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Immunocytochemical study of the ontogeny of the marsupial *Didelphis albiventris* immune system

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ABSTRACT

Marsupials have considerable merits as models for studying the developmental dynamics of the mammalian immune system, but until recently there has been a conspicuous lack of specific immune probes to facilitate such studies. To begin a precise study of the ontogeny of the marsupial *Didelphis albiventris* we have used cross-reactive polyclonal antibodies raised against evolutionarily highly conserved peptides which form part of the antigen specific receptor complexes of human differentiated lymphocytes. Moreover, because of antigen receptor conservation, the antibodies also recognise specifically the immunocompetent T and B lymphocytes of other species including those in the organs of the opossum. Use of the antipeptide antibodies together with other cross-reacting antibodies has allowed us to study the cellular immunology of T and B cells and antigen presenting cells (APC) during the development of thymus, skin, lymph nodes and spleen in the Brazilian white-belly opossum. The molecular nature and identity of the T cell antigens detected in opossum tissues were confirmed by immunoblotting. These findings indicate that it is now possible to exploit these antibody probes for comparative mammalian studies, and indeed to investigate interesting features of the opossum, such as reaction of the immature immune system of the pouch young to antigenic stimulation.

Key words: Skin; thymus; mesenteric lymph nodes, spleen; *Didelphis albiventris*.

INTRODUCTION

Cluster of differentiation (CD) antigens, CD79a and CD79b are recognised respectively by rabbit polyclonal antipeptide antibodies termed mb-1 and B29 (Mason et al. 1992). These B lymphocyte specific antigens represent parts of the invariant heterodimer molecules respectively of ~ 40 and ~ 37 kDa which associate with the immunoglobulin receptor molecules of human B lymphocytes. Recent studies by Coutinho et al. (1993, 1994) have demonstrated that these antibodies together with a rabbit polyclonal antipeptide antibody to human CD3, a pan T cell antigen receptor associated specific marker (Mason et al. 1989), will recognise specifically the homologues of B and T cell populations of the marsupial *Didelphis albiventris*. Further studies by Coutinho et al. (1993) have demonstrated that a polyclonal rabbit antihuman IgA and a mouse monoclonal antihuman HLA-DR antibody will also recognise the respective homologues of the opossum. These recently defined antibody probes are thus facilitating studies of the developmental dynamics of the opossum immune system. Using immunocytochemistry and immunoblotting we show here the precise development of immunocompetent (antigen receptor expressing) T and B lymphocytes and APC in the developing organs of the opossum.

MATERIALS AND METHODS

Immunocytochemistry

Didelphis albiventris opossum young measuring 10, 12, 14, 24, 29, 45, 48, 50, 58, 60, 75 and 80 mm

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Table. *Antibodies used to recognise T and B cells and class II MHC markers in opossum tissues*

	Specificity	Source	Working dilution
Primary layer antibodies			
Rabbit polyclonals			
Anti CD3	Pan T cells	Dako (A452)	1:60
Anti mb-1 (CD79a)	B cells	Gift from Prof. Mason	1:1000
Anti B29 (CD79b)	B cells	Gift from Prof. Mason	1:200
Anti IgA	Immunoglobulin A	Behring (ORCI 05)	1:200
Mouse monoclonal			
Anti HLA-DR	MHC class II DR locus product	Dako (M746)	1:40
Secondary layer antibodies			
Biotin labelled porcine antirabbit immunoglobulin	Primary layer rabbit immunoglobulin	Dako (E353)	1:300
Biotin labelled porcine antimouse immunoglobulin	Primary layer mouse monoclonal	Dako (E354)	1:200
Tertiary layer			
Streptavidin–biotin Horseradish peroxidase complex	Biotin labelled secondary antibodies	Dako (K377)	As per kit instructions

crown–rump length (CRL), weanling, juvenile and adult animals, captured in the city of Belo Horizonte (Minas Gerais State, Brazil), were used. The animals were killed humanely by chloroform anaesthesia in accordance with the protocols of the Institute Brasileiro de Meio Ambiente which controls the use of wild animals for scientific research in Brazil.

The opossum young measuring between 10 and 24 mm CRL were fixed in Bouin's fluid and embedded whole in paraffin. Serial sections (5 μm) were prepared and the selected sections were mounted on poly-L-lysine subbed slides. Larger animals were dissected and samples of skin, thymus, mesenteric lymph nodes and spleen were embedded in paraffin, sectioned and mounted on slides as mentioned above. The sections were dewaxed in xylene and rinsed in alcohol. A fresh solution of methanol (90 ml) H_2O_2 30% w/v 100 vol (3 ml), for 15 min, was used to block endogenous peroxidase. After a running water rinse the sections were immersed in Tris buffered saline (TBS), two changes of 5 min each.

