Article

Thymic Nurse Cells Support $CD4^-CD8^+$ Thymocytes to Differentiate into $CD4^+CD8^+$ Cells

Aidong Li[1], Xueli Liu[2], Baochun Duan[3] and Jie Ma[2, 4]

Thymic nurse cells (TNCs) represent a unique microenvironment in the thymus for T cell maturation. In order to investigate the role of thymic nurse cells during T cell differentiation, a TNC clone, RWTE-1, which formed a typical complex with fetal thymocytes *in vitro* was established from normal Wistar rat. Hanging drop culture method was applied to reveal the interaction between TNCs and thymocytes. Our result revealed that eighty percent of immature $CD4^-CD8^+$ cells differentiated into $CD4^+CD8^+$ cells after a 12-hour hanging drop culture with RWTE-1. However, in a 12-hour culture of immature $CD4^-CD8^+$ cells with or without RWTE-1 supernatant, only 30% of the cells differentiated into $CD4^+CD8^+$ cells spontaneously. This observation led to the conclusion that RWTE-1 cell has the capacity to facilitate immature $CD4^-CD8^+$ thymocytes to differentiate into $CD4^+CD8^+$ T cells by direct interaction. *Cellular & Molecular Immunology*. 2005;2(4): 301-305.

Key Words: thymic nurse cell, T cell differentiation, hanging drop culture

Introduction

Bone marrow-derived progenitor cells migrate into the thymus where they mature from a $CD3^-CD4^-CD8^-$ triple negative (subcapsule), through immature $CD3^-CD4^-CD8^+$ single positive (or, in some mice, $CD3^-CD4^+CD8^-$) to $CD3^-CD4^+CD8^+$ double positive (cortex), and eventually the mature $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ single positive (medulla) phenotype (1). In this differentiation process, the thymic microenvironment which is constructed by non-lymphoid components plays an important role (2, 3). Among these components, thymic nurse cells (TNCs) have been considered a most intriguing, specialized microenvironment for the study of T cell differentiation, maturation, and negative or positive selection phenomena (4).

Lots of data have been generated about the multi-function of thymic nurse cells. Some investigators have shown that TNC has endocrine capability (5), implying that TNC is involved in T cell differentiation by secreting humoral factors. Others have demonstrated that TNCs can support the growth of fetal thymocytes in the presence of IL-2 (6). Li et al. found some TNC clones have the capacity to bind $CD4^+CD8^+$ thymocytes and to support them to differentiate into $CD4^-CD8^+$ T cells *in vitro* by direct contact (7). However, contradictory studies showed that apoptosis occurred in some thymocytes cocultured with TNCs (8). All these studies indicated the heterogeneity of TNCs and its importance in T cell differentiation in the thymus.

To clarify the role of TNCs during T cell differentiation, we established a TNC clone, RWTE-1, from Wistar rat and cocultured it with fetal thymocyte subpopulations by handing drop technique. The data showed that immature $CD4^-CD8^+$ thymocytes were facilitated to differentiate into $CD4^+CD8^+$ T cells within RWTE-1 cells. In another experiment, we proved that RWTE-1 could not produce a humoral factor to influence T cell differentiation.

In this paper the role of the specific interactions between thymic epithelium and thymocyte in the T cell differentiation process is discussed. Evidence is presented that thymic nurse cells support immature single positive cells to differentiate into double positive cells.

Materials and Methods

Antibodies

Mouse anti-rat thymic epithelial antibodies RE-4D8, RE-5C6, RE-6D6, RE-1D4, and RE-12B2 were used to determine the phenotype of RWTE-1 (9). Mouse anti-rat antibodies, 3B3,

[1]Central Laboratory, Cancer Institute and Hospital, Chinese Academy of Medical Sciences, PUMC, Panjiayuan, Beijing 100021, China;

[2]State Key Laboratory of Molecular Oncology, Cancer Institute and Hospital, Chinese Academy of Medical Sciences;

[3]Department of Animal Research, Cancer Institute and Hospital, Chinese Academy of Medical Sciences;

[4]Corresponding to: Dr. Jie Ma, State Key Laboratory of Molecular Oncology, Cancer Institute, Chinese Academy of Medical Sciences, PUMC, Panjiayuan, Beijing 100021, China. Tel: +86-10-877-04478, Fax: +86-10-877-88451, E-mail: jiema2000@yahoo.com.

