen by enterocytes (Campbell et al., 1999). Because the infused OVA reaches the supramammary lymph node in 1 h, infusion of a soluble antigen appears nearly equivalent to parenteral immunization. This stimulated serum IgG1 and IgG2 responses, with little evidence for a response within the MG itself (Nashar et al., 1991). In similar fashion, infusion of ewes with soluble antigen results in significant antibody synthesis by cells in the supramammary lymph node (Watson, 1984). Thus, intramammary infusion with soluble proteins induces a poor antibody response both locally and systemically (Watson and Lascelles, 1975). The draining nodes for the MG are the internal mammary lymph nodes along the sternum in primates, swine, and many other mammals. The superficial inguinal (supramammary) lymph nodes are most important for species with ventral glands such as ruminants. Thus, the ruminant response to infusion of soluble antigen appears to be a nonmucosal response but rather is a response by draining lymph nodes outside the MG. Antibodies from these cells or their migration to the gland parenchyma can explain the response of milk to infused soluble antigen in ruminants.

In contrast to OVA, particulate antigens such as *S. uberis* largely remain in the ducts and lumen of the MG. Those that reach lymph nodes do so later than OVA. In contrast to OVA, only weak IgG1 and IgA responses to *S. uberis* appear in serum, and the serum IgA response is short-lived. However, IgA responses to *S. uberis* in milk were sustained. This agreed with studies by Guidry et al. (1994), who used killed *S. aureus* and observed IgA response in bovine milk that increased and was sustained throughout lactation. Lee et al. (1978) also found that live *Brucella abortus* gave a

vigorous local IgA response. Local infusion with bacteria can also result in IgM responses in milk (Lascelles and McDowell, 1970) and IgM is an effective opsonin of mastitis-causing bacteria (William and Hill, 1982). Kennedy and Watson (1981) observed vigorous IgG1 responses in milk after mammary infusion of *S. aureus*, but a poor IgG2 response. These findings indicate that there is indeed a local IgA response and some IgG1 response in the ruminant MG to bacterial antigens, but that infusion with soluble proteins primarily leads to a systemic response. These observations are consistent with earlier organ cultures studies (Butler et al., 1972) and with the index of relative occurrence (Figure 2) in that some IgA, IgM, and IgG1 antibodies in the mature milks of domesticated ruminants are locally produced.

Both intraperitoneal and intramammary immunization with OVA or *B. abortus* results in a significant increase in the number of antigen-specific IgA cells in the lamina propria of the jejunum. This suggests that antigen may relocate from the mammary gland to the intestine, where an IgA response is generated from gut-associated lymphoid tissue. These findings provide further evidence for interaction between the gut and mammary gland of sheep when the MG encounters antigen (Sheldrake et al., 1985).

In similar experiments in mice and using OVA, a local IgA response was seen in milk but not in blood (Nashar et al., 1988). The authors concluded that intra-MG immunization can provoke a local IgA response in rodents and that a systemic IgG response is not a major source of IgG in milk as is the case in ruminants. Consistent with this study, local infusions of antigen (ferritin) late in pregnancy increases the number of plasma cells in the immunized MG and the local IgA response (Bennell and Watson, 1979). The biodiversity of the immune systems of rodents and ruminants MG suggests that caution is needed in the extrapolation of the effect of local immunization of the MG. In any case, the presence of lymphocytes and APCs in the MG suggests the potential for a genuine local immune response in the MG.

Local immunization of the MG may also benefit the suckling neonate. With this in mind, the MG may represent a better route of immunization than the gut (Section “Predicting the Origin of Igs in Lacteal Secretions”) because less antigen is needed and it may escape the enzymic degradation of the gut. In piglets, intramammary immunization can mimic mucosal gut immunization if the Ag is infused in the teat canal at appropriate times. Intramammary inoculation with live attenuated TGEV in pregnant sows leads to the induction of persistently high levels of neutralizing IgG antibodies in milk, whereas inoculation of the MG during lactation results in the production of IgA antibodies (Bohl et al., 1972).