The specific primary layer polyclonal or monoclonal antibodies, their specificity, source and working dilutions are described in the Table. Streptavidin–biotin–horseradish peroxidase system was used as previously described by Coutinho et al. (1993). All steps of the immunocytochemical procedures used followed the protocols as described by Coutinho et al. (1994).

As a negative control the primary antibodies were substituted on separate sections by TBS. The counterstaining of the sections was done in Harris's haematoxylin for 5 s.

Immunoblotting

Thymuses were extracted from adult animals, washed and macerated in saline; the sample was fully disrupted by ultrasonication (on ice, 2 × 2 min) with a Lab-Line-ultratip. The extract was then lyophilised. Opossum and human lymphocytes were isolated from heparinised blood by isopaque density gradient centrifugation. Lymphocytes were collected and resuspended in saline, ultrasonicated and lyophilised.

Discontinuous SDS-polyacrylamide gel electrophoresis was performed using the system of Laemmli (1970); samples (50 μg of total protein) were prepared in 1 × Laemmli sample buffer and boiled for 3 min prior to loading on the gel. Protein separation was performed using a 4% stacking gel and 12% resolving gel, at 16 °C for 18 h at 30 V constant voltage. After separation, protein was electrophoretically transferred from the gel to Amersham Hybond C-extra membrane. After transfer the membrane was washed in transfer buffer 1 × 5 min and dried at room temperature. For antibody probing the membrane was rewetted in H_2O then incubated, with shaking, for 1 h in TBS containing 0.05% Tween 20 (TBS-Tween) and 5% non-fat milk powder (Marvel). This was replaced with fresh solution containing the rabbit antihuman CD3 antibody (1:100 diluted from manufacturer's stock) and incubated for 1 h at room temperature. The membrane was washed × 4 in TBS-Tween (15 min per wash). The second antibody incubation was for 1 h in TBS-Tween containing a 1:5000 dilution of a goat antirabbit IgG-alkaline phosphatase conjugate (Sigma). The membrane was washed × 4 in

