Two Stages of Increased IgA Transfer During Lactation in the Marsupial, *Trichosurus vulpecula* (Brushtail Possum)

Frances M. Adamski and Jerome Demmer

*J Immunol* 1999; 162:6009-6015; 
http://www.jimmunol.org/content/162/10/6009

References

This article cites 35 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/162/10/6009.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Two Stages of Increased IgA Transfer During Lactation in the Marsupial, *Trichosurus vulpecula* (Brushtail Possum)\(^1,2\)

Frances M. Adamski\(^3\) and Jerome Demmer

The polymeric Ig receptor (pIgR) and J chain molecules are involved in the transfer of IgA across the mammary gland epithelia into milk. The J chain binds two IgA molecules to form dimeric IgA, and the pIgR transports this complex through epithelial cells. We report here the cloning of the first marsupial homologues for the pIgR and J chain from the brushtail possum. Marsupial young are born after a short gestation and are less developed than eutherian newborn. The pouch young is completely dependent on milk as its sole source of nutrition during early lactation and this phase can be considered to be equivalent to an external gestation. Two periods of increased expression of pIgR, J chain, and IgA heavy chain mRNAs were observed in the mammary gland during lactation. The first occurs for a brief period after birth of the pouch young and is likely to reflect IgA transfer via the colostrum. The second period of increased expression, which is unique to marsupials, occurs after the early lactation period and just before young exit the pouch. We propose that this represents a second colostral-like phase at the end of the external gestation.


Marsupial young are born after a short gestation, which in the possum is only 17 days (1). Their early pouch-life is considered to be an external gestation as many of the developmental changes that occur in utero for eutherian mammals take place during this time. In particular, lymphoid organs of the marsupial neonate are immature and pouch young are unable to elicit an immune response for some time after birth (2, 3). Lactation in the brushtail possum extends for ~200 days, during which milk composition and the pouch young’s behavior changes. In early lactation (first 80 days), the marsupial young is confined to the pouch where it remains attached to the nipple, receiving milk of unique protein, carbohydrate, mineral, and fat composition (4–6). During the switch phase (days 80–120 of lactation), the milk composition changes to become similar in composition to that of eutherian mammals. With the change in milk composition, growth of the pouch young increases, and they undergo changes such as fur growth, their activity increases, suckling is more intermittent (after day 80), and they begin to leave the pouch (after day 115).

At birth, eutherian young are immunologically naive and they receive immune protection by ingestion of Igs secreted into the colostrum until they are capable of mounting their own immune response. In mammals such as humans, where IgG is transferred predominately via the placenta, IgA is the major Ig transferred via the colostrum (7). For marsupials, little is known about Ig transfer via the placenta or mammary gland. In the newborn of several marsupials, no Igs were detected in their serum until they had suckled for the first time, suggesting that in these species there is only postnatal transfer of Igs (8–10). In contrast, Igs have been detected in the serum of newborn of the tammar wallaby (*Macropus eugenii*) before suckling (11). The transfer of Igs in utero has not been investigated for the possum. It has been shown that newborn possums are unable to mount a humoral response for at least 14 days after birth (12). Thus postpartum transfer of both IgG and IgA is likely to be important for immune protection of the possum young. A study of three brushtail possums demonstrated that Igs ingested by 50- and 98- but not 145-day-old pouch young were subsequently detected in their serum (13). This suggests that, although older pouch young can mount their own immune response, they remain receptive to the passive immunity offered by their mothers milk, at least into the switch phase of lactation.

Passively transferred IgA functions to protect mucous membranes (respiratory and gastrointestinal) of the newborn. In eutherian mammals, lymphocytes associated with mammary gland epithelium secrete IgA as a dimer joined by the J chain (14). Dimeric IgA is transported across the epithelial cells into the milk by the polymeric Ig receptor (pIgR)\(^4\) and is released at the apical membrane by proteolytic cleavage of the extracellular domain (secretory component (SC)) (15). It has been suggested that the SC protects dimeric IgA from proteolytic cleavage in mucosal environments (16). The possum IgA heavy chain (C\(\alpha\)) DNA sequence was published recently by Bevlov et al. (17).

