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Immunological protection of the vulnerable marsupial pouch young: two periods of immune transfer during lactation in *Trichosurus vulpecula* (brushtail possum)

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Abstract

Marsupial young are born with an underdeveloped immune system and are dependent upon passively acquired immune protection provided by the mother's milk. Colostrum and milk samples were collected from the brushtail possum throughout lactation and the concentration of secretory IgA (sIgA), IgG and transferrin was determined by Western blotting. Two periods of immune transfer were identified. The first, a colostral phase, occurs immediately after birth and involves sIgA, IgG and transferrin. During the early lactation stage, pouch young receive milk of a unique composition as they undergo developmental changes in the pouch that occur in utero for eutherian mammals. At the end of this external gestation, the composition of the milk changes (switch phase) to resemble that of eutherian mammals in the late lactation phase. The second transfer of immunity consists of IgG and transferrin, and occurs during the switch phase prior to maturation of the immune response.

Keywords: Colostrum; Lactation; IgG; IgA; Marsupial; Transferrin; Immunity; Tight junctions

1. Introduction

The young of mammalian species receive immunity passively from the mother via either the placenta, the mammary gland or both, for protection against pathogens until their immune system has matured and they are able to elicit their own immune response [1,2]. Colostrum is a specialised mammary gland secretion that is produced for a short period after birth (<48 h) and contains various components important for immune protection. These include lymphoid cells, cytokines, immuno-modulating substances (casein derivatives), growth factors, hormones and immunoglobulins [3]. It is recognised that passively acquired immunity is important for the

Abbreviations: dIgA, dimeric IgA; sIgA, secretory IgA; SC, ecretory component; Tf, transferrin; SA, serum albumin.

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health of the neonate. For example, newborn pigs which did not receive colostrum from the sow showed nearly 100% mortality during the first 3 weeks of life [4].

Colostrum contains high concentrations of immunoglobulins, IgA and IgG, and the relative concentrations of these vary between species. Ingested IgA protects against pathogens in the gastrointestinal tract, whereas IgG is transported across the gut epithelium into the circulatory system of the young. For primates IgA is the predominate Ig transferred through the colostrum, with IgG being transferred via the placenta prior to birth. The colostrum of ungulates, which have no prenatal IgG transfer, contains a high concentration of IgG as well as IgA, whereas rodents transfer IgG by both mechanisms, and the IgG concentration of their milk is intermediary [1,2]. Specific transporters are used to actively transfer these immunoglobulins across the mammary gland epithelium into milk. The polymeric immunoglobulin receptor (pIgR) transfers dimeric IgA (dIgA), which is secreted by lymphocytes associated with the mammary gland epithelia [5]. The mammary gland epithelial IgG-transporter has not been characterised.

Reproduction in marsupials differs markedly from that of eutherian mammals. Marsupial young are born after a short gestation (17 days in the possum, *Trichosurus vulpecula*) at an immature stage of development, and during the early lactation phase (days 1–80) they undergo developmental changes that occur in utero for eutherian mammals [6,7] (Fig. 1). For this reason the early lactation phase of marsupials may be considered as an external gestation. The composition of milk during early lactation is unique and must contain all the nutrients required by the developing pouch young. Milk composition changes around mid-lactation (switch phase, days 80–120) to become more like that of eutherian mammals during late lactation (days 120–200) [8–10]. Around the switch phase, the pouch young also undergoes physiological changes that are suggestive of a second birth; suckling becomes intermittent, their fur begin to grow, their eyes open and they begin to leave the pouch (Fig. 1).

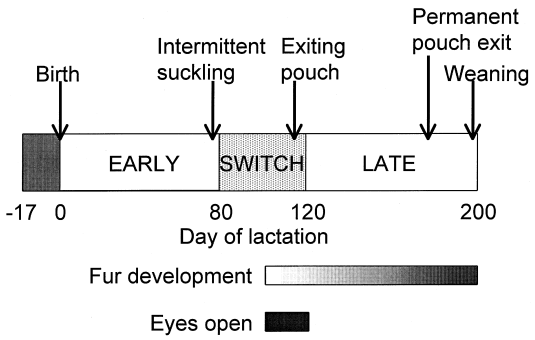

Fig. 1. Three stages of possum lactation. The early, switch and late phases of lactation are shown with respect to day of lactation. Various events that occur during lactation are indicated. Notation of these events was carried out by Terry Fletcher (Landcare, Lincoln, New Zealand) during sampling from the possums used in this study.

