INSTITUTO OSWALDO CRUZ

Doutorado em Biologia Celular e Molecular

O MICROAMBIENTE TÍMICO EQÜINO: CARACTERÍSTICAS MORFOLÓGICAS EM ANIMAIS NORMAIS OU PORTADORES DE ANEMIA INFECCIOSA EQÜINA

ELLEN CORTEZ CONTREIRAS

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Orientadores: Prof. Dr. Wilson Savino

Prof. Dr. Henrique Leonel Lenzi

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EXAMINADORES:

Prof. Drª. Suzana Corte-Real Farias - **Presidente**
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Prof. Dr. Elmiro Rosendo do Nascimento

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"A maior recompensa para o trabalho do homem

não é o que ele ganha com isso,

mas o que ele se torna com isso."

John Ruskin

"Nunca houve uma noite ou um problema que

pudesse derrotar o nascer do sol ou a esperança."

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"Senhor, para uma melhor integração dos homens

entre si e convosco, quero fazer da ciência um diálogo,

da minha aula um lar, dos meus alunos amigos,

da minha vida um dom."

Joaquim Sfredo

Os amigos
São tão amigos, que voltam.
São tão fraternos, que se unem.
São tão simples, que cativam.
São tão desprendidos, que doam.
São tão dignos, que amam, compreendem e perdoam.

Os amigos
São tão necessários, que sempre se fazem presentes.
São tão grandes, que se distinguem.
São tão dedicados, que edificam.
São tão preciosos, que se conservam.
São tão irmãos, que partilham.
São tão sábios, que ouvem, iluminam e calam.

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São tão raros, que se consagram.
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São tão importantes, que não se esquecem.
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Texto retirado do livro "Uma pausa para Deus"

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ABREVIATURAS E SIGLAS

AIE	Anemia Infecciosa Eqüina
CD	*cluster of differentiation* – marcador de superfície designando linhagem ou estágio de diferenciação, reconhecido por um grupo de mAb
CD3	Complexo protéico associado ao receptor clonal para antígeno da célula T (CD3-TCR)
CD4	Molécula acessória de células T, que serve como marcador fenotípico relacionado às funções auxiliar/indutora. Co-receptor para MHC II
CD8	Molécula acessória de células T, que serve como marcador fenotípico relacionado à função citotóxica. Co-receptor para MHC I.
CD11a	Leucócitos. Adesão (liga-se a ICAM-1,-2)
CD25	Cadeia α do receptor de IL-2
CD34	Ligante para L-selectina, expresso em precursor de células hematopoéticas
CD44	Receptor para componentes de matriz extracelular, hialuronato e fibronectina
CD54	Membros das ICAMs, co-receptor para LFA-1
CD58	Molécula de adesão: co-receptor para CD2
CD90	Membro da super-família gênica das imunoglobulinas, sendo marcador de células T
DN	Timócitos duplo-negativos $CD4^-8^-$
ECM	Matriz extracelular
FN	Fibronectina
G-CSF	Fator estimulador de formação de colônias de granulócitos
GM-CSF	Fator estimulador de formação de colônias de granulócitos e macrófagos
IL	Interleucina
LFA-1	Antígeno de função linfocitária-1 (CD11a)
MHC	Complexo principal de histocompatibilidade
PTR	Células fagocitárias do retículo tímico
SP	Timócitos simples-positivos: $CD4^+$ ou $CD8^+$
TCR	Receptor clonal de células T
TEC	Células epiteliais tímicas
TNC	Célula *nurse* do timo
VLA	*very late antigen* – antígeno de aparecimento tardio

VLA-4	Receptor de fibronectina ($\alpha4\beta1$) pertencente à família das integrinas; reconhece porção da molécula derivada de um "splicing" alternativo
VLA-5	Receptor de fibronectina ($\alpha5\beta1$) pertencente à família das integrinas; reconhece o tetrapeptídeo RGDS
VLA-6	Receptor de laminina ($\alpha6\beta1$) pertencente à família das integrinas; reconhece a região E8 da laminina obtida por fragmentação enzimática

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RESUMO

Neste trabalho, estudamos timos de equinos, incluindo aspectos morfológicos e o microambiente tímico em fetos, animais normais após o nascimento, e eqüinos com Anemia Infecciosa Eqüina (AIE). Utilizamos 64 animais em diferentes idades. Os timos foram analisados por técnicas histológicas, imunohistoquímica para detecção de proteínas de matriz extracelular tais como, fibronectina, laminina e colágeno tipo IV, e ainda microscopia eletrônica.

Nos animais após o nascimento, classificamos a involução tímica dependente da idade em cinco graus. Atrofias graus I e II ocorriam predominantemente entre 6 a 18 meses de idade; atrofia III, de 18 meses até 4 anos de idade; atrofias IV e V, de 4-5 anos de idade até 18 anos. Esta atrofia não ocorre uniformemente, no mesmo timo, demonstrando variação local de um lóbulo para outro, sugerindo variabilidade de microambiente.

Espaços perivasculares (PVS) foram observados contendo, linfócitos os quais formavam uma camada celular ou eram dispostos em cordões, sugerindo comunicação funcional com a camada periférica de células epiteliais do compartimento intraparenquimal. A matriz extracelular no timo eqüino, apresenta distribuições definidas na cápsula, septos e espaço perivascular (colágenos intersticiais, proteoglicanos, fibras elásticas e fibronectina); membrana basal lobular e vascular (laminina e colágeno tipo IV); intersticial ou intraparenquimal colágeno tipo III e fibronectina. Isto é semelhante com o que foi observado em timo de outras espécies de mamíferos.

Hematopoese intratímica não linfóide é um acontecimento freqüente em cavalos. Eosinófilos se diferenciam dentro do timo uma vez que formas imaturas como mielócitos e metamielócitos foram detectados. Eosinopoese foi observada em timos eqüinos, em todas as

idades estando, entretanto em menor número em animais idosos. Eosinófilos imaturos e maduros foram encontrados em várias regiões dos lóbulos tímicos (dispersos ou formando agregados), particularmente nos espaços perivasculares, nas regiões cortical e medular. É interessante destacar que grânulos de eosinófilos não apresentam o típico cristalóide como outras espécies de mamíferos.

Avaliamos também fetos eqüinos. Aspectos morfológicos foram descritos, mais em relação ao aparecimento seqüencial de certos eventos fundamentais, assim como a definição cortico-medular, e maturação dos corpúsculos de Hassall. Esses são similares àqueles descritos em humanos e outras espécies animais. Entretanto, os fetos eqüinos, apresentavam intensa eosinofilia intratímica e hematopoese de outras linhagens. Adicionalmente, vasos linfáticos bem definidos repletos de linfócitos foram vistos nos timos fetais. Nossos resultados demonstraram que comparando várias características morfológicas com timos de outros mamíferos, o timo fetal eqüino exibe aspectos particulares, sugerindo representar um interessante modelo adicional para estudos de hematopoese não linfóide intratímica em mamíferos; assim como a origem e destino de linfócitos encontrados dentro de vasos linfáticos tímicos.

Finalmente, estudamos timos de cavalos com Anemia Infecciosa Eqüina. Observamos uma severa e acelerada atrofia tímica, com formação de grandes corpúsculos de Hassall cistificados, assim como um aumento da deposição dos componentes de matriz extracelular e da rede vascular quando comparados aos timos de animais normais.

Concluindo, nosso estudo enfatizou ainda a importância de se analisar vários modelos animais, de forma a evitarmos o viés de percebermos o sistema imune baseando-se somente ou, em sua maioria, no modelo de camundongo.

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ABSTRACT

In this work, we studied morphological aspects and the equine thymic microenvironment in fetuses, and in the normal post-natal development, as well as in horses undergoing Equine Infectious Anemia (EIA).

This study comprised 56 animals in different ages. These thymuses were analyzed by conventional histology, immunohistochemistry for detection of extracellular matrix proteins.

In post-natal animals, we classified the equine age-dependent thymic involution or atrophy in five grades. Atrophies of grades I and II occurred predominantly from 6 to 18 months old; atrophy III, from 18 months to 4 years old; atrophies IV and V, from 4-5 to 18 years old. This atrophy does not occur uniformly, even in the same thymus, showing local variation from one lobule to another, thus suggesting microenvironmental variability. Perivascular spaces (PVS) were observed and lymphocytes formed a cell layer or were arranged in strands, suggesting a functional communication with the peripheral layer of epithelial cells from the intraparenchymal compartment.

The extracellular matrix in the equine thymus presented four basic distribution profiles in capsular, septal and perivascular (interstitial collagens, proteoglycans, elastic fibers and fibronectin); lobular and vascular basement membrane (laminin and type IV collagen); interstitial or intraparenchymal type III colagen and fibronectin. In general, this is similar to what has been previously seen in the thymus of other mammalian species.

Intrathymic non-lymphoid hematopoiesis is a frequent event in horses. Eosinophils differentiate within the equine thymus since immature forms such as myelocytes and metamyelocytes are often detected. Eosinopoiesis were observed in the equine thymus in all ages being however less numerous in the older animals. These immature and mature eosinophils were found in various regions of the thymic lobules (scattered or forming

clusters), particularly in the perivascular spaces, both in the regions cortical and medular. Interestingly, eosinophil granules do not exhibit the typical crystalloid from other mammalian species.

We also evaluated the equine fetal thymus. The morphological aspects described, plus the sequential appearance of certain fundamental events, such as cortical-medullar definition, the appearance and the maturation of Hassall's corpuscles, are similar to those described in humans and other animals species. However, the equine fetal thymuses show intense intrathymic eosinophilia and hematopoiesis of other lineages. Additionally, clear-cut lymphatic vessels full of lymphocytes were seen in these fetal thymuses. Our results show that despite sharing several morphological features with the thymus from other mammals, the equine fetal thymus exhibits particular aspects, suggesting that it may represent an interesting model for further studies on mammalian intrathymic non-lymphoid hemopoiesis as well as the origin and fate of lymphocytes found within thymic lymphatic vessels.

Finally we studied thymuses from horses undergoing equine infectious anemia. we observed a severe an accelerated thymic atrophy, with formation of large cystic hassall's corpuscles, as well as an augmentation in the deposition of extracellular matrix components and the vascular network when compared with normal animals.

In conclusion, our study also emphasizes the importance of analyzing various animal models, in order to avoid a skew view of the immune system, based only or mainly on the mouse model.

1. INTRODUÇÃO

O timo é um órgão linfóide primário, especializado em um complexo processo de seleção, maturação e expansão de células precursoras dos linfócitos T (revisado em Miller, 1994), os quais, uma vez diferenciados, migram para os órgãos linfóides periféricos, localizando-se nas chamadas regiões timo-dependentes. Esse processo ocorre no contexto tecidual do chamado microambiente tímico, uma rede tridimensional essencialmente formada por células epiteliais (TEC), células dendríticas, macrófagos, fibroblastos e elementos da matriz extracelular (ver revisões Boyd *et al*., 1993; Savino, 1994; Anderson *et al*., 1996). A diferenciação de timócitos é um processo pelo qual precursores derivados inicialmente do fígado e, posteriormente**,** da medula óssea proliferam, reorganizam os genes e expressam os receptores de células T correspondentes, passam por processos de seleção positiva e negativa, originando células T maduras que vão representar o chamado repertório de células T (ver revisão Owen *et al*., 1999).

Estudos cinéticos favorecem a idéia de que essas células deixem o órgão numa via ordenada, conforme proposto por Scollay & Godfrey (1995). O papel de interações celulares mediadas por matriz extracelular é fundamental neste processo (Savino *et al*., 1993; Savino & Dardenne, 2000), tendo sido postulado que o substrato molecular dessa migração seja um arranjo tridimensional de matriz extracelular (Savino *et al*., 1996).

A importância do timo foi demonstrada primeiramente por Miller (1961), quando camundongos neonatos timectomizados até o 2^{o} dia de vida não rejeitavam enxertos de pele heterólogos, apresentavam linfopenia e desenvolviam um quadro de imunodeficiência, sendo susceptíveis a infecções (George & Ritter, 1996).

É interessante notar que grande parte do conhecimento foi gerado utilizando-se modelos experimentais murinos e ainda em humanos, sendo relativamente escasso o conhecimento sobre outros mamíferos, e em particular animais de grande porte.

É nesse contexto que se insere nosso trabalho, o qual corresponde a uma análise morfológica e imunohistológica de timos de eqüinos normais, e daqueles portadores de infecção pelo vírus da Anemia Infecciosa Eqüina.

No entanto, antes de abordarmos os aspectos específicos da metodologia aqui utilizada e os resultados obtidos, julgamos ser relevante rever uma série de conceitos relativos à morfologia do timo, particularmente seu compartimento microambiental, esperando com isso melhor contextualizar nosso trabalho.

1.1 Evolução da Família *EQUIDAE* e Classificação Taxonômica do Gênero *Equus*

A família *EQUIDAE* fornece um dos registros mais completos da evolução em uma série animal, incluindo cavalos, asnos, onagros e zebras atuais do Velho Mundo. Grande parte de seu desenvolvimento ocorreu na América do Norte, mas os cavalos aí se extinguiram no fim do Pleistoceno (ou início do Recente) por causas desconhecidas. Os cavalos selvagens do oeste norte-americano, aí existentes nos últimos cinco séculos, descendem de animais que escaparam dentre os trazidos pelos primeiros exploradores e colonizadores (Storer *et al*., 1979).

A origem dos cavalos é desconhecida. O registro inicia-se com *Hyracotherium Eohippus*, no Eoceno inferior da América do Norte e Europa (53 milhões de anos atrás). Era um habitante de florestas, alimentando-se de folhas, com cerca de 28 cm de altura, pescoço e cabeça curtos e dentição completa de 44 dentes pequenos, de coroa baixa, raiz fechada e desprovidos de cemento. As patas anteriores tinham 4 dedos funcionais e as posteriores apenas 3, sendo o primeiro e o quinto representados por finos ossos rudimentares. O *Miohippus* do Oligoceno (37 milhões de anos atrás) tinha o tamanho de um carneiro, com molares mais altos, mas ainda de raízes fechadas e com três dedos funcionais em cada pata; os dedos laterais eram menores e apenas um rudimento do quinto persistia na pata anterior. No Mioceno já se haviam desenvolvido diversas linhagens (*Parahippus*, *Merychippus*), algumas comedoras de ramos, outras pastadoras (Storer *et al*., 1979).

O *Anchitherium* era um membro persistente de cavalos mascadores. Durante o Plioceno, diversos grupos distintos de cavalos (*Pliohippus*, etc.) pastavam nas planícies da América do Norte. Alguns se estenderam à Eurásia e *Hippidion* à América do Sul, no Plioceno há 5 milhões de anos, originando o último alguns gêneros de pernas curtas que não sobreviveram ao Pleistoceno (18 milhões de anos). Os dedos laterais eram curtos, não tocando o chão. Os pré-molares e molares eram mais longos, com raízes mais curtas, esmalte mais dobrado e cemento entre as dobras. Finalmente durante o período Plioceno (5 milhões de anos), na América do Norte, desenvolveram-se os primeiros cavalos de um único dedo, espalhando-se posteriormente por todos os continentes com exceção da Austrália. No Pleistoceno, havia dez ou mais espécies (de *Equus*) de diversos tamanhos na América do Norte; desapareceram todos, porém, nos tempos pré-históricos. A evolução dos cavalos acompanhou as mudanças conhecidas das paisagens terciárias, de florestas úmidas e pradarias bastante secas (Storer *et al*., 1979).

A chave de classificação taxonômica do gênero *Equus* é a seguinte:

Reino	Metazoa (= Animalia)
Sub-reino	Eumetazoa
Filo	Chordata
Subfilo	Vertebrada
Superclasse	Gnatostonata
Classe	Mammalia
Subclasse	Theria
Infraclasse	Eutheria
Ordem	Perissodactyla
Família	Equidae
Gênero	*Equus*
Espécie	***Equus caballus***

Os animais da espécie *Equus Caballus* possuem um dedo funcional com casco em cada perna (nas formas recentes); habitam planícies abertas ou desertos, e se alimentam de gramíneas. Foram o principal meio de transporte e de trabalho para o homem durante séculos, com 50 ou 60 raças domésticas, desde o pônei Shetland com 1,10 m de altura no ombro e pesando apenas 136 kg até as raças Shire e Percheron com 1,85 m de altura e pesando até 1.200 kg ou mais. As éguas possuem placenta do tipo epitéliocorial e a gestação é de aproximadamente 11 meses. A vida média dos eqüinos é de 30 anos.

1.2 Organização Histológica e Filogenia do Timo

Em humanos, os dois primórdios tímicos originam-se no final da 4ª semana de gestação na forma de proliferações endodérmicas na extremidade ventral dos prolongamentos das terceiras bolsas faríngeas. Essas proliferações endodérmicas formam tubos ocos que invadem o mesoderma subjacente, derivado da crista neural, transformando-se, após, em cordões sólidos ramificados. Esses cordões constituem os primórdios dos lóbulos tímicos (Larsen, 1997).

As células epiteliais podem ter, dependendo de sua localização, origem ecto ou endodérmica, enquanto o mesênquima é responsável por outras células do microambiente (Von Gaudecker, 1991; Larsen, 1997).

Por suas dimensões e sua situação anatômica, o timo humano normal não pode ser palpado e não é visível nas radiografias cervicotorácicas. Seus tumores malignos ou benignos, contudo, manifestam-se radiograficamente através de opacidades de volume variável, às vezes medianas e mais freqüentemente lateralizadas. O órgão pode ser observado diretamente por mediastinoscopia, com retirada de material para biópsia. O timo sofre involução gradativa, quase desaparecendo em indivíduos idosos (Steinmann *et al*., 1985; George & Ritter, 1996).

Em mamíferos adultos se localiza acima do coração, embora sua posição em outros vertebrados possa variar. Em eqüinos, em particular, o timo está no espaço mediastínico pré-cardial ventralmente à traquéia e aos grandes vasos. Projeta-se caudalmente ao epicárdio e pode se estender cranialmente para a região do pescoço. Somente pequenos remanescentes do timo ativo podem ser encontrados aos pares em cavalos de até seis meses de idade. Em cavalos de seis anos de idade o timo pode ser observado, histologicamente, no tecido adiposo retroesternal (Venzke, 1986).

Nos mamíferos em geral, o timo é um órgão bilobado com os lobos unidos intimamente por um tecido conjuntivo denso modelado, o qual encapsula o órgão como um todo. A cápsula também penetra profundamente para dentro do órgão, formando septos que dividem parcialmente o órgão em lóbulos. Desses septos derivam trabéculas que levam a vascularização e inervação para o órgão.

Os lóbulos tímicos são constituídos por duas áreas nítidas que são o córtex, o qual forma a maior área externa; e a medula, localizada centralmente. Cada área possui uma composição celular linfóide e microambiental típica (ver revisão Ritter & Crispe, 1995). A grande densidade celular da região cortical, devido ao empacotamento de timócitos (imaturos, em diferentes estágios de diferenciação) contrasta com a menor celularidade na região medular (linfócitos maduros), permitindo uma visualização mais nítida da rede de células epiteliais e fagocitárias (Lampert & Ritter, 1988). Entre o limite dessas regiões, observamos a junção córtico-medular, local de entrada de vasos e nervos no parênquima tímico.

O rudimento tímico em camundongos, forma-se em torno do 10° dia de vida embrionária, e nesta fase passa a ser povoado por precursores linfóides, ocorrendo a definição dos compartimentos córtico-medular (Owen & Ritter, 1969; Ritter, 1978; Fontaine-Perus *et al*., 1981), e por células epiteliais e fibroblastos, além de macrófagos e células dendríticas, estas de origem hematopoética. Os macrófagos distribuem-se através do córtex e medula, sendo responsáveis pela fagocitose de restos celulares (Surh & Sprent, 1994). As células dendríticas

predominam na região medular, podendo alcançar áreas córtico-medulares, tendo acesso a timócitos imaturos (Kyewski *et al*., 1987; Shortman & Vremec, 1991).

De um ponto de vista filogenético, o timo aparece tardiamente, sendo restrito a espécies de vertebrados, mas já estando presente em peixes, incluindo tubarões e arraias (Chondrichthyes) cujo primeiro ancestral apareceu a 400 milhões de anos atrás (Manning, 1979). No entanto, nas diversas classes de vertebrados, observa-se uma diferença em relação à posição do órgão (sobre as guelras nos peixes; bilateral em anfíbios, répteis e aves, e supra-cardíaco na maioria dos mamíferos), o que provavelmente representa diferentes graus de migração do primórdio tímico a partir de sua localização inicial nas bolsas faríngeas (Ritter & Crispe, 1992). Além disso, em termos da organização histológica do órgão, a configuração "cortico-medular" dos lóbulos tímicos só irá se estabelecer de forma definitiva a partir de anfíbios.

Por outro lado, é importante destacar que, independentemente da espécie ou classe de vertebrados estudadas, o timo é a principal fonte de diferenciação de linfócitos T.

1.3 Diferenciação Intratímica de Linfócitos

Entre a chegada de precursores no timo e a saída de linfócitos T imunocompetentes do órgão, existe intrinsicamente um importante processo de migração, concomitante a várias interações celulares e moleculares. As alterações genéticas e bioquímicas advindas desses processos caracterizam a diferenciação intratímica de células T, resultando em um dos eventos mais interessantes e complexos da fisiologia do sistema imunitário.

O processo de maturação de células T pode ser separado em três estágios seqüenciais: 1) entrada de células precursoras do fígado fetal ou medula óssea para o timo (importação); 2) diferenciação dos timócitos, sob influência de células não linfóides do microambiente tímico; 3) saída de células T do timo para os órgãos linfóides periféricos (exportação).

Os pró-timócitos, originam-se de precursores com atividade hematopoética e se tornam comprometidos com a linhagem T após entrarem no timo. Essa entrada provavelmente se dá por diapedese através da cápsula ou pelos vasos sangüíneos na junção córtico-medular, após a definição da região medular**,** utilizando-se mecanismos dependentes de matriz extracelular e de moléculas de adesão (Boyd & Hugo,1991; Dunon & Imhof, 1993). As moléculas CD44 e VLA-6 (a integrina $\alpha 6\beta 1$) estão diretamente implicadas nessa migração transendotelial (revisado em Patel & Haynes, 1993).

A colonização do timo parece dar-se em forma de ondas, as quais ocorrem durante o período embrionário (a primeira coincidindo com a iniciação da linfopoese no timo, sendo separada da próxima onda por um período refratário) e, provavelmente, também durante o período pós-natal (Jotereau *et al*., 1987; Coltey *et al*., 1987; Ezine, 1989; von Gaudecker, 1991). No camundongo, os progenitores iniciam a colonização do timo em torno do 10° e 13° dia de vida fetal, originando os linfócitos T produzidos até a 1ª semana pós-natal, enquanto aqueles que chegam ao timo depois do 13° dia de vida fetal produzirão uma segunda geração de linfócitos T, a qual começa a substituir a primeira geração a partir de 7 dias de vida. Novos precursores migrarão para o timo por toda a vida do indivíduo (Donskoy & Goldscheider, 1992). Nas aves, a primeira onda se inicia em torno do 7º dia e no homem, em torno da 9ª semana.

Células tímicas precursoras se originam em sítios hematopoéticos diferentes de acordo com a idade do indivíduo. O primeiro sítio identificado (em aves) é a ilha hematopoética que circunda a aorta dorsal (Le Douarin *et al*., 1984). No último estágio, o saco vitelino, fígado fetal, e a medula óssea do adulto são as principais fontes de precursores (Moore & Owen, 1967).