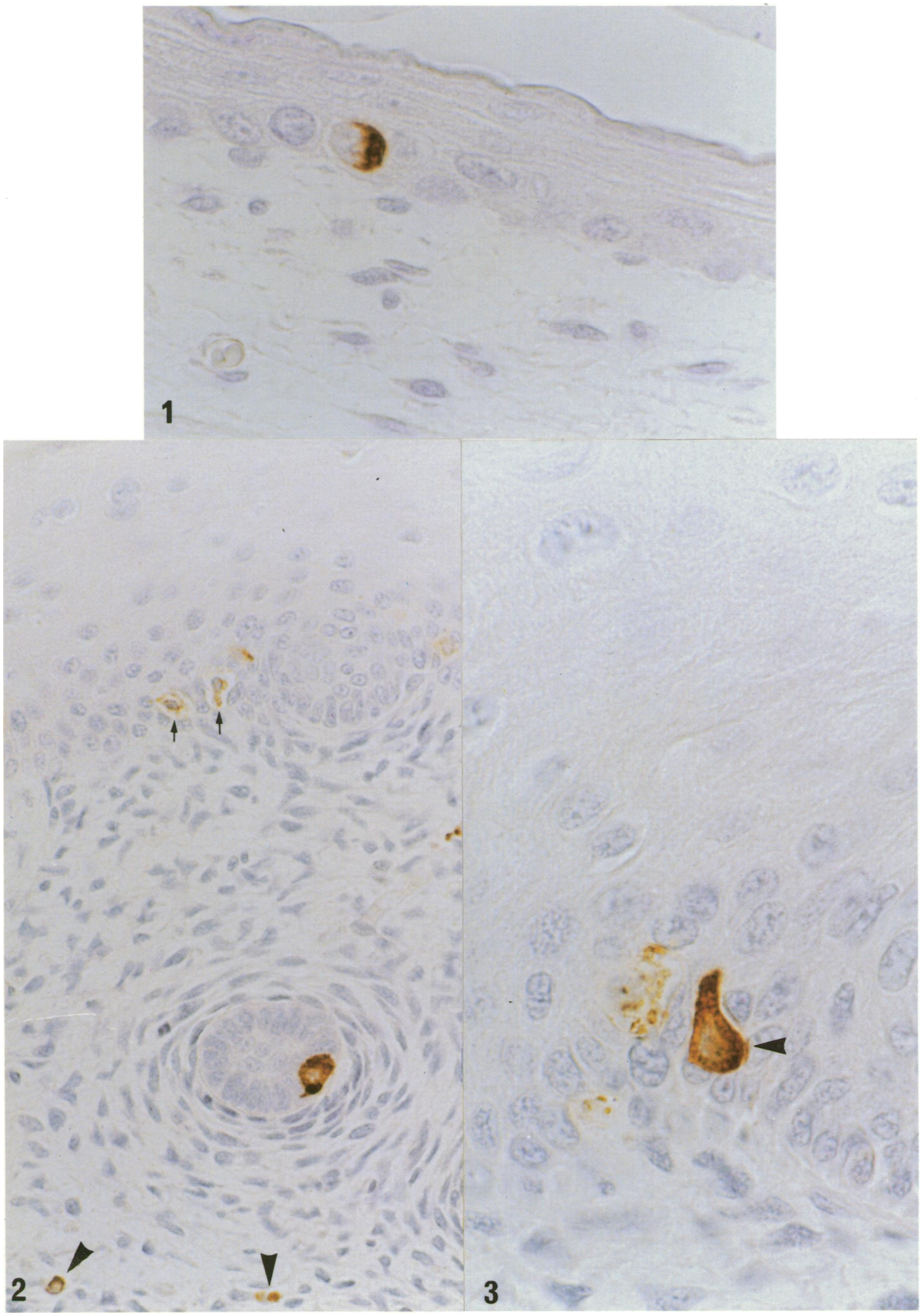

Fig. 1. HLA-DR positive round Langerhans cells is located in 10 mm opossum epidermis. × 2000.

Fig. 2. 24 mm young opossum skin. Arrows indicate Langerhans cells. One HLA-DR positive cell is present in a hair follicle primordium. Arrowheads indicate HLA-DR positive macrophages. × 400.

Fig. 3. Arrowhead indicates an HLA-DR positive Langerhans cell in a 29 mm young opossum epidermis. × 2000.