The immune system of newborn eutherian mammals matures soon after birth and thus the need for maternally derived immune protection is transitory. In contrast, the lymphoid organs of marsupial young do not mature until the end of the early lactation stage when the pouch young first leaves the teat [11–15]. Pouch young are able to elicit a limited immune response soon after birth (day 14 in the possum), but their immunoglobulin serum concentrations do not reach adult levels until the switch phase of lactation. These findings suggest that marsupial pouch young are dependent on immune protection acquired passively from the mother's milk for an extended period after birth [13]. For marsupials, little is known about immunoglobulin transfer via the placenta or the mammary gland. In several marsupial species no immunoglobulins were detected in the serum of the new born pouch young until after they had suckled for the first time, suggesting that there is only postnatal transfer of immunity [16–18]. In contrast, immunoglobulins have been detected in the placenta of the tammar wallaby (*Macropus eugenii*) and the serum of their newborn prior to suckling [19].

Transfer of immunoglobulins in utero or via the mammary gland has not been investigated for the possum. Since newborn possums are unable to mount a humoral response for some time after

birth, post-parturition transfer of immunoglobulins is likely to be important for their immune protection. A study of three brushtail possums demonstrated that IgG ingested by 50 and 98, but not 145, day old pouch young were subsequently detected in their serum [20]. Thus, possum pouch young are receptive to passive immunity offered by their mother's milk into the switch phase of lactation. Colostrum is a clear viscous secretion and has been observed for several marsupial species including the possum [14]. The concentration of IgG in colostrum of the tammar wallaby and hill kangaroo (*Macropus robustus*) is low (0.4–1 mg/ml), however, there was a dramatic increase in the serum IgG concentration of the neonate upon suckling for the first time, suggesting that pouch young acquire IgG passively from the colostrum [13,19]. Immunoglobulin A was also detected in tammar wallaby colostrum, however, its importance for immune protection was not established [19]. We have recently shown that expression of mRNAs that encode dIgA and the pIgR are elevated in the possum mammary gland during the first week of lactation, which is consistent with a colostral phase involving IgA transfer [21]. A second period of elevated expression of these mRNAs in the mammary gland was observed around the switch phase and this may represent a second phase of immune transfer in the possum.

As an extension of this work, secretion of IgA and IgG into possum milk during lactation was analysed. The concentration of IgA was elevated in mammary gland secretions obtained at the beginning of lactation, but the concentration of IgG was relatively low. The concentration of IgG, but not IgA was increased in milk produced during the switch phase of lactation.

2. Materials and methods

2.1. Expression of recombinant proteins and generation of antisera

GST-J chain fusion protein: The *Bam*H I–*Eco*R I restriction fragment from pGEM-Teasy possum J chain clone [21] was cloned into pGEX-2T (Pharmacia Biotech, Uppsala, Sweden).

GST-Cα fusion protein: The *Apa* I–*Sal* I restriction fragment from pGEM-Teasy possum Cα clone [21] was end-filled with T4 DNA polymerase and cloned into the *Sma* I site of pGEX-2T (Pharmacia Biotech).

SC-HIS fusion protein: SC of possum pIgR was engineered as follows. PCR fragment A: nucleotides 149–702 of possum pIgR were amplified using 5′ primer = CAT ATG GCT TTC TTC CTT GCC TG and 3′ primer = TCA GGG CCC ACT TGT TCC TG. PCR conditions were annealing at 58°C, with 80 s extension for 30 cycles using the cDNA clone as a template [21]. PCR fragment B: nucleotides 1166–1945 of the pIgR was amplified using 5′ primer = AGA GCT CTA CAG CTC TTT GTC and 3′ primer = CTC GAG AGT GCT CCC CAA ATC TGT AG. PCR conditions were annealing at 58°C, with 80 s extension for 30 cycles. Both products were cloned into pGEM-Teasy (Promega, Madison, WI, USA) and verified by DNA sequencing. Fragment A was subcloned into *Nde* I–*EcoR* I sites of pET 23b (Novagen, Madison, WI, USA), followed by fragment B into the *Sac* I–*Xho* I restriction site and finally the *Apa* I–*Sac* I restriction fragment from pIgR cDNA clone.

