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Development of the immune system and immunological protection in marsupial pouch young

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Abstract

At birth the tissues of marsupial immune system are underdeveloped. The young animal is not immunocompetent. Histological and immunohistochemical studies of pouch young epithelial tissues provide a clear picture of tissue development but the timing of onset of immunocompetence awaits definition. The survival of the neonatal marsupial in a microbially rich environment is dependent on maternal strategies, including immunoglobulin transfer via milk and, in some species, prenatally via the yolk sac placenta. It is also likely that pouch secretions play a role. This review summarizes our current knowledge of the pathway of immunological development in marsupials and the protection and threats afforded by the pouch environment.

Keywords: Ontogeny; Marsupials; Immunocompetence

1. Introduction: the adult metatherian immune system

From an anatomical perspective adult marsupials possess an array of primary and secondary lymphoid tissues similar to those seen in eutherian mammals. The thymus, a pivotal organ in immune function, was first described by Symington [1] and Johnstone [2] in a range of marsupials including *Didelphis pusilla, Thylacinus* spp, *Dasyurus viverrinus, Antechinomys lanigera, Parameles gunni, Trichosaurus vulpecula* and *Phascolarctos cinereus*. It is now clear that the anatomical location of the thymus varies amongst major taxonomic groups within the Marsupialia. All the American and the Australian polyprotodont species (Caenolestidae, Dasyuridae, Didelphidae, Thylacinidae, Notoryctidae and the Peramelidae) possess only a thoracic thymus, while most diprotodonts (Burramydae, Phalangeridae, Petauridae, Phascolarctidae, Tarsipedidae and the Macropodidae) but not the Vombatidae, possess both a cervical and thoracic thymus. Yadav [3] attributed the presence of a cervical thymus to the small, restrictive

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size of the thoracic cage in the developing quokka (*Setonix brachyurus*), whilst Ashman et al. [4] believed it could be attributed to the presence of a deep, moist pouch and associated microbiologically enriched environment. Whatever the reason for this dichotomy, in animals that possess both a cervical and thoracic thymus it is the former which is the larger and more important.

There also appear to be some differences in the number and distribution of lymph nodes in different marsupials. Within the group of New World polyprotodont opossums, Azzali and Di Dio [5] failed to detect significant numbers of lymph nodes associated with the small and large intestine in both male and female *Didelphis azare* and the common opossum (*D. marsupialis*). They concluded that the lymphatic system of these marsupials was not as developed as eutherians of the same size. In contrast, Bryant and Shrifine [6] reported that the pouchless opossum (*Marmosa mitis*) had 27 mesenteric lymph nodes, *D. azare*, 44 and the Virginian opossum (*D. viginiana*), 45. Stone et al. [7] reported difficulties in locating mesenteric nodes in the grey short-tailed opossum (*Monodelphis domestica*) and a comparable situation was reported for the Australian dusky antechinus (*Antechinus swansonii*) and the brown antechinus (*A. stuartii*) by Poskitt et al. [8] but not for the koala (*P. cinereus*), the ringtail possum (*Pseudocheirus peregrinus*) or the brushtail possum (*T. vulpecula*) [9]. Hanger and Heath [10] described the mesenteric lymph nodes of the koala as being oval in shape, smaller in size, but greater in number than those found in the opossum but fewer than observed in macropods.

Histological, and more recently, immunohistochemical studies, have been conducted on lymphoid tissues of a number of marsupials. Block [11] described the spleen of adult Virginian opossum as primarily a myeloid organ, with unusual sinusoid-like areas, whilst Hayes [12] reported tissue with extensive trabeculae and nodular masses separated from the red pulp by a marginal zone and populated primarily by lymphocytes. Coutinho et al. [13] examined the spleen of the white-eared opossum (*D. albiventris*), using cross reactive antibodies to the B-cell markers, CD79 a and b, and the T-cell marker, CD3 and to HLA-DR. They found HLA-DR positive cells and CD3 positive T-cells in the germinal centres and scattered through the splenic cords, while B-cells were distributed at the periphery of the follicles and in the splenic cords and sinuses. Poskitt et al. [8] found the spleen of the Australian polyprotodont, the brown antechinus, to have well structured red and white pulp comparable to eutherian mammals. A similar situation has been described in the tammar wallaby [14]. In contrast, Stone et al. [7] described poorly developed sinuses in the spleen of the grey short-tailed opossum.