Received Jul 18, 2005. Accepted Aug 5, 2005.

Table 1. Phenotypical comparison of CD4 depleted fetal thymocytes after 12-hour culture

Phenotype	% of positive thymocytes after 12-hour culture		
	Medium	Condition medium*	Hanging drop
$CD4^+CD8^-$	1	1	2
$CD4^-CD8^+$	77	75	10
$CD4^+CD8^+$	20	24	80
$CD4^-CD8^-$	2	2	8

*50% medium + 50% supernatant of RWTE-1 cells.

RTH-7 and 10B5, which recognize rat CD5, CD4 and CD8 antigen respectively, were used to separate thymocytes (10).

Preparation of thymic nurse cells

Thymic nurse cells were isolated as described elsewhere (11). Briefly, fragments obtained from fetal Wistar rats were enzymatically dissociated using 0.25% trypsin at 37°C for 30 min. TNC complexes were purified from total thymic cells by serial sedimentations on 50% fetal calf serum (FCS). Then the cells were cultured in RPMI 1640 medium supplemented with 20% FCS. The adherent cells were cloned by limited dilution.

Immunofluorescence study

RWTE-1 cells were incubated with various anti-thymic epithelial antibodies for 1 h at 4°C. The cells were then washed twice with PBS, and incubated with fluorescein labeled antibody against mouse Ig for another 30 min at 4°C. Thereafter, the cells were washed three times and subsequently assessed by a FACS analyzer (Becton Dickinson).

In the double staining study, 1×10^6 thymocytes were simultaneously stained with FITC-labeled anti-rat CD8 antibody as well as PE-labeled mAb against rat CD4.

Separation of the subsets of thymocytes

Thymocytes were first treated with mouse anti-rat-CD4 mAb and/or mouse anti-rat-CD8 mAb for 1 h at 4°C. Then the cells were washed by PBS and incubated with anti-mouse IgG labeled magnetic beads. The cells unconjugated with the beads were separated by a magnetic column and used for subset study.

Hanging drop culture

Two-week pregnant rat was sacrificed and the thymuses of the fetuses were removed. The thymuses were smashed in PBS and the suspension of thymocytes was derived by passing through a cell filter. The subsets of embryonic thymocytes and RWTE-1 cells were seeded in a Terasaki plate (Nunc Inc.) at a ratio of 40:1. Then the plate was turned over and incubated for 12 h. After a 12-hour culture, the cells of each well were collected and smeared on slides using a cytospinner (Sakura Inc., Tokyo). The cells engulfed at least two thymocytes were considered to be positive.

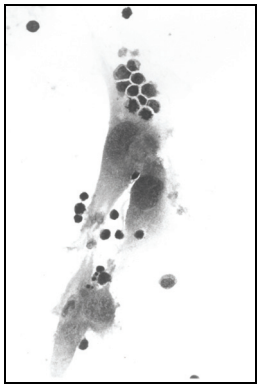

Figure 1. Microphotograph of RWTE-1 cells. Attachment of thymocytes to RWTE-1 cells could be observed.

Immunohistochemical staining of intra-TNC thymocytes

Phenotypes of intra-TNC thymocytes were determined after 12-hour handing drop culture by immunohistochemical staining. The smears of TNC complexes obtained by a cytospinner were used for microscopy observation.