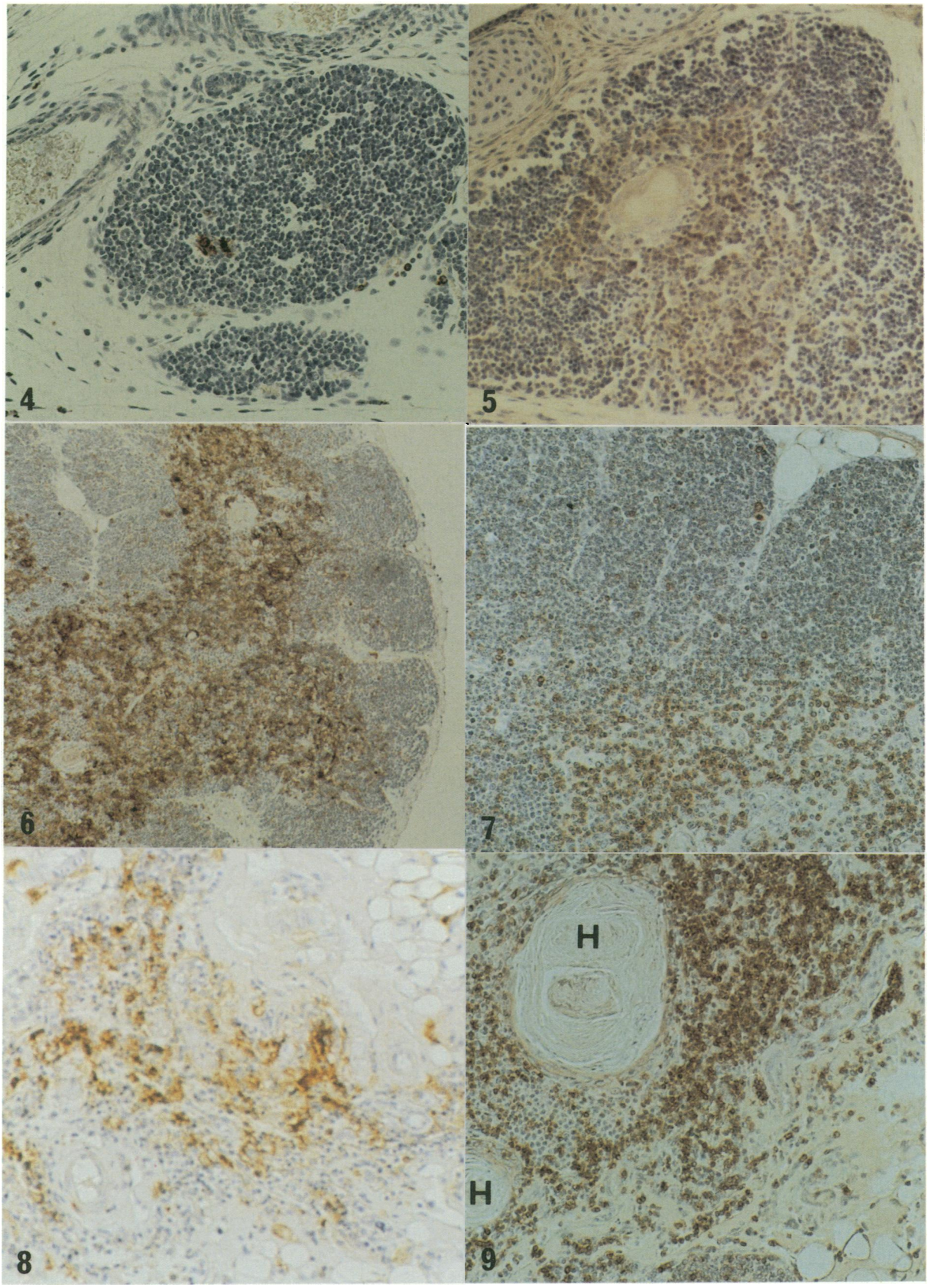

Fig. 4. HLA-DR positive cells are present in a 12 mm opossum thymus. × 175.

Fig. 5. CD3 positive thymocytes have accumulated inside a 14 mm opossum thymus. × 200.

Fig. 6. HLA-DR positive cells are concentrated in the thymus medulla of a 24 mm opossum. × 100.

Fig. 7. 24 mm opossum. Numerous CD3 positive thymocytes are located in the thymus medulla. × 175.

TBS-Tween (15 min per wash). Alkaline phosphatase activity was detected using nitro-blue tetrazolium and BCIP in Tris buffered saline (pH 9.6).

RESULTS

Skin

In 10 mm CRL newborn opossum, round cells expressing HLA-DR were observed in the epidermis of the front limbs and face. Indeed of all the skin sites examined at this stage, only these two sites were positive for HLA-DR. In 14 mm CRL animals the HLA-DR positive intraepithelial cells have short processes. The cells were noted in the dermal connective tissue and amongst epithelial cells of the hair primordia. In older animals the dendritic aspects of those cells were well defined (Figs 1–3).

Thymus

In newborn 10 mm CRL opossum young the thymus was formed by a cluster of small cells with rounded nuclei. The cells were HLA-DR and CD3 negative. Strongly stained HLA-DR positive cells were first detected in 12 mm animals (Fig. 4); at the 14 mm stage of development weakly stained CD3 positive thymocytes were present within the thymic medulla, beyond the blood–thymus barrier, but near to blood capillaries (Fig. 5). In 24 mm opossums the cortical and medullary thymic histological structures were well established; HLA-DR positive reticuloepithelial cells were concentrated in the thymic medulla or as isolated cells in the cortex. CD3 positive lymphocytes were present in greater number in the medulla than in the cortex where nonstained lymphocytes were predominant (Figs 6, 7). The 24 mm thymus pattern persisted in larger lactant, weanling and adult animals. Hassall's bodies were HLA-DR negative.

In 1 presumed old opossum the lobular structure of the thymus had disappeared; HLA-DR positive APC were noted in fat-cell rich connective tissue along with isolated accumulations of CD3 positive cells. The observed Hassall's bodies were HLA-DR negative (Figs 8, 9).

Mesenteric lymph nodes

The presence of HLA-DR and CD3 positive cells in the mesenteric lymph nodes was first observed when the young opossum reached 75 mm CRL (Figs 10, 11). In weanling, juvenile and adult animals HLA-DR APC were noted in the germinal centre of the follicles and in the cords and sinuses (Fig. 12). CD3 positive T cells were scattered through the germinal centre and follicular corona, but were noted to be concentrated in the paracortex (Fig. 13). CD79a, CD79b and IgA positive B cells were observed in the periphery of the follicles and in the medullary cords and sinuses (Figs 14, 15).

