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THYMIC NURSE CELLS ARE THE FIRST SITE OF VIRUS REPLICATION AFTER INOCULATION OF THE RADIATION LEUKEMIA VIRUS

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Abstract—The induction of thymic lymphomas by inoculation of the Radiation Leukemia Virus (RadLV) requires interactions between RadLV, lymphoid cells and thymus microenvironment. The possible localization of this interaction within the peculiar lymphoepithelial complexes called 'thymic nurse cells' (TNCs) has been investigated. Electron microscopic studies, as well as *in vitro* experiments using a very sensitive infectious centre detection assay demonstrated that most of the first virus producing cells after RadLV inoculation are located within the TNCs. Most of these structures belong to the thymus subcapsular zone. They contain lymphoid cells with the phenotype of the major (cortical) thymocyte population. Data support the view that a limited subpopulation of subcapsular immature thymocyte can act as specific targets for productive infection with RadLV. Furthermore, the initiation of virus replication appears related to the interaction between the immature thymocyte and the 'nurse cells' microenvironment.

Key words: Thymic nurse cells, Radiation Leukemia Virus, virus replication.

INTRODUCTION

IN C57BL/Ka mice, thymic lymphomas are induced by inoculation of the Radiation Leukemia Virus (RadLV), a retrovirus originally extracted from radiation induced lymphomas of the same mouse strain (for review see [14, 15]). The selective localization of lymphomas within the thymus suggests a specific interaction between lymphoid target cells for RadLV and thymic microenvironment [1, 15]. Target cells are found in bone marrow, spleen and thymus; however, both virus replication and lymphoma development depend upon the presence of the thymus [16, 17, 18]. In the thymus, target cells, representing about 1/50000 of the total population of thymocytes, belong to a limited fraction of the dividing blast cells; they display phenotypic characteristics suggestive of transitional forms between prothymocytes and cortical blast cells [5]. After inoculation of RadLV, viral antigens and budding virus particles were first detected in only a few blast cells of the thymus subcapsular zone [5, 9]. Therefore, it has been proposed that RadLV acts specifically upon the early stages of intrathymic T cell differentiation [1, 5, 9, 14].

Intrathymic T cell differentiation is thought to result from interactions between immature lymphoid cells and the non-lymphoid stromal cells [6, 26]. The lymphoepithelial thymic nurse cell (TNC) complexes, which were recently described in dissociated mouse thymuses [24] might represent a critical step in the evolution of differentiating T cells.

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Abbreviations: *FACS*, fluorescence activated cell sorter; *FCS*, fetal calf serum; *FITC*, fluorescein isothiocyanate; *I.T.*, intrathymically; *PBS*, phosphate buffered saline; *RadLV*, Radiation Leukemia Virus; *TNC*, thymic nurse cell.

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Therefore, we undertook to investigate whether TNCs are also involved in the development of RadLV induced thymic lymphomas. In this report, we shall demonstrate that most of the TNCs belong to the subcapsular zone and that intra-TNC lymphoid cells display the same phenotypic characteristics as cortical thymocytes. We shall also prove that TNCs are involved in the process of virus replication occurring early after inoculation of RadLV.

MATERIALS AND METHODS

Animals. Four-week-old C57BL/Ka mice of both sexes were used.

Virus. RadLV/VL3 was obtained from the culture fluids of the BL/VL3 cell line derived from a RadLV-induced C57BL/Ka thymic lymphoma [21]. The biological and serological properties of this virus isolate were described [11]. Intrathymic injections were made in both lobes of the thymus of mice with 0.05 ml of virus preparation with a titre of 10^7 infectious particles/ml as estimated by an *in vitro* immunofluorescence assay [20].

