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ymic Nurse Cells: A Site r Interactions Between diation Leukemia Virus d Thymus Immature mphoid Cells

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INTRODUCTION

In the low leukemia C57BL/Ka strain of mice, thymic lymphomas are induced experimentally by exposure to appropriate doses of whole-body irradiations or by inoculation of Murine Leukemia Viruses (MuLV) (19). One of these retroviruses is the Radiation Leukemia Virus (RadLV) originally extracted from radiation induced thymic lymphomas in the same mouse strain (24). Four components at least are essential to induce thymic lymphomas by RadLV (20): (a) the virus (RadLV); (b) susceptible target cells for neoplastic transformation; (c) the thymic microenvironment; and (d) a genetically susceptible and histocompatible host.

The biologic, serologic and structural characteristics of RadLV have been widely documented (8,9,27). However, the mechanism of RadLV oncogenicity is still unknown. The presence of transforming sequences in the genome of RadLV, in particular, although suggested by a recent report (27) has not yet been definitely demonstrated. Target cells susceptible to productive infection and to neoplastic transformation by RadLV have been identified in bone marrow, spleen, and thymus (3,21). However, overt virus replication and lymphomatous development occur selectively in the thymus (10,11,21). Interestingly, both phenomena are first detected among the blast cell population of the thymus outer cortex (or subcapsular zone). This subset of large lymphoid cells represents the first stage of the intrathymic pathway of T-cell differentiation, apparently under the control of the so-called thymic microenvironment (6).

Many investigations have unsuccessfully attempted to reproduce in vitro the neoplastic transformation of lymphoid cells by RadLV; therefore, most of our knowledge of the interactions between RadLV, susceptible target cells, and thymus microenvironment is based on the study of early virus replication after the infection of lymphoid cells by RadLV. Target cells susceptible to productive infection by RadLV display several phenotypic characteristics compatible with those of transitional forms between prothymocytes and the blast cell population of the outer cortex (4).

One could expect that target cells belong to the lymphoid cell population, which is normally associated with the epithelial cells within the so-called thymic nurse cells (TNCs). These particular lymphoepithelial complexes have been recently described by Wekerle and Ketelsen (34) in thymus cell suspensions obtained by enzyme dissociation and sedimentation at 1*g*. As proposed by these authors (34,35), TNCs could act as specific sites for the immigration of T-cell precursors; the intimate

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contacts between the immature T lymphocytes and epithelial cell membranes would trigger the first steps of intrathymic T-cell differentiation and proliferation. The experiments summarized in this chapter were undertaken to further characterize the phenotype and the origin of the lymphoid cell population associated with TNCs, and to determine their possible role in RadLV induced lymphomagenesis.

MATERIAL AND METHODS

Animals

The experiments employed C57BL/Ka mice of both sexes. Congenic (Thy.1-1) C57BL/Ka mice (called BL/1.1 for convenience) that were developed by M Lieberman at Stanford University were used in some experiments.

Virus

RadLV/VL3, a thymotropic (T+), leukemogenic (L+) virus biologically and serologically identical to RadLV was obtained from the culture fluids of the BL/VL3 cell line derived from a RadLV induced C57BL/Ka thymic lymphoma (9,26). Intrathymic injections were made in both lobes of the thymus of mice with 0.05 ml of virus preparation with a titer of 10^7 infectious particles per milliliter as estimated by an in vitro immunofluorescence assay (25).

Thymus Dissociation and Isolation of the TNCs

In each experiment, thymuses of 40 to 60 mice were minced into small pieces with scissors. The fragments were dissociated by several incubations in the presence of dispase or collagenase (18). The first two cell suspensions obtained with this procedure contained only small lymphocytes and were called PBS suspensions and dispase suspensions. They represented, respectively, 45% to 55% and 23% to 30% of the whole thymus cell population (Table 23.1). The remaining 14% to 19% of thymus cells, designated as starting cell suspension were used for the isolation of TNCs. The cell suspension was submitted to several successive runs of sedimentation at 1g (34). A supernatant and a pellet were recovered after each sedimentation; cells contained in the pellet were used for the following run of sedimentation; this procedure was repeated until a satisfactory enrichment for TNCs was reached. In the last fraction obtained, TNCs represented 70%–80% of the cells. It was calculated that each young adult thymus contained 15,000 to 20,000 thymic nurse cells.

