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THYMIC NURSE CELLS ACCOUNT FOR THE THYMUS DEPENDENCY OF PRELEUKEMIC CELLS IN MICE AFTER INOCULATION OF RADIATION LEUKEMIA VIRUS

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Abstract—Inoculation of Radiation Leukemia Virus (RadLV) into C57BL/Ka mice induces thymic lymphomas after a 3–6 month latent period. The leukemogenic process requires a sequence of events from the productive infection of susceptible target cells and induction of preleukemic cells to irreversible neoplastic transformation. Preleukemic cells were detected in the thymus during the first week following virus injection. The thymus dependency of these cells was shown to depend transiently upon peculiar lymphoepithelial complexes called "Thymic Nurse Cells" (TNCs). Indeed, the first preleukemic cells appearing in the RadLV-inoculated thymuses were observed selectively within TNCs. They remained closely associated with these complexes during the first 2 or 4 weeks. Later on, TNCs disappeared almost completely whereas non-TNCs associated preleukemic cells were found. Lymphoepithelial interactions within TNCs were thus required for the initial events of RadLV-induced lymphomagenesis. The subsequent TNCs depletion expressed a disturbance of thymic lymphopoiesis in relation with the neoplastic process.

Key words: Thymic nurse cells, radiation leukemia virus, preleukemic cells.

INTRODUCTION

INOCULATION of the thymotropic, leukemogenic Radiation Leukemia Virus (RadLV) induces thymic lymphomas in C57BL/Ka mice after a 3–6 month latent period [7, 17, 20]. Target cells for infection by RadLV, which belong to a subset of immature thymocytes [3], start sustaining viral replication in the subcapsular zone of the thymus where they are "engulfed" within the so-called "Thymic Nurse Cells" (TNCs) [13]. These lymphoepithelial complexes are normally involved in the early steps of intrathymic lymphopoiesis [14, 19, 25, 26]. Later on, virus production spreads to the entire cortex [7]; meanwhile, potential leukemia inducing cells, designated as "preleukemic" cells by Haran Ghera [12], appear (Goffinet *et al.*, in press). These preleukemic cells are dependent on host factors and thymic microenvironment for their further evolution into thymic lymphomas [10, 12, Goffinet *et al.*, in press].

Interactions between the progeny of lymphoid target cells and thymic stromal cells (e.g. epithelial cells, macrophages, interdigitating cells) might explain the influence of thymic microenvironment on the "pre-" leukemogenic process, either by cell-to-cell contact or by production of soluble factors. In this report, we present evidence that at least some of these critical interactions occur within the cortical lymphoepithelial complexes, called "Thymic Nurse Cells".

Abbreviations: FACS, fluorescence activated cell sorter; *FITC,* fluorescein isothiocyanate; *PBS,* phosphate buffered saline; *RadLV,* radiation leukemia virus; *TNC,* thymic nurse cell.

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MATERIALS AND METHODS

Animals

Four-week old C57BL/Ka (Thy-1.2) and 2-month old congenic C57BL/Ka (Thy-1.1) (called BL/1-1 for convenience) mice were used.

Virus

The thymotropic, leukemogenic RadLV/VL3 was prepared and inoculated intrathymically as previously described [8, 13].

Isolation of "Thymic Nurse Cells"

TNCs were obtained by enzyme dissociation of pooled thymuses and repeated 1 g sedimentation following already published procedures [13, 25]. The method yielded several cell fractions, designated SPBS, S start, S1, S2, S3, TNCs. Indeed, S start was the cell suspension obtained after completion of enzyme dissociation; "TNCs" was the last fraction obtained along the sequential 1 g sedimentation procedure. As controlled by phase contrast microscopy, only the S start and the TNCs fractions actually contained TNCs. The total cell number and the TNCs number per thymus were estimated.

Detection of preleukemic cells

The method described by Boniver *et al.* [4] was followed. Briefly, bone marrow or thymus cell suspension collected from RadLV inoculated C57BL/Ka (Thy-1.2) donor mice were inoculated intrathymically into 400 R whole body irradiated thymus bearing congenic BL/1-1 recipient mice. These animals were sacrificed when moribund. The difference in Thy-1.2 antigens allowed the identification of the donor or recipient origin of the tumors. Leukemias of donor origin indicate a transfer of preleukemic cells with the cell inoculate.

Immunofluorescence staining

Anti-Thy-1.2 or anti-Thy-1.1 monoclonal antibodies (New England Nuclear, Liege, Belgium) and FITC-conjugated goat anti-mouse IgM antibodies (Nordic Laboratories, Leuven, Belgium) were used as described for immunofluorescence staining and FACS IV analysis (Becton Dickinson, Sunnyvale, California, U.S.A.) [21].

