DEFRESNE MP_14

Thymus 6, 324–333 (1984)
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Thymic Nurse Cells and thymic repopulation after whole body sublethal irradiation in mice

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Key words: Thymic Nurse Cells (TNCs), thymic lymphopoiesis

Summary. Thymic Nurse Cells (TNCs) are lymphoepithelial complexes which are thought to play a role in the early stages of the intrathymic differentiation pathway. Therefore, their repopulation kinetics were analyzed in mice after sublethal whole-body irradiation. Changes of the number of TNCs per thymus were parallel with the evolution of the whole thymocyte population. Particularly, a first wave of TNCs restoration was followed by a secondary depletion and a final recovery. This suggests that TNCs restoration is related to the proliferating progeny of intrathymic radioresistant thymocytes. When normal bone marrow cells were grafted intravenously after irradiation, no secondary depletion was found. This pattern of restoration was obviously related to thymic repopulation by cells which were derived from the inoculated bone marrow. Homing studies with FITC labelled bone marrow cells showed that inoculated bone marrow cells did not penetrate TNCs early after irradiation. Later on, when immigrant cells started to proliferate, they were found preferentially within TNCs before spreading in the whole thymus.

The results indicate that interactions between immature thymocytes and epithelial cells within TNCs are critical for the first steps of intrathymic lymphopoiesis.

Introduction

The first steps of intrathymic lymphopoiesis apparently require interactions between immature lymphoid cells and thymic stromal cells [1–4].

A peculiar lymphoepithelial complex, first described as 'Thymic Nurse Cell' (TNC) by Wekerle & Ketelsen [1, 5] appears as an example of such critical interactions. Indeed, several recent observations support the view that TNCs are involved in the early stages of the intrathymic T-cell differentiation pathway. First of all, the expression of H2-K, H2-D and Ia determinants on the TNC external membrane led to the assumption that the epithelial component of TNCs might be involved in the acquisition of 'self recognition' by differentiating T lymphocytes [1–3]. Secondly, TNCs were found preferentially in the subcapsular zone of the thymus [2, 6] where immature thymocytes divide actively [7, 8]. Thirdly, the lymphoid blast cells engulfed in TNCs contain membranous alkaline phosphatase [9, Goffinet *et al.*, submitted], an enzyme found on foetal thymocytes [10, Goffinet *et al.*, submitted] and in bone marrow precursors [Lenaerts *et al.*, in prep.] as well as nuclear terminal deoxynucleotidyltransferase [9] previously des-

cribed in bone marrow prothymocytes and in a few thymic subcapsular lymphoblasts [11, 12]. Finally, after inoculation of the thymotropic, leukemogenic Radiation Leukemia Virus [13, 14], the first virus-producing cells which belong to a very immature subset of cortical blast cells [15, 16] were predominantly found among the lymphoid cells associated with TNCs [6]. Close interactions between thymocytes precursors and thymic epithelium thus seem to occur within TNCs. These lymphoepithelial complexes might therefore undergo significant changes during thymus repopulation after sublethal whole-body irradiation, a model which has been frequently used for the study of thymic lymphopoiesis. Here we report on the kinetics of TNCs in sublethally whole-body irradiated mice and on their interactions with bone marrow T cell precursors in marrow radiochimeras.

Materials and methods

Mice. Two-month old C57 BL/Ka mice of both sexes were used. Four-week old congenic Thy-1.1. C57BL/Ka mice (called BL/1.1 for convenience) which were developed by M. Lieberman at Stanford University were used as donors of bone marrow cells for BL/1.1 (Thy-1.1) irradiated mice.

Irradiation. Mice were whole-body irradiated with 400 rads. The irradiation was delivered by an X-ray machine (Stabilivolt Siemens, 190 kV, 18 mA, HVL: 0,5 mm Cu) at a dose rate of 160 rads per min.

Restoration. In the 'homing' experiments, bone marrow cells were incubated for 20 min at 37°C in a solution of 30 μg/ml of fluorescein isothiocyanate in phosphate buffered saline (PBS) solution [17, 19]. They were then washed in foetal calf serum (FCS) and PBS before being used for restoration. Aliquots of 15.10^6 cells were injected intravenously within 2 h following X-ray exposure.

In the 'repopulation' experiments, bone marrow suspensions were prepared in PBS supplemented with 5% FCS. Aliquots of 5.10^6 cells were injected intravenously within 2 h following X-ray exposure.

