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CELLULAR ASPECTS OF RADIATION LEUKEMOGENESIS IN C57 BL/Ka MICE: ALTERATIONS TO THYMIC MICROENVIRONMENT AND LYMPHOPOIESIS

M. P. DEFRESNE, A. M. RONGY, R. GREIMERS and J. BONIVER

Laboratory of Pathological Anatomy, University of Liège, Belgium

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Abstract—After a leukemogenic split dose course of irradiation, thymic nurse cells (TNCs) disappear. We have correlated this with the loss of an epithelial cell surface antigen (recognized by monoclonal antibody ER-TR3 and tentatively identified as Ia). In addition, epithelial cells have lost their capacity to interact with fetal thymocytes *in vitro*. Marrow grafting early after irradiation, that prevents the development of lymphomas, restores thymic nurse cells and thymocyte population. Such reconstitution and lymphoma prevention were not observed when marrow grafting was performed later (1 month after irradiation) during the preleukemic period.

Key words: Radiation-induced thymic lymphoma, thymic nurse cells, lymphoepithelial interactions *in vitro*, thymic lymphopoiesis.

INTRODUCTION

WHOLE body X irradiation of young C57BL/Ka mice with fractionated doses (4×1.75 Gy at weekly intervals) results in thymic lymphoblastic lymphomas in the majority of animals [1]. The cellular processes leading to the development of the thymic tumors involve interactions of the oncogenic agent (radiation) and the T-cell differentiation pathway. The leukemogenic agent acts on one (or several) set(s) of cells in the lymphohaemopoietic system. These *target cells* then transform into potential neoplastic (leukemic) cells, or *preleukemic cells*, which progress to frank neoplasia during a latent period of 3–6 months (or more). Then, autonomous *lymphoma cells* proliferate and give rise to thymic tumors [2].

The presence of *preleukemic cells* was demonstrated by a transplantation assay *in vivo*; these cells have the ability to give rise to lymphomas after transfer into histocompatible mice [3]. The experimental procedure involved transplantation of cells, removed from 'preleukemic' mice, into intact whole body sublethally irradiated (400 rads) hybrid recipients (one parental strain matching the donor strain). Genotype analysis (based on H-2 differences) indicated tumors originating from the grafted cells. More recently, congenic mice bearing the Thy-1.1, or Thy-1.2 antigen were used and the origin of lymphomas assessed by immuno-labelling [4]. By definition, *preleukemic* cells are collected from animals, which do not show any clinical or anatomical manifestation of the lymphoma.

Using the transplantation assay, preleukemic cells were demonstrated in bone marrow and/or thymus in several models of spontaneous—or induced lymphomas—as in SJL/J mice after treatment with dimethylbenzanthracene, in AKR mice and in C57BL mice exposed to various leukemogenic agents, such as dimethylbenzanthracene, whole body split dose irradiation, or several variants of RadLV [3–15]. The phenotype of preleukemic cells in thymus has not been defined. In bone marrow, as demonstrated in several models [10, 13, 16, 17], they share the characteristics of prothymocytes.

For progression to lymphoma growth, preleukemic cells require several conditions [2, 9, 10]. First of all, they give rise to tumors, only within the thymus; they cannot grow within thymectomized animals. Secondly, some damage to the host seems to be important: preleukemic cells grow only if the hosts have been irradiated before transplantation.

Our previous studies were devoted to define the components of thymic microenvironment that control the progression of PLCs. Indeed, thymic nurse cells (or TNCs), which are lymphoepithelial complexes presumably involved in T-lymphocyte differentiation [18–21], disappear almost completely after a split dose of irradiation [22]. In contrast, they reappear in the thymus of marrow reconstituted irradiated mice, which do not

Abbreviations: BM, bone marrow; *FCS*, fetal calf serum; *FITC*, fluorescein isothiocyanate; *mAb*, monoclonal antibody; *PBS*, phosphate buffered saline; *PLCs*, preleukemic cells; *RadLV*, radiation leukemia virus; *TNCs*, thymic nurse cells.