RESULTS

The first series of experiments were performed in order to determine the site of origin of "preleukemic" cells in C57BL/Ka mice after an intrathymic inoculation of RadLV. Table 1 reports a representative experiment, showing that "preleukemic" cells were detected in the thymus on day 5 whereas in the bone marrow they were not found until day 9. Similar results were obtained in accompanying independent experiments as well as

TABLE 1. PRELEUKEMIC CELLS IN THE THYMUS AND IN THE BONE MARROW AFTER RADLV INOCULATION*

Time after RadLV inoculation (days)	Leukemia development in recipients inoculated with Thymocytes			Bone marrow cells		
	Incidence	Leukemia origin†		Incidence	Leukemia origin†	
		Donor	Recipient		Donor	Recipient
2	4/10	—	3	1/10	—	1
5	7/10	2	5	0/10	—	—
9	9/10	5	3	7/10	3	2
16	8/10	3	5	3/10	2	1
23	4/10	1	3	5/10	3	2
30	3/10	3	—	3/10	3	—

*Four-week old C576BL/Ka mice were inoculated intrathymically with RadLV/VL3; groups of 5 mice (donor) were sacrificed at different time intervals; aliquots of 5×10^6 thymus or bone marrow cells were injected intrathymically into 400 R irradiated 2-month old thymus bearing BL/1 mice. Two recipients were used for each cell inoculation. The origin of the lymphomas developing in the BL/1 recipients was done by immunofluorescence analysis with anti-Thy-1.1 and anti-Thy-1.2 monoclonal antibodies;

†Only lymphomas of donor origin derive from the inoculated preleukemic cells.

in cases in which RadLV has been inoculated intravenously (data not shown). Thus, the first preleukemic cells appearing after virus injection were located within the thymus.

Next, investigations were undertaken to establish a possible correlation between intrathymic preleukemic cells and TNCs. The evolution of the number of lymphoepithelial complexes per thymus was determined in mice which had been injected intrathymically by RadLV. Simultaneously, the presence of "preleukemic" cells was analyzed in the various cell fractions obtained along the procedure of TNCs isolation.

As shown in Fig. 1, the number of TNCs recovered per thymus decreased drastically after virus inoculation; it reached the lowest values, i.e. about 1000 per thymus, on day 60 whereas in thymuses from age matched normal mice it was still about 13,000. Thymuses however were still "normal" or subnormal in terms of weight and total cellularity. No TNCs were recovered from thymuses showing evidence of lymphoma.

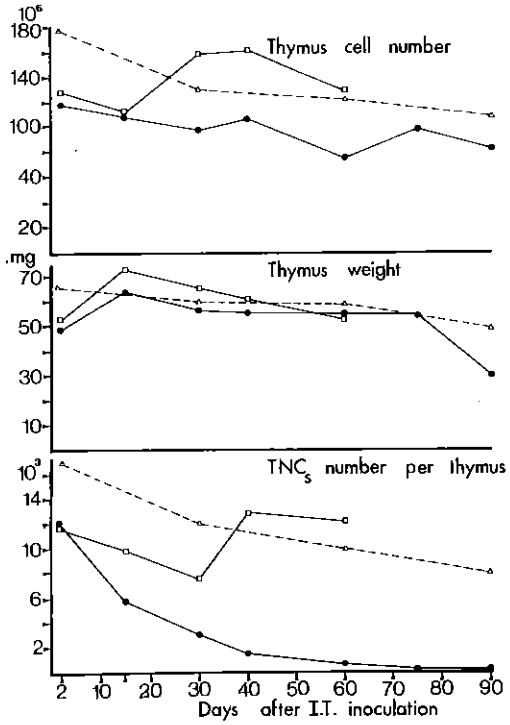

FIG. 1. Evolution of thymus cell number, thymus weight and TNCs number per thymus in C57BL/Ka mice after intrathymic inoculation of RadLV. (●——●) RadLV-inoculated mice; (□——□) PBS-inoculated mice; (Δ- - - -Δ) untreated mice.

Table 2 reports two independent experiments on the localization of preleukemic cells in the successive fractions obtained along the isolation of TNCs. During the first few weeks following inoculation of RadLV (i.e. 2 weeks in experiment 1, 4 weeks in experiment 2), preleukemic cells were found exclusively in cell fractions which contained TNCs, i.e. the "S start" and "TNCs" fractions.

Later on, preleukemic cells were detected also in cell suspension (i.e. SPBS, S1, S2, S3) without TNCs, as controlled carefully by phase microscopy.

DISCUSSION

These results indicate that "preleukemic" cells (or potential leukemic inducing cells) appear in the thymuc of C57BL/Ka mice as early as 2–5 days after inoculation of the

TABLE 2. PRELEUKEMIC CELLS IN THE CELL SUSPENSIONS RECOVERED ALONG THE ISOLATION OF TNCs*

Time after RadLV inoculation (days)	Origin† of lymphomas in recipient mice inoculated with:													
	SPBS		SStart		S1		S2		S3		S4		TNCs	
	Don.	Rec.	Don.	Rec.	Don.	Rec.	Don.	Rec.	Don.	Rec.	Don.	Rec.	Don.	Rec.
Exp. 1														
2	—	3	1	2	—	3	—	2	—	2	—‡	2	2	—
15	—	1	—	2	—	2	—	3	—	2	nd	nd	1	1
30	1	2	1	2	2	1	2	—	nd	nd	nd	nd	2	—
45	—	3	2	2	—	3	2	1	nd	nd	nd	nd	2	—
60	3	—	2	—	—	2	2	—	nd	nd	nd	nd	1	—
Exp. 2														
2	—	4	—	4	—	3	—	5	—	3	nd	nd	2	—
15	—	2	1	2	—	3	—	2	—	2	nd	nd	1	1
30	nd	nd	1	2	—	2	—	5	—	2	nd	nd	2	—
40	nd	nd	1	3	—	4	1	1	—	4	nd	nd	2	—
60	nd	nd	2	1	1	1	4	1	nd	nd	nd	nd	2	—
110	nd	nd	4	—	2	—	2	—	nd	nd	nd	nd	2	—