Immune transfer to the possum neonate via the milk has not been studied in great detail. In this work, we have cloned and characterized the first marsupial homologues of the pIgR and J chain genes. Analysis of the expression pattern of the pIgR, J chain, and C\(\alpha\) mRNA suggests that there are two stages of increased IgA transfer during lactation in the possum, after birth and during the switch phase.

\(^4\) Abbreviations used in this paper: pIgR, polymeric Ig receptor; IgA heavy chain, C\(\alpha\); SC, secretory component; RACE, rapid amplification of cDNA end; CDR, complementarity-determining region.
Materials and Methods

RNA purification

Mammary gland total RNA. Mammary glands were collected as described previously (6), and the date of lactation was based on the size of the pouch young (18). The frozen mammary tissues were ground under liquid nitrogen by mortar and pestle, and RNA was extracted using the guanidinium acid phenol chloroform method (19) with the modification of reducing the recommended amount of tissue per volume of reagent by one half. White blood cell total RNA. RBCs were lysed by the addition of 12 ml of lysis solution (150 mM NaCl, 10 mM KHCO₃, and 0.1 mM EDTA) to 4.5 ml of heparin-treated possum blood, incubating on ice for 20 min. White blood cells were harvested by centrifugation and washed with 1× PBS before RNA isolation as described above.

RT-PCR and 5′ rapid amplification of cDNA end (RACE)

Random or oligo-dT primed cDNA was generated using the Superscript preamplification system (Life Technologies, Gaithersburg, MD) as per the manufacturer’s instructions. PCR was performed using Platinum Taq (Life Technologies) or Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The 5′ RACE was performed as described by Life Technologies using Superscript II reverse transcriptase and RNase H from Life Technologies and terminal transferase kit and RNase T1 from Boehringer Mannheim. Primer-specific cDNA was purified using the BRESAclean DNA purification kit (Bresatec, Thebarton, Australia). Upstream primers, abridged anchor primer and abridged universal amplification primer, used in the PCR reactions are as described by Life Technologies. All PCR products were cloned into pGEM-Teasy (Promega, Madison, WI), and at least two clones of each were DNA sequenced.

plgR. RT-PCR was performed with 5′ primer (GAG GCA AGA TGA AGA GAT CT) and 3′ primer (GGA TCC GYT GAY TGY TAY). PCR conditions were annealing at 37°C for 3 cycles, then 55°C for 27 cycles with 60s extensions. The 5′ RACE was performed with GSP1 (CTG GCA TAG AAA CTT GG), GSP2 (AGG GCA CAG TTC AAY AAR TG) and GSP3 (AGG CTC CAG CAA CTT TAA GTC). PCR conditions for PCR1 were annealing at 50°C for 10 cycles then 55°C for 20 cycles for 4-min extensions, and for PCR2 were annealing at 57°C for cycles with 2.5-min extensions.

J chain. RT-PCR was performed with 5′ primer (GAG GCA AGA TGA AGA GAT CT) and 3′ primer (GGA TCC GYT GAY TGY TAY). PCR conditions were annealing at 58°C for 25 cycles with 80s extensions.

IgA. RT-PCR was performed with 5′ primer (GAG GCA AGA TGA AGA GAT CT) and 3′ primer (GGA TCC TAY TGG TGY AAR TGG). PCR conditions were annealing at 37°C for 3 cycles, then 55°C for 27 cycles with 60s extensions. The 5′ RACE was performed with GSP1 (CTG GCA TAG AAA CTT GG), GSP2 (AGG GCA CAG TTC AAY AAR TG) and GSP3 (AGG CTC CAG CAA CTT TAA GTC). PCR conditions for PCR1 were annealing at 50°C for 10 cycles then 55°C for 20 cycles with 4-min extensions, and for PCR2 were annealing at 57°C for 26 cycles with 2.5-min extensions.