GST-fusion proteins were over-expressed in DH5α (Gibco-BRL, Gaithersburg, MD, USA) as per manufacturers instructions (Phamacia Biotech). The SC-HIS fusion protein was over-expressed similarly in BL21(DE3) bacterial cells (Stratagene, La Jolla, CA, USA). All the fusion proteins were purified from inclusion bodies essentially as described previously [22]. The bacterial cell pellet from a 400 ml culture was resuspended in 10 ml of sonication buffer, frozen at −80°C and the thawed cells were lysed by sonication in the presence of a protease inhibitor cocktail (Sigma-Aldrich). Inclusion bodies were recovered by centrifugation (8000 × *g*, 4°C, 10 min) and washed four times with RIPA buffer before solubilization in 4 ml of 2% SDS. Solubilized protein was diluted 5-fold into 0.1 M Tris–HCl pH 7.5, clarified by centrifugation (20,000 × *g*, 4°C, 20 min) and dialysed against 1 × PBS.

Antisera was raised in New Zealand's White rabbits as described by Grigor et al. [9], by subcutaneous injection with 400 μg of the fusion protein in Freund's complete adjuvant, followed by two injections of 400 μg of antigen in Freund's incomplete adjuvant at 3–4 week intervals. Approximately 3 weeks after the final injection, the rabbits were anaesthetised with pentobarbitone and bled out. The serum was clarified by centrifugation and stored in aliquots at −20°C.

2.2. DNA sequencing

DNA sequencing reactions were carried out by the DNA Sequencing Unit (Massey University, Palmerston North, NZ) using an ABI Automated DNA sequencer.

2.3. Western blotting analysis

Western blotting was performed as described by Sambrook et al. [23], hybridising in 5% dried skimmed milk powder, 1 × PBS. Serum dilutions were 1/2000 for anti-Cα, anti-J chain and anti-IgG (gift from Bryce Buddle, AgResearch Wallaceville); 1/1000 for anti-SC, $1/1.2 \times 10^5$ for anti-possum serum albumin [9] and 1/1000 anti-human occludin (Zymed Laboratories, San Francisco, CA, USA). Secondary antibody, HRP conjugated goat anti-rabbit (Sigma-Aldrich), was used at 1/2500–1/5000 dilution and the signal developed using the SuperSignal Substrate Western Blotting kit (Pierce Chemical, Rockford, IL, USA). Antibodies were removed by first washing with stripping solution (2% SDS, 50 nM Tris pH 7.6, 100 mM β-mercaptoethanol (three changes of 10 min each at room temperature) and then four times with 1 × PBS before hybridising with a different antibody. Purified possum sIgA (gift from Robyn Midwinter, AgResearch Wallaceville) and IgG (gift from Don Otter, Dairy Research Institute) were used to generate standard curves on the Western blots used to quantify the concentration of IgA and IgG in milk samples. The volume of milk sample loaded was adjusted so the signal intensity was within the linear part of the standard curve. Unknown samples were loaded in duplicate and Western blots were repeated. Autoradiographs were scanned and volume analysis performed using Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA).

2.4. Collection of milk and serum samples

Female brushtail possums that were known to have mated, due to the presence of sperm in the urine [24] were checked for the presence of pouch young at 16–18 days post-mating to obtain new born pouch young. For milk samples obtained between days 0 and 18 of lactation the pouch young were removed from the teat and euthenased. For milk samples across lactation (day 20–190 of lactation) the pouch young were placed in a humidified incubator at 25°C and returned to the teat after sampling. One to six hours after removal of pouch young, female possums were anaesthetised by fluothane/O_2 inhalation and injected intravenously with 2 i.u. oxytocin. Milk was collected into glass capillary tubes or eppendorf tubes and stored at −20°C. Blood was collected from the tail of anaesthetised possums using a 5 ml non-heparinised vacutainer (Becton Dickinson, Franklin Lakes, NJ) and the clarified serum was stored at −80°C. Serum lactose concentration was assayed using an enzymatic method as described previously [25].

2.5. Preparation of mammary gland protein samples

Mammary glands were collected from feral possums as described previously [10] and the date of lactation was based on the size of the pouch young [26]. The frozen mammary tissues were ground under liquid nitrogen by mortar and pestle and 400 mg homogenised in 1 ml of buffer (0.1 M HEPES pH 7.9, 15 mM MgCl, 0.1 M KCl, 1 mM vanadate, 0.5 mM DTT, 0.4 mM PMSF). Protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce Chemical).