Antibodies and Complement (C). In some experiments, bone marrow cells were depleted of T cells by treating the cell suspensions with a $1/10^4$ final concentration of monoclonal anti-Thy-1.2 antibody (New England Nuclear, Liège, Belgium) for 30 min at 4°C and C for 45 min at 37°C. The source of C was rabbit C (Cederlane Laboratories, Canada).

Cell surface typing and FACS analysis. Monoclonal anti-Thy-1.1 and anti-Thy-1.2 antibodies (New England Nuclear, Liège, Belgium) were used for cell surface typing by indirect immunofluorescence. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgM (Nordic, Leuven, Belgium) or FITC-

conjugated sheep F(ab′)2 anti-mouse Ig (NEN) was used as a second step antibody. Cell suspensions were analyzed with a fluorescence activated cell sorter (FACS IV) (Becton Dickinson, Sunnyvale, California) as described [20]. *In situ* staining of individual permeabilized TNCs was performed following previously published procedure [2].

Thymus dissociation and TNCs isolation. In each experiment, TNCs were isolated from 5 to 10 thymuses. Thymuses were minced with scissors and washed for 10 min in PBS. The fragments were dissociated by repeated incubations in the presence of Dispase, Collagenase and DNase. TNCs were isolated from the resulting suspensions by successive runs of 1 g sedimentation by using a slight modification [6] of the method originally described by Wekerle & Ketelsen [1]. After the isolation procedure cell numbers were scored in each fraction, the percentage of TNCs was defined in the last cell suspension, and the number of TNCs per thymus was then calculated.

Cultures. To recover lymphoid cells from TNCs, the isolated TNC complexes were cultured in RPMI 1640 medium (Gibco Bio Cult Ltd, Ghent, Belgium) supplemented with 10% heat inactivated FCS, 2mM/ml L-glutamine, 1% non-essential amino-acids, 1 mM/ml sodium pyruvate, 150 U/ml penicillin and 0.075 mg/ml streptomycin, and grown in 30 mm Petri dishes (Lux Scientific Corporation, Sanbio, The Netherlands). After a 20 h incubation period, lymphoid cells were released and collected.

Results

Evolution of TNCs in 400 rads irradiated mice

The kinetics of TNCs repopulation was studied after a sublethal irradiation of 400 rads. Groups of five to ten mice were sacrificed between the 2nd to the 40th post-irradiation day. Thymus weight, total thymus cell number and TNCs numbers per thymus were measured. As seen in Fig. 1, TNCs disappeared during the early rapid involution. The first phase of thymic regrowth started within 4–5 days after irradiation and preceded the reappearance of TNCs which occurred only on day 11. Thymic repopulation continued until the 15th day post-irradiation and was accompanied by an increase in the number of TNCs. The secondary thymic atrophy which started two weeks after irradiation was associated with a decrease in the TNCs number. The second and final phase of thymus regeneration started on the 20th day, again preceding the increase of TNC number.

The evolution of TNCs number per thymus was clearly parallel to that of the total thymus cellularity. TNC restoration was therefore closely related to thymocyte regeneration.

The data raised the question of the possible relationship between TNCs and the progeny of intrathymic radioresistant thymocyte precursors and marrow-derived prothymocytes which normally ensure thymus repopulation

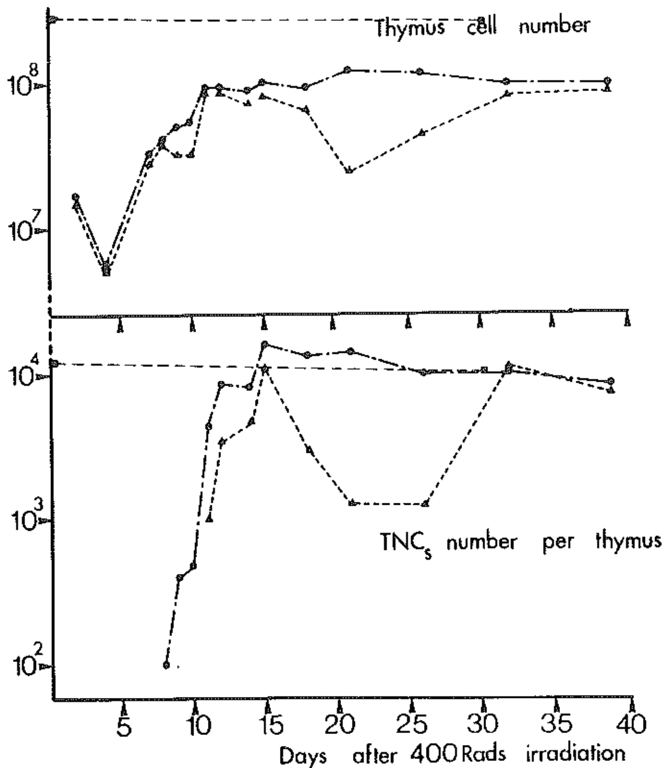

Figure 1. Evolution of TNCs and of thymus cell number in 400 rads irradiated mice (▲----▲), in 400 rads irradiated bone marrow reconstituted mice (●----●) and in non-treated mice (■----■).

after sublethal irradiation, respectively, during the first and the second phase of regeneration [21–27].