TABLE 23.1. Cell suspensions obtained during the isolation of thymic nurse cells

Cell suspension	Percent of whole thymocyte population
PBS suspension	45–55
Dispase suspension	23–30
Starting cell suspension	14–19
1st supernatant	8–11
2nd supernatant	2–5
3th supernatant	0.95–1.23
4th supernatant	0.30–0.82
5th supernatant	0.12–0.18
Lymphoid cells recovered from TNCs	0.07–0.12

Recovery of Lymphoid Cells Associated with TNCs

The pellet containing TNCs was suspended in RPMI 1640 supplemented with 10% FCS and antibiotics and incubated for 20 to 24 h at 37°C in 5% CO_2 atmosphere. Under these conditions, the epithelial cells rapidly attached to the bottom of the petri dish, resulting in the release of the lymphoid cells associated with TNCs. Lymphoid cells were then recovered and washed. In the normal thymus, 9 ± 1.4 lymphoid cells per TNC could be recovered, about 20% of them were blast cells. The number of lymphoid cells associated with TNCs represented 0.07% to 0.12% of the whole thymus cell population.

Thymus Repopulation Experiments

Two-month-old C57BL/Ka mice were irradiated with 400 R and immediately grafted intravenously with 5×10^6 normal bone marrow cells from 1-month-old BL/1.1 mice. Groups of about 50 mice were sacrificed on day 9, 11, 12, 13, and 15. Thymuses were removed and used to prepare TNCs. The various fractions obtained along the isolation procedure were plated in culture for 20 h to allow the release of lymphoid cells from TNCs. The percentages of donor- and recipient- type cells in each cell suspension were then scored by immunofluorescence staining for Thy-1.1 and Thy-1.2 antigens.

Membrane Immunofluorescence Studies

For cell surface typing in the thymus repopulation experiments, thymus cell suspensions were incubated with monoclonal α-Thy-1.1 and α-Thy-1.2 antibodies (New England Nuclear, Dreieich, West Germany) and then with FITC conjugated goat antimouse IgM (Nordic Laboratory, Leuven, Belgium) at a dilution of 1:40 as a second step antibody (17). To determine the phenotype of lymphoid

cells associated with TNCs, monoclonal α-Thy-1.2, α-Lyt-1 and α-Lyt-2 antibodies (Becton Dickinson, Mountain View, Ca, USA) were used with FITC conjugated goat antirat IgG (Nordic Laboratory, Leuven, Belgium) as a second step antibody. The cells were analyzed with a fluorescence activated cell sorter (FACS IV, Becton Dickinson, Sunnyvale, Ca, USA) or with a Leitz UV microscope.

TdT (Terminal Deoxynucleotidyltransferase)

Tdt expression was detected by immunofluorescence using an immunoadsorbent-purified rabbit antibovine TdT serum and a fluorescein conjugated F(ab')^2 goat antirabbit IgG (Terminal transferase immunofluorescence assay kit, BRL Rockville, Maryland, USA).

ULTRASTRUCTURAL DEMONSTRATION OF MEMBRANE ALKALINE PHOSPHATASE

Aliquots of TNCs enriched pellets were fixed for 30 to 60 min in 0.1 M Na-cacodylate buffered 2.5% glutaraldehyde solution (pH 7.4) and subsequently placed into the incubation medium for 30 to 45 min, according to the procedure described by Reale and Luciano (30). As controls, the specific inhibitor L-p-bromotetramisole (5) or KCN (1mM) was added to the incubation mixtures. After postfixation in buffered 1% OsO_4 solution during 30 min at 4°C, routine procedures were used for processing the pellets for electron microscopy.

RESULTS

Phenotype of Thymic Nurse Cells and of TNC-associated Lymphoid Cells

Morphology. As seen with a transmission electron microscope (Fig. 23.1), TNCs appear as large lymphoepithelial complexes. In fact, lymphoid cells are located within invaginations of the epithelial cell cytoplasm. About 20% of them are blast cells. Mitotic figures are frequently observed.

With the scanning electron microscope (Fig. 23.2), TNCs look like spheres: generally, the external epithelial membrane is continuous and displays reliefs corresponding to the lymphoid cells located within the epithelial cell. In about 10% of TNCs, there are communications between these lymphoid cells and the extracellular spaces.