Spleen

In the spleen of opossum young smaller than 80 mm CRL no cellular structures reacted with the antibodies used. In 80 mm CRL opossum APC HLA-DR positive cells made their first appearance around the splenic arteries (Fig. 16). Also in 80 mm animals the splenic corpuscles were unformed; however, small arteries branching from the trabecular arteries possessed sheaths of CD3 positive T lymphocytes (Fig. 17). In the sheaths the most peripheral lymphocytes were CD3 negative and did not express CD79a, CD79b or IgA. Mixed with the positive CD3 T lymphocytes were scattered HLA-DR positive APC (Fig. 18).

In weanling, juvenile and adult animals the results were similar; HLA-DR positive cells were concentrated in the germinal centre of the follicles or scattered in the splenic cords of the red pulp (Fig. 19); CD3 positive lymphocytes were present in the thymus-dependent areas or as isolated cells in the red pulp, B cells expressing CD79a and CD79b were distributed at the periphery of follicles, in the splenic cords and sinuses and within the lumen of blood vessels (Figs 20, 21). Isolated IgA positive immunocytes were located in the red pulp cords.

Figure 22 demonstrates by immunoblotting that the antihuman CD3 (pan T cell) antibody recognises an appropriate CD3 homologue of ~ 24–26 kDa in the opossum.

DISCUSSION

With the exception of the presence of HLA-DR positive cells in the newborn opossum epidermis, our studies show that up to the 2nd day of pouch life the Brazilian white-belly opossum is devoid of the

Fig. 8. In the thymus of an old animal, where no lobular structure is observed, clusters of HLA-DR positive cells are present in fatty connective tissue. × 175.

Fig. 9. Accumulation of randomly distributed CD3 positive thymocytes in an old opossum thymus; H, Hassall's body. × 175.