In contrast to Ag infusion, injection of live or killed pathogens or soluble antigens differs from the natural means of antigen exposure and is essentially parenteral,

not a mucosal, immunization. Nevertheless, injecting virus into the MG of pregnant sows resulted in virus-specific IgA immune response in the milk (Salmon, 1995). Antibody activity was also detected in adjacent MG, albeit at lower levels. Thus, the virus may naturally spread among glands or to the gut where the gut–MG axis was activated, or lymphocytes stimulated in one gland may have moved to another, as described by Husband et al. (1996). Intramammary injection of sows with ferritin induces an IgM response, whereas the injection of this molecule and OVA together with complete Freund’s adjuvant lead to local granuloma formation but also resulted in predominantly local IgA response and the presence of antigen-reactive lymphocytes in both peripheral blood and milk (Evans et al., 1982). This indicates that although antigen injection is not biologically relevant, it can nevertheless result in an apparent local response at least in monogastrics (Lee et al., 1992).

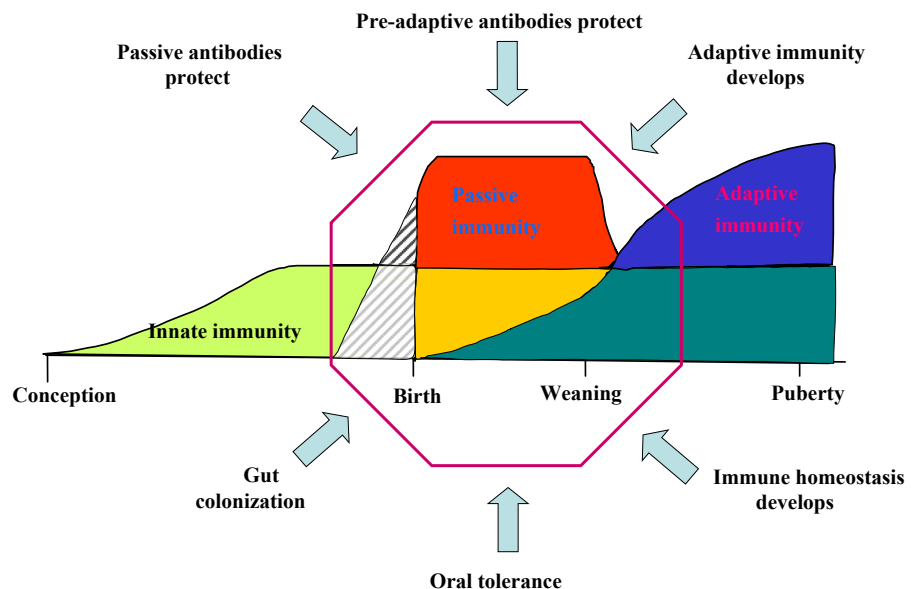
The Adaptive Cellular Immune Response

Adaptive cellular immune responses in the MG are mediated by effector T cells, after encounters with APC in conjunction with co-activator molecules. Stimulated CD4 and CD8 cells can differentiate into a number of different effector T cells such as Th1, Th2, Th17, and regulatory T cells (Treg). Most relevant may be the Th17 lineage that is responsive to extracellular pathogens at mucosal surfaces (Aujla et al., 2007). Because most mastitis-causing bacteria (staphylococci, streptococci, and enterobacteria) are extracellular pathogens, it might be expected that Th17 lymphocytes have an important role in the defense of the MG. Target cells for the Th17 signature cytokines IL-17A, IL-17F, and IL-22 are MEC and fibroblasts. These cells respond by producing self-defense proteins and chemokines that

attract and activate particularly neutrophils but also other leukocytes such as Th1 cells (Kolls and Khader, 2010). Transcripts of IL-17A were found in leukocytes attracted in milk by chronic *S. aureus* or streptococcal mastitis (Bruno et al., 2010; Tao and Mallard, 2007) or by immune-mediated mammary inflammation (Riollet et al., 2006). Human and bovine MEC express the receptor for IL-17A and IL-17F, and bovine MEC respond to these two cytokines by producing antibacterial factors and chemokines (Bougarn et al., 2011). Thus, IL-17 and IL-17–producing cells may be especially important for the defense of the MG.