O desenvolvimento de linfócitos tímicos é completamente dependente dessas células precursoras (Owen & Ritter, 1969). Embora em fases iniciais do desenvolvimento elas penetrem através da cápsula, tão logo o timo é plenamente vascularizado, entram via vênulas na região da junção córtico-medular. Uma simples célula precursora (*stem cell*) é capaz de dar origem a todas as diferentes subpopulações de células T durante o desenvolvimento do timo. Já no órgão, sob influência do microambiente tímico, os precursores começam a sofrer uma intensa modulação na expressão gênica de proteínas de membrana, as quais traduzem estados fenotípicos e funcionais importantes no desenvolvimento da célula T (Nikolic-Zugic, 1991).

Foram identificadas várias proteínas de membrana, expressas durante a diferenciação intratímica. Dentre essas proteínas destacamos o receptor clonal de células T (TCR), o complexo CD3, as moléculas CD4 e CD8, além do antígeno Thy-1 (CD 90), a cadeia α do receptor para a IL-2 (CD25) e o antígeno CD44 (Sprent, 1989; Fowlkes & Pardoll, 1989).

A organização e a expressão do TCR são necessárias para que a célula T reconheça o antígeno em associação com o complexo principal de histocompatibilidade (MHC) próprio. O TCR é um heterodímero polipeptídico composto por duas cadeias, $\alpha\beta$ ou $\gamma\delta$, as quais são codificadas por um rearranjo de famílias gênicas (Hedrick *et al*., 1984; Marrack & Kappler, 1987; Strominger, 1989), e cujo ligante é o complexo formado por um peptídeo ligado ao MHC

(Marrack & Kappler, 1986, 1987; Grey *et al*., 1989, Ashwell & Klausner, 1990, Chien & Davis, 1993; Kruisbeek, 1999).

O complexo CD3 é formado por um conjunto de proteínas que se associa ao TCR, sendo os dois complexos conjuntamente expressos na superfície celular. Essas proteínas do CD3 são cruciais na ativação da célula T, ativando, após o reconhecimento antigênico, a via de sinalização celular (Ashwell & Klausner, 1990; Rothenberg, 1992).

As moléculas CD4 e CD8 são glicoproteínas transmembranosas, ditas acessórias ou co-receptores, por estarem envolvidas no processo de diferenciação e ativação de células T, apresentando especificidade de interação com moléculas de classe II e classe I do MHC, fenômeno esse relacionado ao evento de seleção intratímica de células T. Essas interações também determinam uma ligação mais eficiente do TCR com o complexo antígeno-MHC (ver revisão Anderson *et al*., 1996).

Ao longo da diferenciação intratímica de linfócitos, células duplo-negativas para a expressão de CD4 e CD8 passam a expressar essas moléculas (sendo agora chamadas de timócitos duplo-positivos), além de CD3 e TCR, e serão então expostas aos processos de seleção. Essa etapa ocorre a partir de processos de rearranjos e expressão gênicas nos timócitos, sendo fundamental a expressão do TCR na superfície da célula (Blackman *et al*., 1990). O fenômeno de seleção define quais os possíveis receptores (aproximadamente 10^{12}) gerados no processo de rearranjo de genes do TCR que serão capazes de reconhecer peptídeos próprios e estranhos em associação com moléculas do MHC. Para tal, dois processos de seleção são propostos: a) <u>seleção positiva</u>, onde timócitos com TCRs que ligam adequadamente moléculas do MHC do próprio organismo sobrevivem e aqueles com TCRs que não se ligam morrem; b) <u>seleção negativa</u>, onde timócitos expressando TCRs com alta afinidade de ligação para complexos antígeno/MHC do próprio organismo são eliminados (revisado em Fowlkes & Pardoll, 1989; Robey & Fowlkes, 1994).

Cerca de 95% dos timócitos são eliminados durante os processos de seleção do receptor de células T. Ocorre apoptose, um mecanismo de morte celular programada, onde endonucleases clivam o DNA em vários fragmentos. Esses pequenos fragmentos apoptóticos são comumente vistos dentro de macrófagos tímicos (von Boehmer *et al*., 1989).

Na diferenciação intratímica de células T ocorrem as seguintes etapas**:** as células passam inicialmente através do estágio duplo-negativo (DN; $CD4^-8^-$) e dependem da expressão da cadeia β do receptor de célula T (TCR). Essa fase controla o estágio de progressão de células DN para duplo-positivas (DP; $CD4^+8^+$), sendo dirigida pelo complexo pré-TCR, que consiste da cadeia

TCR β, cadeia pré-T α e componentes do complexo de sinalização CD3 (von Boehmer & Fehing, 1997). A segunda etapa é regulada pelo complexo TCR αβ expresso em células DP, que interagindo com o complexo peptídeo/MHC nas células do microambiente tímico, dirige a maturação das células T para o estágio de simples positivas (SP; $CD4^+$ e $CD8^+$) (Marrack & Kappler, 1997).

Em camundongos, o estágio duplo-negativo (DN) pode ainda ser sub-dividido em quatro sub-estágios distintos, baseados na expressão de CD44 e CD25. Inicialmente, os timócitos são $CD44^+CD25^-$. Na fase seguinte, as células se apresentam com fenótipo $CD44^+CD25^+$. Subsequentemente (pré células T precoces e tardias), a expressão de CD44 é progressivamente perdida. No estágio precoce, as células apresentam fenótipo $CD44^-CD25^+$. Nesta fase aparece o pré-TCR (seleção β) e inicia-se a fase pré-T tardia onde as células são $CD44^-CD25^-$ (Owen *et al*., 1999). Aqui, muitos estudos têm mostrado que a via de receptor de IL-7 tem papel obrigatório no desenvolvimento das células T. A IL-7 tem potente contribuição para a sobrevida, diferenciação e proliferação de precursores de células T (Kruisbeek, 1999). Em camundongos geneticamente deficientes para IL-7 ocorre uma redução do número de células DN $CD44^+CD25^+$ (Rodewald *et al*., 1997). Foi demonstrado ainda nesta fase, através da purificação de células DN $CD44^+$ e DN $CD44^-$, que estas podem se reagregar a células estromais purificadas, e sua progressão para linfócitos SP depende de fibroblastos (Owen *et al*.,1999).

Durante a fase DN de desenvolvimento de células T, foram identificadas integrinas (α4β1, α5β1) no desenvolvimento dos timócitos associados à matriz extracelular (Utsumi *et al*.,1991). Células T imaturas são capazes de se ligar a constituintes de matriz indicando que os receptores das integrinas são ativos (ver revisões Savino *et al*., 1993, 2000; Anderson *et al*., 1997). Foi demonstrado ainda em camundongos geneticamente deficientes para o gene da integrina β1, que células linfóides precursoras tem a produção de linfócitos T bloqueadas. Também, em camundongos "nocautes" para o gene da integrina α4, fica prejudicado o desenvolvimento de células T depois do nascimento (Arroyo *et al*., 1996).

1.4 Microambiente Tímico

O microambiente tímico é um compartimento não-linfóide heterogêneo, constituído de rede tridimensional composta por vários tipos de células, dentre as quais a mais frequente é a célula epitelial tímica (ver revisão Boyd *et al*., 1993; Anderson *et al*., 1996; Savino & Dardenne, 2000). Essa rede de células epiteliais é permeada por várias outros tipos celulares, tais como

fibroblastos, células dendríticas e macrófagos, e ainda matriz extracelular. A região cortical, corresponde essencialmente a uma rede de células epiteliais, densamente preenchida por timócitos, enquanto que na região medular aparecem células epiteliais distintas das corticais, bem como macrófagos e células dendríticas originadas da medula óssea. Nesta região, a trama é mais densa, sendo ocupada mais esparsamente pelos timócitos.

1.4.1 Células Epiteliais

As células epiteliais tímicas (TEC) são as mais numerosas do microambiente tímico, formando uma trama tridimensional em todo o parênquima tímico (revisado em van Ewijk *et al*., 1999). Apresentam características estruturais típicas dos epitélios por possuirem filamentos intermediários formados por citoqueratinas, desmosomos entre as mesmas, e exibir uma membrana basal nas áreas em contato com o tecido conjuntivo. No entanto, o epitélio tímico se apresenta como um tecido heterogêneo, no qual podem ser observadas variações morfológicas e antigênicas, sugerindo que tal heterogeneidade reflita funções distintas (revisado em von Gaudecker, 1991; Boyd *et al*., 1993). De fato, o próprio padrão de citoqueratinas no epitélio tímico é complexo e único, com co-expressão de citoqueratinas típicas de epitélios simples e estratificado. Foi ainda demonstrada uma diversidade interespecífica na distribuição de diversas citoqueratinas nos lóbulos tímicos (Meirelles de Souza *et al*., 1993).

De um ponto de vista morfológico as TEC podem apresentar-se isoladamente sob a forma de células estreladas, denominadas *células retículo-epiteliais*, ou formando arranjos globulares com linfócitos, caracterizando os chamados *complexos linfoepiteliais "nurse*", ou formando arranjos concêntricos denominados *corpúsculos de Hassall* (estruturas altamente queratinizadas que podem sofrer degeneração ou calcificação, variando consideravelmente em tamanho de acordo com as espécies), ou sob a forma de *cistos*, que aparecem durante a involução natural ou acidental do órgão (revisado em von Gaudecker, 1991; Boyd *et al*., 1993; Villa-Verde *et al*., 1995).

As TEC são bem maiores que os linfócitos, possuem núcleo grande e vesiculoso, e no citoplasma, o complexo de Golgi e o retículo endoplasmático granular apresentam-se em geral bem desenvolvidos (Pfoch *et al.*, 1971). Podem-se observar também grânulos citoplasmáticos eletron-densos que representam grânulos de secreção endócrina (Savino e Santa-Rosa, 1982).

Funcionalmente, as células retículo-epiteliais podem produzir e secretar vários fatores solúveis [interleucina-1 (IL-1), IL-3, IL-7, fator estimulador de formação de colônias de

macrófagos e granulócitos (GM-CSF)], e ainda hormônios tímicos (timulina, timosina α1, timopoietina e fator tímico humoral-γ2) (ver revisão Savino e Dardenne, 2000). Esses fatores são auxiliares no processo geral de diferenciação intratímica de linfócitos.

Além disso, as TEC expressam em sua superfície antígenos codificados pelo complexo maior de histocompatibilidade (MHC) de classes I e II, cuja interação com o TCR expresso na membrana dos linfócitos determina nestes respostas relacionadas à sua seleção positiva ou negativa.

As células epiteliais interagem como os timócitos via moléculas de adesão tais como LFA-3 (CD58) que se liga ao CD2 nos timócitos e ICAM-1 (CD54) que se liga ao LFA-1 (CD11a) de timócitos (revisado em Patel & Haynes, 1993).

Conforme detalhado abaixo, as TEC produzem ainda moléculas de matriz extracelular (ECM), que também são utilizadas em interações com os timócitos através de receptores específicos expressos na membrana de ambos os tipos celulares (revisado em Savino & Dardenne, 2000).

1.4.1.1 Heterogeneidade do Epitélio Tímico

Como mencionamos acima, o epitélio tímico apresenta marcante heterogeneidade morfológica e antigênica, a qual foi inclusive demonstrada com o uso de uma série de anticorpos monoclonais (mAb) gerados contra células do próprio microambiente tímico. A partir desses dados, foi proposto o conceito de subpopulações de TEC, e uma classificação imunofenotípica das mesmas, segundo os chamados grupos de imunomarcação de TEC (CTES - *clusters of thymic epithelial cell staining*) (Kampinga *et al*., 1989). Os cinco maiores "clusters" marcadores de TEC foram identificados como: 1) CTES I, marcadores pan-epiteliais e reconhecendo todo o epitélio tímico; 2) CTES II, com especificidade para as TEC localizadas nas regiões subcapsular/perivascular e a maior subpopulação de células epiteliais medulares; 3) CTES III, que detectam moléculas especificamente expressas por células epiteliais corticais, incluindo os complexos linfoepiteliais *nurse*; 4) CTES IV, que marcam corpúsculo de Hassall e as células epiteliais medulares, e 5) CTES V, específicos para corpúsculos de Hassall e algumas vezes o epitélio medular à sua volta.

Outros estudos (Savino e Dardenne, 1988a, 1988b; Colic *et al*., 1989) sugeriram a existência de subpopulações de TEC, segundo sua expressão diferencial de citoqueratinas. Posteriormente, no entanto, foi mostrado, através do uso de uma série de diferentes mAb anti-

citoqueratinas, que a heterogeneidade do epitélio tímico poderia variar de acordo com a espécie estudada ou fase do desenvolvimento em uma dada espécie (Meireles de Souza *et al*., 1993). Esses dados, juntamente com outros, mostram que citoqueratinas normalmente expressas na medula dos lóbulos tímicos podem também ser expressas por células corticais em determinadas patologias, tais como infecção chagásica experimental (Savino *et al*., 1989) e diabetes autoimune (Savino *et al*., 1991). Baseados nessas observações, foi formulado o conceito de plasticidade do epitélio tímico interpretando-o de modo mais dinâmico, susceptível a variações decorrentes de estímulos diversos (Meireles de Souza & Savino, 1993; Lannes-Vieira *et al*., 1994).

No estudo sobre a heterogeneidade do epitélio tímico, foi isolado *in vitro* um complexo denominado célula *nurse* do timo (TNC), estrutura linfoepitelial, na qual uma célula epitelial tímica é capaz de conter um número variado de timócitos, predominantemente de fenótipo imaturo $CD4^+/CD8^+$, e de localização cortical nos lóbulos tímicos (ver revisão Villa Verde *et al*., 1995). As TNCs são capazes de secretar hormônios tímicos e citocinas, expressam proteínas de MHC classe I e classe II e produzem proteínas de ECM, sugerindo que no seu interior existam condições microambientais especiais que influenciam passos específicos no processo de diferenciação intratímica de células T (Villa Verde *et al*., 1995).

A heterogeneidade do epitélio tímico também pode ser evidenciada na medula, através das estruturas denominadas corpúsculos de Hassall. Esses correspondem a agregados de células epiteliais tímicas, que sofrem um processo de queratinização centrípeta, com degeneração das células mais centrais. Nesse sentido, os corpúsculos de Hassall expressam queratina de alto peso molecular e compartilham outros antígenos com queratinócitos epidermais (Lobach, 1985; Laster & Haynes, 1986; Laster *et al*., 1986). Seu tamanho varia em diferentes espécies: por exemplo, no camundongo são pequenos e de difícil definição, enquanto que no homem são abundantes, grandes e facilmente reconhecidos. Em humanos, o aumento do número desses corpúsculos foi observado em doenças infecciosas agudas. Gilhus *et al*., (1985) postularam que esses corpúsculos participam fagocitando e removendo células mortas do timo. Além disso, atividade de produção de hormônios tímicos também foi localizada em suas células (Savino *et al*., 1984).

Outros tipos de células (linfócitos, plasmócitos, macrófagos) podem ser encontradas nos corpúsculos de Hassall. Além disso, em indivíduos idosos podem ser encontrados (e mesmo predominar) corpúsculos de Hassall císticos (Gilhus *et al*., 1985). A natureza epitelial dessas células formadoras da parede de cistos pode ser confirmada pela presença de queratinas em seu citoplasma (Savino e Dardenne, 1988a). Radiograficamente, os cistos são massas definidas de

densidade uniforme, e em humanos podem variar de 1 a 18 cm de diâmetro e em seu interior é observado um colóide semelhante a uma mucosubstância ácida (Oksanen, 1972).

1.4.2 Macrófagos e Células Dendríticas

Macrófagos e células dendríticas podem ser identificados no timo (Timens *et al*, 1988; Nakahama *et al*., 1990), constituindo pequenas populações de células microambientais, de origem hematopoética. De fato, essas células parecem estar intimamente relacionadas, pois expressam alguns antígenos comuns e podem derivar do mesmo precursor (mielóide CD34+) proveniente da medula óssea (Duijfestijn e Barclay, 1984). As células dendríticas estão localizadas na medula e na junção córtico-medular (Shortman e Vremec, 1991), sendo elementos-chave no estabelecimento de seleção negativa dos timócitos (ver revisão Nossal, 1994). Nesse sentido, estudos utilizando anticorpos monoclonais demonstraram que as células dendríticas apresentam alto nível de MHC classe II e moléculas CD1, além de ainda expressarem fracamente a molécula CD4 (Landry *et al*., 1989). Contudo, existe uma célula dendrítica linfóide, cuja a origem não está bem determinada, que parece originar-se de precursor linfóide CD34+ (Banchereau *et al*., 2000). Células dendríticas tímicas murinas, expressam ainda antígenos típicos de células T como CD2, CD8α e CD25 (revisado em Ardavín, 1997).

Monócitos do sangue migram para o timo pela região córtico-medular onde eles se transformam em macrófagos. Os mesmos são facilmente identificados no córtex e também estão presentes na medula, podendo ser encontrados na cápsula, septos interlobulares e espaços perivasculares (ver revisão Kendall, 1981, 1991). Os macrófagos estão envolvidos em fagocitose de células apoptóticas (Surh & Sprent, 1994). Fragmentos apoptóticos são encontrados predominantemente em macrófagos corticais, sendo raros na medula (Sarrazin *et al*., 1989; Kendall, 1990). Alguns macrófagos e células dendríticas interdigitantes podem funcionar na apresentação de antígenos para linfócitos.

Todos os macrófagos expressam molécula MHC classe I na sua superfície. Em contraste, somente 50 a 75% expressam MHC classe II (Nabarra & Papiernik, 1988; Beller & Unanue, 1979). A expressão de classe II parece ser uma propriedade potencial de todas as subpopulações de macrófagos tímicos (provavelmente induzidas pelos produtos de células T), já que macrófagos classe II são encontrados em todas as áreas do timo.

1.4.3 Fibroblastos

No timo, os fibroblastos estão localizados principalmente na cápsula, septos e regiões perivasculares do microambiente tímico. De ponto de vista funcional, dados experimentais recentes sugerem a participação dessas células na diferenciação precoce de timócitos duplo-negativos (DN), dependem da apresentação da IL-7 através de moléculas de ECM produzidas por elas (Anderson *et al*., 1997).

1.4.4 Células Mióides

Em 1888, células tímicas com características de músculo estriado foram primeiramente reconhecidas em sapos e salamandras. Em 1905, foram observadas por Hammar em timos humanos, que ele chamou de células mióides (ver revisão Cheng & Turpen, 1995). Ultra-estruturalmente, possuem arranjos de microfilamentos e placas densas típicas de músculo estriado. As estriações transversais podem inclusive ser facilmente identificáveis em aves e répteis, onde são relativamente numerosas. Além disso, por métodos imunohistoquímicos, foi detectado forte marcação para anticorpos para actina e miosina de músculo estriado (Drenckhahn *et al*., 1979), além de outros componentes, tais como mioglobina, desmina e o componente M de creatina quinase e beta-enolase (Cheng & Turpen, 1995; Detrich III *et al*., 1995).

Por microscopia eletrônica foram observadas conexões desmosomais entre células mióides e células epiteliais, (Bielinska *et al*., 1996). Essa íntima relação tem sido citada como evidência que ambas derivam de um precursor comum (Dzierzak & Medvinsky, 1995; Bielinska *et al*., 1996).

Células mióides têm uma distribuição irregular através do timo. São, na sua maioria, encontradas em agrupamentos na medula e estão com freqüência intimamente associadas a corpúsculos de Hassall (Drenckhahn *et al*., 1979; Suster & Rosai 1992). Embriologicamente, em humanos, elas aparecem no 8º mês de gestação.

Alguns investigadores reportam que células mióides tendem a aumentar em número com a atrofia do parênquima tímico. A função dessas células ainda é desconhecida. Podem estar relacionadas com miastenia grave (van der Geld, 1966; Henry, 1966). Há uma teoria que postula que essas células apresentam antígenos de músculo esquelético dentro do timo para induzir tolerância própria (Shivdasani *et al*., 1995).

1.4.5 Hematopoese Intratímica Não-Linfóide

Além da diferenciação de linfócitos T, outros elementos sangüineos podem estar presentes no timo, tais como mastócitos, eosinófilos, neutrófilos e plasmócitos, formando uma população menor. Essas células estão localizadas em áreas do tecido conjuntivo tal como o septo fibroso e espaços perivasculares (ver revisão Bodey *et al*., 1999). Eosinófilos são mais numerosos no timo de recém-nascidos e crianças do que em timo dos adultos (Bhathal, 1965; Dourov, 1982). No timo fetal humano precursores granulocíticos e eritróides já foram descritos no tecido conjuntivo e em volta de pequenos vasos do córtex (Taylor & Skinner, 1976). Além disso, em algumas espécies de aves já foi observada eritropoese intratímica, formando pequenos grupos, circundados por células da rede epitelial tímica (Kendall, 1995).

Granulócitos foram localizados no cortex tímico, na região subcapsular, no cortex externo e no tecido conjuntivo do septo e cápsula. Várias colônias granulocitopoéticas localizadas no tecido conjuntivo exibiram eosinófilos e neutrófilos em várias formas de diferenciação e maturação celular (Bodey *et al*., 1998).

Os mastócitos são regularmente encontrados no septo e na cápsula do tecido conjuntivo do timo. Eles aumentam em número na involução do timo e algumas vezes também em timite, quando podem ser encontrados nos lóbulos. Em certas espécies, como nos caninos, são muito numerosos (Henry *et al*., 1981).

Assim, apesar de existirem relativamente poucos estudos sobre hematopoese não-linfóide no timo, há indícios de que tal evento de fato ocorra, pelo menos em algumas espécies, inclusive em humanos (Bodey *et al*., 1998).

1.5 Expressão Intratímica de Ligantes e Receptores de Matriz Extracelular

A matriz extracelular (ECM), além de ser suporte para as células, compreende um conjunto de fatores dinâmicos da diferenciação celular (Kornblihtt & Gutman, 1988). As moléculas de ECM ligam-se às células através de receptores específicos expressos na membrana plasmática e interagem direta ou indiretamente com o citoesqueleto e com outros sistemas intracelulares, podendo estar relacionadas com processos de diferenciação, proliferação, morte e/ou migração celular (Kornblihtt & Gutman, 1988).

A presença de componentes de ECM no timo foi inicialmente estudada por métodos histológicos, tendo sido descrita a distribuição de fibras reticulares e fibras elásticas (Henry, 1967; Savino, 1982). Por exemplo, demonstrou-se a existência de trabéculas e uma fina trama de fibras reticulares nas regiões medular e perivascular, e no delineamento de membranas basais lobular e vascular (Henry,1967).

Posteriormente, utilizando-se métodos imunohistoquímicos em estudos realizados em timos humanos (Berrih *et al*., 1985) e de camundongos (Lannes-Vieira, *et al*., 1991) foi observada uma distribuição de colágeno tipo I restrita aos espaços intersticiais da cápsula, septos e vasos sanguíneos, enquanto, colágeno tipo IV, fibronectina e laminina estavam presentes nas membranas basais, formando ainda uma fina rede na região cortical, e uma rede mais espessa na medula do órgão. Esse padrão é filogeneticamente conservado, tendo sido visto em diversas ordens de mamíferos (Meireles-de-Souza *et al*., 1993). Ainda assim, nada se encontra na literatura sobre a expressão de componentes da ECM em timo de eqüinos.