3. Results

3.1. Secretory IgA and IgG are components of possum milk

Antibodies were generated against components of secretory IgA (sIgA); the J chain, secretory component (SC) and IgA heavy chain (Cα), by cloning regions of these genes for expression as GST or HIS-tag fusion proteins in *E. coli*. The over-expressed proteins were purified from inclusion bodies and antisera was raised in rabbits. To demonstrate that both sIgA and IgG are present in possum milk, total milk proteins were separated by SDS-PAGE and analysed by Western blotting (Fig. 2). The IgG antibody picked up both Cγ and the Ig-light chains (upper and lower bands, respectively), indicating that IgG was present in possum milk. Dimeric IgA, two IgA molecules joined by the J chain, is transported across epithelial cells by the pIgR which is cleaved at the apical membrane leaving its extra-cellular domain, the secretory component, attached to dIgA. Three molecules of sIgA; Cα, J chain and SC were shown to be present in possum milk, indicating that dIgA was actively secreted into possum milk (Fig. 2).

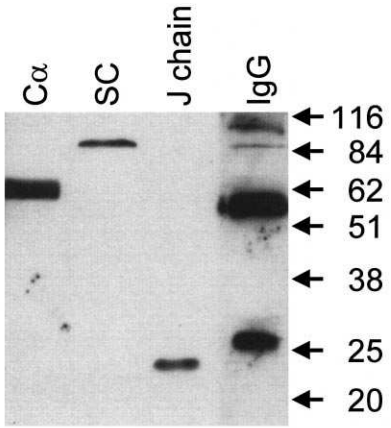

Fig. 2. Both sIgA and IgG are present in possum milk. Protein from 2 μl of possum milk (day 90 of lactation) were separated by denaturing SDS PAGE and transferred to nitrocellulose membrane for Western blotting. Strips containing the separated proteins were probed with antisera against Cα (IgA heavy chain), secretory component (SC), J chain and IgG (both Cγ and immunoglobulin light chains are detected). Position of the molecular weight (kDal) protein markers is indicated.

3.2. Two periods of immunoglobulin transfer during lactation in the possum

To determine the period of colostrum formation and immune transfer during lactation in the possum and which immunoglobulins constitute this secretion, milk samples were collected throughout lactation from three possums at precise intervals from day 20 until the end of lactation (day 190). Mammary gland secretions collected earlier than day 20 (days 0–18) of lactation came from individual possums. Samples collected at the beginning of lactation (days 0–2) were clear yellow viscous secretions and did not contain casein, consistent with being colostrum and low casein mRNA expression in the possum mammary gland at parturition (results not shown) [10]. The changing concentration of sIgA (Cα and SC) and IgG across lactation was determined by Western blotting (Fig. 3). The results for a collection series from one of the possums analysed, with the addition of samples collected at days 0, 10 and 18 of lactation are shown in Figs. 3A and B. Analyses of samples collected at the beginning of lactation are shown in Fig. 3c, alongside day 40 and 131 milk samples from a second possum. It is not expected that removal of the PY from the teat will effect the final concentration of proteins in the milk sample collected at this or future time points.

Both Cα and SC were detected in all milk samples indicating that active secretion of dIgA into milk continues throughout lactation. The concentration of Cα at days 20–40 of lactation was ~1.6–1.9 mg/ml and at days 131–152, ~0.56–0.6 mg/ml. There was a higher level of Cα and SC, ~2- and 5-fold, respectively, in secretions obtained at the beginning of lactation (days 0–1) compared to the remainder of lactation (Fig. 3). This suggests that at the beginning of lactation a greater proportion of IgA is actively transferred and is consistent with elevated expression of