Another manifestation of cell-mediated immunity is so-called delayed-type hypersensitivity (DTH). Type IV DTH is mediated by CD4+ Th1, Th17 cells and CD8+ cytotoxic lymphocytes. The contribution of DTH to mastitis manifestations has long been suspected. A special form of chronic *S. aureus* mastitis in cows is characterized by the appearance of nodules at the base of the teat, which are in fact typical granulomas containing clusters of staphylococci. Thus, natural infections or deliberate sensitization augmented the reactivity of the MG to luminal infusion of bacterial extracts (Targowski and Berman, 1975). De Cueninck (1979) demonstrated that an antigen-specific inflammatory response in the MG could be produced by parenteral sensitization with OVA. The inflammatory response was characterized by the influx of neutrophils into the lumen of the MG. This type of antigen-specific response to OVA or to tuberculin coincided with cutaneous DTH and could be transferred to naïve animals by peritoneal exudate cells or lymphoid cells but not by antibodies (Nonnecke and Targowski, 1984). Besides neutrophils, mononuclear leukocytes including activated CD4+ and CD8+ lymphocytes were recruited into the MG in increasing proportions with time post-challenge (Riollet et al., 2001).

FIGURE 5 The critical window of immunological development. Factors affecting events within the octagon window are indicated with arrows. Passive immunity (red) when superimposed on innate immunity (green) yields orange. Similarly, developing adaptive immunity (blue) when superimposed on both passive and innate immunity yields light blue. The lined triangle before birth applies only to species, e.g., humans and mice, in which passive immunity also takes place in utero (see Figure 1). (See color plate section.) Modified from Butler et al. (2006).



Preparturient Immunosuppression of Innate Immunity

Preparturient immune suppression in cows occurs a few weeks before and a few weeks after parturition, during the critical window of immune system development (Figure 5). During this time a wide range of immunological dysfunction occurs, including impaired neutrophil and lymphocyte functions, the results of which are increased incidence of various diseases including mastitis (Kehrli et al., 1989; Mehrzad et al., 2001; Shuster et al., 1996). During this period neutrophils show a significant loss of the homing receptors CD62L and CD18, affecting their ability to enter the site of infection (Burton et al., 1995), which thus affects the prevalence of mastitis in periparturient animals.

The immune suppression seen during the periparturient period has been shown to be the product of both parturition and lactation. Myeloperoxidase is critical to the microbial killing mechanisms of neutrophils (Winterbourn et al., 2000) and activity wanes 2–3 weeks before parturition and is restored 3–4 weeks after parturition (Cai et al., 1994; Kehrli et al., 1989). In mastectomized cows there is a decrease in myeloperoxidase activity before calving that returns to normal several weeks faster than in control animals. Hypocalcemia is common in periparturient dairy cows and is associated with an increase in an animal's susceptibility to mastitis and many other physiological changes (Kimura et al., 2006; Curtis et al., 1983).

THE ROLE OF LACTEAL SECRETIONS IN PASSIVE IMMUNITY

The Critical Window of Neonatal Immune Development

The critical window is illustrated in Figure 5. Some events that occur during this period lie outside the focus of this chapter, e.g., immunological tolerance, but may still depend on antibodies and immune regulatory factors present in colostrum/milk. The adjuvant effect of MAMPs from colonizing gut flora has an important role but their impact may be modulated by passive antibodies or other factors in colostrum/milk.