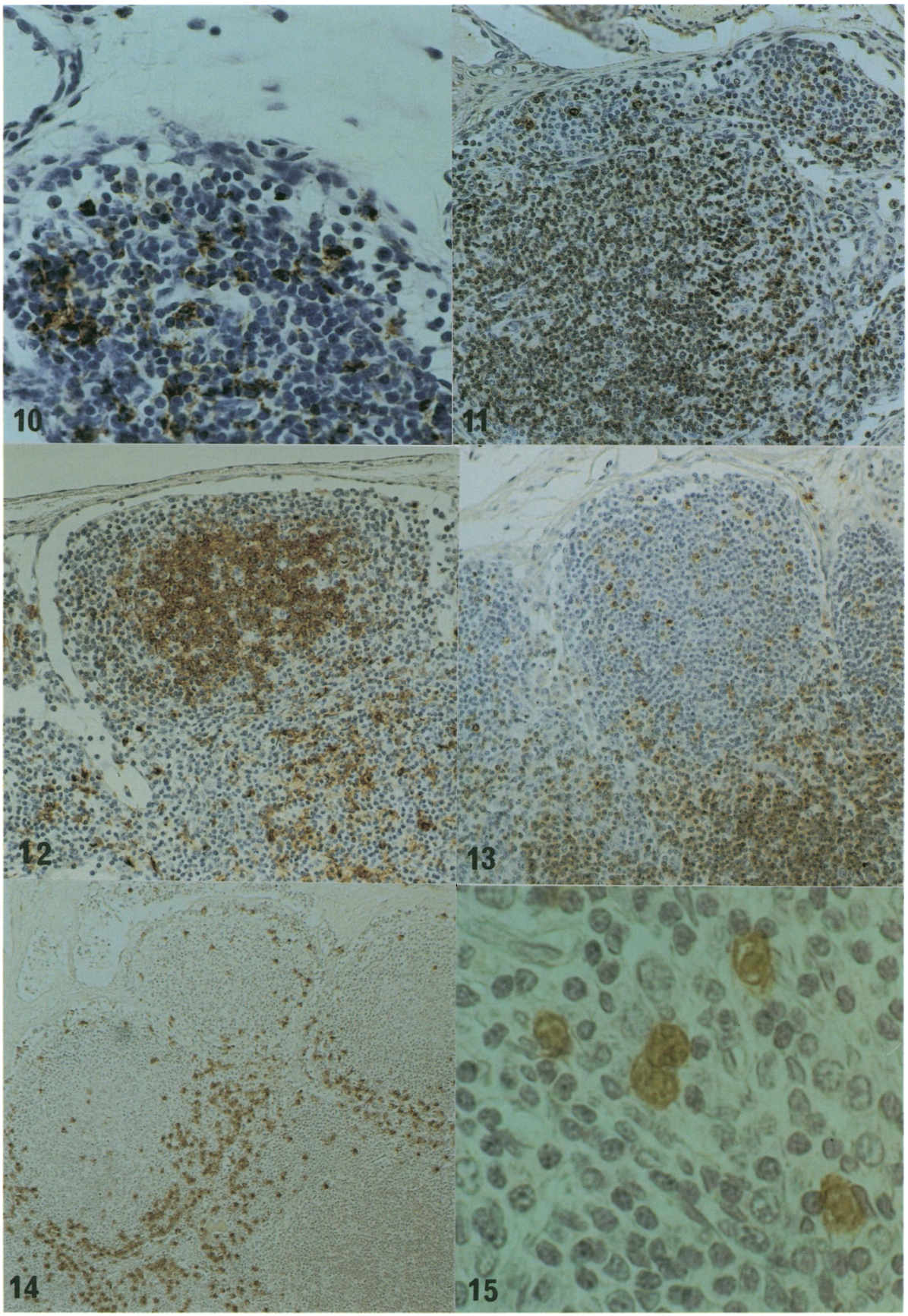

Fig. 10. 75 mm opossum mesenteric lymph node presenting HLA-DR positive cells. × 350.

Fig. 11. CD3 positive T cells are present in a 75 mm opossum mesenteric lymph node. × 175.

differentiated immunocompetent cellular components involved with the systemic immune response. These results support functional immunological studies demonstrating that at birth marsupials are essentially immunologically incompetent (Hubbard et al. 1991).

When the newborn opossum reaches the marsupium it attaches to a maternal nipple. The particular arrangement of the intrinsic tongue musculature and the cellular contiguity and imbrication between the nipple and opossum mouth cells are responsible for the fetuses' adhesion and suckling (Coutinho et al. 1967). The epithelial contiguity and imbrications may thus favour the situation where only proteins present in the milk may be captured by the young. This may diminish entry of potentially antigenic foreign proteins at a time when the opossums' immune system is not fully competent.

Our results indicate the likely sequential maturation of the organs involved in the opossum immune response. Epidermal Langerhans cells are the first immunocytochemically detected HLA-DR positive APC to appear in the opossum young. This observation is interesting insomuch that the newborn opossum skin comes into contact with foreign antigens during its migration from the cloaca to the marsupium and later in the marsupium. It could be speculated that these early APC may have a functional role in the interaction between foreign antigens and the development of the opossum immune system. Langerhans cells have been reported to differentiate very early during human fetal development (Sewell et al. 1986).

APC and CD3 positive T cells are present in 12 mm CRL opossum thymus, i.e. at 2 d of pouch life. These findings support the morphological studies of Rowlands et al. (1964) that in *Didelphis virginiana* thymic lymphocytes are first identified in the 2nd day of pouch life. When the opossum fetuses reach 24 mm the thymus cortex and medulla are histologically and immunologically differentiated; at this stage of development positive HLA-DR APC and CD3 positive T cells have been described as present in the lamina propria of the duodenum. Identified Peyer's patches containing HLA-DR positive dendritic cells and CD3 positive T cells were observed in 45 mm CRL opossum duodenum (Coutinho et al. 1994). As CD3 positive T cells were observed in the mesenteric lymph nodes of opossums measuring 75 mm, whilst in spleen they were identified in animals longer than 80 mm, it is surmised that T lymphocytes migrating from the thymus first home to the intestinal mucosa, and after the differentiation of the Peyer's patches, they colonise the mesenteric lymph nodes. Subsequently there is homing of CD3 positive T cells to the spleen. The presence in the spleen of immunocompetent cells of B lineage was only observed after weaning, a fact that supports the finding of Cutts & Krause (1982) who reported that prominent germinal centres are first present in weanling and adult *Didelphis virginiana.*

Recent studies have shown in the lamina propria of 60 mm CRL opossum duodenum the presence of CD79b and IgA positive B lymphocytes. In 80 mm opossum duodenum and ileum B cells expressing CD79b, CD79a and IgA were present in the Peyer's patches and in the small intestine lamina propria (Coutinho et al. 1994). In the current study mesenteric lymph nodes and splenic B cells were first observed in weanling ($\sim$ 120 mm) animals; the homing to these lymphatic organs of B lineage cells probably follows a progressive sequence (correlating with the increases in CRL) subsequent to the differentiation of APC cells and the homing of T cells to those organs. The finding of IgA-positive B cells within the mesenteric lymph node is in keeping with the predominance of this B cell isotype in such sites in all mammalian species studied. This also reflects the role of IgA immunoglobulins as the major antibodies in the secretory immune system.