Thymus dissociation and isolation of the TNCs. In each experiment, 40–60 mice were killed by cervical dislocation. Thymuses were aseptically removed, put in sterile cold phosphate buffered saline (PBS) containing 5% heat inactivated fetal calf serum (FCS) (Gibco Bio Cult. Ltd., Ghent, Belgium) and minced into small pieces with scissors. Thymus fragments were placed into a trypsinisation bottle and shaken for 5 min in 50 ml of cold PBS containing 5% FCS. The supernatant containing only small thymocytes was removed; this cell suspension represented 80% of the whole thymic population; it did not contain any detectable TNCs; it was called 'PBS fraction'. The remaining fragments were shaken twice, each time for 20 min in 50 ml of Dispase solution (Dispase II, Boehringer, Mannheim; 1.2 Uml in PBS) to which was added 2 ml DNase solution (deoxyribonuclease I, Boehringer, Mannheim; 0.02 mg/ml in PBS). The dissociation was achieved by two or three incubations, each lasting 20 min, in 50 ml of Collagenase solution (Collagenase Worthington CLSIV, Millipore Corporation; 40 U/ml in PBS) to which was added 2 ml of DNase solution. The cell suspensions obtained after the enzyme dissociation were pooled and designated 'starting cell suspension'. This suspension, which represented 15–20% of the whole thymic cell population, was resuspended in RPMI 1640 medium (Gibco Bio Cult. Ltd, Ghent, Belgium) and layered on top of 10 ml 30% FCS in PBS. TNCs were isolated by four or five runs of sedimentation at 1 *g* according to the method previously described by Wekerle and Ketelsen [24]. Each step yielded a supernatant ('top fraction') and a pellet. This latter one was submitted to repeated runs of sedimentation until a satisfactory enrichment with TNCs was obtained. Cell recovery and enrichment with TNCs in the successive fractions obtained by this procedure in a representative experiment are shown in Table 1.

Subcapsular cell staining in the thymus. Subcapsular cell staining in the thymus was performed according to the method described by Scollay *et al.* [23]: thymuses were immersed for 12 min in a solution of 400 μg/ml of fluorescein isothiocyanate (Calbiochem Behring Corporation, La Jolla, California, U.S.A.) in PBS. They were then washed in FCS and PBS before being used either for frozen sections or for TNC isolation.

Cell surface phenotype and FACS analysis. Monoclonal α-Thy-1.2, α-Lyt-1 and α-Lyt-2 antibodies (Becton Dickinson, Mountainview, U.S.A.) were used for cell surface typing by indirect immunofluorescence [12] and analyzed with a FACS IV [22]. FITC conjugated goat anti-rat IgG (Nordic Laboratory, Leuven, Belgium) was used as a second step antibody at a dilution of 1/40.

BL/RL12-NP assay. The detection of small numbers of RadLV producing cells in lymphoid cell suspensions was made by a sensitive *in vitro* infectious centre detection assay as previously described [3].

Nylon wool column separation. This was performed to obtain non-adherent thymocytes and remove macrophages using the method described by Julius *et al.* [13].

Cultures. The cells were suspended in RPMI 1640 medium (Gibco Bio Cult. Ltd., Ghent, Belgium) supplemented with 10% heat inactivated FCS, 2 mM/ml L-glutamine, 1% non-essential amino-acids, 1 mM/ml sodium pyruvate, 150 U/ml penicillin and 0.075 mg/ml streptomycin; they were grown in 30 mm Petri dishes (Lux Scientific Corporation, The Netherlands).

Transmission electron microscopy. Samples were prepared for examination with an EM 301 Philips electron microscope as described [2].

TABLE 1. TNC ISOLATION BY REPEATED 1 *g* SEDIMENTATION: CELL RECOVERY AND ENRICHMENT WITH TNCs IN THE VARIOUS FRACTIONS OBTAINED AFTER EACH SEDIMENTATION IN A REPRESENTATIVE EXPERIMENT

Step	FCS (ml)	Sedimentation time (min)	% Cells in the 'top' fraction (compared with the whole thymic population)	% Cells in the serum fraction and in the sediment (compared with the whole thymic population)	% TNCs in the sediment
1	10	20	13.9	2.24	1
2	10	15	1.8	0.33	1
3	10	15	0.22	0.07	1
4	5	15	0.04	0.01	80

RESULTS

Subcapsular localization of thymic nurse cells

Dipping thymuses in FITC resulted in the selective staining of the eight to 10 outermost cell layers (Fig. 1). Cell suspensions were prepared from the FITC treated thymuses by enzyme dissociation; they were then fractionated by repeated 1 *g* sedimentation. The percentage of fluorescent cells, i.e. subcapsular cells, was determined in each fraction by fluorescence microscopy. As shown in Table 2, the 'starting cell suspension' contained

TABLE 2. SUBCAPSULAR LOCALIZATION OF THYMIC NURSE CELLS

Cells*	% Fluorescent cells Exp. 1	Exp. 2	Exp. 3
Starting suspension	12	10.7	8.9
1st 'top' fraction	11	6.34	10.5
2nd 'top' fraction	7	4.12	5
3rd 'top' fraction	6	10.74	10.5
4th 'top' fraction	6	12	14.2
5th 'top' fraction	N.D.	N.D.	11.8
TNCs	63	47	52

*The definition of each cell suspension is described in Materials and Methods.