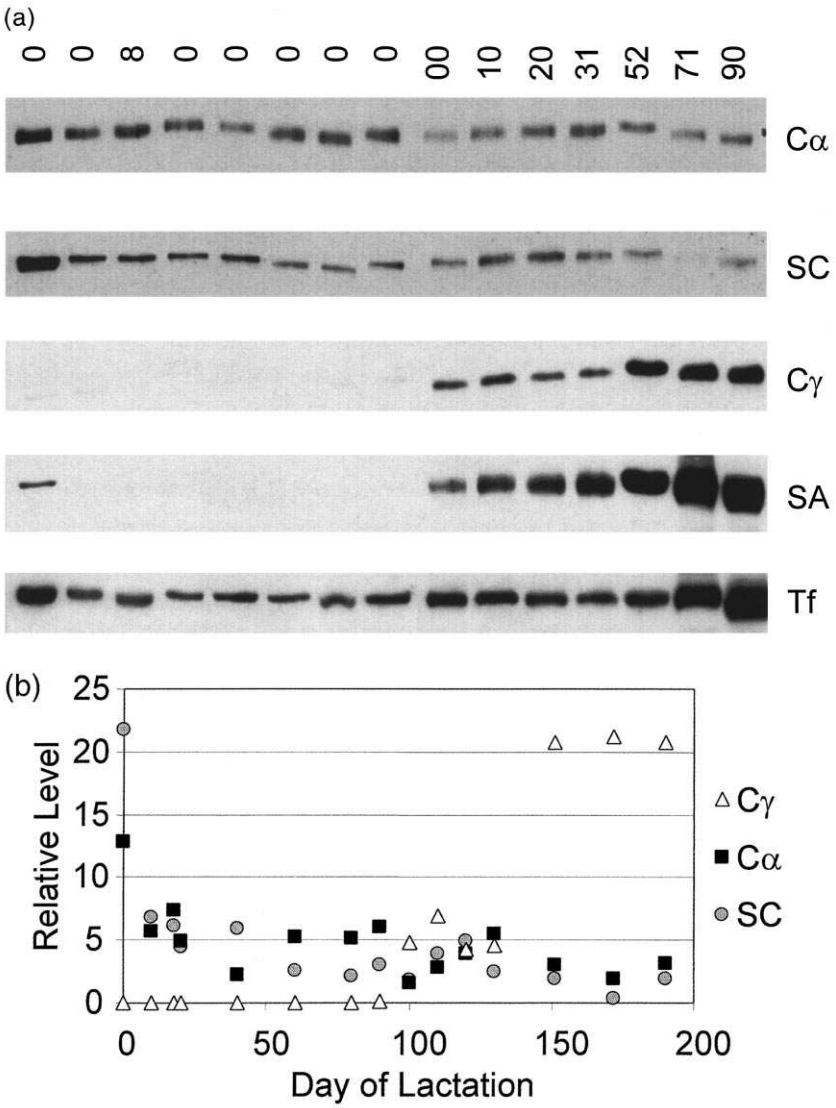

Fig. 3. Concentration of sIgA (Cα and SC), IgG, serum albumin (SA) and transferrin (Tf) in possum mammary gland secretions during lactation. Protein from 0.75 μl of each sample was separated by denaturing SDS PAGE and transferred to nitrocellulose membrane for Western blotting. **A**. Western blot analysing milk samples collected from a single possum (days 20–190) as well as samples collected at day 0, 10 and 18 of lactation. Western blot was probed sequentially for SC, Cα, Cγ and SA. A second blot was used to probe for Tf. The day of lactation is indicated. **B**. Graph showing how the concentration of SC, Cα and Cγ changes during lactation. The relative intensity of the bands in the Western blots (Fig. 3A) was used to determine the relative level of the proteins in the samples collected across lactation. **C**. Western blot to analyse samples collected between days 0–10 of lactation (from individual possums), along side milk samples collected at day 40 and 131 of lactation (from a single possum). Western blot was probed sequentially for SC, Cα, Cγ, SA and Tf. The day of lactation is indicated.

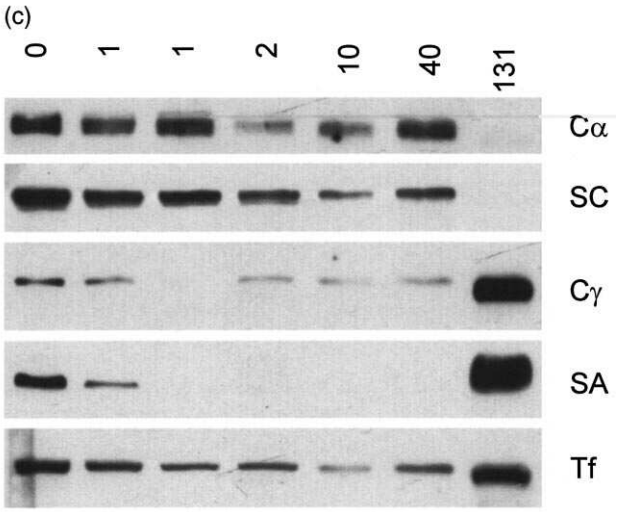

Fig. 3 (*continued*)