Análises *in vitro* demonstraram que as TEC, inclusive as TNCs, produzem colágenos tipo IV, fibronectina e laminina (Berrih *et al*., 1985; Savino *et al*., 1986; Lannes-Vieira *et al*., 1991; Villa-Verde *et al*., 1994) sugerindo que essas células possam ser, ao menos parcialmente, as produtoras da membrana basal intratímica. Além disso, recentemente foi demonstrada a produção de fibronectina por células fibroblastóides de origem mesenquimal (Anderson *et al*., 1997), e ainda a produção de fibronectina, laminina e colágeno tipo IV por células fagocitárias (Ayres-Martins *et al*., 1996). Lannes-Vieira *et al*., (1993) descreveram a expressão do complexo VLA-6 em células epiteliais tímicas murinas, o qual parece ser relevante para a fisiologia da célula microambiental tímica, participando também na diferenciação de células T intratímicas.

Em timos normais humanos, diferentes subpopulações de linfócitos expressam diferentes integrinas para ECM (Ruco *et al*., 1993). A integrina VLA-4 é um receptor de fibronectina expresso particularmente por linfócitos corticais. A interação entre VLA-4 nos linfócitos e fibronectina nas células epiteliais parece ser importante na maturação de células T (Dalmau *et al*., 1999). Outra integrina, VLA-6 liga-se à laminina, um componente da membrana basal, e é expressa por linfócitos tímicos (Wadsworth *et al*., 1992; Lannes-Vieira *et al*., 1993; Ruco *et al*., 1993). Diversos estudos demonstraram a participação direta dos componentes da matriz extracelular tímica e seus receptores nas interações timócitos/células epiteliais. Nestes estudos, foi de grande valia a utilização de linhagens de células epiteliais tímicas e também dos complexos TNC (Lannes-Vieira *et al*., 1993; Lagrota-Cândido, 1994; Villa-Verde *et al*.,1994; Mello-Coelho, 1997). Villa-Verde *et al*., (1994) observaram que fibronectina e laminina aceleram

espontaneamente *in vitro* a liberação de timócitos de células tímicas *nurse* (TNC), e que anticorpos anti-ECM exibem um efeito bloqueador. Foram também demonstradas *in vitro* interações entre timócitos e células não epiteliais fagocitárias do retículo tímico (PTR), e tratamentos com anticorpos anti-matriz extracelular, como por exemplo a fibronectina e seu receptor anti-VLA-5 bloquearam a formação de rosetas entre elas (Ayres-Martins, 1996). O tráfego de linfócitos dentro da TNC (compreendendo o favorecimento e inibição da emperipolese) é afetado por interações envolvendo ligantes e receptores de ECM. Dessa maneira, a análise dinâmica dos complexos de TNC deve ser vista como uma ferramenta relevante *in vitro* para estudos funcionais de distintas moléculas de adesão no tráfego intratímico de linfócitos (Villa-Verde *et al*., 1994).

Foi constatado aumento de ECM no timo de animais submetidos a doenças infecciosas agudas, tais como a raiva (Savino *et al*., 1987), infecção por *Trypanosoma cruzi* (Savino *et al*., 1989), e também em infecções congênitas humanas como sarampo, sífilis e por citomegalovírus (Fonseca, 1991). Foi sempre observada uma correlação positiva entre o aumento da rede de ECM intratímica e o grau de depleção linfocitária do parênquima tímico (ver revisão Savino *et al*., 1991). Além disso, outros padrões de distribuição anormal de ECM intratímica foram detectados em condições patológicas tais como miastenia grave (Savino & Berrih, 1984), síndrome de Down (Fonseca *et al*., 1989), e diabetes autoimune experimental (Savino *et al*., 1991).

1.6 Ontogenia e Involução Tímica

Existem três elementos críticos para o desenvolvimento normal do epitélio tímico, e por extensão, do órgão como um todo: ectoderma da fenda branquial, endoderma das bolsas faríngeanas e mesenquima dos arcos faríngeanos derivado da crista neural. Se algum desses componentes estiver faltando, ocorre uma falha no desenvolvimento tímico (Norris,1938; Cordier& Haumont, 1980; Salaun *et al*., 1986).

Nos mamíferos o tecido do timo origina-se principalmente da terceira, podendo ocorrer uma participação menor da quarta bolsa faríngea. A terceira bolsa faríngea é uma estrutura mais complexa consistindo numa massa epitelial dorsal maciça (origina a paratireoide inferior) e numa porção oca, alongada e ventral, que forma o timo.

Os dois primórdios ou esboços do timo humano surgem ao final da quarta semana de gestação como evaginações endodérmicas ventrais das terceiras bolsas faríngeas. Essas proliferações endodérmicas formam tubos ocos que invadem o mesoderma e, posteriormente,

transformando-se em cordões sólidos que se ramificam. Esses cordões constituem-se nos primórdios dos lóbulos tímicos. O mesoderma subjacente é derivado da crista neural e forma os septos entre os cordões epiteliais endodérmicos (Larsen, 1997). Na ausência da crista neural o timo não se desenvolve.

Assim, uma interação entre a crista neural e componentes endodérmicos dos primórdios tímicos condiciona esses últimos a uma diferenciação subsequente.

Ao longo da vida pós-natal, o timo sofre fisiologicamente um processo de involução, caracterizado por uma grande depleção linfocitária, ocorrendo diminuição do volume cortical dos lóbulos. Aumento do número dos corpúsculos de Hassall e de cistos epiteliais pode também ser observados (ver revisão Savino, 1994).

Dois tipos de involução foram descritos: *aguda*, em resposta a variados tipos de estresse, e *crônica*, que está associada com a idade. O timo é muito sensível a estresse exógenos, incluindo infecções agudas, malnutrição, cirurgias, antibióticos e outras drogas, e ainda situações fisiológicas de estresse como a gestação, lactação, muda em pássaros e metamorfose em anfíbios (ver revisão Clarke e MacLennan, 1986). Os efeitos dessas situações sobre o timo são, na maioria das vezes, mediados por hormônios esteróides, e resultam na morte da maioria dos timócitos corticais por apoptose. A regeneração tímica ocorre assim que o estímulo do estresse é removido.

A involução relacionada com a idade é caracterizada por uma redução progressiva do tamanho e peso do timo, devido a perda de linfócitos tímicos e de células do microambiente (epitélio, células dendríticas e macrófagos). A taxa da involução é mais rápida durante os 10 primeiros anos de vida, depois ela diminui progressivamente. O volume total do espaço peri-vascular e tecido conjuntivo aumentam durante 20-30 anos de vida no homem, sendo mais tarde substituído pelo tecido adiposo, que vem a formar a maior parte no órgão (Steinman, 1986).

1.7 **Alterações no Timo em Doenças Infecto-Parasitárias**

A atrofia é uma das principais características relacionadas ao timo em diversas patologias, entre elas doenças infecciosas (revisado em Savino, 1990; Savino *et al*., 1991). Por exemplo, na fase aguda da infecção experimental por *Trypanosoma cruzi* ocorre uma severa atrofia em paralelo ao aumento de parasitemia (Savino *et al*., 1989). Histologicamente, destaca-se uma redução da região cortical que pode até mesmo desaparecer quando a atrofia é bastante intensa. Outro aspecto marcante é a presença de muitos núcleos picnóticos de timóticos na região cortical remanescente.

Nos animais infectados com atrofia tímica, observou-se ainda um processo de densificação da rede epitelial do órgão. Um estudo mais detalhado desta rede foi realizado através de análise imunohistoquímica com painel de anticorpos dirigido contra diferentes proteínas da família de citoqueratinas. Células reconhecidas pelo anticorpo monoclonal (mAb) ER-TR.5, que em timos normais estavam presentes exclusivamente na medula, passaram a ser encontradas também no córtex subcapsular e interno. Além disso, células $CK8/18^{+}$, normalmente restritas à região cortical, foram detectadas também na medula tímica (Savino *et al*., 1989). É interessante notar que resultados semelhantes foram obtidos em modelos murinos de infecção pelo vírus rábico e ainda pelo *Schistosoma mansoni* (revisado em Savino *et al*., 1992).

No modelo da infecção pelo *Trypanosoma cruzi*, a severa diminuição na celularidade tímica se refletiu principalmente na diminuição de células imaturas de fenótipo $CD4^{+}CD8^{+}$, com aumento na freqüência de células simples-positivas $CD4^{+}CD8^{-}$ e $CD4^{-}CD8^{+}$ e duplo negativas $CD4^{-}CD8^{-}$. Além disso, o aumento na frequência de células $CD3^{hi}$ corrobora a noção de que as células resistentes são em sua maior parte do tipo medular (Leite-de-Moraes *et al*., 1991; Savino *et al*., 1992).

Um dado relevante para a análise da patologia tímica em doenças infecciosas foi a observação de uma expressão maior de antígenos de classe II do MHC concomitante à atrofia que ocorre no timo de animais infectados (ver revisão Savino *et al*., 1992). Outra importante observação foi a detecção de aumento na expressão de proteínas de membrana basal, tais como fibronectina, laminina, colágeno IV e do antígeno reconhecido pelo mAb ER-TR.7 (Savino *et al*., 1989). Esta modulação foi caracterizada por um aumento progressivo de uma rede intra-lobular destas proteínas. Novamente, alteração semelhante foi observada em diversas infecções agudas, experimentais ou humanas (Savino *et al*., 1992).

Considerando o conjunto de dados anteriormente discutidos, pensamos ser relevante o desenvolvimento de um estudo sobre os componentes linfoide e microambiental do timo num processo infeccioso em animais de grande porte como eqüinos.

1.8 Anemia Infecciosa Eqüina

A Anemia Infecciosa Eqüina (AIE) é uma enfermidade que acomete eqüideos, sendo causada por um retrovírus. É caracterizada por persistência viral, mesmo em presença de anticorpos, podendo haver ou não episódios recorrentes de anemia e mudanças

linfoproliferativas, lesões mediadas imunologicamente e variabilidade no curso clínico (Lennette & Schmidt, 1979; Timakov & Zuev, 1980).

Esta doença, também conhecida como febre dos pântanos, foi pela primeira vez, descrita na Europa no final do século 19, tendo, no início do século 20, sido comprovada a filtrabilidade do agente infeccioso. No Brasil, a mortalidade varia em torno de 80%, causando grandes perdas econômicas na eqüideocultura.

O agente infeccioso da AIE é um vírus classificado na sub-família Lentivirinae, família Retroviridae, baseado em sua ultraestrutura, organização genética, atividade de transcriptase reversa e reação sorológica cruzada (Sellon, 1993).

Eqüinos infectados com o vírus da AIE podem apresentar uma síndrome aguda com febre alta, trombocitopenia e/ou anemia, ou uma síndrome sub-aguda para crônica de febre recorrente, perda de peso, edema ventral e grave anemia ou podem permanecer clinicamente normais (Clabough, 1990; Clabough *et al*., 1991). O título viral no soro de animais infectados aumenta com a elevação da febre (Kono *et al*., 1971; Clabough, 1990), podendo o vírus ser detectado no soro, fígado, baço, linfonodos, medula óssea, pulmões e rins (Kono *et al*., 1971; Mc Guire *et al*., 1971; Rice *et al*., 1989; Sellon *et al*., 1992).

Finalmente, cabe ressaltar que as infecções por lentivírus são consideradas exemplos de doenças imunopatológicas em que as alterações patológicas nos tecidos são, na maior parte, indiretamente mediadas por respostas imune e inflamatória do hospedeiro (Trautwein, 1992).

O vírus da AIE se replica primariamente em tecidos que possuem macrófagos maduros, como por exemplo: fígado, baço, linfonodos e glândulas adrenais (Sellon *et al*., 1992). No entanto, até o momento, não se sabe o vírus ocorre em macrófagos e células dendríticas do timo. De modo semelhante, não há dados sobre possíveis alterações nos compartimentos linfóide e microambiental tímicos no curso dessa enfermidade.

2. JUSTIFICATIVA E OBJETIVOS

Nosso estudo visou dar prosseguimento aos trabalhos experimentais sobre o microambiente tímico, já desenvolvidos em outras espécies de animais. Estudamos a morfologia do timo e a matriz extracelular em eqüinos sadios de várias faixas etárias, e aqueles portadores da Anemia Infecciosa Eqüina. A escolha desta patologia deve-se ao fato de que este vírus de evolução lenta, pertencente a família Retroviridae, determina o aparecimento de uma enfermidade que compromete o sistema imunológico com produção de uma anemia normocítica normocrômica.

Nesse sentido, procuramos alcançar no presente trabalho, os seguintes objetivos:

1. Descrever as características morfológicas do timo de eqüinos normais em diferentes idades.

2. Analisar o microambiente tímico de eqüinos normais em diferentes fases do desenvolvimento, comparando-o com os padrões encontrados em outros mamíferos, destacando a distribuição dos componentes da matriz extracelular.

3. Analisar comparativamente o microambiente tímico de eqüinos adultos, infectados pelo vírus da Anemia Infecciosa Eqüina, com timos de eqüinos não infectados.

3. MANUSCRITOS QUE COMPÕEM O CORPO DA TESE

3.1 *The equine thymic microenvironment: a morphological and immunohistochemical analysis*

Neste trabalho estudamos a morfologia do timo eqüino, incluindo os diversos tipos celulares e a matriz extracelular de animais normais de diferentes faixas etárias, demonstrando as alterações do microambiente que ocorrem ao longo da involução natural do órgão.

3.2 *Developmental aspects of the cellular and extracellular matrix components in the equine fetal thymus*

A ocorrência de fêmeas grávidas entre os animais estudados nos proporcionou a oportunidade de estudar fetos eqüinos em diferentes idades gestacionais (2-10 meses). Este estudo nos permitiu evidenciar pela primeira vez na literatura, características morfológicas do timo eqüino em diferentes fases do desenvolvimento embrionário, onde pudemos demonstrar por exemplo que a eosinopoese intratímica nestes animais precede o nascimento.

3.3 *The equine thymus is a special microenvironment for eosinophil lineage*

Durante o nosso estudo, verificamos no timo eqüino a presença de hematopoese não linfóide, principalmente eosinopoese nas fases pré e pós natal. Com esses resultados sugerimos que o microambiente tímico eqüino é também adequado para a diferenciação de eosinófilos, atuando bidirecionalmente e influenciando o microambiente e/ou os compartimentos linfóides do órgão.

3.4 *Morphological changes in the thymus of horses undergoing equine infectious anemia*

A atrofia é uma das principais características relacionadas ao timo em diversas patologias, entre elas doenças infecciosas. Pensamos ser relevante o desenvolvimento de um estudo sobre os componentes linfóide e microambiental em timo de eqüinos infectados com o vírus da Anemia Infecciosa Eqüina (família *Retroviridae*). Esta doença é de notificação obrigatória, podendo, na maioria das vezes, levar o animal a morte, ocasionando grandes prejuízos econômicos. Verificamos que nos animais infectados, a involução do órgão é bastante acelerada, com expansão de corpúsculos de Hassall da natureza cística, e ainda aumento de deposição de matriz extracelular.

THE EQUINE THYMUS MICROENVIRONMENT:
A MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS

Ellen Cortez Contreiras[1,4], Luzia Fátima Gonçalves Caputo[2],
Maria de Nazareth Leal de Meirelles[3], Déa Maria Serra Villa-Verde[1],
Henrique Leonel Lenzi[2] and Wilson Savino[1]

[1]Laboratory on Thymus Research, Department of Immunology,
[2]Department of Pathology and [3]Department of Ultrastructure and Cell Biology
Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil;
[4]Department of Morphology, Biomedical Institute,
Fluminense Federal University, Rio de Janeiro, Brazil.

Correspondence:
Wilson Savino
Laboratory on Thymus Research,
Department of Immunology,
Oswaldo Cruz Institute, Oswaldo Cruz Foundation,
Av. Brasil 4365 - Manguinhos,
21045-900, Rio de Janeiro, Brazil.

Tel: (55) (21) 280-1486, (55) (21) 598-4326, (55) (21) 598-4327
Fax: (55) (21) 280-1589, (55) (21) 590-9741
e-mail: savino@ioc.fiocruz.br --- savino@gene.dbbm.fiocruz.br

Abstract

The present work aimed to provide morphological and phenotypic data regarding the organization of the equine thymic microenvironment along with the post-natal development of the horses. This study comprised 42 normal animals (18 males and 24 females), aging 6 months-18 years. Thymuses were analyzed by histological techniques, immunohistochemistry for detection of extracellular matrix proteins (ECM) (fibronectin, laminin, type IV collagen) and electron microscopy.

The general analysis of the data allowed to classify the equine thymic involution or atrophy in five grades, which progress more or less sequentially along with ageing. Atrophies of grades I and II occurred predominantly from 6 to 18 months; atrophy III, from 18 months to 4 years; atrophies IV and V, from 4-5 to 18 years. It is important to point out that the thymic atrophy does not occur uniformly, even in the same thymus, showing local variation from one lobule to another, thus stressing the microenvironmental variability. Frequently, lymphocytes in perivascular spaces formed a perilobular layer or were arranged in strands, suggesting a functional communication with the peripheral layer of epithelial cells from the intraparenchymal compartment.

Hassall's bodies exhibit frequent, cyst formation, glandular (mucous) metaplasia, and signs of degeneration and reabsorption by macrophage-PAS cells and giant cells.

Extracellular matrix proteins presented four basic distribution patterns in capsular, septal and perivascular (interstital collagens, proteoglycans, elastic fibers and fibronectin); lobular and vascular basement membranes (fibronectin, laminin and type IV collagen); interstitial or intraparenchymal (type III colagen and fibronectin) and pericellular (proteoglycans).

Intrathymic non-lymphoid hematopoiesis is a frequent event in equines. Eosinopoiesis was the most predominant non-lymphoid lineage produced, and eosinophils were found in both the perivascular spaces and intraparenchymal compartments.

Another interesting feature in the equine thymus is the presence of prominent lymphatic vessels, full of lymphocytes, and that may represent an important pathway of thymocyte exportation.

The present study shows that the equine thymus: presents a general morphological and involutional characteristics similar to other mammals, although exhibiting some particular characteristics, as exemplified by the prominent non-lymphoid hemopoiesis. This study also emphasizes the importance of analyzing various animal models, in order to avoid a skew view of the immune system, based mainly on the mouse model.

Introduction

Despite horses have been used as one major immunologic serum "factory" in early immunology, the morphological profiles of their lymphoid organs, and more particularly the thymus remain poorly studied. This organ is located in pre-cardial mediastinum, overlies the pericardium and may extend cranially to the cervical region reaching sometimes the thyroid gland (Venske, 1986). It usually does not present, like in humans, a clear distinction between the right and left lobes. It becomes larger around the second months of age, then beginning to gradually regress or involute, so that after three years of age, it is generally constituted most by adipose tissue (Dyce *et al*., 1997).

The thymic arteries are branches of common carotid and internal thoracic; the veins drain to internal jugular and thoracic veins, and the lymphatic vessels carry the thymic lymph to cranial sternal lymph nodes (Venske, 1986).

There is an increasing characterization of monoclonal antibodies (mAb) to cell surface molecules of equine leukocytes, defining mainly the T and B subpopulations. For instance, the monoclonal antibodies (mAb) CVS5, CVS4 and CVS8 recognized only T equine lymphocytes in peripheral blood. In the thymus they detect CVS4, CVS8 double-positive cells. Biochemical characterization suggested that CVS5, CVS4 and CVS8 recognize the equine homologues of CD5, CD4 and CD8, and that the characteristics of these antigens are similar to those of other species (Lunn *et al*., 1991; 1995).

Eight murine mAb were used to identify the equine CD8α on CD8β chains and to define the expression of these chains on lymphocytes from various lymphoid tissues. CD8α was a 39 kDa protein and CD8β was a 32 kDa protein. Both chains were expressed on most of the $CD8^+$ T lymphocytes in the peripheral blood, spleen, thymus, mesenteric lymph nodes and ileal intraepithelial lymphocytes (Tschetter *et al*., 1998).

Three populations of cells in the equine thymus were distinguishable by mAb UCF6 G-3 target antigen density, suggesting increasing stages of T-cell maturation, and a pan-T-cell marker, CD5, was also expressed in low number of B lymphocytes (Blanchard-Channel *et al*., 1994). Interestingly, hybridization analysis using human probes suggested that the equine CD4 and CD8α genes are more closely related to the human than to the murine counterparts (Grunig *et al*., 1994).

To establish standard procedures for naming equine leukocyte surface molecules, the equine molecules equivalent to human molecules were designated EqCD followed by the CD number, e.g. EqCD4, EqCD5, EqCD8, etc (Kydd *et al*., 1994). Equine NK cells may be identified by an $EqCD3^-$ $EqCD8^+$ phenotype (Lunn *et al*., 1995), and the equine hyperexpression of the MHC class-II antigens on T cells is an indication of activated lymphocytes (Bendali-Ahcene *et al*.,1997).

Despite the progress made to phenotypically define horse leukocytes, there are scarce data in the literature about the morphologic characteristics of the equine hemopoietic microenvironments, (particularly the thymus), which certainly restrains the biological or morphofunctional general view of the equine immune system. The present work aimed to provide morphological and phenotypic data regarding the organization of the equine thymic microenvironment along with the post-natal development of the animals.

Material and Methods

Animals

This study comprised 42 normal horses (18 males and 24 females), aging 6 months-18 years. At least 5 specimens from each age range were studied. Animals were obtained at Federal Rural University of Rio de Janeiro (Department of Parasitology, Rio de Janeiro, Brazil), and were handled according to the ethical rules established by the governmental ethics committee of EMBRAPA (National Brazilian Agency for Agricultural Research). Additionally, all horses used in this study were checked for the presence of infectious equine anemia virus, and only serologically negative, as ascertained by the commercial Coggin's test were used throughout this study (Lab. Bruch, São Paulo, Brazil).

Histology and electron microscopy

When used for histological techniques, thymus fragments were fixed in Carson's Formalin-Millonig, embedded in paraffin. Five μm thick sections were stained with a variety of histological or histochemical techniques, whose general features are summarized in the table. Stained material was then analyzed under bright field or confocal laser microscopy (He/Ne Laser, LSM-410 model, Zeiss, Germany).

For ultrastructural analysis, tiny thymus fragments ($1mm^3$) were fixed in 2.5% glutaraldehyde in 0.1M Na cacodylate buffer, pH 7.2, for 1h, rinsed in the same buffer and postfixed with 1%

OsO_4, dehydrated through an ascending series of acetone and embedded in Epon 812. Ultrathin sections were picked up on 300-mesh copper grids, contrasted with uranyl acetate and lead nitrate, and examined using a Zeiss EM 10C transmission electron microscope (Germany).

Immunohistochemistry

Some thymus fragments, once excised were immediately frozen in liquid nitrogen, and then kept in deep freezing conditions (-80 ^{o}C) until use. Frozen sections (5μm thick) were fixed in cold acetone, washed in PBS and subjected to indirect immunofluorescence as currently done in our laboratory (Villa-Verde *et al*., 1994). Briefly, specimens were subjected to a given primary antibody for one hour, extensively washed in PBS and exposed to the fluorochrome-labeled second antibody for a further hour. After washing, slides were mounted and analyzed under confocal laser microscopy.

Distinct primary antibodies were applied to study the horse thymic microenvironment. The anti-pancytokeratin polyclonal serum was used to reveal the whole thymic epithelial network, as previously demonstrated (Savino *et al*., 1982). Furthermore, we evaluated three typical basement membrane-associated ECM proteins, laminin, fibronectin and type IV collagen (Centre de Radioanalyse, Pasteur Institute, Lyon, France). The presence, and conserved distribution of these molecules in the thymuses from several mammalian species have been previously reported by our group (Berrih *et al*., 1985; Lannes-Vieira *et al*., 1991; Meireles de Souza *et al*., 1993). All immunesera were applied at 1/200 dilution.