Cell Surface Antigens. The expression of the Thy.1, Lyt-1, Lyt-2 cell surface antigens was analyzed in lymphoid cells released from TNCs. As

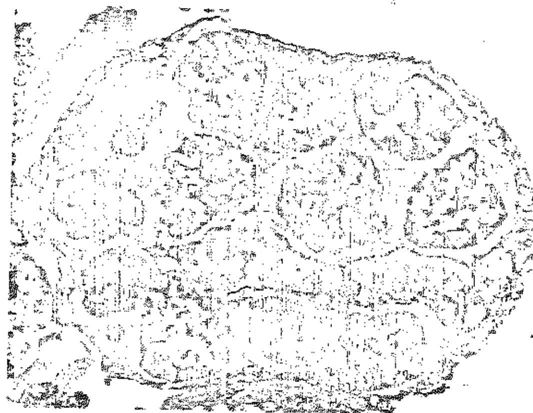

FIGURE 23.1. Transmission electron micrograph of a thymic nurse cell containing intact lymphoid cells. N = nucleus of the epithelial TNC. Magnification, 4500.

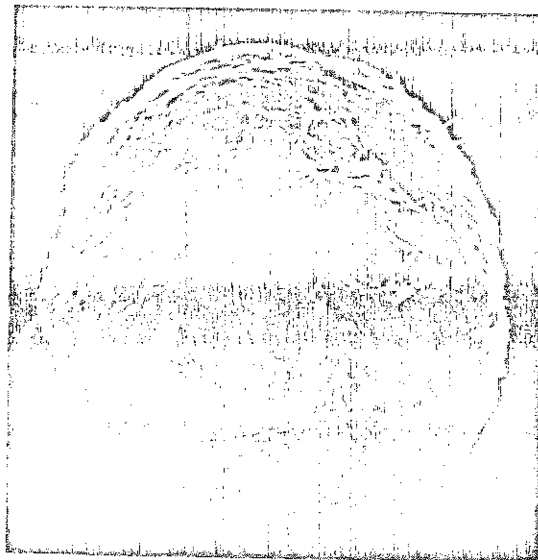

FIGURE 23.2. Scanning electron micrograph of a thymic nurse cell with a "continuous" epithelial membrane. Magnification, 6000.

reported elsewhere(18), it was shown that the cell surface phenotype of TNC-associated lymphoid cells was similar to that of the major cortical thymocyte repopulation.

TdT (Terminal Deoxynucleotidyltransferase). TdT is a DNA polymerase acting in the absence of any known template (2). The enzyme is distributed in immature cells of the lymphoid system (13,14).

TdT expression was determined by immunofluorescence in lymphoid cells recovered from TNCs and from the various cell suspensions obtained along the TNC isolation procedure. TdT was present in almost all of the cells tested. In the TNC associated lymphoid cells, TdT positive cells dis-

played a heterogeneity of fluorescence pattern. Some TdT+ cells showed a mixed nuclear and cytoplasmic pattern of fluorescence, as ordinarily seen in cortical thymocytes, other TdT+ cells displayed a nuclear pattern as observed in bone marrow TdT+ cells and in large lymphoid cells of the thymus outer cortex (13,14). These data indicate that most of TNCs derive from the thymus cortex; moreover, the nuclear pattern of some TdT+ cells suggests that some of them contain immature thymocytes and originate in the subcapsular zone.

Alkaline Phosphatase. APase is expressed on the membrane of lymphoid cells in the thymus of fetuses and in thymic lymphomas induced in adult mice by irradiation or by inoculation of RadLV (23). In the thymus of normal adult mice, the enzyme is detected only on very scarce cortical lymphoid cells that are in close contact with epithelial cells (12).

This latter observation led us to look for the expression of APase in lymphoid cells associated with TNCs. As shown in Figure 23.3, one could observe significant enzyme activity on the membrane of some lymphoid cells located within TNCs. The reaction was negative in controls (ie, no substrate in the incubation medium) or in samples treated in the presence of inhibitors (bromotetramisole or KCN). About 20% of TNCs contained at least one lymphoid cell with alkaline phosphatase activity. No APase activity was detected in free lymphoid cells.

Localization of Thymic Nurse Cells in the Thymus

As mentioned above, TNCs are spherical structures recovered from thymuses after enzyme digestion and selective cell separation. No such structures have been described so far in the thymus in situ, suggesting that the shape of TNCs relates to membrane rearrangement during the isolation procedure.