Library screening

The EcoRI-EcoRI DNA fragments from clones containing plgR and J chain RT-PCR products were purified and used to generate ³²P-radiolabeled probes with the Redprime random primer labeling kit (Amersham, Aylesbury, U.K.). These were used to screen the possum early lactation mammary gland cDNA library (20) using methodology previously described (21). Recombinants were purified to single plaques and the internal plasmid (pBS; Stratagene, La Jolla, CA) was excised with ExAssist helper phage (Stratagene) following recommended procedures. The resulting plasmids were characterized by DNA sequencing.

DNA sequencing

DNA sequencing reactions were conducted by the DNA Sequencing Unit (University of Waikato, Hamilton, New Zealand) using an automated DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequence data was collated using the SEQUENCE II program of the Lasergene software package (DNASTAR, Madison, WI).

Northern blotting

Total RNA (6 μg) was denatured in the presence of formamide and formaldehyde and then resolved by gel electrophoresis in 1.5% agarose, 0.6 M formaldehyde gels (22). RNA was transferred to Hybond N membrane (Amersham) by capillary blotting. Blots were hybridized overnight at 55°C with ³²P-radiolabeled cDNA probes in Church and Gilbert hybridization solution (22) following 30 min prehybridization. Blots were washed in 2× SSC/0.1% SDS at 55°C, then 1× SSC/0.1% SDS for 20 min each. Blots were exposed to Kodak XAR-5 film (Rochester, NY) at −80°C with two intensifying screens. The possum 18S rRNA probe is an unpublished DNA fragment (400 bp) amplified by RT-PCR (J. Demmer, GenBank AF089722).

Results

Isolation of possum plgR gene

A 156-bp DNA fragment corresponding to the possum plgR was amplified by RT-PCR using mammary gland total RNA (28 days lactation) and oligonucleotide primers designed to conserved regions of the plgR coding sequence. This fragment was used to screen an early lactation possum mammary gland cDNA library, and 12 positive cDNA clones were isolated. Of these, three recombinants containing different-sized inserts were characterized by DNA sequencing. All three recombinants contained plgR sequence, and the DNA sequence of the largest cDNA clone (2066 bp) was determined for both DNA strands. This recombinant contained the majority of the coding sequence (from nt 782, Fig. 1).

The remainder of the coding and 5′ untranslated sequences were obtained by the 5′ RACE procedure. The possum plgR is a 733-aa polypeptide including the 17-aa signal peptide. It shares 44–48% amino acid identity to human, murine, bovine, and rat plgR sequences, which is lower than that observed between these species (56–80%) with the exception of the rabbit plgR sequence, which has low homology with the possum plgR (40%) as well as eutherian plgR sequences (43–47%).

The marsupial plgR sequence was compared with that of eutherian species to determine whether or not important functional domains have been conserved. The possum plgR contains all five extracellular Ig-like domains, including the cysteine residues involved in intramolecular disulfide bonds (Fig. 1). The 23-aa residue polymeric Ig-binding domain, located in the first Ig-like domain, is involved in the initial noncovalent interaction with polymeric Ig (23). In the case of dimeric IgA, this is followed by the formation of a disulfide bridge between one of the Cα chains and the fifth Ig-like domain of the plgR (24). In the possum plgR, both the plg-binding domain (Fig. 2A) and the cysteine residues involved in the formation of the covalent interaction are conserved (Fig. 1, circles). This suggests that the mechanism for receptor-Ig interaction has been conserved in marsupials.

The possum plgR transmembrane domain is also highly conserved within the 10-aa core (Fig. 2B, boxed region). It has been suggested that this region may be involved in transducing a signal, generated by ligand binding, to the cytoplasmic membrane leading first to endocytosis and then transcytosis of the receptor to the apical membrane (25). The region of lowest sequence conservation was in the linker region (domain VI; Fig. 1) connecting the Ig-like and transmembrane domains. This region is the most variable between all the plgR sequences with respect to size and sequence (25).