Appropriate secondary antibody (diluted 1/100) corresponded to the goat anti-rabbit Ig coupled to fluoresceinisothiocyanate (Biosys, Compiegne, France).

Results

General morphological features and age-dependent involution of the equine thymus

Weight of both male and female equine thymuses showed a large range of variation within the various age groups. There was no correlation between animal weight and relative thymus weight (Fig. 1), and the correlation between the animal age and relative thymus weight presented a soft alometry (Fig. 2). The mean of the male and female thymic weights of all ages was 29 and 20 grams respectively, and the maximum male and female weight were 150 Kg (2 years old) and 140,5g (6 years old), respectively.

Microscopically, the postnatal equine thymuses showed lobules divided by two basic compartments: the *Intraparenchymal Compartment* (IPC) or Thymic Epithelial Space (TES) and the *Extraparenchymal Compartment* (EPC), containing the Perivascular Spaces (PVS). Each lobule consisted of a peripheral, darkly staining lymphocytic cortex and an inner, paler medulla, containing the distinctive Hassall's corpuscles (HC) (Fig.3). Although appearing very different in stained sections, the cellular components of cortex (the first anatomical region) and medulla (the second anatomical region) were similar, in that in both sites there is a mixture of T-lymphocytes and epithelial cells; the lymphocytes being enclosed in a mesh formed by the cytoplasmic processes of epithelial cells, constituting the IPC or TES compartment. The third anatomical region of the equine thymus, was the perivascular space (PVS) for its location adjacent to the blood vessels. The PVS was separated from TES by a basement membrane and did not contain developing thymocytes. PVS together with the capsular and septal regions not vascularized comprised the extraparenchymal compartment (EPC) (Figs.4-5). The boundaries between cortex and medulla were variable, being often not very well defined even in the young 6 months and 1 year old foals.

Among the 42 horses, ranging from 6 months to 4 l/2 years of age, only 13 presented a better defined cortex, showing, in general, a precocious atrophy, which became constant from five years onward. From 6 months to 18 years of age, the thymuses gradually involuted from a mild (grade I) to a very advanced atrophy (grade IV, V) of the TES compartment, associated with an increase in the extraparenchymal compartment, mainly the PVS. The different levels of atrophy presented the following characteristics:

Grade I: Diffuse cortical reduction with focal cortical disappearance, normal medulla and normal extraparenchymal compartment (Figs.6-8); **Grade II:** Disappearance of cortical layer; exacerbation of the epithelial network, intermingled with still abundant lymphocytes; lobules back to back and discrete increase of the extraparenchymal compartment; focal cortico-medullary inversion may occur (Figs. 9-10); **Grade III:** Cortical-medullary inversion, characterized by the formation of an epithelial band, surrounding a lymphocytic cortex, rich in large PAS^{+} macrophage cells, acquiring a profile similar to a fetal internal primordial cortex (Fig. 11); lobules with varied cellularity and size with loss of clear lobular definition; augmentation of the extraparenchymal compartment, mainly the perivascular space (PVS); which contained larger number of lymphocytes, sometimes forming a perilobular layer; increase in the number of mast and plasma cells; exacerbation of the reticulin network in the PVS and in the old cortical area (Fig. 12). **Grade IV**: Lobules cordonal-like, constituted by strips of epithelial cells, sometimes

tending to pseudoglandular arrangements, intermixed with small lymphocytes; striking augmentation of the extraparenchymal compartment, with abundant lymphocytes in PVS that sometimes formed a thin perilobular layer (Fig. 13); **Grade V**: Similar to grade IV, presenting thinner strips of epithelial cells (Fig. 14); less number of lymphocytes; more frequent pseudoglandular arrangements (Fig.15); remarkable increase in the extraparenchymal compartment, with large amount of adipose tissue and less cellular PVS (Fig. 16).

As expected, cortical thymocytes in all ages were predominantly of small and median size, although some large thymocytes could be seen in the external cortex, close to the epithelial cells lining the capsule or septa of the lobules. The epithelial cells, as shown in the classification of different levels of atrophy, formed a diffuse mesh, or lobular peripheral bands, or longs strips with or without pseudoglandular arrangements.

Hassall's bodies were heterogeneous and varied from small and monocellular to solid multicellular with small central cavity, or large cysts, lined by pseudostratified or glandular epithelia, presenting mucous secretory or ciliated cells (Fig. 17). Additionally, an intracavity papillary projection could be seen in one specimen, corresponding to a 30 months old female (Fig. 18). Hassall's body cavities or cysts contained lymphocytes, monocytes/macrophages, eosinophils and apoptotic detritus. As ascertain by PAS and alcian blue stainings, neutral glycoproteins and proteoglycans with low and high sulfatation could be seen in these structures (Fig.19). The Hassall's body cavities appeared from the age of 18 months onwards, and cysts as well as glandular transformation were more frequent in atrophic thymuses, mainly after 4-5 years of age. Sometimes, the Hassall's bodies were full of large number of unidentified dead (apoptotic) cells showing replacement of the epithelial covering by giant macrophage cells (Fig. 20). Nineteen percent of the atrophic thymuses from 6 to 18 years old equines did not present Hassall’s bodies. The appearance of PAS-positive macrophage cells was more prominent in the thymuses with atrophies types IV or V. They were large, presented PAS and alcian blue pH 2,5 positive granules and were located in residual medulla (Fig. 21).

The PVS was very dynamic, being detected in thymuses of all ages, and its cellularity was inversely proportional to the regression of Thymic Epithelial Space (Fig.13). However, in some cases of very advanced atrophy, the PVS was reduced due to replacement by adipose tissue, that increased from 5 years onwards.

High endothelial venules were identified in PVS from 6 months until 9 years of age in 15% of the thymuses (6 in 41), and were more evident in 30 months old animals (Fig. 22). The PVS contained lymphocytes, eosinophils, monocytes-macrophages, mast cells, plasma cells, rare

neutrophils and basophils. Mature and immature eosinophils were located in PVS (Fig. 23), and in the cortex, interacting with epithelial cells (Fig. 24). Eosinopoiesis was more active until 30 months old, eosinophil apoptosis predominated in 5 and 7 years, and the eosinophils, even mature, disappeared or were very scarce from 13 years onwards. Mature eosinophils were predominant in the medulla, surrounding and/or within Hassall's corpuscles.

Mast cells were also prominent in the PVS in most of the thymuses, being also present in the cortex. Foci of mastocytosis were detected in the PVS of five years old thymus, where the mast cells were round to oval, clear or alcian-blue positive (pH 1.0), safranine negative, tightly packed and mixed with few eosinophils (Figs. 25-26).

Mature plasma cells in the PVS, cortex and medulla were also present in variable number in most of the thymuses, being sometimes very numerous in involuting organs. They were often located with eosinophils and/or mast cells or mixed with perivenular lymphocytic aggregates. Small groups of immature plasma cells were identified in PVS and cortex (Fig. 27).

Erythropoietic foci were found within PVS until 9 years old thymuses (Fig. 28), while megakaryocytes (Fig. 29) were observed only in one thymus (4 ½ years), and monocytosis was also a rare event in the PVS.

The presence of lymphatic vessels in PVS, full of lymphocytes, was a constant and noticeable event in thymus of all ages. In serial sections, they appear to begin in the medulla as small and closed terminal sacs (Fig. 30). Blood thymus vasculature was supplied by arterioles that entered the base of the septa in the region of the cortico-medullary junction, and which gave rise to intraparenchymal sets of vessels. Some capillaries that supplied the cortex in direction to the capsule (cortex-septal communicating vessels).

Intrathymic distribution of extracellular matrix molecules

Extracellular matrix was composed by carboxylated more than sulfated proteoglycans (diffuse in the TES compartment), fibronectin, laminin and type IV collagen, seen in interstitium of the parenchyma, capsule, septa, vessels and fine cortical fibers (Figs. 31-36) and interstitial collagens (I and III) in capsule, adventicial of vessels, fine fibers of collagen 3 were also identified in the TES compartment.

Elastic fibers were scarce, did not increase in number after oxidation of specific stain, and were detected only in and around vascular walls and close to lobular basement membrane. During the progression of the thymic atrophy, the capsule never disappeared, although it became tortuous,

and a remarkable increase in the argyrophilic fibers network occurred, mainly in the PVS, together with an augmentation of thick interstitial collagen fibers around the vessels. Surprisingly, the huge deposition of ECM fibers in the extraparenchymal was not necessarily accompanied by increase in the intraparenchymal compartment. However, very often the atrophic lobules exhibited an increase in the number of penetrating fibers, thickening of the intraparenchymal vascular walls and less frequently, they also expressed a dense network of argyrophilic fibers. The amount of connective tissue in the extraparenchymal compartment was inversely proportional to the lipomatous transformation. Ultrastructural aspects of the post-natal thymuses are further shown in the figures 39 to 50.

Discussion

The postnatal equine thymuses exhibits the general thymic architecture described in humans and other mammals, showing however some quantitative and/or qualitative peculiarities. The lobules were divided by two basic compartments, subdivided in four anatomical regions: **Intraparenchymal Compartment** (IPC) or **Thymic Epithelial Space** (TES), in which we can identify the cortex (1^{st} anatomical region) and medulla (2^{nd} anatomical region), and **Extraparenchymal Compartment** composed by Perivascular Space (3^{rd} anatomical region), together with the capsule and non-vascularized septal regions (4^{th} anatomical region) (Steinmann *et al*., 1985; Bofill *et al*., 1985; Levine & Bearman, 1981).

The general analyses of the data allowed to classify the equine involution or atrophy in five grades, which progress more or less sequentially according to the animal ages. Atrophies of grades I and II occurred predominantly from 6 to 18 months; atrophy III, from 18 months to 4 years; atrophies IV and V, from 4-5 to 18 years. These results indicate that the atrophy III seems to be related to the beginning of the puberty (around 18 months for females and 24 months for males), and the progression to atrophies IV and V was coincident, initially, with the period when the corner (the third incisor on either side of each jaw) baby teeth were shed and the permanent incisors have erupted, i.e., the occurrence of the last dental shed. This general thymic behavior was not rigid but variable, although not depending on anti-helminth treatment (data not shown), being possibly influenced by hormones, neuropeptides and the nutritional status of the animals. These molecules modulate the expression of a variety of molecules in both epithelial and lymphoid components, as for example major histocompatibility complex gene products by

microenvironmental cells and the extracellular matrix-mediated interactions, influencing the thymocyte-epithelial cells interactions (reviewed by Savino & Dardenne, 2000).

Similar to human thymus, the equine thymus during aging, the size of intraparenchymal compartment decreases whereas the extraparenchymal compartment increases, including the perivascular spaces (Steinmann, 1986; Bodey *et al*., 1997), where high endothelium venules have been identified. Interestingly, recent studies have shown that, in normal situation, intra-PVS lymphocytes are likely correspond to peripheral mature cells, which migrated from the periphery (CD1a, CD45RO+, CD38-/low, TIA-1+ cytotoxic granules) (Haynes *et al*., 1999; Haynes & Hale, 1998; Flores *et al*., 1999). Taken together, these findings suggest that aging equine thymus may contain a "peripheral" lymphoid component within PVS. Nevertheless *in vivo* bromodeoxyuridine pulse chasing suggest that, at least in the nonobese diabetic mouse, where giant perivascular spaces are seen, intra-PVS cdells do correspond to mature thymocytes that are progressively being accumulated in the organ (Savino *et al*., 1991; 1993).

The frequent presence of variable number of plasma cells in PVS with plasmacytogenesis foci implies the participation of B cells. The presence of B cells located clearly within the human thymic medulla raises the question of whether those cells arose in the medulla, in contrast to PVS B cell that may come from the periphery (Flores *et al*., 1999).

Although the thymic atrophy level was more advanced after puberty, thymopoietic thymic epithelial space actually began to atrophy by the age of 6 months. This is in agreement with Steinmamm *et al*., (1986) which also observed that the human thymus begins to atrophy at age of one year, and shrinks in volume by approximately 3% per year through middle age, then shrinks by < 1% per year thoughout the rest of life (Steinmann *et al*., 1985; Steinmann, 1986).

The pathophysiology of thymic atrophy is very complex and multifactorial, including deficiencies affecting rearrangement of the TCR during intrathymic T cell development (Aspinall, 1997), loss of self peptides on thymic epithelial MHC molecules (Hartwig & Steinmann, 1994), aging of thymic stroma with loss of trophic cytokines produced in the thymic microenvironment (George & Ritter,1996; Hirokawa *et al*., 1982; Leiner *et al*., 1984; Utsuyama *et al*., 1991), and aging of the stem cell population (Tyan, 1977; Kadish & Basch, 1976). Cytokine production and thymic endocrine function are also hormonally controlled and a bidirectional circuitry seems to exist since thymic-derived peptides also modulate hormonal production (Savino & Dardenne, 2000). It appears that locally produced thymus cytokines influenced by systemic hormone production, may actively suppress thymopoiesis (Haynes *et al*.,

2000, Savino & Dardenne, 2000). Probably, different cytokine scenarios or combinations interfere on transcription factors, modulating the T cell quiescence or activation.

It is important to point out that the thymic atrophy does not occur uniformly, even in the same thymus, showing local variation from one lobule to another, thus stressing the microenvironmental variability. The montage of different microenviromental scenarios also explains the focal intrathymic plasmacytogenesis, eosinopoiesis, mastocitopoiesis, erythropoiesis, megakaryopoiesis, lymphocytosis and emergency of HEV in PVS. Frequently, the PVS lymphocytes formed a perilobular layer or were arranged in strands, suggesting a functional communication with the peripheral layer of epithelial cells from thymic epithelial space (TES).

Eosinopoiesis was the predominant non-lymphoid lineage produced inside the equine thymus, and eosinophils were found in both the PVS and TES compartments. One natural candidate for explaining this phenomenon would be an intrathymic release of IL-5, that may be particularly high in the equine thymus. IL-5 was readily detectable in most normal human thymus tissues from 2 years old or even younger patients. IL-5 mRNA was undetectable in thymus derived from patients 3 years of age or older (Flores *et al*.**,** 1999). In our material, eosinophils only disappeared or were rarely seen in thymuses from 13 years old onwards, when there was advanced atrophy. Thus suggests persistent high levels of IL-5 in the horse thymus, an issue that deserves further investigation.

In addition to eosinophils, in some animals we found foci of erythropoiesis, and even more frequently, local confluence of eosinophils, mast cells and plasma cells. We observed an inverse correlation between the number of eosinophils and mast cells. These cell types present different regulatory mechanisms: the eosinophil lineage requires IL-3, IL-5, GM-CSF (Granulocyte Macrophage-Colony Stimulating Factor), whereas mast cells depend on IL-3, IL-4, IL-9, IL-10 and SCF (Stem Cell Factor) (Hultner *et al*., 1990, Miyajima *et al*., 1992). Again, the determination of the cytokine profile in the horse thymus will be certainly useful in order to better understanding the issue of non-lymphoid hemopoiesis in the horse thymus.

All the intrathymic mast cells were alcian blue positive; safranine negative, suggesting that they are comprised in the mucosal mast cell phenotype, although horse mast cell populations are not well characterized.

One thymus (female, 5 years old) presented nodular aggregates of mast cells, characterizing an intrathymic mastocytosis (Figs.25,26).

During the thymic involution, the epithelial cells initially formed peripheral lobular bands, and in the grades IV and V of atrophy they were frequently arranged in long and thin strips, developing pseudoglandular transformations (endodermal component of epithelial cells?)

One histological hallmark of equine thymus was the great morphological variability of the Hassall's bodies and their tendency to undergo cystic degeneration and mucous or glandular metaplasia. They were lined by flattened cuboidal, pseudostratified or ciliated columnar with globlet epithelial cells, acquiring, sometimes, a mucous acinus-like configuration. Some cysts were ruptured, devoid of epithelial, isolated or fused, full of cellular debris surrounded by volumous macrophage giant cells. We interpreted the PAS + macrophage cells not only as macrophage full of insoluble and indigestible lymphocytic residues that did not migrate to the Hassall's bodies (Siegler, 1964), but also as macrophages that take part in the digestion of Hassall's corpuscle contents.

The lymphatic vessels were very conspicuous, containing of lymphocytes in most of the thymuses, even in atrophic ones, suggesting their importance as one lymphocyte exportation pathway to the periphery. Interestingly, such a conspicuous thymic lymphatic vasculature is not commonly seen in thymuses from other mammals, and certainly deserves further investigation, since horses may correspond to an excellent animal model for studying the molecular mechanisms involved in thymocyte exportation through lymphatic vessels.

The histoarchitecture, distribution and composition of extracellular matrix in equine thymuses was very similar to the aspects already described in other mammals, emphasizing, however, the absence or scarce elastic fibers in the intraparenchymal compartment, in opposition to the diffuse and frequent pericellular distribution of proteoglycans.

The ECM presented four basic distributive pattern: a) capsular, septal and perivascular (interstital collagens, proteoglycans, elastic fibers and fibronectin); b) lobular and vascular basement membrane (laminin and type IV collagen); c) interstitial or intraparenchymal (type III colagen and fibronectin) and d) pericellular (proteoglycans).

During involution, both the lymphoid tissue and the perivascular space decreased, and fatty atrophy develops (Kornstein, 1995; Henry, 1992; George & Ritter, 1996; Steinmann *et al*.**,** 1985). In fact, the lipomatous atrophy is likely a metaplastic event arised from a reprogramming of undifferentiated mesenchymal cells present in perilobular connective tissue along a new pathway. This is likely brought about by changes in signals generated by mixtures of cytokines, growth factors, and extracellular matrix components that compose the cell microenvironment.

In conclusion, the present study shows that the equine thymus: a) presents a general morphological and involutional characteristics similar to other mammals (including the enahncemet of extracellular matrix deposition). This involutional process is not homogeneous, even within the same thymus, and begins before the puberty (6 months of age); b) expresses high endothelial venules in PVS and more infrequently in the medulla; c) the lymphatic vessels are prominent and may represent an important pathway of thymocyte exportation; d) Hassall's bodies exhibit frequent, cyst formation, glandular (mucous) metaplasia, and signs of degeneration and reabsorption by macrophage-PAS cells and giant cells; e) intrathymic non-lymphoid hematopoiesis is a frequent event, showing eosinopoiesis, erythropoiesis and mastocypoiesis; and f) B cells increase in number during the thymic involution, mainly in the PVS, presenting mature plasma cells and foci of plasmacytogenesis.

The results raised some questions that deserve to be addressed in the future, such as the functional significance of the glandular metaplasia in the Hassall's bodies, the lymphatic vessel pathway for thymocyte exportation and the control mechanisms that allow in the horse thymus non-lymphoid hematopoiesis to take place. Finally, this study also emphasizes the importance of analyzing various animal models, in order to avoid a skew view of the immune system, based only or mainly on the mouse model.

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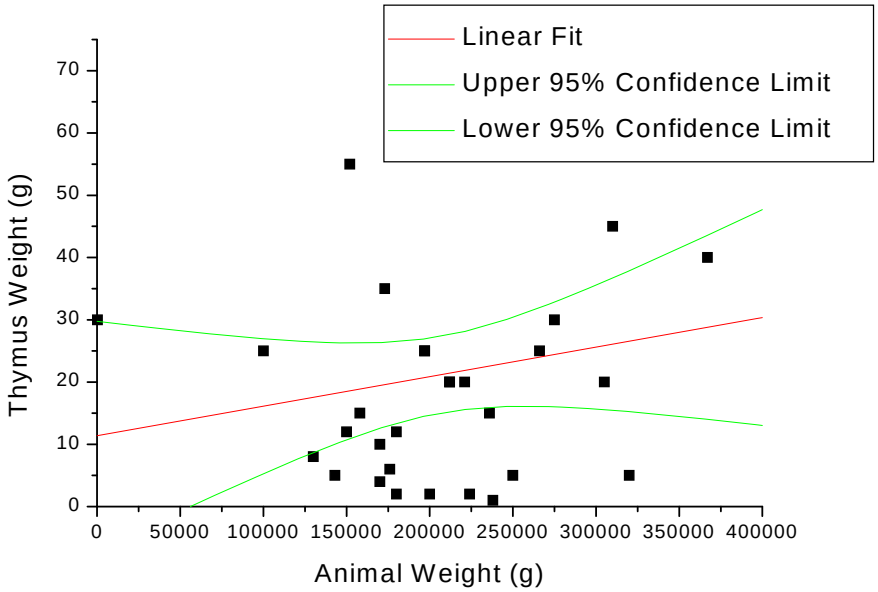

Figure 1 – Linear Regression with 95% confidence Band, of Animal Weight and Thymus Weight, demonstrated a soft positive alometry with this two variables.

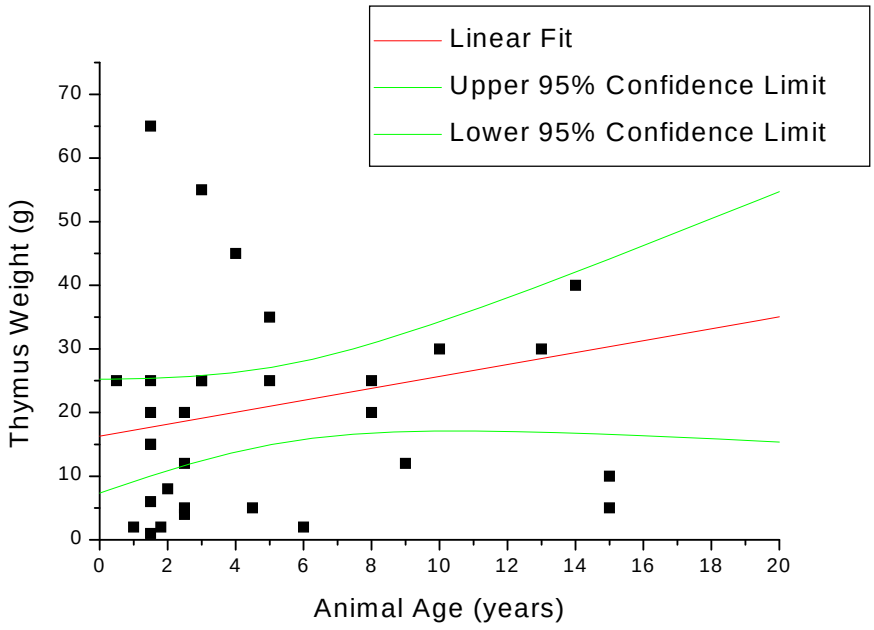

Figure 2 – Linear Regression with 95% confidence Band, of Animal Age and Thymus Weight, demonstrated a soft positive alometry with this two variables.

Legends of Figures

Figure 3 - Normal (2 years old) equine thymus stained by Lennert's Giemsa, showing clear cortico-medullary definition and thin interlobular septum (x 80).

Figure 4 - Normal (2 years old) equine thymus showing a PVS full of vessels, surrounding by cells, externally confined by lobular basement membrane. (methenamine silver + periodic acid staining, x 200).

Figure 5 - Normal (2 years old) equine thymus showing transversal sections of PVS in the medullary region limited by sheath-like basement membrane. The dark cells correspond to macrophages full of glycoproteins. (methenamine silver + periodic acid staining, x 200).

Figures 6-7 - Normal (6 months old) equine thymus showing grade I atrophy characterized by focal persistence of the cortex (Fig. 6), alternating with areas showing significant decrease in the amount of cortical thymocytes (Fig. 7). Medullary region is diffusely infiltrated by eosinophils, which are more concentrated close to a small Hassall's body. (Lennert's Giemsa (Fig. 6) and haematoxylin-eosin stainings (Fig. 7) (x 125).