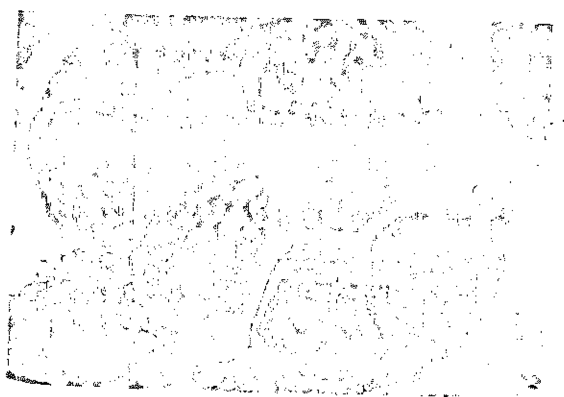

FIGURE 23.3. Localization of alkaline phosphatase in lymphoid cells associated with TNC. Ultrathin sections stained with uranyl acetate only. Magnification, 6000.

The following observations indicate that most TNCs originate in the outer cortex of the thymus. First of all, as mentioned above, the nuclear localization of TdT in some of the TNC-associated lymphoid cells suggests that these cells belong to the outer cortex blast cell population.

Selective labeling of the thymus subcapsular cells was performed by the method of Scollay et al (31). One could stain selectively with fluorescein isothiocyanate the 5% to 10% of cells located in the most external cell layers of the thymus. Sixty percent to 70% of the TNCs isolated from such thymus cell suspensions were labeled by fluorescein, demonstrating that most TNCs originate in the subcapsular zone. Details of the experiments are reported elsewhere (18).

Thymic Nurse Cells: A Site for the Proliferation of Direct Progeny of T-cell Precursors

To investigate the origin of lymphoid cells associated with TNCs, a study of thymus repopulation after irradiation and bone marrow grafting was undertaken. Adult C57BL/Ka mice were exposed to a 400-R dose of X-irradiation and then injected intravenously with 5×10^6 normal BL/1.1 bone marrow (see *Materials and Methods*). The pattern of thymus repopulation in such chimeras has been described in many reports (1,7,33).

After a first wave of regeneration due to the multiplication of surviving thymocytes, the lymphoid cells derived from grafted marrow (donor type cells) start to proliferate at detectable levels from day 8 onward. The evolution was followed by immunofluorescence detection of the Thy-1.1 donor type and Thy-1.2 recipient type cell surface antigens (Table 23.2). The frequency of donor type cells was about 10% on day 15 and reaches 80% on day 20 (data not shown).

TABLE 23.2. Thymus repopulation of 400-R treated C57BL/KA mice grafted with normal BL.1-1 bone marrow cells

Day post irradiation	Percentage of Thy.1-1 donor type cells in thymus
8	0.005
9	0.005
11	0.250
12	1.500
13	2.800
15	11.000

FIGURE 23.4. TNC isolated 4 days after intrathymic inoculation of RadLV. A virus particle (arrow) is budding from the membrane of a blast cell. N = nucleus of the epithelial TNC. Magnification, 15,000.

To test the hypothesis that the progeny of T-cell precursors is found among the TNC-associated lymphoid cells (35), the percentage of donor type cells was scored in the lymphoid cell population released from TNCs and in the various cell suspensions obtained along the isolation procedure (Table 23.3). On day 9 after irradiation, the percentages of donor type cells were much higher in the last supernatant (40×) and in the TNC fraction (85×) than in the whole thymocyte population. It must be noted that under these experimental conditions, these two fractions represented less than 0.2% of the total thymic cells. Later on, the percentages of donor type cells in the TNCs became similar to those scored in other fractions and in the whole thymocyte population.

These data indicate that after irradiation and bone marrow grafting, the first lymphoid cells derived from grafted marrow appear preferentially in a subset of the blast cell population of regenerating thymus, at least partially, associated with TNCs. This preferential localization is transient. The results support Wekerle's hypothesis that TNCs could act as a site of cellular interaction, resulting in the proliferation and the differentiation of T-cell precursors when they enter the thymus.