The plgR cytoplasmic domain contains signaling sequences for basolateral targeting, endocytosis, and transcytosis (26, 27), and this region of the possum plgR is highly conserved with eutherian sequences (68–78% identity). Signals required for basolateral targeting of the de novo plgR reside in a region proximal to the transmembrane domain. Three amino acid residues have been previously defined as being important for basolateral signaling in the rabbit plgR (Fig. 2C, underlined sequence) (28). However, these three residues are not conserved in the possum plgR, suggesting that either the possum receptor uses a different mechanism for basolateral targeting or that the basolateral signaling sequence is larger than that previously characterized. Interesting, amino acids immediately distal to these residues are highly conserved between...
FIGURE 1. Possum plgR: DNA and deduced amino acid sequence. Nucleotide and amino acid numbers are indicated to the right. The signal peptide is italicized, and the transmembrane region is in bold. Boundaries of the Ig-like domain and the domain linking these and the transmembrane domain are numbered (I–V and VI, respectively). Conserved cysteines are indicated by asterisks, and cysteines involved in bringing about the covalent bond between the first of these cysteines and the Ig heavy chain are circled. The basolateral targeting (baso) and endocytosis (endo) signal sequences are indicated. GenBank accession no. AF091137.
Cloning of the possum Ca variable domain

The possum Ca variable and constant domains were amplified by 5’ RACE and RT-PCR, respectively, using total RNA from possum white blood cells and oligonucleotide primers based on the known possum Ca constant domain nucleotide sequence (17). The DNA sequence was determined for two clones containing the variable domain, and a high degree of amino acid variation was found in the complementarity-determining regions (CDR1, CDR2, and CDR3-D-J) (V-1 and V-2; Fig. 4). As expected, there were also amino acid differences in the framework regions and in the leader peptide sequence. The CDR3-D-J region is assembled by recombination of the germ line V (variable), D (diversity), and J (joining) segments resulting in the differences in amino acid identity and length seen in this region. The amino acid sequence of the constant domains (CH1–CH3) of the New Zealand possum (C-2) differed from that obtained from the Australian possum (C-1) at three amino acids: Asn144 to Asp, Arg 193 to Gly, and Asn 399 to Lys (Fig. 4). A recent study of the V\textsubscript{\textbeta} repertoire of the marsupial Monodelphis domestica (South American short-tailed opossum) demonstrated the presence of two V\textsubscript{\textbeta} families that are phylogenetically most similar to the group III family in vertebrates (33). The variable domains of the possum Ca are most homologous to the MdoV\textsubscript{\textbeta1} family (results not shown).

Expression of the IgA, J chain, and plgR mRNA in the mammary gland during lactation

Dimeric IgA is expressed by lymphocytes associated with the mammary gland epithelium. The expression pattern IgA in the mammary gland during lactation was determined by analysis of Ca and J chain mRNA expression. Northern blots of total RNA extracted from mammary glands at different stages of lactation were hybridized with the J chain and IgA probes (Fig. 5A). The expression of both these mRNAs was increased for two short periods during lactation: at the beginning of lactation (days 1.5–6) and, unexpectedly, again in the switch phase (days 106, 110, and 115). Expression of the J chain mRNA during early lactation (days 31–94) was demonstrated using RT-PCR (Fig. 5C). This suggests that there is expression of dimeric IgA during this period of lactation, albeit at a low level.

If the transfer of IgA into milk is increased for these two periods of lactation, then expression of the plgR, which transports dimeric IgA across epithelial cells, is also likely to be increased at these times. The plgR mRNA was found to be expressed throughout lactation (Fig. 5A). Importantly, we find that there are two periods during lactation when expression of this mRNA was increased: at the beginning of lactation (days 1.5–6) and again from the switch phase of lactation until the end of lactation (days 106–189). It

Isolation of possum J chain gene

A 400-bp DNA fragment corresponding to the possum J chain was amplified by RT-PCR using possum mammary gland total RNA (18 days lactation) and oligonucleotide primers designed to conserve regions of the J chain. This DNA fragment was used as a probe to isolate three cDNA clones from the early lactation possum mammary gland cDNA library. Two of these contained the entire J chain coding sequence, and the DNA sequence was determined for the largest cDNA clone (1620 bp; Fig. 3). The possum J chain is 160 aa in length and has a leader peptide of 22 aa. The possum J chain amino acid sequence has 63–68% identity with eutherian sequences, which is similar to that observed between eutherian species (67–80%). The cysteine residues that form intra- and intermolecular disulfide bonds are conserved (Fig. 3) (32).
would appear that the pattern of pIgR mRNA expression coincides with that of the J chain and Ca, with expression being highest at the beginning of lactation and again from the latter part of the switch phase.