Histological studies on nodal tissue, other than that associated with the alimentary tract, are few. Haynes [15] described the cervical lymph nodes of the adult fat tailed dunnart (*Sminthopsis crassicaudata*) as having both a subcapsular and peritrabecular sinus and medullary cords and sinuses, but containing large numbers of mast cells, particularly in the medullary cords. Pabst and Gehrke [16] reported a dense accumulation of lymphocytes in one female *D. domestica* in the position where tonsils would normally be found. Likewise Bryant and Shrifine [6] observed tonsils in the pouchless opossum and Hemsley et al. [9] reported tonsils, similar to those found in eutherians, in the ringtail possum, the brushtail possum and the koala. Gut associated lymphoid tissues consisting of aggregated lymphoid follicles in the Peyers Patches of the small intestine have been observed in the opossum, the white-eared opossum [17] and the koala [9]. In the same study caecocolic lymphoid patches were also detected. They had well developed caps, but the domes were not prominent. In the koala intrafollicular invaginations were observed but were not present in either of the possums. CD3/ CD5positive T-cells were present throughout the epithelium and CD79b positive B-cells were detected in the epithelium and the cap and dome regions [9].

Coutinho et al. [17] using the cross reactive antibodies described above, observed that the mesenteric nodes of adult white-eared opossum appeared to lack a dome region, whilst the lymphoid follicles themselves were enclosed by lymphatic sinuses and lacked a mantle zone. In

contrast Hemsley et al. [9] observed distinct mantle zones in the mesenteric lymph nodes of the ringtailed possum, the brushtailed possum, and the koala. They concluded that the cellular distribution resembled that seen in eutherian mammals.

Marsupials produce an array of immunoglobulins similar to those seen in eutherian mammals. IgM and IgG isotypes have been detected in the Virginian opossum [18], the brushtail possum [19], the quokka [20], the koala [21], and in the macropods, the hill kangaroo (*Macropus robustus*), the grey kangaroo (*M. giganteus*) and the tammar wallaby (*M. eugenii*) [22]. IgA has been detected in milk of the brushtail possum [19] the quokka [20] and the tammar wallaby [22]. Most recently the genes encoding IgG, IgM , IgA and IgE and been isolated and sequenced in the tammar wallaby, the brushtail possum and the grey short-tailed opossum [23–25], as have the genes regulating junctional diversity in immunoglobulins, RAG1 and TdT [26,27] and the polymeric Ig receptor and the J chain of IgM and IgA [28]. In our laboratory we have recently sequenced the constant region of the T-cell receptor α and β genes [29] and, as documented in others papers in this volume, sequence data has now been obtained for some of the immunoregulatory cytokines.

In 1993 Lynch et al. [30] reported the isolation of a putative complement component 3-like protein from the quokka and recently the presence of a classical and alternate system of complement activation, similar to that seen in eutherian mammals, has been documented in the adult grey short-tailed opossum [31]. However, most functional studies of the marsupial immune system have provided problematic and often inconsistent results. Summarized in a review by Stone et al. [7] these studies suggest a limited capacity for allogenic reactivity, a poor capacity for immunoglobulin class switching and an apparent lack of the memory response. In addition, the level of immunoglobulin production appears to be lower in marsupials. Low secondary and anamnestic responses to bacterial flagella and bacteriophage antigens have been reported in the grey short-tailed opossum [32], the quokka [33] and the koala [34]. In these animals the average titre observed in the secondary response was 1.5 times that of the primary response compared to 100-fold increases seen in eutherians.

Immunoglobulin class switching appears to be delayed and reduced in marsupials compared to eutherians. Rowlands [35] found that IgM dominated the secondary responses of the Virginian opossum. Low levels of IgG were detected 40 days after primary immunization and then only after the animal had received a second immunization. Similar observations have been made in the grey short-tailed opossum [32,36]. Marsupials appear to respond differently to antigens presented as hapten–carrier complexes. Both the grey short-tailed opossum [7] and the quokka [37] display a secondary response which is independent of the carrier.

Recently, studies of experimental infection of the brushtail possum with *Mycobacterium bovis* have suggested a lowered capacity for both a humoral [38] and cell-mediated response [39].

However, most work of immune function was undertaken nearly 30 years ago and has been largely confined to the American polyprotodonts. The current availability of immunohistochemical and molecular protocols, provides the tools for a reinvestigation of these phenomena, particularly in Australian species.