TABLE 23.3 Thymus repopulation in 400-R irradiated C57BL/KA mice grafted with normal BL.1-1 bone marrow cells: enrichment for Thy.1-1 donor type cells in various fractions obtained by TNC isolation[a]

Cell suspension[b]	Day post irradiation				
	9	11	12	13	15
PBS	1	1	1	0.70	1
Dispase	1	1	1		1
Starting	12	2.10	1.55	1.42	1.25
Supernatant 1	1	1.45	1.16	3.20	1.22
Last supernatant	40	4.20	1.45	1.10	1.26
Lymphoid cells released from TNC	85	6.70	1.80	1.10	0.78

Thymic Nurse Cells: A site for Early Virus Replication After RadLV Inoculation

The possible role of TNCs in the interactions between RadLV and susceptible target cells was investigated in experiments in which we looked for the presence of virus-producing cells in TNCs early after the inoculation of RadLV in mice. In a first series of experiments, TNCs were isolated on day 2 and 4 after an intrathymic inoculation of virus. They were fixed and prepared for examination with an electron microscope. These TNCs looked smaller than normally. Indeed, they contained less numerous lymphoid cells (4.1 ± 1.4/TNC) than normal TNCs (9 ± 1.4/TNC). This lymphoid cell population represented about 0.07% of the entire thymocyte population. Thirty-five percent of the TNC-associated lymphoid cells were blast cells. Type C virus particles were observed on the membrane of a few lymphoid blast cells located in the TNCs. The various cell fractions obtained along the isolation procedure were also examined. It was seen that scarce blast cells in the last supernatant (which contained about 90% blast cells) exhibited virus particles. No evidence of virus production was detected in the other fractions.

Next, we attempted to determine quantitatively whether the virus-producing cells were preferentially located in TNCs. For this purpose, an infectious center detection assay was used, which quantifies in vitro the RadLV-producing cells in cell suspensions previously treated in vivo or in vitro with RadLV (3). Thymuses were removed on day 2 after the intrathymic inoculation of RadLV. TNCs and the various cell suspensions obtained through the procedure were incubated for 24 h at 37°C; the free lymphoid cells recovered from each fraction were then cocultured with the BL/RL12-NP indicator cells in order to determine the frequency of infectious centers. At this time interval after the injection of the virus, only 1 in 30,000 cells could act as infectious centers (3); as shown in Table 23.4, the frequency of infectious centers was 300 times higher in the lymphoid cells released from TNCs than in the entire thymus cell population: about 1 in 100 cells produced viruses. On the other hand, the frequency of infectious centers was also rather high in the last supernatant, which contained about 90% of blast cells. The results clearly demonstrate

TABLE 23.4. Virus replication in thymus lymphoid cells on day 2 after in vivo inoculation of RadLV[a]

Suspension	Relative frequency of infectious centers[b]
PBS	1
Starting	1
Supernatant 1	0.3
Supernatant 2	1
Supernatant 3	1
Supernatant 4	3–10
TNC lymphoid cells	100–300

[a]Virus replication detected by the BL/RL12-NP co-culture assay 24 h after various cell suspensions were prepared.
[b]Calculated as ratio of absolute frequency of infectious centers in each cell suspension to that observed in whole thymocyte population. This latter value ranged between 1/10,000 and 1/30,000.

the preferential localization within TNCs of the first virus-producing cells after the inoculation of RadLV.

DISCUSSION

The data presented here extend the observation of Wekerle and his colleagues (34,35) on the so-called Thymic Nurse Cells. Our results strongly support the view that these lymphoepithelial complexes play an important role, perhaps specific, in intrathymic lymphopoiesis. Moreover, TNCs have been shown to act as preferential sites for the interactions between leukemogenic RadLV and susceptible target cells.

Our observations strongly suggest that lymphoid cells associated with TNCs (or at least some of them) belong to a subset of immature thymocytes corresponding to the earliest stage of the intrathymic T-cell differentiation pathway. That the majority of TNCs originate in the subcapsular zone and contain lymphocytes with a cell surface phenotype similar to that of cortical thymocytes (18) has been demonstrated. The pattern of fluorescence in a few TdT positive intra-TNC lymphocytes is that described in bone marrow T-cell precursors and in large lymphoid cells of the thymus outer cortex (13,14). The experiments on thymus repopulation after irradiation and bone marrow grafting indicate that lymphoid cells located in TNCs derive directly from marrow T-cell precursors. Indeed, when lymphocytes derived from the grafted marrow start to proliferate in recipient thymuses, there is a remarkable, although transient, enrichment for donor-type cells in the TNC associated lymphoid cell population, compared with the total thymus. These results support the hypothesis (34,35) that TNCs are a site for the proliferation of migrating T-cell precursors.