mRNAs encoding dIgA and pIgR in the mammary gland during the first week of lactation [21]. Expression of the pIgR protein in the mammary gland was high throughout the early lactation phase decreasing from the switch phase to below the level of detection by the end of lactation (Fig. 4). This suggests that active dIgA secretion into milk continued throughout the early lactation phase. Previous analysis of pIgR and dIgA mRNA expression in the mammary gland suggest that there is a second period of increased dIgA secretion into milk during the switch phase [21]. Milk samples were collected from three additional possums at 5-day intervals between day, 100 and 125 of lactation to determine if IgA concentration in possum milk was elevated at this time. No increase in Cα or SC concentrations in milk was observed during the switch phase. This apparent inconsistency between mRNA expression and milk concentration could be due to the increased milk production at this

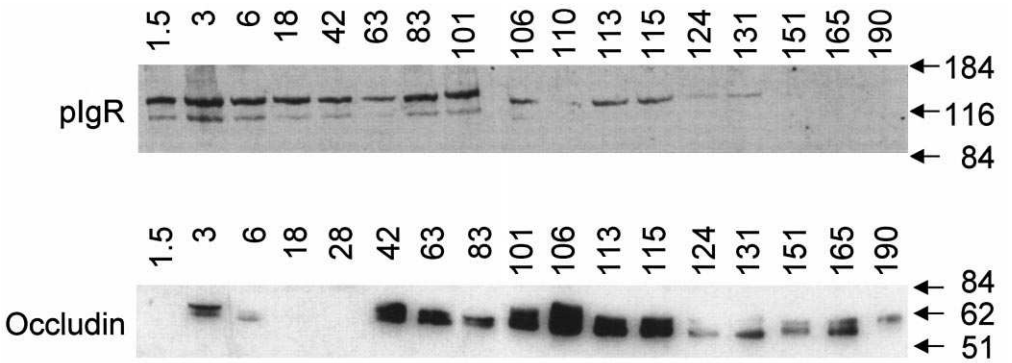

Fig. 4. Expression of the pIgR and occludin in the possum mammary gland during lactation. Total protein (1.4 μg) was separated by denaturing SDS-PAGE and transferred to nitrocellulose membrane for Western blotting. Separate membranes were probed for pIgR and occludin. Day of lactation and position of molecular weight marker (kDal) is indicated.

time having a dilution effect (results not shown).

The IgG milk concentration was low but constant throughout early lactation and was not elevated in secretions obtained at parturition (Fig. 3). The concentration of IgG in milk increased from the switch phase of lactation (from day 100) and this was confirmed by analysis of the possum series of milk samples collected at 5-day intervals through the switch phase (results not shown). A second significant increase in IgG milk concentration was observed at the end of lactation (days 152–190), however, this increase may be due to leakage from the serum due to increased permeability of the epithelia, a phenomenon observed at the end of lactation [27]. The concentration of Cγ at days 20–40 of lactation was 0.68–0.85 mg/ml and at days 131–152, 2.6–5.6 mg/ml. These concentrations of IgG in possum milk are similar to that observed for the tammar wallaby and hill kangaroo of approximately 1 mg/ml at the beginning of lactation and rising to about 6 mg/ml during late lactation [13,15].

3.3. Serum albumin and transferrin are components of possum colostrum

Grigor et al. [9] demonstrated that the concentration of both serum albumin (SA) and transferrin in milk increases significantly during late lactation. These results were confirmed in our experiments and the analysis was extended to mammary gland secretions obtained at the beginning of lactation (Fig. 3). Serum albumin was not detected in milk during early lactation except for days 0–1 and the increase in milk concentration during the switch-late lactation phases mirrored that observed for IgG (Fig. 3). A similar pattern of secretion was observed for transferrin except that transferrin is expressed at a significant level during early lactation (Fig. 3). This suggests that SA and transferrin are also important constituents of colostrum formed at the beginning of lactation (days 0–1), and during the switch phase (days 100–120; Fig. 3A).