The Protective Role of Passive Antibodies in Lacteal Secretions

The classification of mammals on the basis of passive immunity is illustrated in Figure 1 and discussed in Section “The Level and Origin of Lacteal Igs”. The paradox that farm animals absolutely require colostrum but infants do not is apparent when the different pathways for the transfer of maternal IgG are realized (Figure 1). Perhaps with the exception of ruminants, the value of continuous

long-term suckling is associated with IgA in mature milk. The value of breastfeeding is demonstrated in the prevention of otitis media (Harabuchi et al., 1994), diarrhea (Ruiz-Palacios et al., 1990; Stoliar et al., 1976), and other childhood illnesses (France et al., 1980; Hanson and Winberg, 1972; Howie et al., 1990). Because there is no absorption of lacteal IgA, protection must be mediated in the infant's GI tract. Thus, protection to otitis media would suggest another mechanism. In ruminants, protection in the GI tract is not restricted to IgA because IgG1 remains the major Ig in mature bovine milk and can also be protective although less effective than IgA in enteritis (Table 1; Bernard et al., 1990; Fahey et al., 1981; Snodgrass et al., 1980; Stone, 1970). The therapeutic value of bovine immune milk antibodies for humans has been controversial for nearly 50 years and will not be discussed here. However, passive antibodies do not protect against Cryptosporidia in children (Sterling et al., 1991), mice (Moon et al., 1988), and calves (Harp et al., 1989). Rather, colostrum-mediated protection could result from non-antibody factors in such cases as respiratory syncytial virus in ferrets, cotton rats, and infants (Nandapalan et al., 1987; Prince et al., 1983; Suffin et al., 1979b).

Uptake of Igs by the Gut of the Neonate

Patterns of Absorption by the Gut

In human infants, a small amount of IgA and other milk proteins is absorbed by the gut of newborn for 12–24 h after birth (Ogra et al., 1978). The small amounts of intact proteins and Ig absorbed by the infant are probably insignificant for protective passive immunity but could be important in antigenic sensitization (Ogra et al., 1977; Walker, 1987). As described, newborn ungulates absorb all proteins nondiscriminately during the first 12–24 h after birth (Figure 1). Thereafter, a phenomenon called gut closure ends this process. Absorption and closure have been studied for >40 years and have been periodically reviewed (Baintner, 2007; Brambell, 1970; Danielsen et al., 2006; Kraehenbuhl et al., 1979). Cessation moves as a wave from duodenum to jejunum to ileum (Murata and Namioka, 1977). Group II mammals (Figure 1) are not a homogeneous group and probably should be divided into two to three subgroups. Whereas rodents selectively absorb IgGs, dogs and cats appear to adsorb all immunoglobulins much like group III mammals (Yamada et al., 1991). Adsorption ceases 12 h postpartum in the dog (Gillette and Filkins, 1966) but it may continue longer in cats. In mink and ferrets, it may continue for 8–20 days (Porter, 1965; Suffin et al., 1979a). Although FcRn is important to gut absorption in rodents (see below), it is unlikely that FcRn is the IgG transport receptor in the piglet or ruminant gut because all proteins, not just IgG, are absorbed. Thus receptor-mediated uptake of IgG in rodents

fails to reveal a mechanism that can explain the nonspecific absorption of all Igs in large farm animals. Rather, achieving high levels of IgG in the sera of suckling ungulates depends on having high levels of IgG in colostrum and the ability of the newborn to suckle, not on the preferential absorption of IgG in the gut (Klobasa et al., 1987; Takimori et al., 2011; Werhahn et al., 1981).