Correspondence to: Dr M. P. Defresne, Laboratory of Pathological Anatomy B23, University of Liège, Sart Tilman, B-4000 Liège, Belgium.

develop lymphomas [22]. The depletion of nurse cells indicates that the complexes between lymphocytes and epithelial cells do not form any longer, as a result of a damage either to their lymphoid, or epithelial component. In fact, several studies on TNCs indicate that these complexes contain early thymocytes that derive from marrow thymic precursors [4, 18–21], themselves altered by a split dose irradiation [11, 23]. Moreover, several observations suggest that the leukemogenic course of irradiation also induces alterations of the thymic stroma itself. Activation of endogenous ecotropic retroviruses in thymic epithelial cells has been reported [24]. Furthermore, one month after the last dose of X rays, grafting normal bone marrow cells does not result in repopulation of the preleukemic thymus [11, 22], suggesting that thymic stromal cells are unable to support T lymphopoiesis.

In this communication, we report two still preliminary series of experiments designed to investigate further the alterations of thymic stroma. To this end, we used an *in-vitro* assay to test the capacity of epithelial cells to interact with immature thymocytes and to form lymphoepithelial complexes. The expression of an antigen thought to play a role in these interactions was also studied in preleukemic thymuses. We show that both the function and the phenotype of epithelial TNCs is altered after fractionated irradiation. Furthermore, we report some data which suggest that the progression of preleukemic cells in the altered thymic microenvironment may require disturbances to thymic lymphopoiesis.

MATERIALS AND METHODS

Mice

C57BL/Ka mice (Thy-1.2) and their congenics BL/1.1 (Thy-1.1) were raised in our animal colony. They were a gift from Drs H. S. Kaplan and M. Lieberman (Stanford University).

Irradiation

For lymphoma induction, mice were irradiated with four whole-body doses of 1.75 Gy applied at weekly intervals. The irradiations were performed under the following conditions: Stabilivolt Siemens, 190 kV, 18 mA, filter: 0.5 mm Cu, focal distance: 35 cm, dose rate: 1.6 Gy/min.

Bone marrow grafting

Bone marrow cells from normal one-month old mice were suspended in phosphate buffered saline (PBS) supplemented with 5% fetal calf serum. Aliquots (0.25 ml) containing 10^7 cells were injected intravenously within 3 hr after the last irradiation.

Numeration of marrow derived thymocytes

Thymocytes from marrow reconstituted animals were treated with monoclonal anti Thy-1.1 or anti Thy-1.2 antibodies and then with a fluórescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig antiserum. They were analysed by flow cytometry with a FACS IV (Becton Dickinson, Sunnyvale, CA) [25].

Isolation of TNCs

TNCs were obtained by enzyme dissociation of pooled thymuses and repeated 1-g sedimentation according to an already published procedure [18–26].

In-vitro *reconstitution of lymphoepithelial complexes*

To evaluate the capacity of epithelial TNCs to form complexes with immature thymocytes, we used a method developed by Nachayama and Wekerle (personal communication). Isolated thymic nurse cells were resuspended in RPMI 1640 culture medium (Gibco Bioculture Ltd, Ghent, Belgium), supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 1% non essential amino-acids, 1 mM sodium pyruvate, 150 μ/ml penicillin and 75 μg/ml streptomycin. After a 24-h incubation in Petri dishes (Lux Scientific Corporation, The Netherlands) at 37°C in a 5% CO_2 atmosphere, the epithelial cells stuck to the plastic surface; the lymphocytes were released by the TNCs and collected. The remaining epithelial cells were incubated for an additional period of four days. They were treated twice with trypsin (0.25% in PBS) for 20 min at 37°C and resuspended in culture medium supplemented with 10% FCS. The epithelial cells were then mixed with thymocytes obtained from thymuses recovered from 16-day old embryos in a ratio of one epithelial cell for 10 thymocytes. Twenty-microlitre aliquots of this cell suspension were incubated for 6 h in a Terasaki plate. The percentage of epithelial cells forming lymphoepithelial complexes in such experimental conditions was then estimated.