*Four-week old C57BL/Ka mice, which had been injected intrathymically with RadLV/VL3, were sacrificed at various time intervals; thymuses were prepared for the isolation of TNCs (see Materials and Methods) and thus yielded several cell suspensions; only the "S start" and the "TNCs" fractions contained TNCs; aliquots of 2×10^5–5×10^6 cells of each suspension were injected intrathymically into 400 R treated congenic BL/1.1 recipients. Two or three recipients were used for each cell inoculation. The origin of the ensuing lymphomas was defined as mentioned in Table 1.

†Only lymphomas of donor origin derive from the inoculated preleukemic cells.

‡nd: no cells in the fraction.

thymotropic, leukemogenic, RadLV. Moreover, at the beginning of the latent period preceding the development of thymic lymphomas, "preleukemic" cells are selectively associated with epithelial cells within the so-called TNCs. These data further extend the previously proposed concept that specific actions of RadLV on target thymocytes under the dependency of the thymic microenvironment account for the selective thymotropism of this retrovirus and of similar variants [3, 7, 10, 13, 16]. Indeed, previous studies clearly demonstrated that target cells for productive infection by RadLV belong to a subset of immature thymocytes and start to sustain virus replication when they are within TNCs [3, 11, 13].

Thus, the early stages of intrathymic leukemogenesis obviously require interaction between the progeny of target cells and epithelial cells, just like interactions between primitive lymphoid cells and epithelial cells are involved in the first steps of intrathymic lymphopoiesis [14, 19, 25, 26]. The intimate mechanisms of such interactions are still unknown.

Taken together, the observations suggest, although they do not demonstrate, that RadLV induced preleukemic cells belong to a subset of immature thymocytes at the earliest step of the intrathymic T-cell differentiation pathway. Their selective residence within TNCs for several weeks might indicate that, unlike the thymocyte population engaged in virus replication [7, 13], the progeny of the first preleukemic cells cannot migrate outside TNCs. Asymmetrical division (and thus acquisition of self renewal), blockade of differentiation or failure of migratory properties might account for this intriguing phenomenon.

The fact that, later on, preleukemic cells were found *not* associated with TNCs suggests that such cells can spread in the whole thymocyte population and escape interactions with

the epithelial cell component of TNCs. However, during the second month after RadLV inoculation, preleukemic cells are still "thymus-dependent". Indeed, they grow only in thymus bearing recipients, and not in thymectomized mice (Goffinet *et al.*, in press). Thus, it appears that the thymus dependent preleukemic period must be divided into two successive phases; during the first one, preleukemic cells are closely related to TNCs whereas during the second one they require interactions with still unknown other components.

Interestingly, the appearance of preleukemic cells was followed by a progressive but drastic decrease of TNCs number per thymus. A loss of TNCs appears as a constant and critical component in the pathogenesis of murine thymic lymphoma. Indeed, TNCs are no longer demonstrable in AKR spontaneous thymic tumours [18] or in C57BL/Ka radiation induced lymphomas (Houben-Defresne and Boniver, in preparation).

Thus, thymus outer cortex, where most of TNCs originate from [13, 14, 19] undergoes a drastic evolution during the preleukemic period after inoculation of RadLV [3] or a leukemogenic course of irradiation in C57BL/Ka mice [2, 15, 24] or in AKR mice [1, 22, 23].

Depletion of TNC associated lymphoid cells might account for the disappearance of TNCs. Inefficient interactions between epithelial cells and lymphoid cells might explain the observed lymphoid depletion. Indeed, as indicated by recent investigations, the intra-TNC lymphoid cell population contains the progeny of T-cell precursors, either derived from bone marrow prothymocytes or from the intrathymic pool of the radioresistant thymocyte population (Houben-Defresne *et al.*, submitted). It has been assumed that contact between these immature thymocytes and epithelial cell membranes could trigger the former ones to proliferate and to differentiate thereafter [25, 26]. A shortage of precursors or a modification of the epithelial cell membrane might thus lead to the observed disappearance of TNCs. As postulated recently in AKR leukemogenesis [27], changes of virus expression in the RadLV infected thymus epithelium [5, 6, 9, 13] may interfere with normal thymocyte differentiation and account for the foredescribed observations.

In conclusion, the present study clearly demonstrates the involvement of a certain type of thymus epithelial cells in the pathogenesis of thymic lymphomas. The mechanisms by which these cells drive the evolution of target cells to frank neoplasia may be critical for understanding the thymotropic leukemogenicity of RadLV.

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