Results

Establishment of the TNC clone RWTE-1

Using the method described above, 7 clones were obtained from the thymus of Wistar rat. One of these clones, RWTE-1, which could form typical complexes in the hanging drop culture was chosen for further study. When RWTE-1 cells and thymocytes cultured through hanging drop technique, most of RWTE-1 cells could not adhere to the substratum. After 6-12 h incubation, they formed typical complexes virtually identical to those obtained from enzyme-digested fresh thymuses. With a laser confocal microscope, we cut the complexes and proved that the thymocytes were present in the cytoplasm but not on the surface of RWTE-1 cells.

When cultured in dish, RWTE-1 cells had an epithelial appearance, which were polymorphic, large, less dense and with one or two large nuclei (Figure 1). Fine tonofilaments in the cytoplasm were also found under transmission electron microscope (12).

Membrane antigen determination

The surface determinants of RWTE-1 were examined by FACS with monoclonal antibodies established in our laboratory (8). Among five anti-thymic epithelial antibodies (RE-4D8, RE-5C6, RE-6D6, RE-1D4, RE-12B2), RWTE-1 was only reactive to mAb RE-4D8, which is an antibody recognizing a common antigen expressed by thymic epithelium (Figure 2). This indicates that these antibodies are not specific to RWTE-1. To induce MHC antigens on the surface of RWTE-1, we cultured RWTE-1 with 100 U/ml recombinant mouse interferon-γ. After 1 day stimulation, MHC class I and class II molecules were highly expressed (Figure 3). Intercellular cell adhesion molecule-1 (ICAM-1)

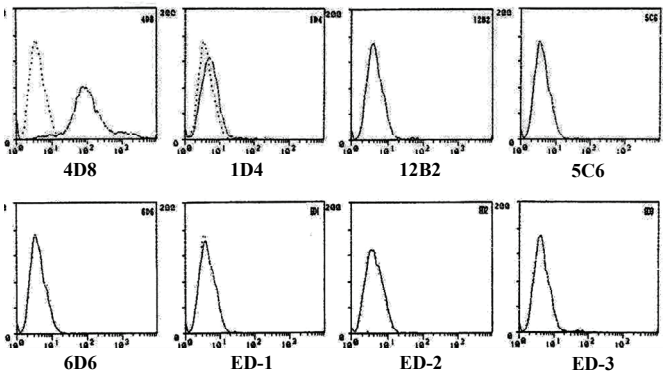

Figure 2. Antigen expression on the surface of RWTE-1 cells. Epithelial antigens defined by monoclonal antibodies against 4D8, 1D4, 12B2, 5C6, and 6D6. Monoclonal antibodies ED-1, ED-2 and ED-3, which recognize antigens expressed on macrophages were negative on RWTE-1 cells.

was detected on the surface of RWTE-1 cells, whereas macrophage markers ED1, ED2 and ED3 were negative (Figure 2). These results confirmed that RWTE-1 cells are epithelial oriented.

Fractionized cells in hanging drop culture

In hanging drop culture, we found that the fetal thymocytes but not the adult thymocytes can be engulfed into RWTE-1 cells. This implied that RWTE-1 cells could harbor immature thymocytes. To investigate which cell subset could be entrapped into RWTE-1, the fetal thymocytes were separated into three groups by mouse anti-rat CD4 antibody, mouse anti-rat CD8 antibody and goat anti-mouse IgG magnetic beads. After passed through a magnetic column, the unbound cells were collected. The $CD4^+$ cells were eliminated by mAb RTH-7 and $CD8^+$ cells were cleared out by mAb 10B5. When mAb RTH-7 and mAb 10B5 were used simultaneously, both $CD4^+$ and $CD8^+$ cells were eliminated. Accordingly, the subset of enriched $CD4^-CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ was obtained, respectively. As each subset of the enriched cells was incubated with RWTE-1 cells in the hanging drop culture, it showed that 12% of the enriched $CD4^-CD8^+$ cells, which contained $CD4^-CD8^+$ and $CD4^-CD8^-$ cells, could be engulfed (Table 2). No engulfment of the enriched $CD4^+CD8^-$ and $CD4^-CD8^-$ subsets was observed. Furthermore, the enriched peripheral $CD4^-CD8^+$ cells were also examined. None of these cells was engulfed. Therefore, we speculated that thymocytes engulfed by RWTE-1 cells were immature $CD4^-CD8^+$cells.