Figure 8 - Normal (6 months old) equine thymus showing lobules back-to-back with scarce reticular fibers in the parenchyma, expressed more in vascular walls. (Gomori's reticulin staining, x 125).

Figure 9 - Normal (18 months old) equine thymus showing grade II atrophy with loss of the cortical layer and PVS containing lymphatic vessels full of lymphocytes. (Lennert's Giemsa staining, x 125).

Figure 10 - Normal (18 months old) equine thymus showing one lobule with cortico-medullary inversion, exemplified by formation of peripheral epithelial cell band, in which some plasma cells are seen. The PVS compartment presents lymphocytes and mature eosinophils. (Lennert's Giemsa staining, x 310).

Figure 11 - Eighteen months old equine thymus exhibiting a grade III atrophy with lobules showing cortico-medullary inversion, with prominent exacerbation of the epithelial network, particularly the subcapular epithelial cell layer. The decrease in thymocyte numbers is accompanied by significant increase of lymphocytes in the PVS, where mast cells are also seen. The perilobular lymphocytes form rows around the lobule, close to the epithelial cells. (Lennert's Giemsa staining, x 310).

Figure 12 - Exarcebation of argyrophilic fibers in PVS, in the border of the lobules, in the TES compartment and in the vascular walls. (Gomori's reticulin, x 310).

Figure 13 - General view of a 6 years old atrophic equine thymus (grade IV of atrophy) with the parenchyma being reduced to cordonal lobules, presenting remarkable expansion of the PVS with lymphocytic nodular clusters and light lipomatous atrophy. (Lennert's Giemsa staining, x 80).

Figure 14 - Eight years old equine thymus exhibiting grade V atrophy with strands of parenchyma mostly constituted by epithelial cells, interposed by PVS containing mast cells and few lymphocytes. (Lennert's Giemsa staining, x 200).

Figure 15 - Fourteen years old equine thymus exhibiting atrophic lobules with glandular transformation of the epithelial cells, showing vacuolated cytoplasm. (Masson's trichrome staining, x 310).

Figure 16 - Eight years old equine thymus exhibiting atrophic thymus with condensation of the reticular network in the cordonal lobules and augmentation of collagen fibres in the PVS compartment. (Gomori's reticulin staining, x 200).

Figure 17 - Fourteen years old equine thymus exhibiting two Hassall's bodies showing glandular features manifested by the appearance of globlet cell type. (Masson's trichrome staining, x 500).

Figure 18 - Eighteen years old equine thymus exhibiting one Hassall's body covered by pseudostratified epithelium, presenting an intraluminal papillary projection. (Masson's trichrome stainin, x 200).

Figure 19 - Fourteen years old equine thymus exhibiting several Hassall's bodies with glandular transformation containing sulfated proteoglycans and neutral glycoproteins. (alcian blue, pH 1.0 - PAS staining, x 310).

Figure 20 - Fifteen years old equine thymus exhibiting a cystic Hassall's body full of cellular debris with substitution of the epithelial layer by macrophage giant cells. (Lennert's Giemsa staining, x 310).

Figure 21 - Two years old equine thymus exhibiting two small aggregates of macrophage-PAS^{+} cells, containing PAS and proteoglycan positive cytoplasmic granules. (2 years old) (alcian blue, pH 2.5, PAS staining, x 310).

Figure 22 - Perivascular space in the thymus from an eighteen months old horse, showing a high endothelial venule, containing intraluminal lymphocytes, and surrounded by lymphocytes, macrophages and mature eosinophils. (Lennert's Giemsa staining, x 500).

Figure 23 - Eosinopoietic focus in PVS of a 2 years old equine thymus, composed mainly by myelocytes with few metamyelocytes, also showing one eosinophil in mitosis. (Lennert's Giemsa staining, x 500).

Figure 24 - Eosinopoietic focus inserted in the epithelial band of one atrophic lobule from an 18 months old equine thymus, showing close contact between eosinophils and epithelial cells. (Lennert's Giemsa staining, x 310).

Figures 25-26 - Nodular focus of mastocytosis in PVS of a 5 years old equine thymus, characterized by ovoid mononuclear mast cells with very well defined cytoplasmic border, intermixed with some mature eosinophils (Fig. 25). The mast cells present different levels of alcian-blue positivity in the cytoplasm, without expression of safranine positive granules (Fig. 26). (alcian blue-safranine staining, x 310).

Figure 27 - Focus of intralobular plasmacytogenesis mingled with epithelial cells, from an 18 months old equine thymus. The adjacent PVS is rich in lymphocytes, showing also one mast cell. (Lennert's Giemsa staining, x 310).

Figure 28 - Erythropoietic focus in interlobular PVS characterized by erythroblasts in different phases of differentiation and maturation. Eighteen months old equine thymus section stained with Massom's trichrome (x 500).

Figure 29 - Mature megakaryocyte in PVS in close contact with macrophages, lymphocytes and eosinophil. Four ½ years old equine thymus section stained with haematoxylin-eosin LSM, transmission mode (x 500).

Figure 30 - Nine years old equine thymus section stained with Gomori's reticulin, and showing a branched lymphatic vessel in one medulla-located PVS (x 200).

Figure 31 - Immunofluorescence detection of fibronectin in a two years old equine thymus. Fibronectin immunoreactivity is seen around Hassall's bodies, in the vessel walls and as isolated interstitial fibers (x 250).

Figure 32 - Immunofluorescence detection of fibronectin in a two years old equine thymus. Fibronectin immunoreactivity is seen in the PVS interstitium, vascular walls, parenchymal capillaries and isolated intralobular fibers (x 250).

Figures 33-34 - Immunofluorescence detection of laminin in five (Fig. 33) and four (Fig. 34) years old equine thymus section. Laminin is seen in the basement membrane of lobules, vessels from PVS and lobular capillaries, as well as in some intralobular isolated fibers (x 250).

Figures 35-36 - Immunofluorescence detection of type IV collagen, showing that this molecule presents the same distribution as laminin. Immunostainings in figs. 35 and 36 correspond to 2 ½ and 5 years old equine thymus sections, respectively). x 200 (Fig. 35); x 400 (Fig. 36)].

Figures 37-38 - Immunofluorescence detection of cytokeratin in the normal medulla, labeling also round and small Hassall's bodies (Fig. 37), in atrophic lobule (compact arrangement of epithelial cells), and in the wall of cystic Hassall's corpuscle. (Figs. 37 and 38 correspond to specimens from 5 and 8 years old animals, respectively) (x 250).

Figure 39 – Ultrastructural aspects of a two years old equine thymus, showing cortical thymocytes with irregular heterochromatin shape and presence of peripheral or central nucleolus (*). Mitochondria are distributed in one cellular pole (x 15,200).

Figure 40 – Ultrastructural aspects of a two years old equine thymus, showing a medullary region in which the ratio of thymocytes to epithelial cells shifted in favor of the epithelial cells. These TEC have abundant cytoplasm and present dilated processes containing finely floccular material. Their cytoplasmic processes are in close contact with lymphocytes. Collagen fibers, transversally sectioned (*), are also seen between the cells (x 6,400).

Figure 41 – Ultrastructural aspects of a two years old equine thymus, showing a medullary region in which lymphocytes are intermingled with epithelial cell processes, sometimes connected by desmosomes. The cell in the center appears to be a dendritic cell, and the cytoplasm of one macrophage containing lysosomes is also seen (⟶) (x 4,410).

Figure 42 – Ultrastructural aspects of a 18 months old equine thymus, showing a medullary region containing one interdigitating cell and two lymphocytes surrounded by epithelial cell processes, some of them connected by desmosomes (➢) (x 11,500).

Figure 43 – Ultrastructural aspects of a five years old equine thymus, showing a medullary region in which epithelial cells of Hassall's bodies exhibit tight bundles of tonofibrils in the cytoplasm (x 4,500).

Figure 44 – Ultrastructural aspects of a nine years old equine thymus, showing a medullary region in which exhibiting highly keratinized and normally keratinzed epithelial cells exhibiting clear nuclei with peripheral heterochromatin and conspicuous nucleolus, characterizing part of Hassall's body wall. The external region of Hassall's body cell is in contact with thymocytes (x 6,000).

Figure 45 – Ultrastructural aspects of a nine years old equine thymus, showing a medullary region depicting one Hassall's body with central cavity containing cilia. Cross-sectional view demonstrates microtubular arrangement in the cilia (⟶) (x 16,000).

Figure 46 – Ultrastructural aspects of a 18 months old equine thymus, showing two immature mast cells, with clear nuclei (heterochromatin in the periphery) and cell contacts between through their surface folds. The central mast cell presents extensive and close contacts with lymphocytes (x 8,000).

Figure 47 – Ultrastructural aspects of a 9 years old equine thymus, depicting mature mast cells with heterogeneous granular content and surface folds, presenting several focal and close contacts with lymphocytes (x 12,600).

Figure 48 – Ultrastructural aspects of a 5 years old equine thymus, showing one PVS containing two plasmablast, the central one exhibiting dilated granular endoplasmic reticulum sacs. The plasmablast is close to one mast cells (⟶) (x 16,000).

Figure 49. Ultrastructural aspects of a two years old equine thymus, revealing two mature neutrophils (⟶) in the medulla, in contact with medium and large lymphocytes. The central neutrophil presents phagocytized cellular debris in one cellular pole and is in contact with a macrophage (➢) (x 6,200).

Figure 50. Ultrastructural aspects of a 5 years old equine thymus, showing intra-PVS eosinophis that bear extensive contact areas among themselves, and one of them is touching an adjacent mast cell. The eosinophil granules do not present core, crystalloid, or internum and the granular matrix is relatively homogeneous (x 6,400).

Table 1. Staining procedures and corresponding tissue labeling

Stainning Procedure	Tissue specificities	Final Colours	References
alcian blue pH 2.5- PAS	Weakly or non sulphated proteoglycans, hyaluronic acid and sialomucins. Polysaccharides and neutral proteoglycans containing 1-2 glycol grupaments.	dark blue	Lev & Spicer, 1964
alcian blue pH 1.0-PAS	Sulphated proteoglycans Polysaccharides and neutral proteoglycans containing 1-2 glycol grupaments	blue	Lev & Spicer, 1964
Gomori's reticulin	Reticular fibers (Type III, and glycoproteins)	black	Gomori, 1937
Weigert's Resorcin with oxidation without oxidation	 Elastic fibers, oxitalanic fibers Elastic fibers, elauninic fibers	 brown to purple brown to purple	Fullmer & Lillie, 1958 Gawlik, 1965
Masson trichorome	collagens fibers muscles nuclei	blue red blue-black	Masson, 1929
methenamine silver perioacid (PAMS)	basement membrane and reticular fiber	black	Jones, 1951
Lennert's Giemsa	nuclei erytrocytes cytoplasme osinophilic granulae basophilic granulae neutrophilic granulae	blue orange purple red dark purple red	Lennert, 1978
Mayer's hematoxilin and eosin	nuclei cytoplasm most other tissue structures	blue pink to red pink to red	
phosphomolibidic acid and picrosirius	collagen fibers	red (MO)	Dolber & Spach, 1993
alcian blue safranin	mast cell mucous mast cell transition in connective tissue	blue red	Strobel *et al*., 1981

DEVELOPMENTAL ASPECTS OF THE CELLULAR AND EXTRACELLULAR MATRIX COMPONENTS OF THE EQUINE FETAL THYMUS

Ellen Cortez Contreiras[1,4], Ester Maria Mota[2], Maria de Nazareth Leal de Meirelles[3], Wilson Savino[1] and Henrique Leonel Lenzi[2]

[1]Lab. Thymus Research, Department of Immunology,
[2]Department of Pathology and [3]Department of Ultrastructure and Cell Biology
Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil;
[4]Department of Morphology, Biomedical Institute,
Fluminense Federal University, Rio de Janeiro, Brazil.

Correspondence:
Henrique Leonel Lenzi
Department of Pathology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation,
Av. Brasil 4365 – Manguinhos,
21045-000, Rio de Janeiro, Brazil.

Tel: (55) (21) 573.8673; 598.4350; 598.4466
Fax: (55) (21) 573.8673
email: hlenzi@ioc.fiocruz.br

Abstract

We describe in this work some morphological and immunohistochemical characteristics of the equine fetal thymuses, in different phases of gestation. Six equine fetuses with ages varying from two to ten months in gestation were analyzed by histological techniques staining, indirect immunofluorescence and electron microscopy.

The morphological aspects described, plus the sequential appearance of certain fundamental events, such as cortical-medullar definition, the appearance and the maturation of Hassall's corpuscles, are similar to those described in humans and other animals species. However, the equine fetal thymuses show intense intrathymic eosinophilia and hematopoiesis of other lineages. Additionally, clear-cut lymphatic vessels full of lymphocytes were seen in these fetal thymuses.

The expression of extracellular matrix components in equine fetal thymuses reproduced the general aspects already observed in humans and other animal models. There was a clear predominance of interstitial collagens and proteoglycans mainly with low sulfation in the capsula, septae and perivascular space, whereas in the intraparenchymal compartment three distinct extracellular matrix patterns were detected related to basement membrane (lobular and vascular) and vascular walls (laminin, fibronectin and type IV collagen); sparse and isolated intraparenchymal fibers, sometimes connected with the septae or perivascular space (fibronectin, type III collagen), as well as cell surface proteoglycans.

Our results show that despite sharing several morphological features with the thymus from other mammals, the equine fetal thymus exhibits particular aspects suggesting that it may represent an interesting model for further studies on mammalian intrathymic non-lymphoid hemopoiesis as well as the origin and fate of lymphocytes found within thymic lymphatic vessels.

Introduction

It is noteworthy the importance horses have played for the development of immunotherapy and progresses regarding the understanding of immunization mechanisms, mainly related with antibody production (Wells *et al*., 1981).

Studies of equine T-lymphocytes have revealed the presence of phytolectin-responsive T-lymphocyte as early as 80 days of gestation period, and the ability of T cells to recognize and respond to an alloantigen stimulation, by the 100^{th} day of fetal development (Perryman *et al*., 1988). Mature T lymphocytes expressing the Eq T3 antigen were demonstrated in the fetal thymus as early as 75 days of gestation (Wyatt *et al*., 1988).

Despite a certain body of evidence concerning the immunological status of the equine fetus, no data are available on the equine fetal thymuses, particularly its microenvironmental compartment. In this respect, we describe herein some morphological characteristics of the equine fetal thymuses, in different phases of gestation.

Material and Methods

Six equine fetuses with ages varying from two to ten months in gestation (2, 3, 4, 5, 6 and 10 months). from equines females of *Equus caballus*, were obtained at Federal Rural University of Rio de Janeiro (Department of Parasitology, Rio de Janeiro, Brazil). These animals were handled according to the ethical rules established by the governmental ethics committee of EMBRAPA (National Brazilian Agency for Agricultural Research). Thymic fragments were fixed in Carson's Millonig formalin.

Histology and ultrastructure

Thymus fragments, once fixed Carson's Millonig formalin, were dehydrated and routinely processed for paraffin embedding and sectioning. Five 5μm sections were stained by hematoxylin and eosin (H&E), Gomori's reticulin, PAS-alcian blue pH 2.5 and 1.0, Weigert's resorsin-fuchsin (with or without 10% potassium persulfate as oxidant), Lennert's Giemsa (Lennnert, 1978) and Masson's trichrome, periodic acid methanamine silver (PAMS), Picrosirius-polarizing method (Junqueira *et al*., 1979) for brightfield microscopy; sirius red (pH 10.2) and

phosphomolybdic acid-picrosirius red (PMA-PSR) (Dolber & Spach, 1993) for confocal laser scanning microscope (LSM-410, Zeiss).

For electron microscopic examination, sections of ten-month fetus were fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate and postfixed in 1% osmium tetroxide, dehydrated in graded acetone series and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed by an EMS 10B Zeiss electron microscope.

Immunohistochemistry

Indirect immunofluorescence was performed on cryostat sections. Unless stated, all reagents applied for immunohistochemistry were purchased from Sigma Co St. Louis, MO, USA. Acetone-fixed 5-µm thick equine thymus frozen sections were washed in phosphate buffered saline (PBS), pH 7.2, 0.01M (10 minutes) and blocked with PBS/BSA 1% (bovine serum albumin). Specimens were then incubated with antibodies specifically recognizing distinct extracellular matrix components were obtained from Institute Pasteur (Lyon, France). All were polyclonal immunesera produced in rabbits by injection of bovine type IV collagen, fibronectin or laminin isolated from a murine Erlisch sarcoma (Grimaud *et al*., 1980). As demonstrated by the manufacturer, these antibodies do not exhibit any crossreaction (assessed by ELISA) with any other ECM molecule. We also used polyclonal antibody rabbit anti human cytokeratin (Dako, California, USA). These primary anitobdies were applied at dilution of 1:200, except the anti keratin antibody, that was applied at 1:40 for 1 hr (37 °C). Slides were washed in PBS and subjected to the FITC-coupled goat anti rabbit Ig antibody at dilution of 1:100 for 1 hr (37°C). Finally, they were washed in PBS for 5 minutes (3 times), and evaluated under fluorescence microscopy.

As a further set of immunohistochemical labeling, five- micrometer thick sections of thymic tissue from four, six and 10 months fetuses were led to adhere on slides previously coated with 3-Aminopropyltriethoxy silane (APFS). Following deparaffination, they were subjected to microwave at 750W for 10 min for antigen retrieval in 0.01 M citrate buffer, pH 6.0 (H 2800 Microwave Processor, Energy Beam Sciences, Inc.). After background blocking with 8% fetal bovine serum, 2% powder skimmed milk, 2.5% bovine serum albumin in 0.01 m PBS, pH 7.4, primary antibodies to desmin, vimentin, myoglobin, paralbumin and tropomyosin were applied and left for overnight at 4 °C. In some

experiments, the anti-cytokeratin antibody was also used in parafin-embedded tissue. After washing, the specimens were incubated with biotinylated anti-rabbit or anti-mouse immunoglobulins for 45 min at 37 °C. After washing they were incubated with extreptavidin- Cy3 conjugate for 45 min. at 37 °C.

Double immunoenzymatic staining was also done, using specific antibodies to desmin and S-100α or S100β subunits proteins, using biotinilated secondary antibodies and streptavidin-alkaline phosphatase (Signet, Massachussets, USA) and peroxidase, with the enzyme activities developed by fast red and diaminobenzidine, respectively. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 15 minutes.

Results

The two-month fetus presented a thymus composed of multiple initial lobules constituted predominantly by epithelial cells forming peripheral arrangements of two layers of cells, surrounding a primitive cortex, predominantly composed of small lymphocytes, a smaller number of medium-sized and rare large lymphocytes (Figs.1,2). Many lymphocytes were located in close contact with epithelial cells (Figs.3,4). The lobules were not vascularized, but neighboring venous capillaries and small venules were found almost touching their perimeter (Figs.5,6). The stroma surrounding the lobules was loose, comprising fibroblasts, and exhibited fine, delicate collagen and reticular fibers, which thickened towards the contact with the epithelial cells (Fig.7). Elastic and oxythalanic fibers in the perilobular conjunctive tissue coincided topographically with the denser areas of reticular fibers.

In the fetuses with ages of 3 months upwards, the lobules were very distinct and the interlobular septa were thicker, composed of loose conjunctive tissue, rich in fibroblasts and reticular fibers (Fig.8). Perivascular space (PVS) were easily identified and the majority of the cells present were fibroblast-like components, lymphocytes, monocytes, eosinophils and few mast cells (Figs.9,10).

At four and a half months of gestation, the septa were seen to be narrow and after the fifth month, became so thin that the border of one lobule would touch the next (Fig.11). Increased cellularity was seen in PVS, which exhibited fibroblast-like cells, lymphocytes, monocytes, eosinopoietic foci, mast cells and rare basophils. Moreover, lymphatic vessels full of lymphocytes appeared for the first time (Fig.12).

From three gestational months onwards, mature and immature eosinophils were visualized in the PVS, which by the ten-month old fetus were arranged in clusters, close to the lymphatic or vascular vessels (Fig.13). In addition to the eosinopoiesis in the four and five months, erythropoietic foci were also identified, characterized by the presence of erythroblasts. In the septum of the four month fetus, an isolated focus of megakaryopoiesis was detected, as ascertain by the presence of megakaryoblast and mature megakaryocytes (Fig.14).

Mast cells were also observed from the three-month fetus onward, which were generally scarce, staining blue in alcian blue-safranine and in PAS-alcian pH 2.5. They exhibited varied morphology, with heterogeneous cytoplasmatic granules. Many were immature and sometimes located next to the subcapsular epithelial cells, predominating in PVS (Fig.15).

There was a clear cortical-medullary definition in all the fetuses, except for the two-month fetus (Figs.16,17). The reticular-epithelial cells were pronounced and up to and including five months, formed a epithelial band subcapsularly arranged (Fig.18).

Typical figures of apoptosis with picnotic and fragmented nuclei, were seen in all fetal thymuses, and macrophages were found bearing apoptotic debris. Lymphocyte mitoses were also frequent, being more numerous in the four and five month old fetuses. Mature and immature eosinophils were detected permeating the cortical in all fetuses, except in fetus with six months. They were often undergoing degranulation and were arranged in close contact with subcortical reticular-epithelial cells (Fig. 19).

Small lymphocytes predominated in the medullary of all the fetuses, with more irregular and less salient nuclei than in the cortical lymphocytes, but not demonstrating a convoluted nuclear aspect. Picnotic lymphocyte nuclei and mitotic figures were observed, but less frequently than in the cortical. The eosinophils in medullary region were always mature and in small numbers, except in the six-month fetus, where they were numerous and frequently peri or intra-Hassall's corpuscles (Figs. 20-21).

Hassall corpuscles were detected for the first time in the three-month fetus (Fig. 16), which were mono and multicellular, sometimes exhibiting cytokeratin granules, but without forming a central cavity. Larger ones showing a central cavity with cellular debris and expressing alcian blue in pH 2.5 appeared from the sixth month onwards. Macrophage giant cells were seen close and inside some Hassall’s bodies in the six months old fetus (Fig. 22). Epithelial cells of these structures, in four and half, six and ten months old fetuses, expressed cytokeratin, tropomyosin and α and β S-100 proteins (Figs. 23-29). In the same

fetuses, desmin-positive myoid cells were also detected close to Hassall's bodies (Fig. 30). All other markers were negative, including the CD1.

The components of the extracellular matrix exhibited the following characteristics: The septa were centrally composed of interstitial collagen fibers, initially of type III, changing to type I with increasing age. Reticular fibers were distributed in the lobular periphery, septa and PVS. They also appeared around the cortical and medullary vessels and formed isolated and sparse fibers both in the cortex and medulla. Incomplete or discontinuous definition of the basement perilobular membrane was detected by PAMS in the five-month fetuses, becoming continuous as from the sixth month. Carboxylated proteoglycans with or without low sulfation were expressed on the surface of cortical lymphocyte cells, in the septa, capsules and PVS. The proteoglycans developed by alcian-blue pH 2.5 always predominated over those marked in pH 1.0. On cryostat sections from five-month fetus, laminin and fibronectin (Figs. 31-32) were colocalized in the periphery of the lobules, capillary walls and in isolated intraparenchymal fibers.