3.4. Permeability of the mammary gland epithelia during lactation

Tight junctions are formed between mammary epithelial cells to provide a barrier between the blood and milk, thereby preventing exchange of components. It has been demonstrated that during the periparturient and involution stages of lactation the permeability of tight junctions increases allowing passage of molecules [27,28]. Thus, the increase in immunoglobulin concentration seen in milk at these times could be due to leakage when the integrity of the epithelia is compromised. The strength of the epithelial barrier can be determined by measuring serum lactose concentration, which increases when lactose is able to flux from milk into the blood. The serum lactose concentration was below detectable

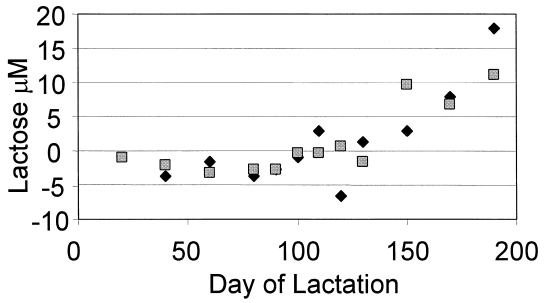

Fig. 5. Changes in serum lactose concentration during lactation in the possum. Graph showing the concentration of lactose in serum during lactation (days 20–190) for two possums (grey squares and black triangles).

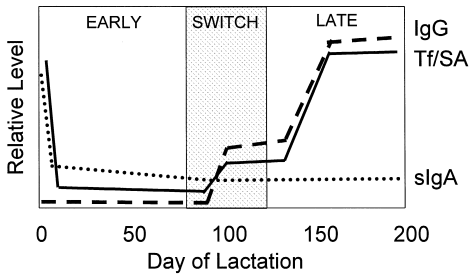

Fig. 6. Schematic diagram showing how the level of sIgA, IgG and transferrin/SA in milk changes with the stage of lactation in the possum. The bar indicates the sequential periods of the early, switch (shaded) and late stages of lactation. The changes in milk concentration of for sIgA, IgG and transferrin/SA during lactation are profiled (not to scale).

levels until day 110 of lactation and rose significantly from day 152 (Fig. 5). Expression of occludin, the major tight junction protein, in the mammary gland is indicative of tight junction formation and maintenance of the barrier. Occludin expression in the possum mammary gland was highest during the latter part of early and through the switch phases of lactation (Fig. 4). These results suggest that the integrity of the tight junctions was maintained during the initial period of increased IgG, transferrin and SA concentrations in milk (days 100–120). The second increase in milk concentration of IgG, transferrin and SA (days 152–190) is likely to be due to leakage, because the epithelia have become more permeable through the decrease in tight junctions.

4. Discussion

In the possum two periods of immune transfer via the mammary gland to the pouch young were identified. The first transfer is a colostral phase and is transitory, occurring at parturition (days 0–1 of lactation) and would provide the new born pouch young with immune protection at this critical time. The immune transfer appears to extend throughout early lactation at a lower level and may be important for protection of the developing pouch young. The second transfer of immunity occurs via the milk during the switch phase (from day 100) and could provide additional protection to the pouch young as it exits the pouch for the first time (second birth).

Four molecules, three with known functions in immunity, were secreted differentially during these periods of immune transfer in possum lactation (Fig. 6). The role of ingested sIgA and IgG are for immune protection of the gut and humoral system, respectively. The function of transferrin in immune protection is to sequester free iron making it unavailable to pathogens [29]. Serum albumin has not been attributed an immune function and a role for this protein in these secretions has not been identified.

Although active transport of sIgA (Cα and SC) into milk occurs throughout lactation in the possum it is elevated during the colostral phase (days 0–1; Fig. 6). Increased sIgA in mammary gland secretions at this time is consistent with previous work showing that expression of Cα, J chain and pIgR mRNAs is elevated in possum mammary gland at the beginning of lactation [21]. The presence of the SC in colostrum and milk and expression of pIgR in the mammary gland throughout lactation demonstrates that dIgA transfer is the result of active transport by the pIgR across the epithelial cells. Attachment of the secretory component to dIgA has a protective function, increasing the stability of dIgA in the gut [30]. The ratio of the IgA-heavy chain to SC in milk increases during lactation suggesting that IgA transfer occurs by additional mechanism(s). Previously we demonstrated that Cα, J chain and pIgR mRNAs were elevated in the mammary gland during the switch phase and suggested that sIgA formed part of a second phase of immune transfer at this time [21]. In this work, we were unable to detect an increase in milk sIgA concentration during the switch phase possibly the result of dilution due to increased production of milk.