Phenotypic change of $CD4^-CD8^+$ cells after 12-hour hanging drop culture

In order to determine the role of RWTE-1 cells in T cell differentiation, the change of T cell differentiation markers on the surface of engulfed thymocytes was monitored after hanging drop culture. As immature $CD4^-CD8^+$ cells could be engulfed by RWTE-1 cells, this subset was hanging drop

Table 2. The engulfment of thymocytes by RWTE-1 cells

Cell fraction	% of engulfment
Embryonic thymocytes	4
Embryonic $CD4^-CD8^-$ thymocytes	1.5
Embryonic $CD4^-CD8^+$ thymocytes	12.5
Embryonic $CD4^+CD8^-$ thymocytes	0
Adult $CD4^-CD8^+$ thymocytes	0
Adult $CD4^+CD8^-$ thymocytes	0

cultured with RWTE-1 cells for 12 h. The CD4, CD8 and CD5 antigens were then detected on cytospin preparations of collected cell suspensions by immunoperoxidase staining.

The expression of CD5 on thymocytes was strongly positive (Figure 4A). This indicated that the engulfed cells were thymocytes. As expected, CD8 expression was also positive (Figure 4B). Interestingly, only a few thymocytes per TNC showed a negative expression of CD4 (Figure 4C). In addition, when both mAb RTH-7 and mAb 10B5 were used in immunoperoxidase staining, all the thymocytes were positive. Calculating all the thymocytes within 200 RWTE-1 cells, we found that 80% of them were $CD4^+CD8^+$ cells. Comparing with the phenotype of the cells before hanging drop culture, there were 80% of $CD4^-CD8^+$ cells gained CD4 determinate after a 12-hour hanging drop culture. This indicated that RWTE-1 cells could promote $CD4^-CD8^+$ cells to differentiate into $CD4^+CD8^+$ cells through direct contact.

Culture of $CD4^-CD8^+$ enriched subset with the supernatant of RWTE-1

To determine whether RWTE-1 cells influence T cell differentiation by producing humoral factors, we cultured $CD4^-CD8^+$ enriched cells in RPMI medium supplemented with 50% supernatant of RWTE-1 cells at 37ºC. The cells cultured in the medium without supernatant were used as a control. After 12 h, cell populations were analyzed by two-color flow cytometry for CD4/CD8 expression. As indicated in Table 1, after a 12-hour culture, $CD4^+CD8^+$cells were evident in both groups. There was not a significant difference between the cells cultured with or without supernate of RWTE-1 cells. In both conditions, enriched $CD4^-CD8^+$ cells resulted in about 20% $CD4^+CD8^+$ cells after 12-hour incubation. These results demonstrated that indirect contact between $CD4^-CD8^+$ thymocytes and RWTE-1 cells was not responsible for differentiation of $CD4^-CD8^+$ cells into $CD4^+CD8^+$ cells in our culture system. The mechanism of spontaneous acquisition of CD4 antigen was not clear. The possible explanation was that 20% of $CD4^-CD8^+$ cells in our system had been selected to differentiate into $CD4^+CD8^+$ cells in the thymus before surgical removal.

Discussion

Until now, the authenticity of the character and function of TNCs has been in question (13). In this study, we

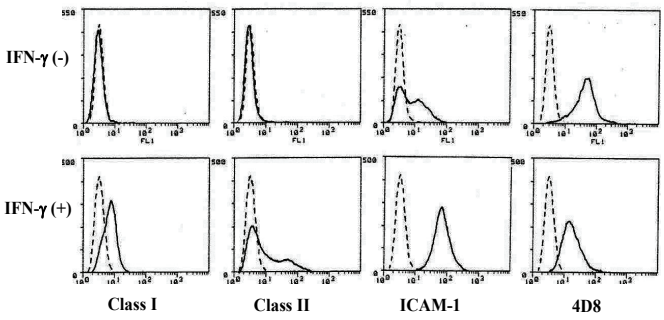

Figure 3. The induction of MHC and ICAM-1 antigens on the surface of RWTE-1 cells. MHC antigens could not be detected in a regular culture condition. After the stimulation with interferon-γ for 48 h, these antigens were detected positive.