The Role of FcRn in Uptake of IgG by the Rodent Gut

Uptake of IgG by the gut of neonatal rats and mice differs from other mammals because it proceeds for 19 days and depends on an epithelial receptor specific for the Fc portion of IgGs (Borthistle et al., 1977; Brambell, 1970), so only IgG is absorbed into blood (Jones and Waldman, 1972). The rat gut can also absorb heterologous IgG and can distinguish subclasses (Guyer et al., 1976; Mackenzie et al., 1983; Morris, 1969). Uptake depends on FcRn that recognizes monomeric IgG and does not require that IgG be glycosylated (Hobbs et al., 1992). The relative subclass levels in mouse milk and pup serum suggest that FcRn in the MG and gut have similar subclass specificity (Guyer et al., 1976). Neonatal Fc receptor has been shown to be present in functional form in the mammary gland of lactating mice and is localized to the epithelial cells of the acini. Analysis of the transfer of Fc fragments and IgG that have different affinities for FcRn indicate that these proteins are transferred in inverse correlation with their binding affinity for FcRn. Thus, in the lactating mammary gland, FcRn appears to have a role in recycling IgG in a mode that may have relevance to FcRn trafficking during the maintenance of constant serum IgG levels. The inverse correlation between transfer of an IgG or Fc fragment into milk and affinity for FcRn has interesting implications for the levels of IgG in the milk that suckling neonatal mice ingest. The IgG1 isotype is present in milk in lower amounts relative to maternal serum levels than IgG2b, which has a lower affinity for FcRn. However, the relative efficiency of transfer across the neonatal intestine, for which a direct correlation with affinity is seen (Medesan et al., 1997), would be predicted to reverse this. The preferential transfer into milk of poor FcRn ligands followed by more efficient neonatal transfer of higher-affinity FcRn ligands might therefore provide a mechanism by which the levels of maternally derived IgG of different isotypes in the neonate are balanced (Cianga et al., 1999).

Whereas intestinal FcRn expression in rodents is limited to the suckling period (Martin et al., 1998), the human FcRn is expressed in both the fetal intestine, where it is involved in IgG uptake from the amniotic fluid into the fetal circulation (Shah et al., 2003) and also in the adult enterocytes, where it serves an important role in intestinal immune surveillance (Dickinson et al., 1999; Yoshida et al., 2004, 2006).

The Mechanism and Consequences of Gut Closure

The exact mechanism leading to gut closure is unknown, although maturation of the lysosomal system is suspected because after closure in piglets only IgG fragments are found in blood (Werhahn et al., 1981; Kraehenbuhl and Campiche, 1969). This was shown using polyvinylpyrrolidone in a number of species (Clarke and Hardy, 1970). Gut closure may be progesterone-associated because it parallels tight junction closure in the mammary epithelium (Nguyen et al., 2001). It may be effected by factors in colostrum (e.g., transforming growth factor- β) that are known to induce accumulation of extracellular matrix production (Xu et al., 1999). It is known that ingestion of protein significantly up-regulates alkaline phosphatases and lactase (Wang and Xu, 1996) that in turn facilitates closure (Stott et al., 1979; Werhahn et al., 1981) and that 13% glucose causes near complete closure (Leece, 1966; Klobasa and Werhahn, 1991). However, this glucose-induced closure was demonstrated in Specific pathogen free (SPF) animals, so that high glucose may facilitate bacterial colonization that may in turn trigger closure.

The decline in Ig uptake by newborn piglets (and other ungulates) in the first few days after birth is not solely dependent on gut closure, but is also a consequence of the precipitous drop in Ig concentration in colostrum. In swine, this is threefold in 12 h and fourfold at 18 h (Klobasa et al., 1987). Because the amount adsorbed in the first 12 h directly depends on the concentration of the ingested Igs (Werhahn et al., 1981), piglets that suckle first at 12 h obtain threefold less Ig in blood. Morbidity and mortality among suckling ungulates can be traced to low blood immunoglobulin levels (Fey, 1971; Logan et al., 1974) such as in severe combined immunodeficiency Arabian horses (McGuire et al., 1976, 1977), resulting from a delay in suckling and/or the availability of Igs in lacteal secretions.

Once closure occurs, ingested antibodies are degraded or act on the surface of enterocytes or within the gut lumen. Immunoglobulin A and IgM (but not IgG) ingested by 2-day-old piglets or IgA ingested by suckling rats become associated with the crypt epithelium (Butler et al., 1981; Nagura et al., 1978), where they may potentially form a specific barrier to bacteria or other antigens at the luminal surface of the epithelial cells.