Venules of high endothelium (HEV) were observed in PVS of the 10 months old fetus, and were surrounded by lymphocytes and eosinophils (Fig. 33). An ultrastructural analysis of this fetal thymus showed thymocytes with irregular nuclei, tending to a cerebriforme or convolute aspect, with cytoplasm indentations, dense peripheral chromatin, visible and frequent nuclear pores and small nucleoli. The cytoplasm was diffusely rich in polyribosomes and one of the cellular poles contained various mitochondria but lysosomes were not detected. A well-defined basement membrane surrounded the reticular-epithelial cells of the cortical and in the cytoplasm, which exhibited developed endoplasmic reticulum and mitochondria. They sometimes surrounded groups of lymphocytes in emperipolesis processes and exhibited various contact points with lymphocytes. These showed dense or clearer cytoplasm, with a greater scarcity of organelles. Macrophages were seen containing sometimes apoptotic debris. (Figs. 34-37).

Discussion

The present study represents to our knowledge the first description of the morphological changes in the equine thymus occurring along with fetal development from two to ten months of gestation. The morphological aspects described, plus the sequential appearance of certain fundamental events, such as cortical-medullar definition, the appearance and the

maturation of Hassall's corpuscles, are similar to those described in humans and other mammalian species. However, the equine fetal thymuses exhibit in a peculiar and intense intrathymic hematopoiesis of other lineages including eosinophils, as well as prominent septal lymphatic vessels and frequent epithelial bands arranged on the edges of the lobules.

The equine thymocytes in equine fetuses are not morphologically different from the thymocytes of other mammals, including humans, and are predominantly small and medium-sized, with scarce occurrences of cells with large profile. In the thymuses of ages between four and five months, evidence of greater activation was detected, shown by an increase in apoptotic cells and cells undergoing mitosis, concurrently with concentrations of eosinopoiesis, erythropoiesis and megakaryopoiesis, together with the appearance of lymphatic vessels full of lymphocytes. Lymphatic vessels with these characteristics indicate the widespread exportation of thymocytes to the periphery. In fact, Smith (1955) observed that intrathymic lymphatic vessels in the form of a sheath accompany the medullary veins and arteries, through which the lymphocytes and in the case of hemorrhage, the erythrocytes also, leave the thymus. Saint-Marie & Leblond (1958) described perivascular lymphatic channels in the medulla of the rat thymus. They concluded that the lymphocytes that penetrate the perivascular channels can reach the circulation by two means: 1) by diapedesis, from these channels, entering closed blood vessels, and 2) traveling through the channels to the lymphatic pharyngeal circulation.

In our study, the lymphatic vessels could only be visualized from four months of gestation onwards, being found in the PVS and apparently originating in the medulla from blind sacs. The presence of these vessels full of lymphocytes varied in number from animal to animal, but the fact that they contain large quantities of lymphocytes, suggests that they are at least in the equine thymus, an important efferent way for thymocyte exportation.

It is important to point out that the thymic lymphatics are essentially efferent (Schooley & Kelly, 1964) and in our material, the presence of lymphocytes inside venules was not detected. In the same way as in normal humans (Sodestrom *et al*., 1970), intraparenchymal high endothelium venules (HEV) were not identified in fetal equine thymuses, independently of the gestational period evaluated. However, HEV were detected in PVS of 10 months old fetus, being surrounded by clusters of lymphocytes. This special type of vessel, which is lined with high endothelium and is associated with lymphocyte blood-to-tissue migration was detected also in thymus from two and half year old horses onward (accompaning manuscript). This observation indicates that the presence of HEV is not

mandatory for intrathymic endothelial-prothymocyte or endothelial-lymphocyte interactions that result in cellular entry from blood into thymus. However, postnatal microenvironmental intrathymic changes may create favorable conditions to stimulate HEV development in the intraparenchymal compartment.

Studies on thin sections of specimens of human normal thymuses also failed to identify HEV similar to those found in lymph nodes, indicating that lymphocytic diapedesis occurs in other types of vessels. This type of venule was only seen in 2/5 of the thymuses of patients with Myastenia Gravis in "paracortical type" areas during the lymph node transformation of the thymuses (Soderstrom *et al.*, 1970). Therefore, the HEV identification in normal human thymus and in thymus of patients with Myastenia Gravis was confined within the PVS (Flores *et al*., 1999).

The Figures 5 and 6 pertaining to 2 months old fetus show accentuated closeness of perithymic vessels to primordial lobular epithelial cells, facilitating blood-thymus interchanges, including intrathymic penetration of circulating pro/prethymocytes. One of the striking aspects of the younger thymuses of three and four gestational month was the presence of subcortical epithelial cells in a band arrangement, which may characterize a special intrathymic microenvironment. Interestingly, this TEC layer has been referred as the "neuroendocrine" epithelium (Ritter & Crispe, 1992; Henry, 1992) sharing expression of some surface molecules with cells derived from neural crest (Mentlein & Kendall, 2000).

The Hassall's corpuscles are very similar to those of other species, including human. They begin as hypertrophied epithelial cells and subsequently become multicellular. From the sixth month of gestation onwards, they become larger, form a central cavity and begin to express proteoglycans with low sulfation. The acquisition of this degree of cell differentiation coincides with the presence of a large number of mature eosinophilis around them, which suggests that the equine Hassall's corpuscles secrete cytokines that are attractant to eosinophils. Two intrathymically produced chemokines, MCP-5 (monocyte chemotactic protein-5) and eotaxin, have an eosinofilotactic action (Rothenberg *et al*., 1995; Jia *et al.* 1996). However, literature makes no reference to any chemokine produced by Hassall's corpuscles, much less with eosinophilotactic activity. Quantitative determination of thymic eosinophilia in swine have shown that the higher quantity of eosinophlis was present, the higher was the numbers of Hassall's bodies calculated per unit area of medulla (Rosario *et al*., 1995). So it is conceivable that secretory cells from equine Hassall's corpuscles produce an eosinophil chemoattractant, whose nature is of course to be determined.

Another aspect deserving discussion is the immunohistochemical detection of S100α and S100β proteins in Hassall's bodies of two fetuses. Usually these proteins are found in dendritic cells of peripheral lymphoid organs. However, S100 proteins were also identified in adipocytes, ecrine sweat glands, salivary glands, myoephitelial cells, and a variety of carcinomas (Drier *et al*., 1987; Schmitt and Bacchi, 1989). Inside the thymus, S100 proteins were also detected in nurse cells of BUF/Mna rats (Ezaki *et al*., 1991) and in human Hassall's corpuscles (Zoltowska, 1991).

The fact that antibodies to S-100 proteins only labeled epithelial cells of thymic medulla (particularly Hassall's bodies) and not dendritic cells, is not so surprising since the cellular distribution of these molecules in mammals varies among species. For instance, in humans S-100 proteins are found in interdigitating dendritic cells, and not macrophages (Ushiki *et al*., 1984); in guinea pigs, they occur in a subpopulation of macrophages (Atoji *et al*., 1991), and in rats, they appear to be confined to the "follicular dendritic cells" both in the lymph node and the spleen. Immunoreactivity for S-100 proteins was also demonstrated in human T lymphocytes (Takahashi *et al*., 1985). Conjointly, thesedata tell us that it will be useful to develop a systematic survey for S-100 proteins in thymuses of various mammals.

A further and important aspect to be discussed herein is related to non-lymphoid hemopoiesis. Except for the animal aging two and a half months, all others equine fetal thymuses exhibited eosinopoiesis, which was more prominent in the 10-months old specimen. Eosinopoietic foci were more frequent in PVS and in outer cortex, sometimes appearing eosinophils in degranulation process or in close contact with cortical epithelial cells. Intrathymic eosinopoiesis is a fact that has been observed in human thymuses since long time ago (Jolly, 1915; Lee *et al*., 1995), although the relevance for the general thymus ontogeny remains unkown.

Erythropoietic foci were also found in the fetal equine thymus, together or not with foci of megakaryopoiesis in one of the five-month thymuses. Erythropoiesis was also observed in the thymuses of humans and pigs (Custer, 1974; Kelemen *et al*., 1979; Bodey *et al*., 1998). Such intrathymic differentiation of various cell lineages seems to depend on various colony stimulating factors (CSF), produced by epithelial cells (Le *et al*., 1988). Yet, although TEC are source of various cytokines in humans, nothing is known for the equine thymus, and further studies on this issue should contribute for a better understanding of why non-lymphoid hemopoiesis in horse thymus is so promiment as compared to other mammalian species.

Mast cells were often seen close to eosinophils. Since these cells produce IL-5 and can contribute to eosinophil survival by releasing GM-CSF (Levi-Schaffer *et al.*, 1998), it is possible that they play a role in the regulation of intrathymic eosinopoiesis in the equine thymus, since early phases of the organ ontogeny.

The expression of ECM components in equine fetal thymuses reproduced the general aspects already observed in humans and other animal models (Berrih et al, 1985; Lannes-Vieira et al, 1991; Meireles de Souza et al, 1993). There was a clear predominance of interstitial collagens and proteoglycans mainly with low sulfation in the capsula, septae and PVS, whereas in the intraparenchymal compartment three distinct ECM patterns were detected, being related to basement membranes and intraparenchymal fibers and cell surface proteoglycans.

Although derived from the analysis of few samples, our data suggest that the equine fetal thymus, despite sharing several morphological features with the thymus from other mammals, exhibits particular aspects, suggesting that it may represent an interesting model for further studies on mammalian intrathymic non-lymphoid hemopoiesis as well as the origin and fate of lymphocytes found within thymic lymphatic vessels.

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Figures

Figure 1 - Equine fetal thymus (2 gestational months) showing initial lobules constituted almost by epithelial cells surrounded by loose stroma and dilated venules and capillaries. (hematoxilin-eosin staining) x 200.

Figure 2 - Equine fetal thymus (2 gestational months) showing lobules with peripheral layer of epithelial cells enclosing a primitive thymocyte cortex. (Lennert's Giemsa staining). x 400.

Figures 3-4 - Equine fetal thymus (2 gestational months) showing close interations between thymocytes and epithelial cells, which are connected themselves by cytoplasm extensions. [Lennert's Giemsa (Fig.3); hematoxilin-eosin stainings (Fig.4)]. x 1,000.

Figures 5-6 – Equine fetal thymus (2 gestational months) showing dilated venular cappilaries very close to lobular periphery. Fig.5 showing also adherence among epithelial cells through filamentous cytoplasm. [hematoxilin-eosin x 1000 (Fig.5); methenamine silver + periodic acid staining, LSM (Fig.6)].

Figure 7 – Equine fetus thymus (2 gestational months) showing interlobular connective tissue rich in a delicate mesh of reticular fibers, some of them internally direct to the superficial layer of epithelial cells that is detached from the basement membrane by artifact. (Gomori's reticulin staining). x 400.

Figure 8 – Equine fetal thymus (3 gestational months) showing lobules very distinct and the interlobular septa initially thick, composed of loose conjunctive tissue. (Gomori's reticulin staining). x 100.

Figure 9-10 – Equine fetal thymus (3 gestational months) showing perivascular spaces (PVS) were easily identified seen in longitudinal . Fig. 9 (Gomori's reticulin x 200). Fig.10 transverse sections. (Gomori's reticulin staining). x 400.

Figure 11 - Equine fetal thymus (5 gestational months) showing thin septa allowing the border of one lobule almost to touch the next. (Gomori's reticulin staining). x 200.

Figure 12 –Equine fetal thymus (4½ gestational months) showing large lymphatic vessel full of lymphocytes in PVS. (Masson's trichrome staining). x 200.

Figure 13 - Equine fetal thymus (6 gestational months) showing eosinophil focus in PVS showing degranulated cells, associated with a local lymphocyte accumulation. (Lennert's Giemsa staining). x 400.

Figure 14 - Equine fetal thymus (4½ gestational months) showing erythroid and megakaryocyte foci in PVS, with lymphocyte engulfed by emperipolesis by one of the megakaryocytes (lymphocyte is surrounded by a clear halo). (Masson's trichrome staining). x 1,000.

Figure 15 – Equine fetal thymus (10 gestational months) showing immature mast cell (⟶). Close to monocyte focus, presenting a promonocyte (⇢). (Lennert's Giemsa staining). x 1,000.

Figure 16-17 – Equine fetal thymuses (3 and 4½ gestational months) showing a well defined boundary between cortex and medulla. Fig.16 shows equine fetal thymus (3 gestational months) the medulla presents Hassall's body and a still prominent extraparenchymal compartment, rich in loose mesenchymal tissue. (hematoxilin-eosine staining). x 100.

Figure 18 - Equine fetal thymus (5 gestational months) showing band arrangement of epithelial cells which is located in the external area of the lobules, interposed between the basement membrane and the cortical thymocytes. (hematoxilin-eosin staining). x 400.

Figure 19 - Equine fetal thymus (10 gestational months) showing immature eosinophils (myelocytes and metamyelocytes) in the peripheral cortex, intermixed with lymphocytes and epithelial cells. (Lennert’s Giemsa staining). x 1,000.

Figure 20 - Equine fetal thymus (10 gestational months) showing large number of eosinophils adjacent to an Hassall’s body, with only few of them intermingled with epithelial cells of the Hassall's corpuscle. (Masson’s trichrome staining). x 400.

Figure 21 - Equine fetal thymus (10 gestational months) showing one Hassall’s body full of apoptotic eosinophils. (Lennert’s Giemsa staining). x 1,000.

Figure 22 – Equine fetal thymus (6 gestational months) showing macrophage giant cells were seen inside Hassall’s bodies. Epithelial cells show weak expression of S100α protein, as revealed by immunoperoxidase assay. x 400.

Figure 23 – Equine fetus thymus (6 gestational months) showing Hassall's bodies positively labeled for the presence of cytokeratin, with concentration of eosinophil (white cells) close to them.

Figures 24-25 - Equine fetus thymus (4½ gestational months) showing epithelial cells of Hassall's bodies exhibiting immunoreactivity to tropomyosin in the cytoplasm.

Figure 26 - Equine fetus thymus (10 gestational months) showing smooth muscle cells in thymic artery wall immunoreactive to tropomyosin. Negative controls did not generate any significant fluorescent signal (not shown).

Figures 27-29 - Equine fetal thymus (10 gestational months) showing Hassall's bodies with epithelial cells immunostained for S-100α (Fig.27) and S-100β (Fig. 28) proteins, as revealed by immunoperoxidase assay (x 400). Figure 29 shows tow unstained Hassall's bodies, in the absence of specific anti-S-100 antibodies). x 1,000.

Figure 30 - Equine fetal thymus (6 gestational months) showing one myoid cell in the medulla immunoreactive for desmin, as ascertained by the anti-desmin monoclonal antibody + alcaline-phosphatase-coupled secondary antibody. x 1,000.

Figure 31 – Immunofluorescence detection of laminin in 5 gestational months old equine fetus. Laminin immunoreactivity is seen in lobular and vascular basement membrane, being absent in the septal connective tissue. x 40.

Figure 32 - Immunofluorescence detection of fibronectin in the thymus of a 5 months old equine fetus. Fibronectin immunoreactivity is seen in septal and PVS connective tissue, vessel walls and in thin trabeculae within the intraparenchymal compartment. x 40.

Figure 33 - Equine fetal thymus (10 gestational months) showing high endothelial venules located in the PVS, surrounded by lymphocyte halo, showing lymphocyte in contact with endothelial cells. (Lennert's Giemsa staining). x 400.

Figure 34 – Ultrastructural aspects of the thymus from a 5 gestacional months, showing several cortical thymocytes, bearing irregular shaped, small nucleoli, few organelae, with polar mitochondria (➢). x 8,060.

Figure 35 – Ultrastructural aspects of the thymus from a 5 months old equine fetus, showing a network of epithelial cells attached among themselves through small desmosomes, two of them in intimal contact with lymphocytes (*). The epithelial cells are surrounded by basement membrane (laminin) (⟶). x 5,890.

Figure 36 –Ultrastructural aspects of the thymus from a 5 months old equine fetus, showing one macrophage with small lysosomes (⟶), in contact with epithelial cell extensions processes. x 8,060.

Figure 37 – Ultrastructural aspects of the thymus from a 5 months old equine fetus, showing one macrophage rich in endoplasmic reticulum and mitochondria, engulfing an apoptotic lymphocyte (*). x 16,800.

THE EQUINE THYMUS IS A SPECIAL MICROENVIRONMENT FOR EOSINOPHIL LINEAGE

Running Title: Eosinophils in equine thymus

Ellen Cortez Contreiras[1,4], Maria de Nazareth Leal de Meirelles[2], Marcelo Pelajo-Machado[3], Wilson Savino[1] and Henrique Leonel Lenzi[3]

[1]Laboratory on Thymus Research, Department of Immunology,
[2]Department of Ultrastructure and Cell Biology and [3]Department of Pathology,
Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil;
[4]Department of Morphology, Biomedical Institute,
Fluminense Federal University, Rio de Janeiro, Brazil.

Correspondence:
Henrique Leonel Lenzi
Department of Pathology
Instituto Oswaldo Cruz - FIOCRUZ
Av. Brasil 4365 - Manguinhos
21045-000, Rio de Janeiro
Brazil.

Tel: (55) (21) 598-4350
Fax: (55) (21) 573-8673, (55) (21) 598-4466
e-mail: hlenzi@ioc.fiocruz.br

ABSTRACT

The association between eosinophils and T lymphocytes is a recognized event, although intratymic interactions involving these two cell types are largely unknown. In this respect, it appeared to be useful to provide the morphological basis for such interactions in the thymus. We then investigated whether eosinophils could be detected in the equine thymus, as well as the morphological and developmental features of these cells in the organ.

Immature and mature eosinophils can be seen in the thymus, being found in various regions of the thymic lobules (scattered or forming clusters), particularly in the perivascular spaces, but also within the parenchyma itself, both in cortex and medulla. Although, their size and shape can vary, their specific granules are particularly large, but do not exhibit the typical crystalloid core seen in eosinophils from other mammalian species.

From an ontogenetic point of view, equine thymic eosinophils can be seen from three gestational months in ahead. In postnatal equines, they are more frequent in very young from 6 months to two years old animals, disappearing or being very scarce in the thymuses of 13 years onwards, when the intraparenchymal atrophy becomes advanced.

Importantly, eosinophils differentiate within the equine thymus since immature forms such as myelocytes and metamyelocytes are often detected.

Taken together, our results lead to the notion that the thymic microenvironment is also adequate for eosinophil differentiation, which in turn may act bidirectionally influencing the microenvironmental and/or lymphoid compartments of the organ.

INTRODUCTION

Eosinophils have many functional hypothetical capabilities, such as interference on tissue remodeling (Hibbs *et al*., 1982); activation and regulation of complement (Weiler & Gleich, 1988); expression of transcabolamim I (Adrouny *et al*., 1984; Zittoun *et al*., 1984) and of melanotransferin (McNagny *et al*., 1996); participation in the reproduction events (Pepper & Lindsay, 1960; Tchernitchin, 1967; Luque & Montes, 1989) as well as effects on coagulation and fibrinolysis (Venge *et al*., 1979); "eokine" production; (Giembycz & Lindsay, 1999); eicosanoid generation (Shaw *et al*., 1985; Bozza *et al*., 1997); cytotoxicity (Davis *et al*., 1984); antigen presentation (del Pozo *et al*., 1992; Weller *et al*., 1993); resolution of edema (Bandeira-Melo *et al*., 2000) and participation in diffuse neuroendocrine system (Weinstock & Blum, 1990 a,b; Weinstock *et al*., 1988). In healthy conditions, eosinophils are mainly localized in submucosal tissue sites.

The association between eosinophils and T lymphocytes is a recognized event, and T-lymphocyte depletion may markedly impair or even abolish the peripheral eosinophilia in some parasitic affections (Basten & Beeson, 1970; Walls *et al*., 1971). Evidence indicates that the factors mediating these events are T-cell-derived cytokines (Sanderson *et al*., 1985), which act selectively on eosinophil production, development and effector functions. The most important of these cytokines appears to be interleukin 5 (IL-5), although granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 also have important effects on eosinophil development, terminal differentiation, function and survival (Campbell *et al*., 1988; Clutterbuck *et al*., 1989; Sonoda *et al*., 1989; Rothenberg *et al*., 1989; Rothenberg *et al*., 1988; Owen *et al*., 1987). Otherwise, eosinophils are clearly multifunctional cells (Lenzi *et al*., 1997) and some of the mediators act by activating or contributing to the activation of target T cells (Weller & Lim, 1997; Behm & Ovington, 2000).

In spite of these data, intrathymic interactions involving eosinophils and microenvironmental cells are largely unknown. This can be a relevant issue since the presence of eosinophils within the thymus has been reported in some vertebrate species, including humans (Bhathal *et al*., 1965; Maxwell, 1985; Aviles-Trigueros & Quesada, 1995; Lee *et al*., 1995; Rosario *et al*., 1995).

Intrathymic T-cell development requires input of precursor cells from either the fetal liver or the bone marrow (Le Douarin & Jotereau, 1975; Jotereau *et al*., 1987). The subsequent differentiation sequence eventually leads to the production of mature, self-tolerant, self-major histocompatibility complex (MHC)-restricted T lymphocytes. In addition to mature thymocytes,

other cells may be generated intrathymically such as B lymphocytes and dendritic cells (Ardavin *et al*., 1993; Wu *et al*., 1995). Nevertheless, it is still unclear whether they all derived *in situ* from a common progenitor, or if distinct committed stem cells are able to colonize the organ. This issue gets still more complicate with the appearance in the literature of data showing not only the presence of granulocytopoiesis (Sin & Saintemarie, 1965), but even cells of the erythroid lineage in the thymus of some vertebrate species, including humans (Kendall & Frazier, 1979; Taylor & Skinner, 1976; Albert *et al*., 1966).

In this respect, it appeared to be useful to provide the morphological basis for such interactions in the thymus. One experimental model, potentially interesting to study this aspect, is the horse, since this animal is known to have very large circulating eosinophils. We then investigated whether eosinophils could be detected in 6 fetal and 42 post-natal equine thymuses, as well as the morphological and developmental features of these cells in the organ.

MATERIAL AND METHODS

Animals

This study comprised a) six equine fetuses of *Equus caballus*, with ages varying from two to ten months in gestation, distributed as follows: 2, 3, 4, 5, 6 and 10 months and b) 42 normal horses (18 males and 24 females), aging 6 months-18 years. At least 5 specimens from each age range were studied. Animals were obtained at Federal Rural University of Rio de Janeiro (Department of Parasitology), and were handled according to the ethical rules established by the governmental ethics committee of EMBRAPA (National Brazilian Agency for Agricultural Research). Additionally, all horses used in this study were checked for the presence of infectious equine anemia virus, as were serologically negative, as ascertained by the commercial Coggin´s test (Lab. Bruch, São Paulo, Brazil).

Histology and electron microscopy

When used for histological techniques, thymus fragments were fixed in Carson's Formalin-Millonig (Carson *et al*., 1973), dehydrated and embedded in paraffin. Five 5μm thick sections then were stained with haematoxylin-eosin and Lennert's Giemsa (1978). To further study

eosinophils under bright field and confocal laser scanning microscopy (LSM-410 model, Zeiss, Germany), Sirius Red (pH 10.2) staining was also performed in selected thymus sections (Bogomoletz, 1980; Luque & Montes, 1989; Vale *et al*., 1997).

For electron microscopy analysis, tiny thymus fragments were fixed in 2.5% glutaraldehyde in 0.1M Na cacodylate buffer, pH 7.2, for 1h, rinsed in the same buffer and postfixed with 1% OsO_4, dehydrated through an ascending series of acetone and embedded in Epon 812. Ultrathin sections were picked up on 300-mesh copper grids, contrasted with uranyl acetate and lead nitrate, and examined using a Zeiss EM 10C transmission electron microscope (Germany).