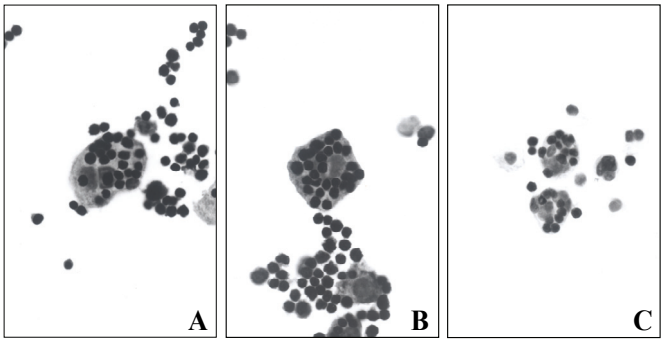

Figure 4. Immunohistochemical staining of intra-TNC thymocytes with anti-rat antibodies (1,000×). After a 12-hour hanging drop culture, the smear of TNC complex was fixed with acetone for 5 min and incubated with mouse anti-rat CD5 (A), mouse anti-rat CD8 (B), and mouse anti-rat CD4 (C) for 1 h. The section was than subjected to the biotin-labeled goat anti-mouse antibody for 10 min and incubated with peroxidase-conjugated streptavidin and submerged in 0.05% (w/v) diaminobenzene in 0.05 mol/L Tris-HCl buffer solution (pH 7.6) for 5 min. Hematoxylin was used for counter staining.

demonstrated the role of TNC clone, RWTE-1, in the process of T cell differentiation. As shown in the figures, RWTE-1 cells formed a characteristic complex structure with fetal thymocytes in hanging drop culture system. From morphological and phenotypic studies, we propose that RWTE-1 clone belongs to CTES type II epithelium. Moreover, macrophage markers ED1, ED2 and ED3 were not labeled by RWTE-1 cells. These findings strongly indicated that RWTE-1 cells were cloned from thymic nurse cells (14-16).

Thymic nurse cell was proved to be an isolated thymic microenvironment for T cell differentiation (14), which plays multi-function during this process (17). According to Brelinska et al. (18), intra-TNC thymocytes were heterogeneous in their phenotypes and represented cells at various stages during their maturation cycle. According to our observation, RWTE-1 cells entrapped immature $CD4^-CD8^+$ thymocytes only. This subset was immature because 1) $CD4^-CD8^+$ cells were obtained from fetal thymus; 2) neither adult nor peripheral $CD4^-CD8^+$ cells could be encapsulated by RWTE-1 cells. Among these immature $CD4^-CD8^+$ cells only 12% of them could be captured by RWTE-1 cells. Although the contamination of $CD4^-CD8^-$ cells contributed to this low encapsulation, whether there are subsets in immature $CD4^-CD8^+$ thymocytes or the entrapping capability of RWTE-1 cells depending on the growth condition remains to be elucidated.

In the stroma, thymic nurse cell has been postulated to be an important docking site in T cell differentiation. It was well accepted that the duration of differentiation prior to the expression of TCR is $CD3^-CD4^-CD8^- \rightarrow CD3^{low}CD4^-CD8^+$/ $CD3^{low}CD4^+CD8^- \rightarrow CD3^{low}CD4^+CD8^+$ (1). However, the precise role of TNC playing in this process is unclear. By coculture thymocytes with TNC monolayer, it was found that TNCs were capable of inducing both mitosis and apoptosis (8). In a similar experiment, RWTE-1 cells were observed to form pseudoemperipolesis with thymocytes and, moreover, mitotic cells were frequently found (12). This implies that RWTE-1 cells may induce the proliferation of thymocytes by direct interaction.