2. Lymphoid tissue development

Marsupials are born after a short gestation in a relatively immature state which has been likened by some researchers to the foetal equivalent of a eutherian mammal. Gestation periods vary from as short as 11 days for the striped-faced dunnart (*S. macroura*) and 13 days for the Virginian opossum, to 32 days in the grey kangaroo, and 35 days in the koala [40].

Studies on the development of primary and secondary lymphoid tissues have been conducted using histological techniques in the quokka [4,41]; the Virginian opossum [11], the opossum [6] and the tammar wallaby [14,42]. Recently immunocytochemical techniques have been used to document specific T- and B-cell populations as

Table 1
Summary of key events in the development of the lymphoid tissue of two American and three Australian marsupials

	Polyprotodonta		Diprotodonta		
Day	*D. virginiana* [11]	*M. mitis* [6]	*S. brachyurus* [4,41]	*M. eugenii* [14,42]	*T. vulpecula* [44]
0	No mature lymphoid tissue Haematopoiesis in liver Primitive cells in blood	No mature lymphoid tissue Haematopoiesis in liver	No mature lymphoid tissue Haematopoiesis in liver	No mature lymphoid tissue Haematopoiesis in liver	No mature lymphoid tissue
1	Lymphocytes in thymus	Lymphocytes in thymus			
2		Lymphocytes in thymus	Lymphocytes in cervical thymus	Lymphocytes in cervical thymus	$CD3^+$-lymphocytes in thoracic thymus
3					
4		Hassalls corpuscles in thymus	Lymphocytes in thoracic thymus	Lymphocytes in nodes	
5 to 7	Hassalls corpuscles in thymus Myeloid tissue in spleen Lymphocytes in nodes		Lymphocytes in nodes Myeloid tissue in spleen		
12		Cortex and medulla in nodes	Hassalls in corpuscles in cervical thymus	Lymphocytes in spleen	
14			Lymphocytes in spleen		
21	Lymphocytes in spleen	Splenic follicles appear	Cortex and medulla in nodes	Cortex, medulla Hassalls corpuscles in cervical thymus	Cortex, medulla in thymus, Large population $CD3^+$ lymphocytes
28	Cortex and medulla in thymus and nodes. GALT present		Cortex and medulla in cervical thymus. Red and white pulp in spleen	Cortex, medulla in thoracic thymus and nodes	Hassalls corpuscles in thymus (day 25)
60	Germinal centres in nodes	Germinal centres	Cortex and medulla in thoracic thymus. GALT present	Germinal centres in nodes Red and white pulp in spleen	
90		Peyer's patches detected 90 days		Lymphocytes in gut	Peyer's patches detected (73 days)

they appear in the white-eared opossum [13], the koala [43] and the brushtail possum [44]. Table 1 compares the developmental pathways observed in these animals.

Despite the significant differences in the length of gestation, birth size and duration of pouch life, all the animals studied followed a similar developmental progression with only small differences in the timing of key events. At birth the liver was the main haematopoietic tissue. No mature lymphoid tissue was apparent and blood contained significant numbers of immature erythroblasts and leucocytes. Unfortunately no immunohistochemical studies have been conducted on the liver or blood of newborns to permit more exact identification of cell types. In tammars, high levels of neutrophils have been detected in blood in the first 2 weeks of postnatal life, and it has been argued that their presence is associated with the need for strategic, non-specific immunological protection [14].

The thymus is the first lymphoid tissue to develop and, in those animals which possess both a cervical and thoracic thymus, it is the former which assumes the dominant role. Lymphocytes first appeared in the thymus during the first few days of life. In both the white-eared opossum and the brushtail possum, CD3 positive T-cells were detected as early as 2 days postpartum [13,44]. By 25 days of age, the thymus of the brushtail possum had differentiated into cortex and medulla and Hassell's corpusles could be observed. The tissue was also densely populated with CD3 positive T-cells and CD79b and IgG positive B-cells [44].

Histological studies show that lymphocytes first appeared in the lymph nodes of the tammar, the quokka and the Virginian opossum between days 4 and 7 after birth (summarized in [42]). While the techniques of immunohistochemistry demonstrated the presence of CD3 positive T-cells at day 48 in the brushtail possum and in 75 mm white-eared opossum young. The subsequent formation of cortex and medulla in lymph nodes occurred first at days 10–12 in the Virginian opossum, at day 14 in the quokka and at day 30 in the tammar. Germinal centres in nodes were not apparent until 2–4 weeks later [13].