8–12% of FITC positive cells; in the last fraction, more than 50% of the TNCs were fluorescent (Fig. 1, inset), demonstrating that most of the TNCs were derived from the thymus outer cortex.

Phenotype of TNC lymphocytes

We undertook next the phenotypic characterization of the lymphoid cells which are associated with the TNCs. TNCs were isolated, resuspended in a culture medium and incubated for 24 h at 37°C in 5% CO_2 atmosphere. Like other epithelial cells, they rapidly stuck to the plastic surface. As a consequence, thymocytes were released into the culture medium (Fig. 2). They were recovered, washed and treated with various monoclonal antibodies as probes and then with FITC conjugated second step antibodies as label. The cell surface antigen phenotype was compared to that of the whole thymic population and to that of some of the top fractions treated in the same conditions. It appeared that all intra-TNC lymphoid cells were Thy-1.2 positive and Lyt-1 positive. Only 75% of them were Lyt-2 positive. This distribution was similar to that observed in the total thymocyte population (Fig. 3) which is mainly composed of cortical cells.

Intra-TNC localization of virus producing cells after I.T. inoculation of RadLV/VL3

The subcapsular origin of both TNCs (see above) and virus replicating cells after RadLV inoculation [5, 9] stimulated us to study the behaviour of TNCs after the inoculation of RadLV.

We examined first, by electronic microscopy, the TNCs and the cell suspensions obtained by repeated sedimentation after enzyme dissociation of thymuses inoculated with RadLV four days before. By electronic microscopic studies, no budding particles were observed in the starting cell suspension or in the first 'top' fractions recovered during the TNC isolation procedure; the last 'top' fractions contained only a few free blast cells with budding particles. Some lymphoid blast cells within TNCs exhibited membrane budding particles (Fig. 4). Free virions and budding particles were also ob-

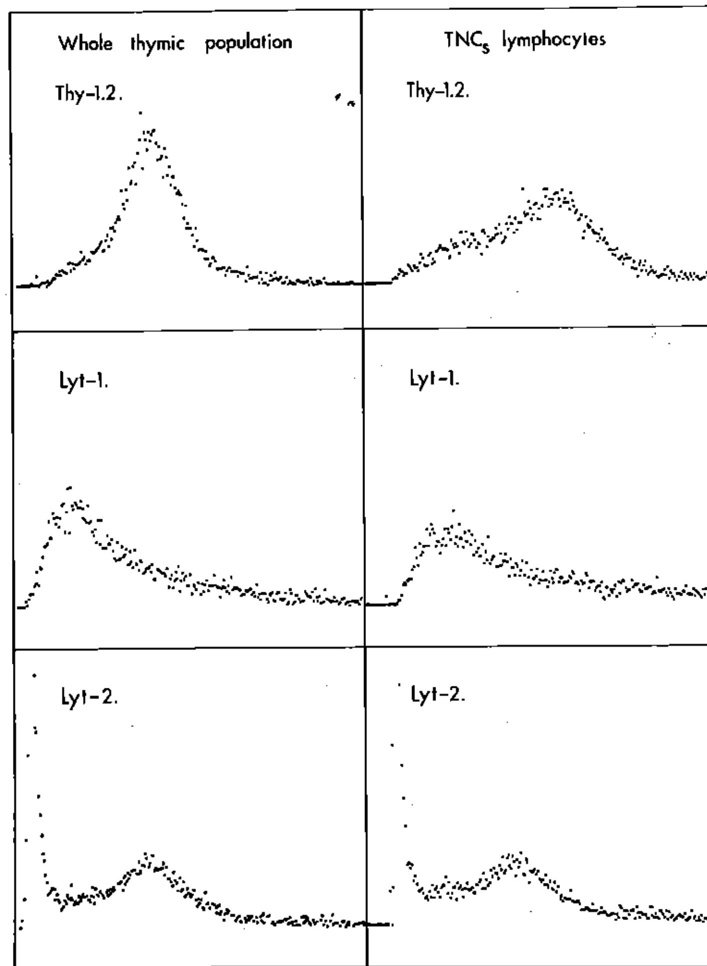

FIG. 3. Cell surface phenotype of the lymphocytes released by the TNCs cultured for 24 h compared with the whole thymocyte population treated in the same conditions.

served in vacuoles of the epithelial TNC (Fig. 5). No such features were ever seen in TNCs isolated from normal C57BL/Ka mice.