As shown in the Table 2, 80% of $CD4^-CD8^+$ fetal thymocytes were differentiated into $CD4^+CD8^+$ thymocytes inside RWTE-1 cells after a 12-hour hanging drop culture. Although 20% of $CD4^-CD8^+$ thymoctyes could gain CD4 determinants spontaneously in a simultaneous course culture, there was a significant difference in term of the percentage of $CD4^+CD8^+$ cells between the two groups. Therefore, we conclude that RWTE-1 cells may facilitate immature $CD4^-CD8^+$ cells to differentiate into $CD4^+CD8^+$ cells by direct contact. Although it was believed that TNC synthesizes and secretes thymic hormones (19, 20) to support the immature thymocytes, this was not found in our present study. $CD4^-CD8^+$ subpopulation of fetus has been considered to be an immature precursor of $CD4^+CD8^+$ cortical thymocytes. Cells of this subset are large, dividing, highly sensitive to cortisone and found in the outer cortex. After a short-term culture, the majority of these cells become $CD4^+CD8^+$ without additional stimulation or growth factor. MacDonald and Wilson reported the similar observation (21, 22). These findings seem different from our result in the *in vitro* culture, in which only 20% of $CD4^+CD8^+$ cells were detected. There are two logical possibilities for this discrepancy. One is that those authors used a purified $CD4^-CD8^+$ subpopulation (purity > 99%), whereas we used an enriched $CD4^-CD8^+$ subpopulation which contains $CD4^-CD8^-$ cells. Alternatively, the difference of thymocytes between rat and mouse may exist.

It has been proposed that the positive selection is a likely feature of TNCs (23). In some reports, TNC represents a selective environment for helper-lineage T cell differentiation and some $CD4^+CD8^+$ subset-bound TNC clones induce their growth and differentiation into mature $CD4^-CD8^+$ T cells. Our RWTE-1 clone influences T cell differentiation at immature $CD4^-CD8^+$ stage. These results prompt us to a

hypothesis that the distinct thymic nurse cells might be responsible for the development of different thymocyte subsets.

Furthermore, it has been demonstrated that the negative selection occurs before $CD4^+CD8^+$ stage of differentiation. RWTE-1 cells prevent some $CD4^-CD8^+$ cells from the negative selection and facilitate them to differentiate into the next phase of maturation. The molecules involved in recognition between RWTE-1 cells and immature $CD4^-CD8^+$ cells remain unclear. More critical experiments need to be done to clarify this issue.

In this study, the conclusion is reached that a TNC clone, RWTE-1, promotes immature $CD4^-CD8^+$ thymocytes to differentiate into $CD4^+CD8^+$ cells by direct contact. The hanging drop culture system provides a useful tool to investigate the role of TNCs in T cell differentiation.

Acknowledgement

This project was supported by the National Hi-Tech R & D Program of China (No.2002AA412131).