A proportion of the IgG1 ingested by calves recycles back into the intestinal lumen where it is available to potentially protect the GI tract against infection (Besser et al., 1988). Gut closure may not prevent recycling because recycling occurs through the crypt epithelial cells, which are known to be responsible for secretory processes in the gut (Newby and Bourne, 1977). The expression of FcRn on duodenal crypt cells in neonatal lambs suggests that FcRn may continue to function in the recycling process (Mayer et al., 2002).

Neonatal Immunoregulation by Colostrum and Milk

Classes of Immunoregulators

Milk and colostrum contain a broad array of components that include factors that can affect the immune response of the suckling neonate. These include mediators such as cytokines, possibly elements of the MFGM, and even immunoglobulins. The immune mediators in lacteal secretions were reviewed in Section “The MG and Its Secretions”. Here, we discuss how their ingestion may modulate the immune system of the suckling neonate.

Immunomodulation by Passive Igs and Antibodies

Studies in rabbits and mice have shown that maternal IgG antibodies can both augment and suppress neonatal responsiveness (Okamoto et al., 1989; Rodkey and Adler, 1983; Rubinstein et al., 1982; Wikler et al., 1980). The suppression of IgE responsiveness is noteworthy (Bednar-Tantscher et al., 2001; Jarret and Hall, 1979; Roberts and Turner, 1983), which might explain the delayed onset of atopy among breastfed infants (Halpern et al., 1973). Suppression in newborn artiodactyls has also been shown (Hammerberg et al., 1989; Hoerlein, 1957; Husband and Lascelles, 1975; Klobasa et al., 1981, 1986; Logan et al., 1974; Muscoplat et al., 1977). In piglets reared in an autosow, ingestion of 3.5 g purified IgG suppresses de novo synthesis of IgG and IgA to the same extent as colostrum (Klobasa et al., 1981). Conventional piglets suckling experienced sows, i.e. those having borne seven to nine litters, experience more suppression of de novo Ig synthesis than those suckling first-litter sows (Klobasa et al., 1986). This might explain why mouse pups suckling SCID mothers showed accelerated development of the gut mucosal IgA system (Kramer and Cebra, 1995) and why secretory immunity develops more rapidly in bottle- and formula-fed infants than in those nursed by their mothers (Sarrinen, 1982; Stephens, 1986).

Mechanisms of Immunosuppression by Passive Maternal Immunoglobulins

The mechanisms of suppression are theoretical: (1) maternal antibodies capture pathogens and foreign antigen preventing them from stimulating the neonatal immune system; (2) natural antibodies in colostrum interfere with colonization by the normal gut flora needed to stimulate neonatal immune competence; and (3) passive maternal IgG saturates FcγRIIβ receptors, which then bind antigen, which then crosslinks to the BCR.

The antibody interference theory has been popular (Van Maanen et al., 1992). Sows routinely immunized with various vaccines induce the production of IgG, IgM, and IgA antibodies that are subsequently transferred to the suckling

piglet via colostrum. Effective interference by these passive antibodies appears to depend on the ratio of ingested antibodies to the invading pathogen (Siegrist, 2003). However, blocking of antibody responses in piglets by maternally acquired immunity does not mean the absence of an immune response by the piglet. Rather, protective cellular immune responses may develop while priming B cells for subsequent exposure to the same antigen (reviewed by Salmon et al., 2009). Another example of antibody interference involved is SIgA in human milk/colostrum against dietary antigens (Rumbo et al., 1998). This IgA may have a role in the control of allergen absorption and contribute to protection of the neonate against the development of allergies of dietary or environmental origin (Welsh & May, 1979).