Myeloid tissue was first observed in the spleen at day 4 in the Virginian opossum, days 5–7 in the quokka and day 12 in the tammar. No CD3 positive T-cells or CD 79b/Ig positive B-cells were detected in either the brushtail possum or the white-eared opossum at birth. Distinct areas of T- and B-cells were not seen until 48 days postpartum in the possum and in 80 mm opossums [13,44]. In tammars such structures were detected at 50 days with adult-like tissue present by 60 days [42].

It is of interest that in newborn white-eared opossums HLA-DR positive cells were detected in the skin around the face and forelimbs but were not present in any other area of skin or in lymphoid tissue [13].

In general, adult tissue structure, and hence implied immunocompetence, was not seen until about half the period of pouch life has elapsed. In tammars this is at approx. 120 days postpartum and coincides with first release of the maternal teat. This situation appears to be the same in both the white-eared and Virginian opossums and the quokka. However, on the basis of immunohistochemical evidence, Baker et al. [44] suggest that the brushtail possum may be able to mount a B-cell response by 48 days postpartum.

3. The development of immunocompetence

The development of immune competence has been documented through analysis of both cell mediated and humoral responses. These studies have been restricted to a few model animals — the quokka, the Virginian and grey short-tailed opossums and the tammar wallaby.

Cell-mediated responses have been studied both in vivo, through responses to graft rejection, and in vitro, through responses of cultured lymphocytes to mitogens and allogenic lymphocytes.

Adult quokkas, opossums and tammars all demonstrate first and second set rejection of skin and tissue grafts [45–49]. The pouch young of the Virginian opossum can accept maternal skin grafts made up to 12 days postpartum. After this

time, however, tissue is rejected suggesting an onset of T-cell responsiveness [45]. In the quokka, Ashman [50] successfully transplanted thymus until 30 days postpartum and Waring et al. [51] reported that successful grafting could occur in animals up to 40 days of age. These graft-rejection studies are limited and somewhat confounded by thymectomy studies. Removal of the thymus in the Virginian opossum, at 10–12 days postpartum, resulted in depressed lymphocyte counts in tissue beds and peripheral circulation [52]. In the quokka removal of the cervical thymus before day 5 [4] or day 10 [46] had significant impact on peripheral lymphocyte counts but ultimately little effect on immune function. In addition, Stanley et al. [33] demonstrated that cervical thymectomy delayed the onset of humoral responses but had no impact on graft rejection in the quokka. These confounding observations have been summarized and analysed in a previous paper [53] and still require investigation.

Proliferative responses to mitogens and allogenic lymphocytes have been examined in a number of adult marsupials. Exposure to the mitogens phytohaemagglutinin (PHA), concanavalin A (ConA) and pokeweed mitogen (PWM) elicited responses in lymphocytes isolated from the quokka [54], the grey short-tailed opossum [55], the koala [56] and the tammar wallaby [unpublished]. In contrast, responses to culture with allogenic lymphocytes were weak or nonexistent in all marsupial species studied including the Virginian opossum [57], the koala [56] and the the grey short-tailed opossum [55,58].

In vitro studies on pouch young lymphocyte responses to mitogens and mixed lymphocyte culture (MLC) have been limited by the size and circulating blood volume of these animals. Baker et al. [44] documented the responses of isolated lymphocytes from older brushtail possum pouch young to the mitogen PHA. They recorded a rise in proliferative response as the animal aged with levels of 83,405 cpm at 100 days rising to 145,425 cpm at 300 days (stimulation indices not given). In contrast, in our laboratory we have used recently developed whole blood techniques to examine both mitogen and mixed lymphoctye responses (MLR) in pouch young tammar wallabies from 7 days to pouch emergence. At day 7 a stimulation index (SI) of 1.9 was observed in response to the mitogen PHA and an SI of 3.8 obtained in mixed lymphocyte culture. This rose to an SI of 12 at day 49 for the mitogen while the SI for MLR remained low at 5. Such responses were comparable to the levels obtained using whole blood from adult animals.