Discussion
This report describes the cloning and characterization of the first marsupial pIgR and J chain cDNAs from the brushtail possum. The pIgR is involved in transport of dimeric IgA across epithelial cells and the J chain functions to dimerize IgA molecules (14, 15). Both of these possum genes are highly conserved with those of eutherian mammals. The mRNAs of pIgR, Ca, and J chain were found to be expressed in the mammary gland throughout lactation, suggesting that there is continuous transfer of IgA into possum milk. Expression of Ca and J chain mRNAs were dramatically increased at two specific periods of lactation, which are summarized in Fig. 6. The first period was at the beginning of lactation, and this would be consistent with IgA transfer via the colostrum as seen in eutherians. The second period of increased mRNA expression occurred...
for a short period (days 106–115) toward the end of the switch phase (80–120 days of lactation). Expression of the plgR mRNA was also increased at these two stages of lactation, with the level of expression at the second period remaining elevated to the end of lactation (Fig. 6). The second period of increased expression of these mRNAs is likely to represent a second period of enhanced IgA transfer via the milk. This second period coincides with the end of the external gestation period of the pouch young’s life and could be thought of as a second colostral phase. Alternatively, the timing of this second period of IgA transfer, just before pouch exit, suggests the intriguing possibility that this could be equivalent to the placental Ig transfer that occurs during late gestation in some eutherian mammals. Both periods of increased IgA transfer would occur when the pouch young are exposed to new pathogens. Indeed, there is a high rate of mortality in the possum pouch young at birth and around the switch phase (34), and increased protection at these stages would be advantageous.

The presence of IgG and IgA in possum milk has not been reported, but is inferred from experiments describing passive transfer of immunity to the neonate and the presence of IgA in the milk of other marsupials (10, 11, 13, 35). We have detected both IgG and IgA in possum milk (Adamski et al., unpublished observations), and although no information regarding their concentration in milk is available, it is likely that both play a role for immune protection of the pouch young. Interestingly, a dramatic rise in the IgG serum concentration was seen at the switch phase in the hill kangaroo (Macropus robustus) pouch young, subsequent to the pouch young beginning to leave the teat and before pouch exiting (36). This increase in serum Ig concentration is thought to reflect the pouch young’s immune response, but may also result from an increase in immune transfer via the milk. The importance of these two periods of immune transfer to the survival of the possum pouch young will require detailed analysis of IgA and IgG concentration in the colostrum and milk throughout lactation.

Colostral secretion of IgA is likely to be controlled by mammotrophic hormones affecting the expression of receptors and IgGs. In mice administration of prolactin, progesterone and estrogen resulted in the development of the mammary epithelia with a concomitant increase in the number of associated IgA-secreting plasma cells and intraepithelia IgA (37). Interestingly, in marsupials including the possum there are two periods when serum concentration of prolactin is elevated, which coincide with increased dimeric IgA and plgR expression in the mammary gland (38). There is a short peak before initiation of lactation and a second period of prolonged elevated serum prolactin concentration around the switch phase, increasing from approximately day 100 in the possum. An alternative possibility is that dimeric IgA expression is stimulated locally by the introduction of novel pathogens by the pouch young. The induction of an IgA Ab response by local challenge of the mammary gland is characteristic of eutherians and has been demonstrated in the quokka, Setonix brachyurus (35). However, this latter explanation would not explain the second period of increased plgR mRNA expression.

In summary, we have cloned and characterized the plgR and J chain of the brushtail possum. The expression pattern of the J chain, Cα, and plgR mRNA during lactation suggests that there are two stages of increased immune transfer in the possum at the beginning of lactation and toward the end of the switch phase. We propose that these represent two colostral phases during lactation, the second being unique to marsupials.

Acknowledgments

We thank Susan Stasiuk for excellent technical assistance, Dr. Tim Day for sampling of possum blood, and Dr. Murray Grigor for helpful discussions.

References