The second theory concerns the effect of passive immunity on gut colonization. Colonization by gut flora is necessary to drive development of the immune system, as evidenced by the more robust response to T-dependent antigens in conventional compared with germ-free animals (Butler et al., 2002, 2005; Dobber et al., 1992; Ohwaki et al., 1976; Woolverton et al., 1992). In rabbits and swine, diversification of the antibody repertoire also depends on microbial colonization (Butler et al., 2011; Knight and Winstead, 1997). Natural antibodies that recognize bacterial polysaccharides such as polyreactive natural IgA antibodies against commensal flora can limit the penetration and adhesion of commensal intestinal bacteria to the neonatal intestinal epithelium (Macpherson et al., 2000; Harris et al., 2006). Human IgA deficiency is often correlated with high concentrations of serum antibodies directed against antigens of the alimentary bolus or of enterotropic bacteria. This suggests that IgA antibodies in the lumen protect against this phenomenon. Although Harris et al. (2006) detected no influence of milk IgA in rodents on the level of gut microbiota, the gradual decrease in the supply of maternal IgA antibodies during the suckling period might explain the parallel increase in bacterial colonization (Inoue and Ushida, 2003; Inoue et al., 2005a; b). More important, these natural SIgA antibodies in milk (Brandtzaeg, 2003) were shown to decrease the spread of microbial pathogens through the population by reducing the pathogen load in the feces.

The third hypothesis revolves around Fcγ receptor-mediated suppression of B cell responsiveness. Swine lymphocytes can be inhibited in vitro by membrane-bound antibodies (Setcavage and Kim, 1978), perhaps indicating suppression through crosslinking of FcγIIB and the B cell antigen receptor (D'Ambrosio et al., 1995; Phillips and Parker, 1983). Apart from this single example, all other positions are only theoretical.

Non-Immunoglobulin Regulation of Neonatal Immune Responses

Cytokines, chemokines, and growth factors are all present in milk and colostrum (Section “The MG and Its Secretions”;

Table 4). The transfer of cytokines from colostrum to the bloodstream of the piglet peaks 2 days after birth, coinciding with the gut permeability period (Nguyen et al., 2007), which suggests that colostrum may provide immune modulators during the critical window (Figure 5; Salmon et al., 2009). Normal milk and colostrum have been shown to stimulate the growth of various cell types in vitro (Klagsbrum, 1978), perhaps the result of the presence of epithelial growth factor (EGF) and colony-stimulating factor (CSF; Table 4). Epithelial growth factor occurs in human, bovine, and porcine lacteal secretions (Jansson et al., 1985; Read et al., 1984; Widdowson et al., 1976). Porcine colostrum contains 1500 ng/mL EGF (Jaeger et al., 1987), and bovine milk 18–320 ng/mL (Xiao et al., 2002; Yagi et al., 1986). Epithelial growth factor is also found in rodents (Grueters et al., 1985; Raabery et al., 1990). Exogenous EGF given orally accelerates intestinal growth (Berseth, 1987; Read et al., 1984) and EGF orally administered to piglets significantly increases jejunal lactase and sucrase activities (James et al., 1987) as well as up-regulating TGF expression (Miettinen, 1993). Insulin-like growth factors (IGFs) are also present in milk. Porcine and bovine colostrum contain 70–350 ng/mL, whereas levels in human colostrum are lower (Xu, 1996). Administration of IGF (1 μ g/d) stimulates brush border enzymes and GI growth (Lemmey et al., 1991; Xu et al., 1994). Detailed reviews of milk growth factors are available (Xu, 1996).

Colostrum and milk also contain various regulatory cytokines (Table 4). In humans, the concentration of active TGF- β is 952 and 178 ng/mL in colostrum and milk, respectively. Based on daily consumption of 700 mL milk, this means that ~125 μ g of active TGF- β daily reaches the infant's GI tract, with about 2.5-fold more reaching the gut daily during the first few days after birth. Transforming growth factor- β 1 and TGF- β 2 are also present in bovine milk (Cox and Burk, 1991; Jin et al., 1991) and the level of TGF- β 1 may reach 248 ng/mL (Ginjala and Pakkaman, 1998). In swine, TGF- β 1 levels are 125–260 ng/mL, drop to 73 ng/mL after 12 h, and become undetectable after 5 days (Xu et al., 1999). Transforming growth factor- β has multiple actions that may be important in the newborn GI tract. First, TGF- β is a switch factor for the expression of IgA so it may stimulate Peyer patch B cells in the neonate to switch from IgM to IgA. Second, TGF- β along with IL-10 suppresses Th1 responses so that it may act to dampen the chronic inflammation of the gut. Some evidence exists for such a role in rats (Playford et al., 1999) in which TGF- β 2 is the predominant isoform and the TGF receptor is expressed in the rat intestine (Zhang et al., 2001). Interestingly, IL-10 levels are also high during the first 80 h of lactation in humans. Finally, TGF- β may have a role in gut closure because it can induce tight junction formation (Xu et al., 1999).