RESULTS

Distribution and morphological features of eosinophils in the fetal equine thymus

From three gestational months in ahead, mature and immature eosinophils were visualized in the perivascular spaces (PVS) (Fig.1), which by the ten-month old fetus were arranged in clusters, sometimes close to the lymphatic vessels. Immature and mature eosinophils were also detected in the cortex of fetal thymuses older than three months. They were often undergoing degranulation and were arranged in close contact with sub-cortical reticular-epithelial cells (Fig.2). The eosinophils in the medulla were always mature and in small numbers, except in the six-month fetus, where they were numerous and frequently located around or inside Hassall's bodies (Fig.3). Interestingly, in addition to the eosinopoiesis, in the thymus of four and five months fetuses, erythropoietic and megakaryopoietic foci were also identified in PVS (Fig.4)

Eosinophils are present in distinct phases of equine thymus development

Since mature eosinophils were consistently found in fetal equine thymus, we asked the question whether their presence could be also seen in distinct phases of the post-natal equine development. In fact, mature and immature (myelocytes and metamyelocytes) eosinophils were found in most of the animals evaluated, with eosinopoiesis being more active from 6 to 30 months of age. Eosinophil apoptosis predominated in the thymuses of 5 and 7 years old horses, and the eosinophils, even mature, disappeared or were very scarce in the thymuses of 13 years onwards, when the atrophy of the epithelial thymic compartment used to be very advanced. Foci of eosinophilopoiesis, with immature eosinophils, were found mainly in the PVS (Fig. 5) and less

frequently in the cortex, where eosinophils were seen in close contact with epithelial cells (Figs. 6-7).

Mature eosinophils, like in the fetuses, predominated always in the medulla, preferentially located around and/or inside Hassall's bodies (Figs.8-10). Very often, eosinophilopoiesis foci were intermixed with immature erythrocytic cells, mature and immature plasma cells (Fig. 11). Some eosinophil clusters exhibit degranulation (Figs. 12-13). When this occurred in the cortex, the released granules were seen in close contact with epithelial and lymphoid cells (Fig. 7). Very often the eosinophils were seen close to and/or surrounding lymphatic and blood vessels (Fig. 14).

Similar to what is seen in many mammalian species, specific granules from horse thymus eosinophils are membrane-bound, as revealed by electron microscopy. Nevertheless, they do not exhibit the typical crystalloid core seen in eosinophils from many other mammalian species (Fig. 15). Yet, in the horse thymus, these eosinophil specific granules can exhibit distinct patterns of electron density: most of them are homogeneously electron dense (H), whereas in others, electron lucent areas (L) can be detected (Fig. 16).

Given the wide distribution pattern of thymic eosinophils in both intraparenchymal and extraparenchymal areas of the organ, close contacts between eosinophils themselves as well as between eosinophils and thymocytes or macrophages were seen (Figs. 17-18).

DISCUSSION

The present work represents a general survey on the morphological and developmental characteristics of eosinophils found in the equine thymus. In this respect, distinct points deserve to be discussed. The first obvious aspect is *per se*, the intrathymic presence of eosinophils and its distribution within the organ. Although relatively poorly studied, the presence of eosinophils has been reported in various vertebrate species. Studies conducted in humans and swines also revealed their presence in extraparenchymal sites, including septa and perivascular spaces (Lee *et al*., 1995; Rosario *et al*., 1995).

Rather unique are the specific granules of eosinophils in equine thymus. In addition to being much larger than their counterparts in other mammals, they do not possess the crystalloid core typically found in other species (Stockert *et al*., 1993). It should be noted however, that such characteristics are not restricted to the equine eosinophils, since eosinophils without a crystalloid internum have been also identified in turtles and lizards (Kelenyi & Nemeth, 1969). Yet, it

remains to be defined the chemical composition of equine eosinophilic specific granules, including if they contain the eosinophil basic protein, classically seen in other mammalian species.

A further aspect deserving discussion concerns the presence of immature forms of the eosinophilic lineage in the equine thymus. The occurrence of myelocytes and metamyelocytes strongly indicates that at least part of the eosinophils found in the thymus is being differentiated in the organ. This is in keeping with the data reported in the human thymus (Lee *et al*., 1995), thus suggesting that the intrathymic differentiation of eosinophils is phylogenetically conserved, at least in mammalian species. Whether a common granulocyte/lymphoid precursor differentiates in the thymus or myeloid *plus* lymphoid precursors independently colonize the thymus and then differentiate into corresponding lineages, is completely undetermined and represents an open field for investigation.

In any case, taken together, the data discussed above strongly indicate that in horses, as in humans and other animals, the thymus can be considered as a physiological site of eosinopoiesis and of mature eosinophil location. Accordingly, and taking into account that in the bone marrow eosinophil differentiation is driven by the local microenvironment, it is conceivable that the thymic microenvironment is also adequate for differentiation of eosinophil lineage. Yet, this issue is to be demonstrated. Besides, experiments are necessary in order to see whether or not mature intrathymically generated eosinophils, physiologically leave the organ (thus similar to mature thymocytes and bone-marrow derived mature eosinophils), or live and die in this particular *niche*. Intrathymic injection of fluorochromes, such as fluorescein isothiocyanate and further analysis of recent thymic emigrants in terms of eosinophil phenotype will hopefully clear this issue. However, the finding of morphological profiles consistent with apoptotic eosinophils inside Hassall's bodies indicates that, at least part of the eosinophil population die in the thymus.

It is interesting to note that in the fetal thymus, in addition to eosinopoiesis, erythropoiesis and megakaryopoiesis were detected. Conjointly, the findings presented herein, related to the equine model, support the concept that, besides the well known lymphopoietic function, the thymus is also site of granulopoiesis (at least regarding eosinopoiesis), erythropoiesis and megakaryopoiesis.

Lastly, the close apposition of eosinophils and lymphocytes in the equine thymus suggest functional bi-directional interactions between these two cell types, including those of paracrine nature. In this respect, it is known that T cell-derived cytokines influence eosinophil differentiation, maturation and activation (Sanderson *et al*., 1985) and reciprocally, eosinophils

are sources of lymphocyte activating cytokines and immunomodulatory neuropeptides (Weinstock *et al*., 1988; Weinstock & Blum, 1990a,b) which probably can interfere in the development of the thymic lymphocytes. In this respect, it is noteworthy the recent data showing that mouse thymus eosinophils are intrathymically recruted during the neonatal period, showing a temporal and spatial association with class-I-restricted selection in the thymus (Throsby *et al*., 2000). These authors demonstrated that the eosinophil is a regulated component of the murine thymus that is recruited in the absence of overt inflammatory stimulus similar to other tissue-marginated eosinophils.

Taken together, the results discussed above lead to the general hypothesis that the thymic microenvironment is also adequate for differentiation of eosinophil, erythroid and megakaryocytic lineages, which in turn may act bidirectionally influencing the microenvironmental and/or lymphoid compartments of the organ.

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LEGENDS OF FIGURES

Figure 1 - Normal (4 ½ gestational months) equine thymus stained by Sirius Red, pH 10.2, showing eosinopoietic focus in PVS constituted by myelocytes and metamyelocytes. x 1000.

Figure 2 - Normal (4 ½ gestational months) equine thymus stained by Lennert's Giemsa, showing metamyelocytes in close contact with cortical epithelial cells. x 1000.

Figure 3 – Normal (10 gestational months) equine thymus stained by Lennert's Giemsa, showing Hassall's body full of apoptotic eosinophils. x 400.

Figure 4 – Normal (4 ½ gestational months) equine thymus showing coexistence of eosinopoiesis and erythropoiesis in PVS. (Lennert's Giemsa). x 1000.

Figure 5 – Normal (2 years old) equine thymus showing focus of eosinopoiesis in PVS interposed between two lobules. (Sirius Red, pH 10.2, LSM). x 400.

Figure 6 – Normal (2 years old) equine thymus showing eosinophils in PVS and also forming a row in the periphery of the cortex of one lobule close to the lobular basement membrane. (Sirius Red, pH 10.2, LSM). x 400.

Figure 7 – Normal (2 years old) equine thymus showing detail of cortical eosinophils in degranulation process, showing released granules in contact with epithelial cells and thymocytes. (Sirius Red, pH10.2, LSM). x 400.

Figure 8 – Normal (2 years old) equine thymus showing mature eosinophils around Hassall's body. (Sirius Red, pH 10.2, LSM). x 400.

Figure 9 – Normal 2 years old equine thymus showing numerous mature eosinophils placed in the medulla. (Sirius Red, pH 10.2, LSM). x 400.

Figure 10 – Normal 2 years old equine thymus showing ruptured Hassall's body presenting a burst-like appearance of the content, surround by mature eosinophils seen in yellow color. (Sirius Red, pH 10.2, LSM. depth code). x 400.

Figure 11 – Normal 6 months old equine thymus showing presence of several plasma cells admixed with eosinopoietic focus in PVS. (Lennert's Giemsa staining). x 310.

Figures 12-14 – Normal (2 years old) equine thymus showing three eosinopoietic foci in PVS showing intense degranulation, one of them located around a vascular vessell (Fig.14). (Sirius Red, pH 10.2, LSM).

Figures 15,16 – Ultrastructural aspects of a 5 years old equine thymus, showing details of different aspects of eosinophil granules, showing granules with eccentric core and homogeneous matrix (H); and with irregular core due to areas of different electron-densities (type L). Small granules (S) seems to be profiles of smooth endoplasmic reticulum and some of them are in direct contact with large ones. x 45,100.

Figure 17 – Ultrastructural aspects from equine thymus, showing advanced eosinophil promyelocyte, touching a macrophage cell with indented nucleus. The immature eosinophil presents Golgi zone (G) and rich endoplasmic reticulum and mitochondria (*). The granules are immature and small, present different shapes and are devoid of crystalloid. x 20,000.

Figure 18 – Ultrastructural aspects of a 5 years old equine thymus, showing three horse intrathymic mature eosinophils in close contact with several thymocytes and with one macrophage. In the right-upper corner there is a part of cytoplasm of one eosinophil showing one granule with an eccentric and round core (⟶). x 8,000.

MORPHOLOGICAL CHANGES IN THE THYMUS OF HORSES UNDERGOING EQUINE INFECTIOUS ANEMIA

Ellen Cortez Contreiras[1,4], Adelaide Lopes Amorim[2],
Maria de Nazareth Leal de Meirelles[3],
Henrique Leonel Lenzi[1] and Wilson Savino[1].

[1]Laboratory on Thymus Research, Department of Immunology
[2]Department of Pathology and [3]Department of Ultrastructure and Cell Biology,
Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil,
[4]Department of Morphology, Biomedical Institute,
Fluminense Federal University, Rio de Janeiro, Brazil.

Correspondence:
Wilson Savino
Laboratory on Thymus Research,
Department of Immunology,
Instituto Oswaldo Cruz - FIOCRUZ,
Av. Brasil 4365 - Manguinhos,
21045-000, Rio de Janeiro,
Brazil.

Tel: (55) (21) 280-1486, (55) (21) 598-4326, (55) (21) 598-4327
Fax: (55) (21) 280-1589, (55) (21) 590-9741
e-mail: savino@ioc.fiocruz.br --- savino@gene.dbbm.fiocruz.br

ABSTRACT

Equine infectious anemia virus (EIAV), a predominantly macrophage-tropic retrovirus, is a lentivirus able to infect horses and cause recurrent episodes of fever, thrombocytopenia and anemia. Experimental infection of foals with this disease results in a progressive infection leading to death, demonstrating the necessity of the host immune system in accomplishing the temporal control of virus replication associated with infection of immunocompetent horses. Despite the likely involvement of T cells in the pathophysiology of Equine Infectious Anemia, to our knowledge no studies have been conducted to evaluate the thymus of EIAV-infected horses.

In the present work, we studied the thymus from EIAV-infected horses, focusing its microenvironmental component. We observed an a severe an accelerated thymic atrophy, with formation of large cystic Hassall'corpuscles, as well as an augmentation in the deposition of extracellular matrix components and the vascular network when compared with normal animals.

In conclusion, the Infectious Anemia accelerated and enhanced the age dependent thymic involution; with lymphocyte reduction, increased extracellular matrix and the vascular network and augmented cystic transformation of Hassall's bodies.

INTRODUCTION

Equine infectious anemia virus (EIAV), is a lentivirus able to infect horses, and that causes recurrent episodes of cell-free plasma viremia with concurrent fever, thrombocytopenia, anemia, edema and lethargy (Kono *et al*., 1973; Montelaro *et al*., 1993). The appearance of clinical symptoms in experimentally infected animals coincided with rapid widespread seeding of viral infection and replication in a variety of tissues. This is a predominantly macrophage-tropic lentivirus, which highlights the potential role of these cells in sequestering lentiviral infections from host immune surveillance (Harrold *et al*., 2000).

The virus is classified as a retrovirus characteristic of RNA viral genome and the presence of a reverse transcriptase or RNA-dependent DNA polymerase (Charman *et al*., 1976; Archer *et al*., 1977). It contains surface *knobs* and a dense, conically shaped core (Matheka *et al*., 1976; Weiland *et al*., 1977). The exterior lipid envelope of the virus is derived from host cell plasma membranes during viral particle maturation (Gonda *et al*., 1978). The surface knobs are virus-specific glycoproteins in the gp90 surface protein and the gp45 transmembrane protein of sequencial EIAV antigenic variants are defined (Montelaro *et al*., 1984; Payne *et al*., 1987) that are probably required for virus penetration of host cells and act as potent immunostimulants (Parekh, *et al*., 1980). The most abundant of the core proteins, p26, consistently evokes a strong humoral immune response in most infected horses and is used as the basis for most serologic diagnostic tests for the virus (Coggins & Norcross, 1970; Issel & Coggins, 1979; Parekh,1980).

The titer of infectious virus in the serum augments with increasing fever (Clabough, 1990; Kono *et al*., 1971) and viral antigen is detectable in almost all tissues, including liver, spleen, lymph nodes, bone marrow, lung and kidney (Kono *et al*., 1971; McGuire *et al*., 1972; Rice *et al*., 1989; Sellon *et al*., 1992). The majority of viral replication during a febrile episode appears to occur in mature tissue macrophage of these tissues, not in circulating blood monocytes (Sellon *et al*., 1992).

The infection results initially in a rapid and dynamic series of clearly demarcated cycles of disease and associated viremia that begin by 3 weeks postinfection and continue at irregular intervals separated by weeks or months (Montelaro *et al*., 1993). The EIAV infection develops in various stages of disease, namely acute, subacute, chronic, and unapparent. Acute and subacute stages are characterized by clinical signs of fever, decreased hematocrite values, anorexia,

depression, and in the more serious cases severe weight loss, edema and death (Kono *et al*., 1973). Episodic outbreaks of clinical illness are of a cyclical nature and often followed by or interspersed with periods of quiescence. Unapparent infection in EIAV-carrier horses may be occur without signs, although the virus can be detected serologically (Coggins *et al*., 1972; Issel & Coggins, 1979).

Experimental infection of foals with this disease results in a progressive infection leading to death, demonstrating the necessity of the host immune system in accomplishing the temporal control of virus replication associated with infection of immunocompetent horses (Perryman, 1988).

Necropsy of an EIAV-infected horse that dies during a febrile episode often reveals generalized lymph node enlargement, hepatomegaly, splenomegaly, accentuated hepatic lobular structure, mucosal and visceral hemorrhages, ventral subcutaneous edema, and vessel thrombosis (Issel & Coggins, 1979, Kono, 1973). Histopathology usually reveals accumulation of lymphocytes and macrophages in periportal areas of the liver, and lymph nodes, adrenal gland, spleen, meninges, and lung (McGuire, 1986). These lymphoproliferative lesions may be the result of spread of virus-reactive T-lymphocytes in an attempt to control infection.

Despite the likely involvement of T cells in the pathophysiology of equine infectious anemia, to our knowledge no studies have been conducted to evaluate the thymus of EIAV-infected horses. This is a relevant issue since the thymus is the central lymphoid organ in which the process of T differentiation takes place. In the present work, we performed a morphological and immunohistochemical analysis of the thymus from EIAV-infected equines, rather focusing in its microenvironmental component.

MATERIAL & METHODS

Animals

This study comprised 8 EIAV-infected *Equus caballus*, race crossbred, varying from 5 to 20 years, including males and females. As controls, thymuses from normal animals with the same age were used, including a control pregnant with the same age. All horses were obtained at Federal Rural University of Rio de Janeiro, Veterinary School, Curral de Apreensão, Parasitology Institute, and handled according to the ethical rules established by the governmental ethics committee of EMBRAPA (National Brazilian Agency for Agricultural Research). Additionally,

all horses used in this study were defined as positive for the presence of EIAV, as ascertained by the commercial Coggin´s test (Bruch, São Paulo, Brazil), that is one highly accepted test to evaluate animals throughout the world. Briefly, this is an agar-gel immunodiffusion test that detects the presence of precipitating antibody in horse sera to determinants of the EIAV group-specific antigens (Coggins *et al*., 1972, Coggins & Norcross, 1970). The ELISA test is used when the horses were seronegative for antibody on the agar-gel immunodiffusion test (Shen *et al*., 1979).

Histology and electron microscopy

When used for histological techniques, thymus fragments were fixed in Carson's Formalin-Millonig (Carson *et al*., 1973), dehydrated and embedded in paraffin. Five 5μm thick sections then were stained with various histological procedures, whose general features are summarized in the table. Specimens were ultimately examined under bright field or confocal laser scanning microscopy (LSM-410, Zeiss, Germany).

For electron microscopy analysis, tiny thymus fragments were fixed in 2.5% glutaraldehyde in 0.1M Na cacodylate buffer, pH 7.2, for 1h, rinsed in the same buffer and postfixed with 1% OsO_4, dehydrated through an ascending series of acetone and embedded in Epon 812. Ultrathin sections were picked up on 300-mesh copper grids, contrasted with uranyl acetate and lead nitrate, and examined using a Zeiss EM 10C transmission electron microscope (Germany).

Immunohistochemistry

In order to process material for immunohistochemistry, freshly-isolated thymus fragments were immediately frozen in liquid nitrogen, and kept in deep freezer conditions (-80^{o}C) until use. Frozen sections (5μm thick) were then fixed in cold acetone, washed in PBS and submitted to indirect immunofluorescence as currently done in our laboratory (Villa-Verde *et al*., 1994). Briefly, specimens were subjected to a given primary antibody for one hour, washed in PBS and exposed to the fluorochrome-labeled second antibody for a further hour. After further washing, slides were mounted and analyzed under confocal laser microscopy.

Distinct primary antibodies were applied to study the horse thymic microenvironment. The anti-pan cytokeratin polyclonal serum was used to reveal the whole thymic epithelial network, as

previously demonstrated (Savino *et al*., 1982). Furthermore, we evaluated three typical basement membrane-associated extracellular matrix proteins, laminin, fibronectin and type IV collagen. The presence, and conserved distribution of these molecules in the thymuses from several mammalian species have been previously reported by our group (Berrih *et al*., 1985; Lannes-Vieira *et al*., 1991; Meirelles de Souza *et al*., 1993). Appropriate secondary antibodies comprised goat anti-rabbit-FITC. As negative controls, primary antibodies were omitted or replaced by unrelated rabbit sera. Significant fluorescent signal were never observed in any negative control (data not shown).

RESULTS

One of the most important morphological features of the EIAV-infected equine thymus was a severe atrophy of the organ, which could be found in five out of six animals studied. We found a very advanced reduction of the intraparenchymal compartment, together with lipomatous atrophy of the extraparenchymal compartment (Figs. 1-2). In one atrophic case, there was still a significant amount of lymphocytes, and sometimes residual medulla was easily identified (Figs. 3-5).

The Hassall's bodies, in four cases, exhibited a huge cystic multilocular transformation, with fusion among the cysts, lined by cuboidal or stratified epithelium, contained amorphous mucous material rich in neutral glycoproteins and proteoglycans with high and low sulfation (Fig. 6).

Together with the severe atrophy, there was exacerbation of the fibronectin, laminin, colagen IV (Figs. 7-9), as well as argirophilic fibers in the capsule and small vessels, and significant increase of interstitial collagens in the adventitia of large vessels, mainly in the tortuous and prominent arteries, compared with normal animals. The PVS collagens in the thymus of 6 years old male equine presented thick and wavy fibers, with corkscrew profile (Figs. 17-18).

We also noticed that in EIAV-infected horses, eosinophils were absent or rare thus in contrast with the high amounts of eosinophils seen in normal age-matched animals (see accompaning paper).

Similarly, mast cells were scarcer in infected horses, as compared to normal equines.

The thymus of the five years old pregnant female was eutrophic, with clear cortical-medullary region, normal pattern of keratin expression in the TES (Fig. 10) conspicuous high endothelium venules (HEV) in PVS (Fig. 11), the medulla (Fig. 12), enlargement of the PVS due high cellularity (Fig. 13), syncytial cells in Hassall's bodies (Fig. 14), numerous eosinopoietic foci (Fig. 15) and less number of erythopoietic and also basophilopoiesis (Fig. 16) foci. Mature eosinophils and basophils were numerous in the medulla, and mast cells were frequent in the PVS

DISCUSSION

The thymus EIAV-infected horses developed atrophy with changes that were similar to, but more exarcebated than normal atrophic thymus, reproducing the same phenomenon previously observed in human with late HIV infection (Savino et al, 1986; Haynes *et al*., 1999; Haynes & Hale, 1998; Schuurman *et al*., 1989). However, while the Hassall's bodies in human HIV infected patients progress to calcification, the equine thymuses showed a tendency to form isolated or multilocular microcysts, resulted from fusion adjacent Hassall's corpuscles. This condition sometimes referred as a Dubois microabscess (Henry, 1978) is definitely not an abscess, being the outcome of leakage of the contents of cystic spaces which are formed within an involuted thymus, traditionally ascribed to congenital syphilis. So far, the mechanisms of the cystic transformation in Hassall's bodies is still unknown. It may depend on excessive mucous secretion or on decrease in the reabsortion of the secreted content. Grossly, the content appears to be similar to that of non infected thymuses, being essentially composed by glycoproteins and proteoglycans (Henry, 1966).

Induction of high endothelial venules (HEV) that are typical for peripheral lymph node observed in the 5 years old pregnant infected female reproduced the same event detected in myasthenia gravis and HIV-1 infected thymuses (Bofill *et al*., 1985; Haynes & Hale, 1998). The appearance of HEV is indicative of lymphocyte importation from periphery.

We have shown in normal post-natal equine thymus (see accompaning paper) that eosinopoiesis is the predominant non-lymphoid myeloid lineage produced inside the organ, in both intraperenchymal and extraparenchymal compartments, and only disappears in animals from 13 years old onwards. Diffently, in EIAV-infected animals, intrathymic eosinophils were very scarce and even absent in animals with six (one) and ten (two) years old. The direct relationship of the thymic eosinophilia with the level of atrophy suggests a uni or bidirectional influence between eosinophils and T-lymphocytes, which remains to be defined. Yet, we can speculate that cytokines may be at the origin of this difference. Several cytokines released by T lymphocytes present selective actions on eosinophil production, development and function. The most important is interleukin 5 (IL-5), although granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 also have important effects on eosinophil development and function (Campbell *et al*., 1988). Accordingly, one may hypothesize that in EIAV infection, intrathymic

eosinophil stimulating cytokines are somewhat defective, resulting in much lower numbers of these cells.