It has been argued that the low level of MLR in marsupials is due to low levels of variability at the major histo-compatibility complex (MHC) Class II loci. In 1976 Fox et al. [57] suggested that *D. virginiana* completely lacked an major histo-compatibility complex (MHC) (Class II) equivalent, given the lack of MLC responsiveness by adult lymphocytes. Using Restriction Fragment Length Polymorphism (RFLP) and southern hybridization analysis, the genes encoding the MHC Class II have been identified in the tammar wallaby [59] and the red-necked wallaby (*M. rufrogriseus*) [60]. Stone et al. [61] demonstrated two MHC Class I loci in the grey short-tailed opossum, whilst Houlden et al. [62] have documented these loci in the koala.

Studies on the capacity of pouch young to mount an humoral immune response have examined both the level and specificity of immunoglobulins present in serum. While immunoglobulins could not be detected in the serum of either newborn Virginian opossums [63] or the quokka [64], opossums aged 8–15 days produced specific immunoglobulins in response to bacterial antigens [65] and to viral antigens by 17 days of age [66]. In the quokka immunization with sheep red blood cells (RBC) elicited immunoglobulin production at 10 days postpartum. In both these animals these responses correlate with the appearance of Hassalls corpuscles in the thymus but occur before mature lymph node tissue is apparent.

4. The pouch environment and maternal protection

The major period of growth and development of marsupials occurs in the maternal pouch or '*marsupium*'. The anatomical structure of the

'pouch' is not consistent across all marsupial groups and varies from a region of underbelly in polyprotodonts, such as in the dasyurids, to the deep moist pouches of diprotodonts like the macropods. In the latter, pouch cleaning, via licking, immediately prior to the birth of the young has been reported and is presumed to reduce bacterial flora. In 1973, Yadav [67] documented the bacterial flora of the pouch of the quokka and raised the possibility of a relationship between birth of a pouch young and changes in the pouch normal flora. He postulated two mechanisms; maternal cleansing via licking and pouch secretions. Subsequently, both Charlick et al. [68] and Old and Deane [69] conducted both a qualitative and quantitative study of microflora changes over oestrus in the quokka and in the tammar wallaby. Both studies noted the persistence of micro-organisms in the pouch, even at the time of birth of the young, although both documented a decline in both abundance and diversity. In contrast, Osawa et al. [70] in a study of bacterial flora of the koala pouch, found that absence of isolates was the norm and presence of culturable bacteria was associated with death of the young.

Clearly, the pouch environment needs to be more closely scrutinized, not only because of the obvious inconsistencies in documenting the microbial threat to the young animal but also to ascertain if the pouch epithelium itself is responsible for modulating microbial growth.

Maternal protection of the neonatal marsupial appears to occur primarily through immunoglobulins secreted into milk. Immunoglobulins could not be detected in the serum of newborn Virginian opossum [63], the quokka [64] and the grey short-tailed opossum [71], but IgG was detected in foetal and newborn sera of the tammar wallaby [72,73]. However, in all these animals the level of IgG rose after suckling and it has been concluded that such serum immunoglobulin is maternally derived. It seems likely that marsupials, like their eutherian counterparts, vary in their capacity for pre- and postnatal transfer of immunoglobulin to their young [74]. The specificity of immunoglobulins transferred via the milk has been documented in both the quokka [64] and the grey short-tailed opossum [75]. Yadav demonstrated the presence of specific antibodies in the serum of pouch young after their mothers had been immunized with bacteriophage and flagella antigens [64]. Furthermore these antibodies persisted until the end of pouch life at 170 days. In the opossum, Jansen et al. [75] demonstrated that maternal immunization against *Trypansoma cruzi* successfully prevented infection in the young animal. It thus appears that maternal immunoglobulins play a significant role in immunological protection of young marsupials not only during the neonatal period but for the duration of development of the lymphoid tissue.

Cells are a common constituent of mammalian milk [76,77]. Their presence and variety have been described in the quokka [78] and the tammar wallaby [79]. In both these marsupials, neutrophils, macrophages and plasma cells were detected at different stages of lactation. It has been postulated that such cells may play a role in immunological protection although their viability after ingestion has not been determined.

5. Conclusions

Although a substantial number of histological and immunohistochemical studies have been conducted on developing and adult lymphoid tissues, our understanding of the development of immunocompetence and maternal protection of the young marsupial is far from complete. In the time that has elapsed since a similar review was undertaken over 10 years ago [53], progress has been constrained by the lack of immunological reagents, sequence data and, to a lesser extent, access to experimental populations. As illustrated in this and other papers in this edition such reagents and protocols are now available. The time is ripe for the metatherian immune system to be as well documented as that of eutherians.

Acknowledgements

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