References

1. Alvarez-Vallina L, Gonzalez A, Gambon F, Diaz-Espada F. Delimitation of the proliferative stages in the human thymus indicates that cell expansion occurs before the expression of CD3 (T cell receptor). J Immunol. 1993;150:8-16.
2. Savin W, Dalmau SR, Dealmeida VC. Role of extracellular matrix-mediated interactions in thymocyte migration. Dev Immunol. 2000;7:279-291.
3. Webb O, Kelly F, Benitez J, et al. The identification of thymic nurse cells *in vivo* and the role of cytoskeletal proteins in thymocyte internalization. Cell Immunol. 2004;228:119-129.
4. Mucci J, Hidalgo A, Mocetti E, Argibay PF, Leguizamon MS, Campetella O. Thymocyte depletion in Trypanosoma cruzi infection is mediated by transsialidase-induced apoptosis on nurse cells complex. Proc Natl Acad Sci U S A. 2002;99:3896-3901.
5. Bodey B, Bodey B Jr, Siegel SE, Kaiser HE. The role of the reticulo-epithelial (RE) cell network in the immuno-neuroendocrine regulation of intrathymic lymphopoiesis. Anticancer Res. 2000;20:1871-1888.
6. Doi H, Nakagawa S, Nagata K, et al. Murine thymic nurse cell clone supports the growth of fetal thymocytes in the presence of interleukin 2. Eur J Immunol. 1991;21:783-792.
7. Li Y, Pezzano M, Philp D, Reid V, Guyden J. Thymic nurse cells exclusively bind and internalize $CD4^+CD8^+$ thymocytes. Cell Immunol. 1992;140:495-506.
8. Hiramine C, Hojo K, Koseto M, Nakagawa T, Mukasa A. Establishment of a murine thymic epithelial cell line capable of inducing both thymic nurse cell formation and thymocyte apoptosis. Lab Invest. 1990;62:41-54.
9. Ma J, Iwaki H, Kikuchi K. Five novel monoclonal antibodies to thymic epithelial cell surface antigens in rats. Chinese Med J. 2002;115:1186-1190.
10. Matsuura A, Ishii Y, Yuasa H, et al. Rat T lymphocyte antigens comparable with mouse Lyt-1 and Lyt-2,3 antigenic systems: characterization by monoclonal antibodies. J Immunol. 1984; 132:316-322.
11. Nihei OK, Campos de Carvalho AC, Savino W, Alves LA. A novel form of cellular communication among thymic epithelial cells: intercellular calcium vave propagation. Am J Physiol Cell Physiol. 2003;285:C1304-1313.
12. Ma J, Iwaki H. Establishment of rat thymic nerse cell clone RWTE-1 and analysis of their role in T cell differentiation. Sapporo Med J. 1994;63:23-33.
13. Vucevic D, Colic M, Popovic P, Gasic S. Different roles of a rat cortical thymic epithelial cell line *in vitro* on thymocytes and thymocyte hybridoma cells: phagocytosis, induction of apoptosis, nursing and growth promoting activities. Dev Immunol. 2002;9:63-72.
14. Pezzano M, Samms M, Martinez M, Guyden J. Questionable thymic nurse cell. Microbiol Rev. 2001;65:390-403.
15. Contreiras EC, Lenzi HL, Meirelles MN, et al. The equine thymus microenvironment: a morphological and immuno-chistochemical analysis. Dev Comp Immunol. 2004;28:251-264.
16. Penninger J, Rieker T, Romani N, et al. Ultrastructural analysis of thymic nurse cell epithelium. Eur J Immunol. 1994;24:222-228.
17. Guyden JC, Pezzano M. Thymic nurse cells: a microenvironment for thymocyte development and selection. Int Rev Cytol. 2003;223:1-37.
18. Brelinska R, Seidel HJ, Kreja L. Thymic nurse cells: differentiation of thymocytes within complexes. Cell Tissue Res. 1991;264:175-183.
19. Bodey B. Neuroendocrine influence on thymic haematopoiesis *via* the reticulo-epithelial cellular network. Expert Opin Ther Targets. 2000;6:57-72.
20. Savino W, Postel-Vinay MC, Smaniotto S, Dardenne M. The thymus gland: a target organ for growth hormone. Scand J Immunol. 2002;55:442-452.
21. MacDonald HR, Budd RC, Howe RC. A $CD3^-$ subset of $CD4^-8^+$ thymocytes: a rapidly cycling intermediate in the generation of $CD4^+8^+$ cells. Eur J Immunol. 1988;18:519-523.
22. Wilson A, Petrie HT, Scollay R, Shortman K. The acquisition of CD4 and CD8 during the differentiation of early thymocytes in short-term culture. Int Immunol. 1989;1:605-612.
23. Cao WM, Murao K, Imachi H, et al. Phosphatidylserine receptor cooperates with high-density liporoptein receptor in recognition of apoptotic cells by thymic nurse cells. J Mol Endocrinol. 2004;32:497-505.