Immunocytochemistry

The specificity of the mouse monoclonal antibody (mAb) ER-TR3 used in this study has been previously described [27]. It is a gift from Dr Van Ewijk. To detect the binding of this mAb on frozen thymus sections, we used a rabbit anti-rat immunoglobulin serum conjugated with horseraddish peroxidase (Dako). The tissue preparation for immunocytochemistry was described previously [28].

RESULTS

A. *Thymic microenvironment*

In-vitro *formation of lymphoepithelial complexes.* These experiments were designed to evaluate the ability of epithelial TNCs from preleukemic mice to form complexes with immature thymocytes *in vitro*.

In a control experiment, TNCs were isolated from normal one-month old mice. After a 5-day culture, the epithelial cells were incubated with fetal thymocytes. We observed that about 20% of the recovered epithelial cells formed new complexes with the immature thymocytes. When examined with a phase contrast microscope or with an electron microscope, these complexes looked like freshly isolated TNCs (Fig. 1).

The same experiment was performed with TNCs isolated one month after the end of a leukemogenic course of irradiation. At that time, all thymuses contain preleukemic cells (Defresne *et al.*, submitted). Only 6% of epithelial TNCs isolated from these preleukemic thymuses were able to form complexes with fetal thymocytes, instead of 23% with age-matched control mice.

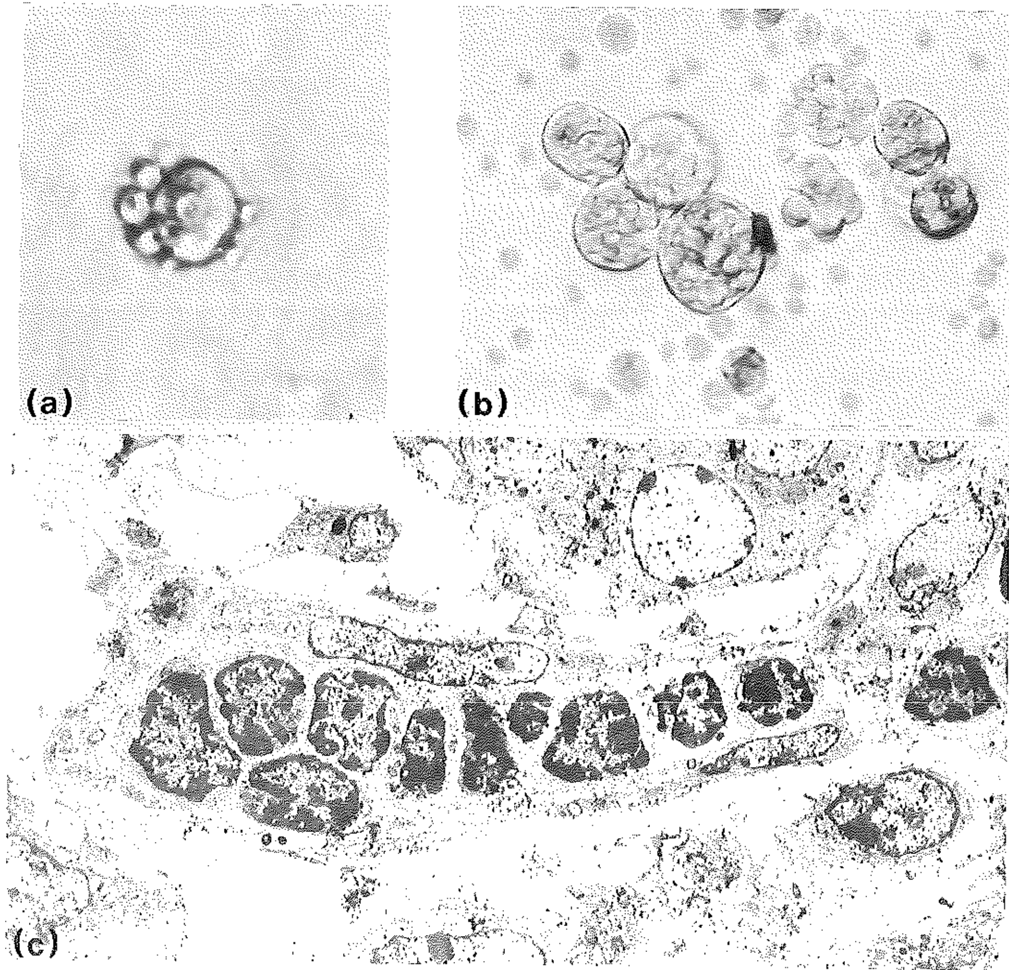
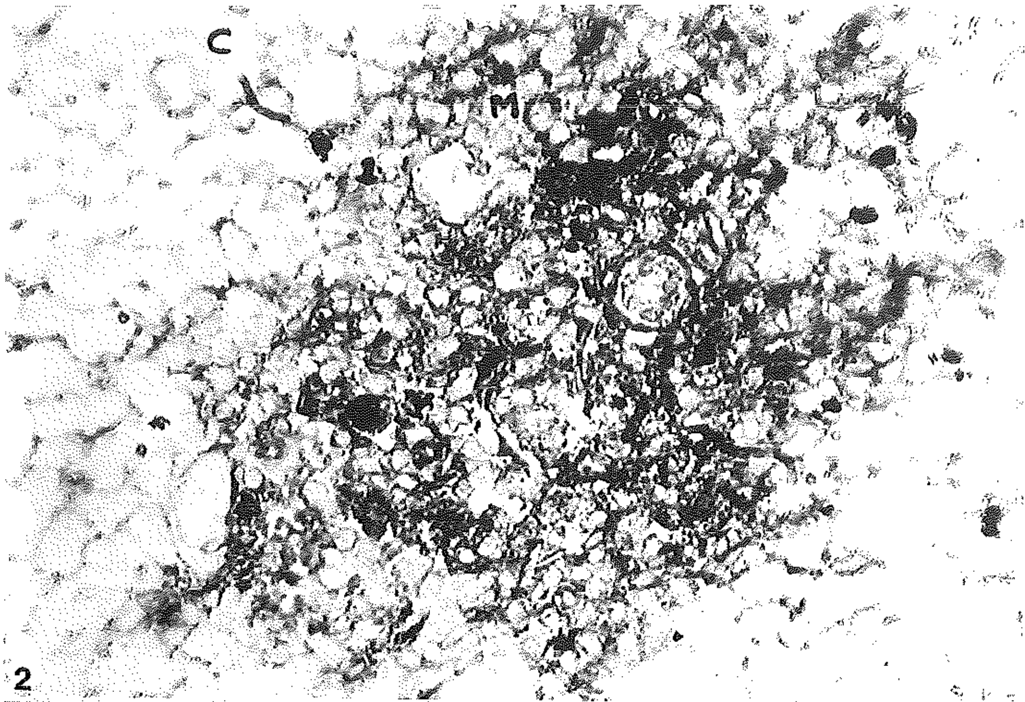