The unusual finding of intrathymic basophilia, together with mast cell infiltration in one thymus (5 years old pregnant female) suggests the local presence of IL-3. Besides IL-3, GM-CSF also induces basophilic differentiation, along with eosinophil lineages (Alam & Grant, 1995). Some authors have identified a common precursor for basophils and eosinophils (Denburg *et al*., 1990), and perhaps the intrathymic functional environment set up by the conjunction between EIA and pregnancy acted on the common precursors, generating both types of cells.

Together with the atrophy, thymuses from EIAV-infected animals exhibited an increase in the deposition of extracellular matrix components and exacerbated the vascular network. This is similar to the response seen in mouse and human acute infections, including *T. cruzi* (Savino et al, 1992).

In conclusion, the Infectious Anemia accelerated and enhanced the age dependent thymic involution; with lymphocyte reduction, increased extracellular matrix and the vascular network and augmented cystic transformation of Hassall's bodies.

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Figures

Figure 1 – Equine Infectious Anemia (14 years old) horse thymus showing the parenchyma replaced by fat, persisting only very thin strands of epithelial cells with small and large cystic Hassall's bodies (Lennert's Giemsa staining, x 80).

Figure 2 – Equine Infectious Anemia (14 years old) horse thymus showing atrophic lobules with cystic Hassall's bodies containing proteoglycans, surrounded by striking lipomatous atrophy also rich in proteoglycans and/or hyaluronic acid (Alcian Blue pH 2.5 – PAS staining, x 80).

Figure 3 – Equine Infectious Anemia (14 years old) horse thymus showing advanced lipomatous atrophy surrounding islands constituted by residual medullary regions, with small cystic Hassall's bodies (Lennert's Giemsa staining, x 80).

Figure 4 – Equine Infectious Anemia (20 years old) horse thymus showing atrophic parenchymal lobe, forming one pseudoglandular arrangement, limited by rows of lymphocytes in residual PVS, largely substituted by lipomatous atrophy (hematoxylin-eosin staining, x 80).

Figure 5 – Equine Infectious Anemia (10 years old) horse thymus showing lipomatous atrophy, residual medullary area and atrophic lobule with lymphocytosis (Lennert's Giemsa staining, x 80).

Figure 6 – Equine Infectious Anemia (6 years old) horse thymus showing bands of PVS surrounding enormous and confluent cystic Hassall's bodies full of dark stained glycoproteins (PAMS staining, x 80).

Figure 7 – Immunofluorescence detection of fibronectin in a six years old Equine Infectious Anemia horse thymus. Fibronectin immunereactivity is seen in mesh mainly in the medulla (x 250).

Figure 8 – Immunofluorescence detection of laminin in a six years old Equine Infectious Anemia horse thymus expressed in the periphery of atrophic lobules and increased in vessels with thick walls. The antibody reveals one vessel communicating PVS with the TES compartment (x 250).

Figure 9 – Immunofluorescence detection of colagen type IV in a six years old Equine Infectious Anemia horse thymus showing the same distribution and intensity as laminin (x 250).

Figure 10 – Immunofluorescence detection of keratin in a five years old Equine Infectious Anemia horse thymus showing intermediate filaments homogeneously expressed in the epithelial network of one preserved lobule (x 250).

Figures 11,12 – Equine Infectious Anemia (5 years old) horse thymus showing prominent high endothelial venules in PVS (Fig.11) and medulla (Fig.12) showing lymphocytes intravascularly located or in passage from the lumen to the tissue (Lennert's Giemsa staining, x 310).

Figure 13 – Equine Infectious Anemia (5 years old) horse thymus showing expanded PVS due to high cellularity, with exarcebation of the reticular fiber mesh (Gomori's reticulin staining, x 200).

Figure 14 – Equine Infectious Anemia (5 years old) horse thymus showing Hassall's body presenting two syncytitial giant cells (Lennert's Giemsa staining, x 500).

Figure 15 – Equine Infectious Anemia (5 years old) horse thymus showing mixed PVS focus of eosinopoiesis and erythropoiesis showing one mitotic eosinophil (Lennert's Giemsa staining, x 310).

Figure 16 – Equine Infectious Anemia (5 years old) horse thymus showing focus of basophilopoiesis in PVS, intermixed with lymphocytes, one plasm cell and lymphocytic syncytial cell (Lennert's Giemsa staining, x 500).

Figures 17,18 – Equine Infectious Anemia (6 years old) horse thymus showing remarkable increase in the interstitial collagen in PVS, forming a cotton-like aspect (Fig. 17). The collagen fibers, in some areas, are tortuous and corkscrew-like (Fig.18). x 310.

Figure 19 – Ultrastructure aspects of a 6 years old Equine Infectious Anemia horse thymus, showing an aspect of PVS with lipomatous atrophy, showing electron dense lipid droplets in adipose cell, adjacent to a fibroblast (⟶) and small lymphocytes with irregular nucleus and very dense heterochromatin (quiescent lymphocytes) (x 10,000).

Table. Staining procedures and corresponding tissue labeling

Stainning Procedure	Tissue specificities	Final Colours	References
alcian blue pH 2.5- PAS	Weakly or non sulphated proteoglycans, hyaluronic acid and sialomucins. Polysaccharides and neutral proteoglycans containing 1-2 glycol grupaments.	dark blue	Lev & Spicer, 1964
alcian blue pH 1.0-PAS	Sulphated proteoglycans Polysaccharides and neutral proteoglycans containing 1-2 glycol grupaments	blue	Lev & Spicer, 1964
Gomori's reticulin	Reticular fibers (Type III, and glycoproteins)	black	Gomori, 1937
Weigert's Resorcin with oxidation without oxidation	 Elastic fibers, oxitalanic fibers Elastic fibers, elauninic fibers	 brown to purple brown to purple	Fullmer & Lillie, 1958 Gawlik, 1965
Masson trichorome	collagens fibers muscles nuclei	blue red blue-black	Masson, 1929
methenamine silver perioacid (PAMS)	basement membrane and reticular fiber	black	Jones, 1951
Lennert's Giemsa	nuclei erytrocytes cytoplasme osinophilic granulae basophilic granulae neutrophilic granulae	blue orange purple red dark purple red	Lennert, 1978
Mayer's hematoxilin and eosin	nuclei cytoplasm most other tissue structures	blue pink to red pink to red	
phosphomolibidic acid and picrosirius	collagen fibers	red (MO)	Dolber & Spach, 1993
alcian blue safranin	mast cell mucous mast cell transition in connective tissue	blue red	Strobel *et al*., 1981

4. CONSIDERAÇÕES FINAIS E CONCLUSÕES

Foram apresentados nesta tese, quatro trabalhos decorrentes de um estudo de 56 eqüinos, incluindo animais de diferentes idades pré e pós-natal, e ainda cavalos portadores de Anemia Infecciosa Eqüina. Ao nosso conhecimento, esse é o estudo mais extenso e detalhado existente na literatura sobre a morfologia de timo eqüino. Foram realizadas análises histológicas, com várias colorações especiais para células e componentes da matriz extracelular; histoquímicas para glicoproteínas e proteoglicanos, e imunohistológicas para detecção de células epiteliais tímicas (incluindo os corpúsculos de Hassall) e de componentes da matriz extracelular. Além disso, realizamos estudos a nível ultraestrutural, com microscopia eletrônica de transmissão. Algumas colorações especiais ou seletivas para eosinófilos e colágenos intesticiais (I e III) e marcações imunohistoquímicas foram também examinadas em microscopia de varredura confocal a laser. Com base nesses estudos uma série de aspectos pode ser definida.

O timo de eqüinos se assemelha ao timo dos demais mamíferos, apresentando, contudo, várias peculiaridades ou variações quantitativas e/ou qualitativas nas várias idades analisadas.

À semelhança dos mamíferos em geral, o timo eqüino apresenta dois compartimentos básicos, subdivididos em quatro regiões anatômicas*:* ***Compartimento Intraparenquimatoso*** subdividido em córtex (1ª região anatômica) e medula (2ª região anatômica), e ***Compartimento Extraparenquimatoso*** constituído por espaços perivasculares (3ª região anatômica), assim como cápsula e septos não vascularizados (4ª região anatômica).

O estudo pós-natal sobre timos de eqüinos que realizamos inicialmente mostrou ainda que o processo involutivo ou de atrofia tímica não é homogêneo, mesmo quando consideramos o timo de um mesmo animal. Além disso, tal atrofia tem início antes da puberdade. Em eqüinos, a detecção frequente de vênulas de endotélio alto (raramente relatadas em timos de outras espécies de mamíferos) sugere intenso tráfego de linfócitos através dessas estruturas.

Outra característica também peculiar em timo de eqüinos é a presença de proeminentes vasos linfáticos, com grande quantidade de linfócitos em seu interior, podendo talvez representar a principal via de exportação de timócitos para a periferia.

Por outro lado, é notável em eqüinos a freqüente e exuberante transformação cística, com metaplasia glandular dos Corpúsculos de Hassall, produzindo grande quantidade de glicoproteínas e proteoglicanos, sugerindo que essas estruturas exerçam funções secretórias.

Também a presença de grande número eosinófilos nos compartimentos extra e intraparenquimatosos foi um evento constante, declinando ou desaparecendo somente em animais

com 13 ou mais anos de idade. É interessante notar que na medula, os eosinófilos tendiam a circundar ou a penetrar no interior de corpúsculos de Hassall. Cumpre frizar que o estudo específico realizado sobre eosinófilos mostrou claramente a existência de formas imaturas, o que sugere fortemente um processo de eosinopoese intratímica. A longa, intensa e precoce presença de eosinófilos maduros e imaturos no timo eqüino sugere a possibilidade de influência bidirecional entre timócitos e eosinófilos, conforme trabalho recente tem funcionalmente evidenciado em timos de camundongos (Throsby *et al*., 2000), alertando para novas e importantes funções imunoregulatórias dos eosinófilos.

Novamente diferindo da grande maioria dos mamíferos, vimos que a hematopoese intra-tímica não linfóide é de fato um evento frequente em timos equinos, principalmente nos PVS, mostrando focos da eosinopoese, eritropoese, mastocipoese e, mais raramente, monocitopoese e megacariopoese. Além disso, a presença de plasmócitos e focos de plasmocitogênese sugere a diferenciação intratímica de células B até o estágio terminal de plasmócitos, dado este também relatado na literatura, principalmente em atrofia involutiva em humanos (Henry, 1992).

No estudo realizado sobre **timos fetais**, foram observados os seguintes aspectos mais relevantes: 1) as células linfóides começaram a habitar o primórdio epitelial tímico antes ou em torno de 60 dias de gestação e não nas 11ª-12ª semanas, como havia sido referido anteriormente (Mackenzie, 1975); 2) o início da maturidade do compartimento intraparenquimatoso, expresso pela definição da região medular e presença de corpúsculos de Hassall, ocorre aos três meses de gestação; 3) notou-se uma grande atividade do espaço perivascular (3ª região anatômica), exemplificada pelo desenvolvimento ou expressão de várias linhagens hematopoéticas (eosinofílica, eritrocítica e megacariocítica), com nítido predomínio da linhagem mielóide eosinofílica. O momento de maior expressão dessas linhagens coincidiu com a evidência morfológica de grande exportação de timócitos através de vasos linfáticos; 4) a maturidade tímica fetal, do ponto de vista morfológico, foi atingida do 6º mês gestacional em diante, quando possivelmente passam a ocorrer interações celulares envolvendo vênulas de endotélio alto no espaço perivascular.

O estudo sobre o timo de animais infectados pelo vírus da **Anemia Infecciosa Eqüina** mostrou uma aceleração do processo de involução tímica devido à idade; tendo provocado ainda acentuado aumento de deposição de matriz extracelular, e também da rede vascular do timo, e levou a uma exacerbacão do fenômeno de transformação cística de corpúsculos de Hassall. Se esses aspectos refletem uma resposta inespecífica devido ao estresse causado pela infecção, ou se correspondem a eventos deflagrados de modo específico pela infecção pelo virus da EIA, é um

ponto ainda sem resposta. De forma similar, será importante analisar se o vírus EIA infecta células tímicas.

Finalmente, nosso trabalho reforça ainda a importância de analisar o timo de vários modelos animais, o que permite uma visão mais abrangente do sistema imune, diferente daquela baseada somente ou principalmente no modelo de camundongo.

5. REFERÊNCIAS BIBLIOGRÁFICAS

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APÊNDICE

6.1. Parâmetros utilizados na análise histológica dos timos de eqüinos

Julgamos que poderia ser útil apresentarmos na forma de apêndice, a tabela que construímos no desenvolvimento da presente tese, e que contém os diversos parâmetros histológicos que foram avaliados nos timos de eqüinos.

Colorações e seus tecidos correspondentes

Colorações	Especificidades de Tecidos	Coloração Final	References
Alcian-Blue pH 2.5- PAS	Proteoglicanos fracamente ou fortemente sulfatadas Polisacarídeos e proteoglicanos neutros contendo grupamentos 1-2 glicol	azul escuro	Lev & Spicer, 1964
Alcian-Blue pH 1.0-PAS	Proteoglicanos sulfatados Polisacarídeos e proteoglicanos neutros contendo grupamentos 1-2 glicol	azul	Lev & Spicer, 1964
Reticulina de Gomori	Fibras reticulares (Tipo III e glicoproteínas)	preto	Gomori, 1937
Resorcina-Fucsina de Weigert com oxidação sem oxidação	 Fibras elásticas, fibras oxitalânicas Fibras elásticas, fibras elaunínicas	 marrom p/ roxo marrom p/ roxo	Fullmer & Lillie, 1958 Gawlik, 1965
Tricrômica de Masson	fibras colágenas músculos núcleo	azul vermelho azul escuro	Masson, 1929
Prata metalamina (PAMS)	membrana basal e fibras reticulares	preto	Jones, 1951
Giemsa Lennert	núcleo eritrócitos citoplasma grânulo eosinofílico grânulo basofílico grânulo neutrofílico	azul laranja roxo vermelho roxo escuro vermelho	Lennert, 1978
Hematoxilina e eosina de Mayer	núcleo citoplasma maioria de outras estruturas teciduais	azul rosa p/ vermelho rosa p/ vermelho	
Ácido fosfomolíbidico e picrosirius	fibras colágenas	vermelho (MO)	Dolber & Spach, 1993
Alcian-Blue safranina	mastócitos mucosos mastócitos transitórios no tecido conjuntivo	azul vermelho	Strobel *et al*., 1981

6.2. Técnicas histológicas que podem ser utilizadas em microscopia de campo claro e/ou de varredura confocal laser

Tendo em vista que as técnicas histológicas para utilização em microscopia confocal a laser, não são usuais, apresentamos abaixo as soluções e os procedimentos relativos às mesmas.

6.2.1 Alcian blue - safranina

Soluções:

Solução de Alcian Blue 1% em HCl 0,7 M (ph 0,5)

Solução de Ácido clorídrico 0,7M

Ácido Clorídrico.....5,8 ml

Água destilada.....94,2 ml

Solução de Alcian Blue a 1%

HCl 0,7M.....100 ml

Alcian Blue.....1 g

Ajustar o pH para 0,5

Solução de Safranina 0,5% em HCl 0,125M:

Solução de HCl 0,125M:

HCl…….....1,05 ml

Água destilada.....98,95 ml

Solução Safranina 0,5%

HCl 0,125M.....100ml

Safranina.....0,5g

Método:

1-Desparafinizar e hidratar os cortes até água destilada.

2- Corar pelo Alcian blue por 30 minutos.

3- Lavar em água destilada para retirar o excesso do corante.

4- Corar pela Safranina de 30 seg. a 1 minuto.

5- Lavar em água destilada.

6-Desidratar, clarear e montar.

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6.2.2. Ácido fosfomolíbdico – Picrosirius (PMA-PSR)

Soluções:

Ácido Fosomolíbdico 0,2%.

Solução de Picrosirius 0,1% pH 2,0:

Solução saturada de Ácido Pícrico (6g em 200 ml de H_2O).....200 ml

Sirius Red F3BAou Direct Red 80.....0,2 g

Ácido Clorídrico 0,01N.

Método:

1- Desparafinizar e hidratar os cortes até a água destilada.
2- Lavar em água destilada por 10 minutos.
3- Colocar as lâminas na solução de Ácido Fosfomolíbdico por somente 1 minuto.
4- Desprezar o Ácido Fosfomolíbdico.
5- Corar pelo Picrosirius por 90 minutos.
6- Lavar em Ácido Clorídrico 0,01N durante 2 minutos.
7- Lavar em álcool 70% durante 45 segundos.
8- Desidratar, clarificar e montar.

Referências:

Paul C. Dolber e Madison S. Spach, Conventional and Confocal Fluorescence Microscopy of Collagen Fibers inthe Heart. The Journal of Histotechnology and Cytochemistry, 41 (1993), 465-469.

Paul C. Dolber e Madison S. Spach, Picrosirius Red Staining of cardiac muscle following Phosphomolybdic Acid Treatment. Stain Technology, 62 (1987), 23-26.

6.2.3. Sírius red pH 10,2 para eosinófilos

Solução:

- Dissolver 0,5g de Sirius Red em 45 ml de Água destilada.
- Acrescentar 50 ml de Álcool Absoluto.
- Adicionar HCl 0,1N, até atingir o pH 10,2.
- Dissolver por agitação e deixar repousar por 2 horas.
- Adicionar lentamente 3 ml de Cloreto de Sódio a 20%.
- Gotejar lentamente o Cloreto de Sódio 20% em baixo de uma luz forte até aparecer o precipitado. Deixar repousar durante a noite e filtrar na manhã seguinte.

Esta solução dura 1 mês à temperatura ambiente. Mas pode durar mais caso fique na geladeira.Quando passar de 1 mês aumentar o tempo de coloração.

Método:

1- Desparafinizar e hidratar os cortes até a água destilada.
2- Corar por 7 minutos pela Hematoxilina de Mayer (cortes com 5μm) e 3 minutos (cortes de 30μm.
3- Lavar em água corrente por 5 minutos.
4- Lavar em água destilada por 2 minutos.
5- Passar pelo álcool 70% por 3 minutos.
6- Corar pela solução de Sirius Red por 1 hora ou mais.
7- Lavar em água corrente por 10 minutos.
8- Desidratar, clarificar e montar em Goma de Damar.

Referências:

Bogomoletz W 1980. Avantages de la coloration para le rouge Sirius de l'amyloide et des éosinophiles. *Arch Anat Cytol Pathol* 28: 252-253.

Luque EH, Montes GS 1989. Progesterone promotes a massive infiltration of the rat uterine cervix by the eosinophilic polymorphonuclear leukocytes. *Anat Rec* 223: 257-265.

6.3 Ficha de registro de dados

Para uniformizar o registro de dados obtidos durante a leitura microscópica das lâminas, elaboramos a ficha especificada a seguir:

N° de ordem: ------- Data de leitura: --------/ ------- / 2000

IDADE: _____ TRATADO: SIM ( ) NÃO ( )

REGISTRO PATOLOGIA:
TIMO **SEXO**: F ( ) M ( )

_____

Nº do animal: _____ Imunocitoquímica: _____ ME: _____
Semi-Fino ( ) Ultra-Fino ( )

1. **PESO**: Animal _____ Timo _____ Baço _____ OBS.: _____

2. **LÓBULOS**: _____

2.1 Definição Córtico-Medular: SIM ( ) NÃO ( )

3. **CÁPSULAS**: constituição: capilares ( ) arteríolas ( ) vênulas ( ) artérias ( ) veias ( ) linfáticos ( ) com ( ) **ou** sem ( ) células

_____

3.1 Espessura: _____

3.2. Nervos: _____

3.3 Resquícios endodérmicos: _____

4. **SEPTOS**: constituição: capilares ( ) arteríolas ( ) vênulas ( ) artérias ( ) veias ( ) linfáticos ( ) com ( ) **ou** sem ( ) células

capilares septo-corticais _____

_____

4.1 Espessura: _____

4.2 Nervos: _____

4.3 Resquícios endodérmicos: _____

5. **CÉLULAS SEPTAIS**: Monócitos ( ) Linfócitos ( ) _____

Mastócitos: Ausente ( ) Presente ( ) Tipos: MMC ( ) CTMC ( ) _____

Eosinófilos: Ausente ( ) Presente ( ) Maduros ( ) Imaturos ( ) _____

Eosinopoese: SIM ( ) NÃO ( ) _____

Tecido hematopoético não eosinofílico: _____

6. **CORTICAL**: _____

6.1 Nervos: SIM ( ) NÃO ( ) _____

6.2 Vasos: SIM ( ) NÃO ( ) _____

6.3 Zona Subcapsular (Arranjo Pseudo-Epitelial): SIM ( ) NÃO ( ) _____

_____

6.4 Células Reticulo-Epiteliais: _____

6.5 Linfócitos: Mensuração P ( ) M ( ) G ( ) _____

6.6 Picnose (Linfólise): SIM ( ) NÃO ( ) _____

6.7 Mitose: SIM ( ) NÃO ( ) _____

6.8 Macrófagos ( ) com detritos apoptóticos ( ) ; PAS cells ( ) ; Nurse Cells ( ) ; Cels. Interdigitadas ( )
Plasmócitos ( ) ; Cels. Mióides (Citoesqueleto Desmina) ( ) ; Linfofagia ( ) ; Folículos Linfóides ( )
Eosinófilos: Maduros ( ) Imaturos ( ) ; Neutrófilos Maduros ( ) Imaturos ( ) ; Eritropoese ( )

_____

7. **MEDULAR**: _____

7.1 Nervos: SIM ( ) NÃO ( ) _____

7.2 Vasos Linfáticos: SIM ( ) NÃO ( ) _____

7.3 Espaço Peri-Vascular: SIM ( ) NÃO ( ) _____

7.4 Células Reticulo-Epiteliais: _____

7.5 Linfócitos: Mensuração P ( ) M ( ) G ( ) _____

7.6 Picnose (Linfólise): SIM ( ) NÃO ( ) _____

7.7 Mitose: SIM ( ) NÃO ( ) _____

7.8 Macrófagos ( ) com detritos apoptóticos ( ) ; PAS cells ( ) ; Cels. Interdigitadas ( ) ; Plasmócitos ( )
Cels. Mióides (Citoesqueleto Desmina) ( ) ; Linfofagia ( ) ; Folículos Linfóides ( ) ; Eritropoese ( )
Eosinófilos: Maduros ( ) Imaturos ( ) ; Neutrófilos Maduros ( ) Imaturos ( ).

8. **CORPÚSCULOS DE HASSALL**: _____

Presença ( ) Ausência ( ) Tamanho (P M G) Sólido ( ) Cavidade ( ) Cistos ( )_____

Células de Intrusão: Eosinófilo ( ) Detritos Celulares ( ) Neutrófilos ( ) Linfócitos ( )_____

Monocelular ( ) Multicelular ( ) _____

9. **COLORAÇÕES ESPECIAIS**: _____

Reticulina:
Cápsula { Externa: _____
Interna: _____

Cortical : Perivascular ( ) Fibras Isoladas ( ) Espaço Perivascular ( ) Trabéculas Migrantes ( )

Picrosirius com polarizacão : col I ( ) col III ( ) _____

PMA- PSR:_____

PAS-AB pH 2,5: _____

PAS-AB pH 1,0: _____

Resorcina-Fucsina S/ O2: _____

Resorcina-Fucsina C/ O2: _____

PAMS: _____

10. **FOTOS**: _____

11. **OBSERVAÇÕES**: _____

12. **CONCLUSÃO**: _____

_____