Our results using immunocytochemical techniques disagree with those of Rowlands et al. (1972) who indicated that lymphocytes appear in the *Didelphis virginiana* spleen between 17th and 20th days in the pouch, while gut associated lymphoid cells are observed later in development. In the *Didelphis albiventris* spleen of animals measuring 29 mm CRL (20 d of pouch life) no immunocompetent cells of T or B lineage were detected. These apparently conflicting findings may be a reflection of the differences in the techniques used; our results were generated using antibodies to immunological markers associated with differentiated/functional lymphoid cells in contrast to Rowlands et al. (1972) who used conventional cell morphological studies. Clearly the latter studies would detect lymphocytes from their first appearance regardless of their differentiation status.

Finally, the immunoblotting clearly demonstrates

Fig. 12. Juvenile opossum lymph node. HLA-DR positive APC are concentrated in the germinal centre. × 175.

Fig. 13. CD3 positive T cells are concentrated in the thymus-dependent paracortex of a juvenile opossum mesenteric lymph node. × 175.

Fig. 14. CD79a activated B lymphocytes are present in the periphery of mesenteric lymph nodules from a juvenile opossum. × 100.

Fig. 15. IgA positive immunocytes in the medulla of mesenteric lymph node from a juvenile opossum. × 1750.

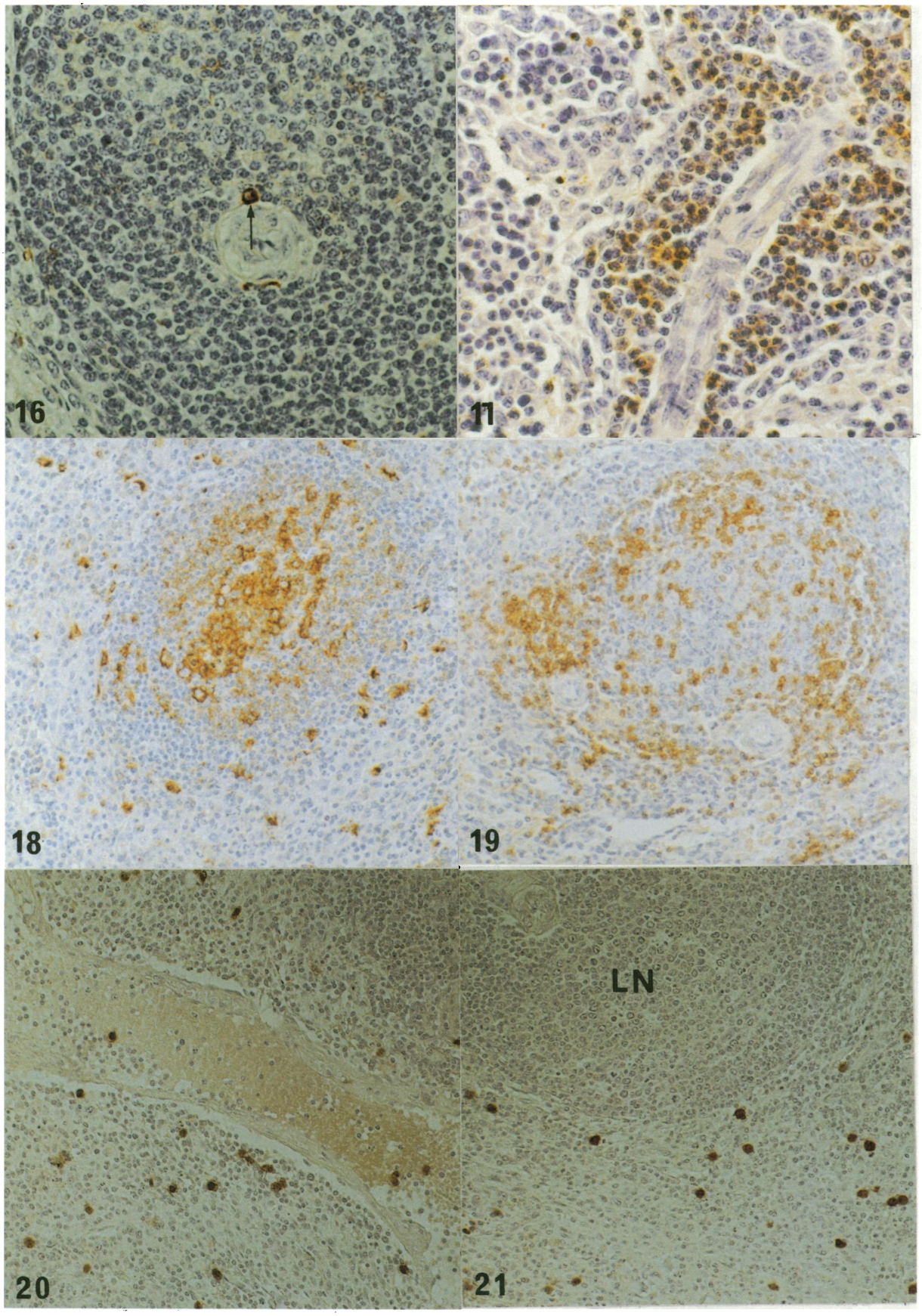

Fig. 16. Arrow indicates an HLA-DR positive APC located near an artery in an 80 mm opossum spleen. ×350.

Fig. 17. CD3 positive T cells form a sheath around a splenic artery of an 80 mm opossum spleen. ×350.