FIG. 1. *In-vitro* reconstituted thymic nurse cells (TNCs). Epithelial TNCs form new complexes when incubated with foetal thymocytes. (a) Association between an epithelial TNC and immature thymocytes after an incubation of 1 h (×360). (b–c). *In-vitro* reconstituted TNCs after an incubation of 6 h (b: ×360; c: ×3000).



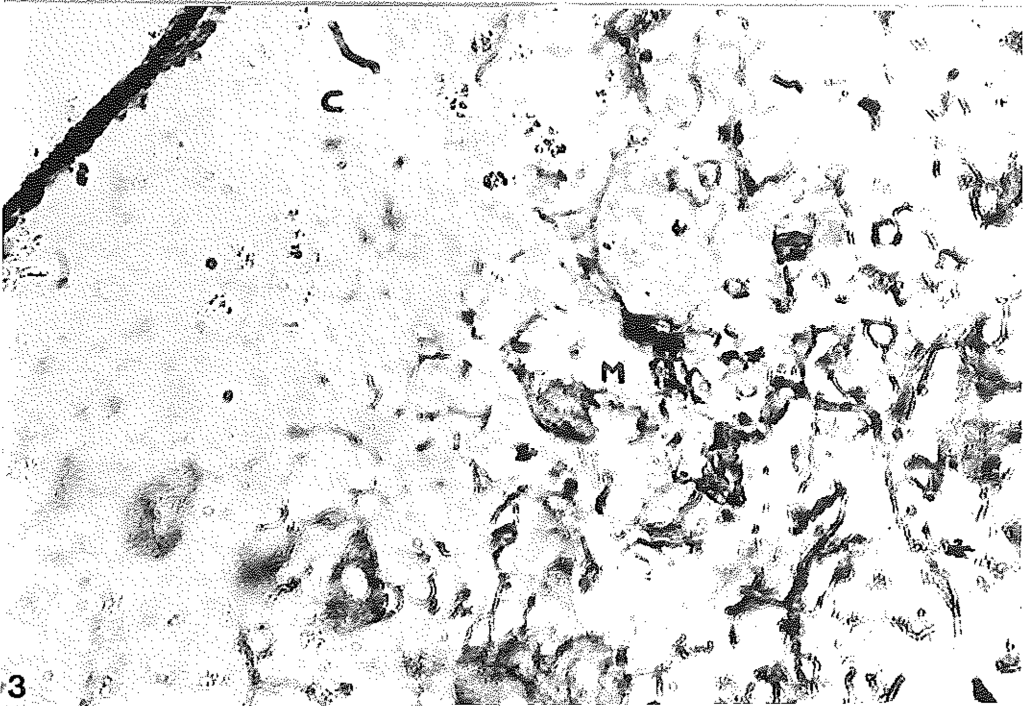


FIG. 2. Immunoperoxidase staining pattern of a frozen normal thymus section incubated with the monoclonal antibody ER-TR_3 (×875) C: cortex, M: medulla.

FIG. 3. Immunoperoxidase staining pattern of a frozen preleukemic thymus section incubated with the monoclonal antibody ER-TR_3. A large cortical area is negative (×875) C: cortex; M: medulla.

TABLE 1

First treatment	Second treatment (1 month after first treatment)	(Thymic lymphopoiesis 7 weeks after the first treatment) Marrow-derived thymocytes†	Nurse cells‡	Lymphoma incidence§
4×1.75 Gy	—		+	+++
4×1.75 Gy+BM*	—	+++	+++	+/−
4×1.75 Gy	BM	+	+	+++
4×1.75 Gy	4 Gy		+	+++
4×1.75 Gy	4 Gy+BM	+	+	++
—	4 Gy		+	+/−
—	4 Gy+BM	+++	+++	+/−

* BM = graft of 10^7 bone marrow cells from normal BL/1.1 mice.
† + ≤10%, +++ ≥ 80%.
‡ + ≤2000/Thymus; +++ ≥ 12000/thymus.
§ +/− <10%; ++ <90%; +++> 90%.

TNCs were also isolated from mice sacrificed one month after a split dose irradiation and marrow grafting. The percentage of cells forming complexes *in vitro* was then estimated at 15%.

Distribution of ER-TR3 positive cells in preleukemic thymuses. The ER-TR3 antibodies detect presumably Ia antigens [27], which are thought to play a role in the interactions between thymic epithelial cells and immature thymocytes [29, 30]. Their distribution was compared in preleukemic thymuses and in age-matched control animals.

The reactivity of ER-TR3 with frozen normal thymus sections has previously been described [27]. This mAb gives a fine reticular staining pattern in the thymic cortex and a confluent staining in the medulla (Fig. 2).

The staining pattern obtained with the same antibody in frozen preleukemic thymus sections differed substantially: indeed, large cortical areas were negative whereas the medulla was still positive in a confluent way (Fig. 3).

B. *Thymic lymphopoiesis*

(Thy-1.2) mice were irradiated with four whole body irradiations of 1.75 Gy at weekly intervals. One group of mice was grafted within 3 h after the last irradiation with (Thy-1.1) marrow cells. One month later, the other mice were either irradiated with 4 Gy, or grafted with normal (Thy-1.1) marrow cells, or irradiated and marrow reconstituted, or not further treated. Five mice from each experimental group were sacrificed 3 weeks later and used for numeration of marrow derived thymocytes (if there was a marrow reconstitution) and of thymic nurse cells.