The following experiments were undertaken to determine whether the early virus producing cells detected by electronic microscopic studies displayed a preferential localization in TNCs. For this purpose, we applied a very sensitive *in vitro* method, the BL/RL12-NP co-cultivation assay, which allows the estimation of the number of virus producing cells in lymphoid cell populations infected by RadLV [3]. Two days after I.T. inoculation of RadLV into 40 C57BL/Ka mice, thymuses were removed, dissociated with enzymes and fractionated by repeated 1 *g* sedimentation. Cells from the various fractions and TNCs were cultured for 24 h. Non-adherent lymphoid cells were recovered and assayed for virus production by co-culture with BL/RL12-NP. Table 3 shows the results of two replicate experiments. The frequency of infectious centres, i.e. virus producing cells, in the whole thymocyte population was $1/3 \times 10^4$, as expected from previous *in vitro* and *in vivo* studies [3, 5, 10]. There were 300 times as many virus producing cells in the lymphoid cell population released from TNCs than in the 'starting cell suspension' or in the 'PBS suspension'. In effect, 1 out of 100 cells recovered from TNCs could act as an

FIG. 1. FITC staining of the subcapsular zone of the thymus: 10–12 cell layers of the outer cortex are labelled (×250). Inset: fluorescent TNC isolated from the FITC treated thymus by enzyme dissociation and repeated 1 *g* sedimentation (×800).

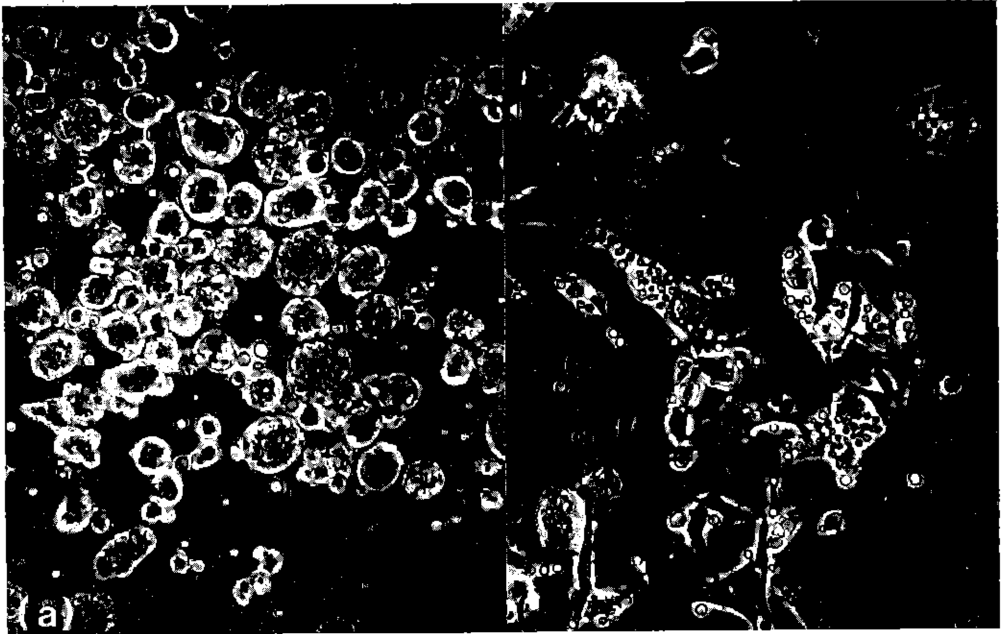

FIG. 2. (a) Suspension of TNCs after the isolation procedure; (b) TNCs in culture after 24 h: engulfed thymocytes were released in the medium (× 184).