The selective thymotropism of RadLV is thought to be related to specific interactions between the virus and the T-cell differentiation pathway (20). Our results provide new evidence that such interactions occur at the earliest stage of intrathymic lymphopoiesis, probably within TNCs. Target cells susceptible to productive infection by RadLV display phenotypic characteristics compatible with that of transitional forms between prothymocytes and the blast cell population of the thymus outer cortex (4); they might be associated with TNCs. The early progeny of these target cells, ie, the first virus producing cells appearing after the inoculation of RadLV, are found preferentially in the thymus subcapsular zone among the immature lymphoid cell population located within TNCs.

Interestingly, target cells for infection by RadLV share several phenotypic characteristics with the thymus lymphoid cells, which are sensitive to the activity of Natural Killer (NK) cells (16). Both might belong to the same subset of immature thymocytes, possibly associated with TNCs. Accordingly, during RadLV induced leukemogenesis, one should expect to find important variations in the sensitivity of thymus lymphocytes to NK cells, and perhaps in the NK activity itself [as shown recently in the case of radiation induced leukemogenesis (29)]. Furthermore, the hypothesis that the precursor NK cells and the T-cell precursors (32) are the same cells has many implications for the study of regulatory mechanisms involved in leukemogenesis.

Alkaline phosphatase (APase) might be a phenotypic marker for differentiating T-cell precursors. In the present work, some intra-TNC lymphoid cells have been shown to be APase positive. They probably correspond to the scarce APase positive thymocytes that have been identified in the young adult thymus cortex (12). Interestingly, rather high numbers of APase positive cells have been described in the 17-day-old fetuses (23). The sixteenth to seventeenth day of fetal life seems to be critical for the ontogeny of cortical thymocyte populations: one can detect the appearance of TNCs (Wekerle and Luckenbach: unpublished data, cited in reference 22) and of TdT positive cells (15) simultaneously with a sharp increase in the number of Thy-1, Lyt-1, 2, 3 positive lymphocytes (28). One might postulate that APase is a differentiation marker for TNC-associated lymphoid cells that are precursors of the major cortical thymocyte population. The fact that the first virus-producing cells after inoculation of RadLV are preferentially located in TNCs (which

contain APase positive cells) and that a high level of APase activity has been detected in RadLV induced thymic lymphomas (23) present a novel hypothesis: the intra-TNC APase positive immature thymocytes might be selective targets for both productive infection and neoplastic transformation by RadLV. This hypothesis is now under investigation in our laboratory.

SUMMARY

In the C57BL/Ka strain of mice, the inoculation of radiation leukemia virus (RadLV) results in the development of lymphomas that are selectively located in the thymus. Target cells susceptible to productive infection by RadLV belong to a restricted subpopulation of immature lymphoid cells corresponding probably to transitional forms between T-cell precursors and subpopular blast cell population (Boniver et al: to be published). Therefore, the possible relationship between RadLV and thymic nurse cells was investigated: TNCs are those peculiar lymphoepithelial complexes recently described by Wekerle and Ketelsen (34), which are supposed to sustain the first step of intrathymic proliferation and differentiation of immature T-cell precursors. By using a selective method for staining the thymus subcapsular zone with fluorescein isothiocyanate (31), it was demonstrated that more than 50% of TNCs belong to the 10 outer cell layers of the thymus cortex. TNC lymphoid cells were recovered from TNCs after a short in vitro incubation, treated with α Thy 1, α Ly 1, α Ly 2 monoclonal antibodies and a second step FITC conjugated serum and then analyzed with a FACS IV: their cell surface phenotype was similar to that of cortical thymocytes. Next, TNCs were analyzed during the first few days after intrathymic inoculation of RadLV. On day 2, very scarce virus-producing cells could be detected in the thymus. Using an in vitro infectious center detection assay, it was shown that only 1 in 30,000 thymocytes could produce RadLV. Most of them were located in TNCs: indeed these complexes contained 300 times more virus-producing lymphoid cells than the entire thymus cell population.

Electron microscopic examination of these TNCs demonstrated that among lymphoid cells, C-type particles were budding only from the surface of blast cells. It is concluded that target cells start sustaining viral replication when they are engulfed in TNCs located in the subcapsular zone. These data agree with the previous observation that RadLV act specifically on a subpopulation of immature lymphoid cells located in the thymus subcapsular zone. It is suggested that susceptibility to productive infection by RadLV could be related to the phenotypic reorganization in T-cell precursors under the influence of the TNC microenvironment.

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