The 3-week interval was chosen because a full thymic repopulation by donor derived (donor) cells or TNCs restoration is generally observed in radio-chimeras at the time [21]. Groups of 10 mice were maintained for determination of lymphoma incidence.

The results of several separate experiments are summarized in Table 1.

Grafting normal bone marrow cells after the end of a split dose irradiation almost totally inhibited the development of thymic lymphomas. Meanwhile, thymus was repopulated by lymphoid cells that were derived from the grafted marrow, and TNCs were restored. There was no lymphoma prevention if marrow grafting was performed one month after the split dose irradiation; there was no thymocyte repopulation by grafted marrow, nor restoration of thymic microenvironment. A weak effect on thymic repopulation and lymphoma prevention was observed when a simple irradiation of 4 Gy was done before the late marrow graft.

DISCUSSION

Our observations on the phenotype of thymic stromal cells and the function of epithelial TNCs *in vitro* suggest strongly that the membranes of cortical epithelial cells are altered in the leukemogenic process after a split dose irradiation. The epithelial TNCs loose their ability to form complexes with fetal thymocytes. This may be due to an injury to the epithelial membrane and thus to the structures involved in the interactions with differentiating immature thymocytes. Alternatively or simultaneously, there could be a decrease in the production of factors or hormones, such as thymulin, which play a role in T-cell lymphopoiesis [31].

The observations with the ER-TR3 monoclonal antibodies may indicate a modification of the epithelial membrane: these antibodies failed to label some cortical stromal cells in preleukemic thymuses. ER-TR3 is thought to detect Ia antigens [27], which presumably play a role in the interactions between stromal cells and immature thymocytes [29, 30], for example within nurse cells [18, 20].

Indeed, the membrane of TNCs normally expresses antigens recognized by ER-TR3 antibodies ([32], Defresne *et al.*, unpublished). It has been suggested that thymocyte precursors are engulfed within these complexes after the recognition of Ia antigens and find there microenvironmental conditions triggering their proliferation and their differentiation [18, 19]. The incu-

bation of epithelial cells with anti-Ia or ER-TR_3 monoclonal antibodies inhibits in fact the formation of TNCs *in vitro* (Wekerle, unpublished; Defresne *et al.*, unpublished). Thus, the present study suggests that the disappearance of TNCs in the preleukemic period can be due to the lack of expression of Ia-like antigens in preleukemic thymus epithelium.

Whether these alterations are due to the leukemogenic irradiation or to the presence of preleukemic cells has not yet been answered. A single non-leukemogenic dose of 4 Gy induces phenotypic alterations of the thymic stroma (Defresne *et al.*, unpublished) as well as a transient disappearance of TNCs [20, 21]. In fact, these alterations are reversible. The observations on marrow reconstituted mice are also interesting in this respect. As previously demonstrated [1], lymphoma development is prevented by a normal bone marrow graft; preleukemic cells were induced in thymus and bone marrow but eventually, disappeared (Defresne *et al.*, submitted) whereas the number of TNCs was restored [22]. When bone marrow graft was done several weeks after split dose irradiation, there was no lymphoma prevention nor restoration of thymic lymphopoiesis. Thus the presence of preleukemic cells, that are resistant to 4 Gy, is concomitant with the thymus unresponsiveness to repopulation by normal marrow derived precursors. Finally, the intrathymic injection of leukemic cells into normal mice induces strong modifications in the phenotype of cortical epithelial cells (Detiège and Defresne, unpublished). Taken together, the data suggest that the interactions of preleukemic cells and the host vary along the process of leukemogenesis. During a first period (a few weeks), there is a radiation injury to several cell populations in thymus and lymphoid tissues. This stage is required for preleukemic cells to "take". It can be made reversible by bone marrow grafting, which restores the altered populations. During a second period (from the second month until the onset of lymphomas) damages to those populations are irreversible, perhaps mediated by preleukemic cells, and are not reconstituted by a late bone marrow graft; whether these alterations play any role on the progression of preleukemic cells has still to be investigated.

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