Another important regulatory cytokine is IL-6. Lacteal IL-6 is produced by mammary epithelial cells and IL-6

concentrations are positively correlated with monocyte numbers in milk (Table 4; Saito et al., 1991). Interleukin-6 concentrations in human colostrum are 10- to 20-fold higher than in blood, and milk levels can be even higher (Saito et al., 1991). Values range from 0.8 to 300 pg/mL (Wallace et al., 1997). Interleukin-6 induces CSF, is involved in B cell growth and differentiation, and is 10- to 100-fold higher in human milk than serum (Hara et al., 1995). Saito showed that secretion of IgA from milk B cells is IL-6 dependent. Whereas the IL-6 levels in lacteal secretions appear to be a simple consequence of the predominance of macrophages in the healthy gland, they could have downstream effects. Neonates ingest milk containing both IL-6 and the macrophages that secrete IL-6. Thus, IL-6 could have a role in the differentiation of both epithelial and lymphoid cells in the neonatal GI tract. The secretion of IL-6 by monocytes/macrophages ingested by the neonate may also be a mechanism for avoiding its degradation in the stomach. However, because IL-6 levels are also elevated in blood and milk of mastitic cattle (Shuster et al., 1993), its role seems primarily related to defense of the gland, rather than to regulation of the neonatal immune system (Hagiwara et al., 2000).

Infection and/or inflammation of the MG would be expected to result in a cytokine profile in colostrum or milk that differs from that of healthy glands. During inflammation, one anticipates an increase in the transcription and secretion of the endogenous pyrogens from macrophages, i.e., IL-6, TNF- α , IL-12, IL-8, and IL-1. The bovine MG responds in the anticipated manner. Figure 4 shows the abrupt rise in TNF- α , IL-1, and IL-8 after infusion of the bovine MG with *E. coli*. Rapid elevation of TNF- α , IL-8, and GM-CSF was also observed in the sheep MG infused with LPS (Waller et al., 1997). This is associated with elevation of IL-6 not only in the gland, but also in serum (Hagiwara et al., 2000) and with increased expression of IL-12 (Taylor et al., 1997), which suggests that the neutrophil influx that is the hallmark of mastitis (Figure 3; see Section “The Mechanism and Consequences of Gut Closure”, above) results from the activation of macrophages/monocytes/DC within the MG. Accepting that the epithelia in contact with potentially colonizing microorganism exist in a mild inflammatory state, subtraction of the cytokine profile of the inflamed gland from that of the normal gland should provide insight into cytokines/chemokines/growth factors that are important in healthy glands, may function in the neonatal GI tract under healthy gland conditions, and are a feature of the inflammatory response of the MG. Whereas the situation regarding the cytokine profile of the infected MG fits nicely with what is generally known about the inflammatory response, the cytokine profile of the healthy gland offers minimal insight into how the cytokine profile of colostrum and milk in healthy animals functions. As discussed above, TGF- β and IL-6 may be needed for the mucosal response of the neonate.

In addition to cytokines, Julius et al. (1988) described a peptide from sheep milk capable of stimulating B cell development in mice (and perhaps also in newborn lambs) and a non-Ig temperature-labile factor in bovine colostrum may suppress de novo synthesis by newborn piglets (Klobasa et al., 1990). Retinoic acid metabolites in bovine colostrum may also serve as major maturation factors in the imprinting of gut lymphoid cells (Mora and von Andrian, 2004; Saurer et al., 2007).

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