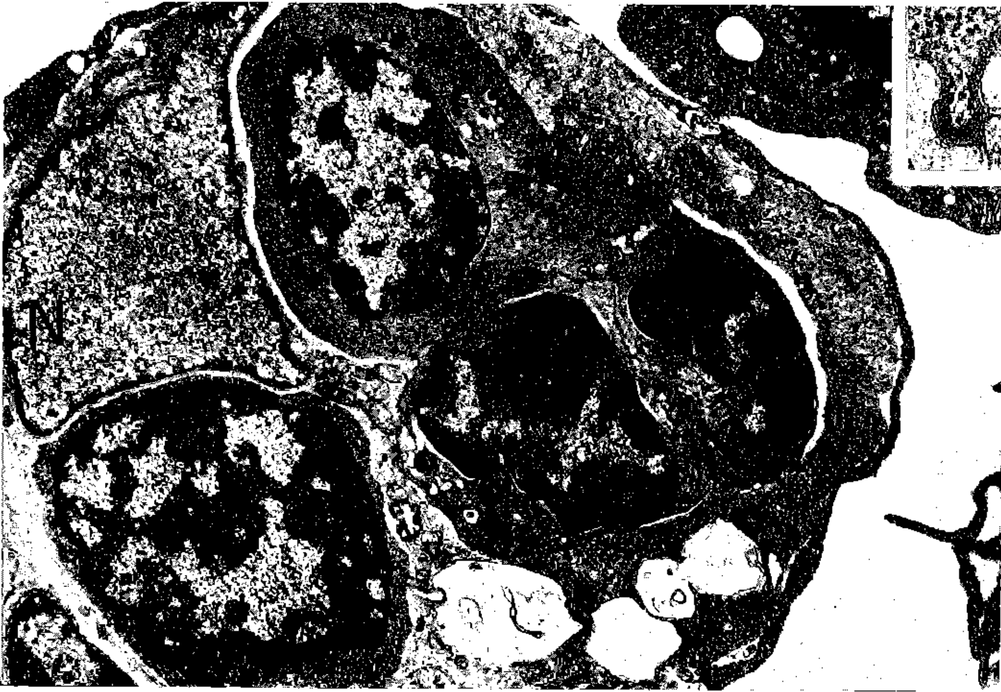

FIG. 4. Budding viral particle (arrow and inset) in a blast cell engulfed in a TNC obtained from thymuses inoculated with RadLV four days before. N: nucleus of the epithelial TNC (× 6250; inset: × 55,625).

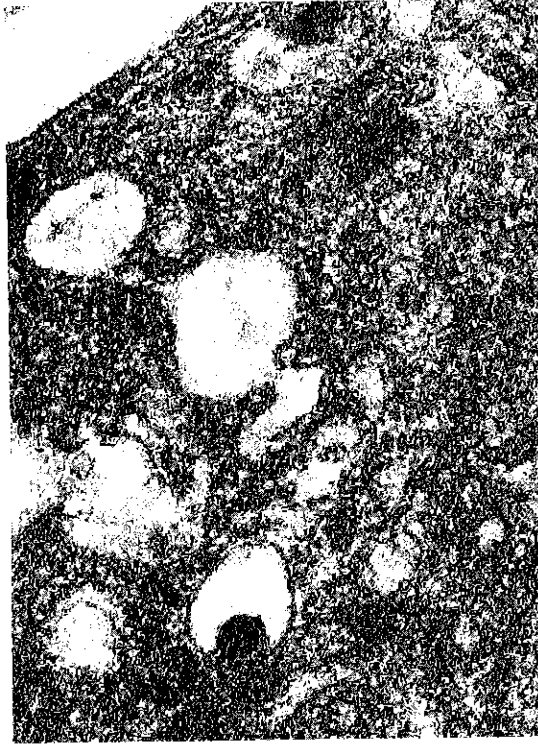

FIG. 5. Budding particle in a vacuole of the epithelial TNC (× 50,000).

TABLE 3. VIRUS REPLICATION IN LYMPHOID CELLS RECOVERED FROM DISSOCIATED THYMUSES ON DAY 2 AFTER INOCULATION OF RADLV AND MAINTAINED 24 h IN CULTURE AFTER THE PROCEDURE OF TNC ISOLATION

Cells*	Frequency of infectious centres Exp. 1	Exp. 2
PBS suspension	$1/3 \times 10^4$	N.D.
Starting suspension	$1/3 \times 10^4$	$1/10^4$
1st 'top' fraction	$1/10^5$	N.D.
2nd 'top' fraction	$1/3 \times 10^4$	N.D.
3rd 'top' fraction	$1/3 \times 10^4$	N.D.
4th 'top' fraction	$1/3 \times 10^3$	$1/3 \times 10^3$
TNCs lymphocytes	$1/10^2$	$1/10^2$

*The definition of each cell suspension is described in Materials and Methods.

infectious centre whereas only 1 out of 30,000 thymocytes did so. The lymphoid nature of the virus replicating cells detected in this experiment was demonstrated by the fact that removal of macrophages by nylon wool column filtration [13] did not change the frequency of infectious centres in the cell population recovered from TNCs (Table 4).

DISCUSSION

The selective localization of RadLV induced lymphomas in the thymus has been considered as a consequence of specific interactions between RadLV, lymphoid target cells and thymus microenvironment [1, 14]. In this report, it could be seen that after inoculation of RadLV, the initiation of virus replication occurs in only a few immature lymphoid cells of the thymus cortex which are closely associated with a specialized epithelial component of the thymic microenvironment, i.e. the so-called thymic nurse cells.