Transfer of IgG during possum lactation was more complex. Although the milk concentration of IgG was not elevated in colostrum compared to the remainder of the early lactation phase, its concentration in milk throughout early lactation was significant. Marsupial pouch young are receptive to IgG ingested throughout the early lactation phase [20]. When immunoglobulins were ingested by a 50- and 98-day old pouch young these were subsequently detected in their serum. Both the tammar wallaby and hill kangaroo have low IgG concentration in mammary gland secretions obtained within 24 h after giving birth [13,19]. Although the serum of the wallaby fetus contains IgG, demonstrating placental transfer of this immunoglobulin, the IgG serum concentration of the neonates increased upon suckling for the first time [19]. Thus, IgG provided via the mammary gland to the marsupial pouch young during early lactation is being exploited for their immune protection.

The milk concentration of IgG increased during the switch phase (from day 100) of pos-

sum lactation and again near the end of lactation, from day 152 (Fig. 6). Given that milk production increases during the switch phase the actual amount of immunoglobulin transferred at this time will be greater than that indicated by the change in concentration. Elevated concentrations of serum proteins in milk can be the result of either an active mechanism or leaking through the epithelial cell layer. The later phenomenon is observed after peak lactation as the mammary gland begins to reduce milk production and remodel (involution) [27,28]. Expression of the tight junction protein occludin in the mammary gland remains high throughout the switch phase and serum lactose concentration does not increase until after day 130 of lactation. This suggests that during the initial increase in IgG milk concentration the integrity of the epithelial layer is intact and that IgG transfer was the result of an active process. Thus, this period represents a second phase of immune transfer during possum lactation. The second increase in IgG milk concentration occurs as the lactose concentration in serum increases and is likely to be the result from leakage though the epithelia from maternal serum into milk.

Increased IgG concentration in milk from the switch phase has also been observed during lactation in the tammar wallaby and hill kangaroo [13,15]. Receiving additional immunity from the mother during the switch phase is timely for the pouch young as they begin to leave the teat increasing their exposure to novel pathogens. In addition, their immune system is only beginning to be able to elicit a mature immune response [13–15].

A third pattern of immune transfer was observed for changes in milk concentration of transferrin and SA (Fig. 6). The concentration of both these molecules was elevated in the colostrum and decreased (undetectable for SA) for the remainder of early lactation. Their milk concentration increased again during the switch phase and near the end of lactation (from day 152) in the same manner as was seen for IgG and was consistent with previous studies on possum and hill kangaroo milk [9,13]. In addition, the pattern of changing transferrin concentration in milk during lactation mirrors that of transferrin mRNA expression in the possum mammary gland, being elevated at the beginning of lactation and again from the switch phase [31]. Thus, transferrin and SA are components of immunity transferred from the mother to the pouch young through the colostrum and during the switch phase.

The two periods of immune transfer would provide immunological protection of the pouch young when they are most vulnerable. The first period of transfer at parturition is especially important as their immune system has not developed and is unable to respond to stimulation [14]. The second period of transfer during the switch phase occurs when the pouch young begins to leave the pouch and would be susceptible to novel pathogens. How then are these periods of immune transfer induced in the possum? In the possum, there is a spike in circulating prolactin concentration just prior to birth, low circulating levels during the remainder of early lactation and this increases again at the beginning of the switch phase peaking in late lactation [32]. Expression of the prolactin receptor mRNA in the mammary gland mimics the pattern of serum prolactin concentration during lactation [33]. Interestingly, the same pattern of mRNA expression was observed for the pIgR, transferrin and late lactation protein in the possum mammary gland [21,31]. This suggests that there is a common mechanism for inducing their expression possibly through the prolactin receptor signalling pathways. Prolactin, as well as other mammotrophic hormones, is important for induction of lactation and colostrum formation. For example, localisation of dIgA producing lymphocytes to the mammary gland epithelia and expression of the pIgR is induced by prolactin [34,35]. In the possum, the first prolactin surge correlates with induction of lactation and colostrum formation at the beginning of the external gestation/early lactation phase. The unique composition and the low volume of milk expressed during early lactation is suited to the developing pouch young. The second surge occurs to induce lactation proper, with milk composition changing to resemble that of eutherian mammals and the

mammary gland developing to produce more milk. The two periods of immune transfer may reflect this phenomenon of inducing lactation twice. The benefit of immune transfer during the colostral phase is undoubted. Immune transfer during the switch phase may provide important immunity to the pouch young as it leaves the pouch or alternatively, it may be a remnant induced by hormones that bring about the change in lactation status.

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