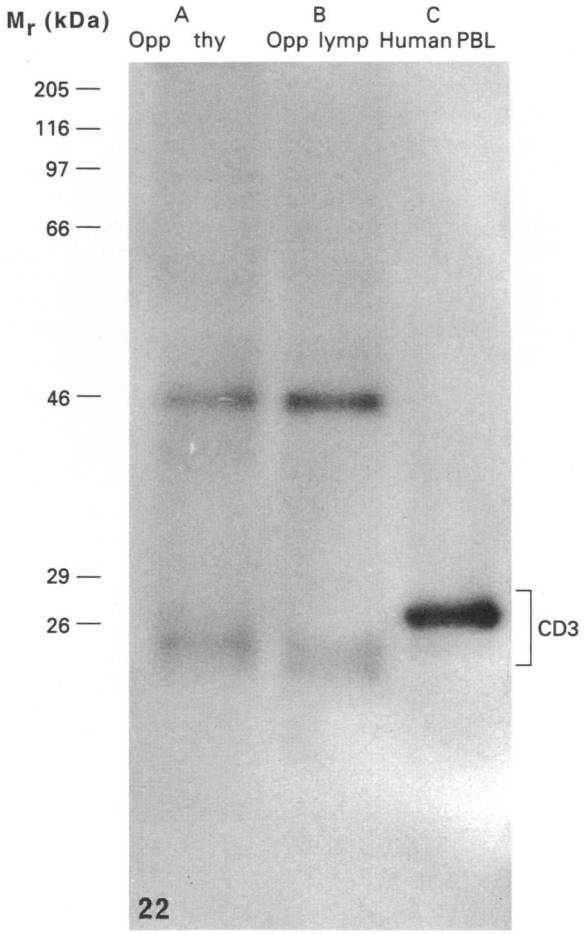

Fig. 22. The antihuman CD3 antibody binds to an ~ 24–26 kDa band from opossum thymus and blood lymphocyte extract (lanes A and B). There is a corresponding ~ 26–27 kDa band indicating the binding of the antibody to the CD3 antigen present in the human peripheral blood lymphocyte extract (lane C). Additional bands of ~ 46 kDa are noted in the extracts of opossum tissues. M_r, molecular weight markers; Opp, opossum; thy, thymus; lymp, lymphocytes; PBL, peripheral blood lymphocytes; CD3, cluster of differentiation antigen 3.

in extracts from opossum lymphocytes a molecule of ~ 24–25 kDa which corresponds to the homologue of the human T cell specific molecule CD3. The additional band seen at ~ 46 kDa with the opossum extract is likely to represent a nonspecific species reactivity, as this ~ 46 kDa band is noted in the absence of a CD3 band when using a control unimmunised normal rabbit serum.

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Fig. 18. HLA-DR positive APC are concentrated in the germinal centre of a splenic lymph nodule from an adult opossum. × 200.

Fig. 19. CD3 positive T cells are concentrated in the corona of a spleen follicle from an adult opossum. × 200.

Fig. 20. CD79b activated B cells are observed in the red pulp and lumen of a splenic trabecular vein from an adult opossum. × 175.

Fig. 21. CD79a positive B cells are observed outside a splenic lymph nodule of an adult opossum spleen. LN, Lymph nodule. × 175.

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