TNCs are particular lymphoepithelial complexes in which lymphoid blast cells and lymphocytes are in close contact with epithelial cell membranes [24, 25]. Wekerle *et al.* [25] have postulated that a formation of TNCs represents an obligatory intracellular differentiation cycle for intrathymic differentiation. According to this hypothesis, lymphoid primitive cells would recognize the antigens expressed by TNCs and hence be triggered into proliferation and differentiation. Our studies support this view. We have also demonstrated that most of the TNCs are derived from the outer cortex; this localization implies that most of the TNC-associated lymphocytes belong to the highly proliferative fraction of cortical thymocytes. Furthermore, recent observations indicate that the early progeny of bone marrow derived T cell precursors are preferentially associated with

TABLE 4. EFFECT OF NYLON WOOL PURIFICATION ON THE FREQUENCY OF VIRUS PRODUCING CELLS IN THYMUS CELL SUSPENSIONS ON DAY 2 AFTER RadLV INOCULATION

Cells tested*	Total population of thymocytes	Nylon wool purified thymus cells
Starting suspension	$1/3 \times 10^4$	$1/3 \times 10^4$
TNCs lymphocytes	$1/10^2$	$1/10^2$

*The definition of each cell suspension is described in Materials and Methods.

TNCs; in effect, in the thymus of the bone-marrow-grafted 400-R-irradiated mice, the first groups of donor-derived lymphoid cells appear in the subcapsular zone; at least, some of these repopulating cells are preferentially located within TNCs (Boniver *et al.*, in preparation).

The lymphoid cells released from TNCs after a 24 h incubation *in vitro* display a Thy-1, Lyt-1, Lyt-2 distribution comparable to that of the total thymocyte population, which is mostly composed of cortical thymocytes. This result is consistent with the aforementioned observation that most of the TNCs are of cortical origin. However, it cannot be excluded from the fluorescence data that there is a relative enrichment for cells with a low Thy-1$^+$, high Lyt-1$^+$, Lyt-2$^-$, phenotype, corresponding to the medullary type of thymus lymphocytes. It is therefore possible that a few TNCs derive from the medulla. Alternatively, thymocytes with such cell surface phenotype may have arisen from cortical intra-TNCs thymocytes during the 24 h *in vitro* incubation.

TNCs play an important role in the initiation of virus replication after inoculation of the thymotropic, leukemogenic RadLV. Indeed, the first virus producing cells were predominantly detected among the lymphoid blast cells associated with TNCS. This localization was demonstrated by electronic microscopic studies and by use of a very sensitive infectious centre detection assay. It was established that the frequency of RadLV producing lymphocytes was 300 times higher in TNCs than in the whole thymocyte population and in most of the fractions obtained during the procedure of TNC isolation. Previous studies have shown that in the thymus, target cells for productive infection by RadLV are scarce (1 out of 30,000–50,000 thymocytes) and belong to a very immature subset of cortical blast cells, which could correspond to transitional forms between prothymocytes and the subcapsular blast cell population [5]. The early virus producing lymphoid blast cells that we have detected within TNCs unequivocally belong to the progeny of these immature target cells.

The present data provide good evidence that TNCs are the primary site of replication of exogenous RadLV. The requirement of the intra-TNC microenvironment for the interactions between RadLV and early thymocytes acting as target cells may therefore account for the selective thymotropism of this retroviral isolate.

The observation of type C particles budding from the epithelial TNC membranes confirms previous data on virus replication in thymus epithelial cells after the inoculation of RadLV [5, 6]. The susceptibility of *in vitro* cultured thymus epithelial cells to RadLV infection has also been demonstrated [4]. It is of interest that virus production occurs in those epithelial cells in which the first virus producing lymphoid cells are located. In AKR mice, the epithelial parts of TNCs express retroviral products in their cytoplasm and on their membrane [17]. Whether or not virus replication in thymus epithelial cells play a role in the leukemogenic process is still an unsolved question.

In conclusion, the data presented here suggest an intimate relationship between the mechanisms involved in the early stages of intrathymic lymphopoiesis and the factors determining T cells susceptibility to infection by RadLV and/or their ability of virus production. Such mechanisms might account for the selective thymotropism of RadLV, in terms of virus replication and perhaps of lymphomatous transformation. These hypotheses are now under investigation in our laboratory.

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