Evolution of TNCs in 400 rads irradiated, bone marrow reconstituted mice

The next experiments were designed to define more accurately the relationship between TNCs restoration and the proliferation of thymocytes derived from thymus-homing prothymocytes. For this purpose, thymus repopulation was studied in marrow radiochimeras. The evolution of total thymus cell, and TNC numbers per thymus was established in C57 BL/Ka (Thy 1.2) mice which were irradiated with 400 rads and immediately grafted with 5.10^6 bone marrow cells from normal congenic BL/1.1 (Thy-1.1) mice. At various time intervals thereafter, the percentages of donor (Thy-1.1) and recipient (Thy 1.2) thymocytes were scored.

As in the 400 rads treated mice, TNCs disappeared rapidly after irradiation in the marrow reconstituted mice (Fig. 1 and 2). They responded earlier than

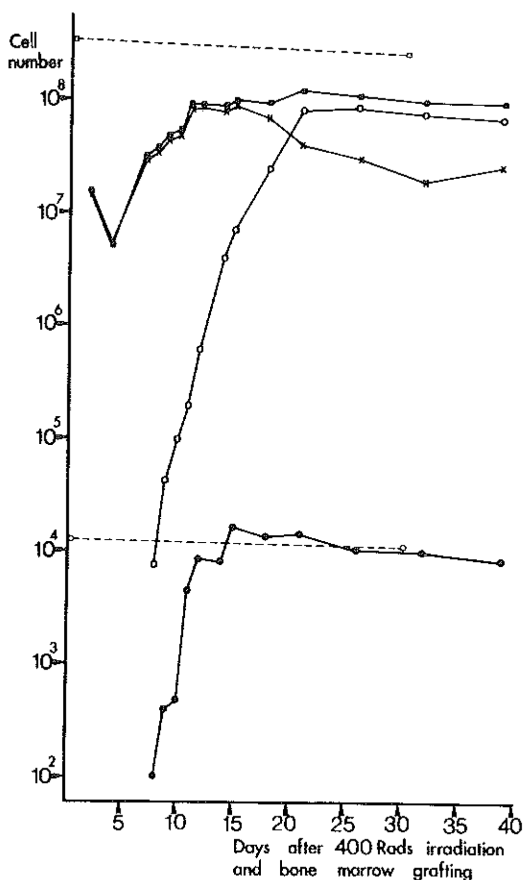

Figure 2. Evolution of TNCs in 400 rads irradiated, bone marrow reconstituted mice: evolution of TNCs number (●—●), of thymus cell number (■—■), donor type cell number (○—○), of recipient type cell number (X—X) in 400 rads irradiated, reconstituted mice. Evolution of TNCs number (○----○) and of thymus cell number (□----□) in non-treated mice.

in non-grafted mice. Indeed they were found as early as day 8 after irradiation. At the same time, the first thymocytes derived from the 'donor' inoculated marrow were detected (Fig. 2). The number of TNCs and of donor-type cells per thymus increased simultaneously (Fig. 2). Later on, no secondary atrophy, nor decrease of TNCs numbers occurred (Fig. 1). This was obviously related to the progressive and complete restoration of thymocyte population by the grafted marrow (Fig. 2), since the host type thymocytes were replaced by 'donor' cells during that time period. Similar results were obtained when BL/1.1 (Thy-1.1) mice were irradiated and reconstituted with marrow cells from normal C57BL/Ka (Thy-1.2) mice depleted of T cells by treatment with monoclonal anti-Thy-1.2 antibody plus C, indicating that thymus restoration is due to the bone marrow prothymocytes.

Here again, after sublethal irradiation and bone marrow grafting, TNCs restoration was closely linked with that of thymocyte population.

Relations between bone marrow precursors cells and TNCs

The foredescribed observations strongly suggest that TNCs are related, at least in part, to the progeny of bone marrow prothymocytes. Two series of experiments were undertaken to analyze these relations in more detail.

First of all, the homing pattern of grafted bone marrow cells was studied by using the method developed by Scollay *et al.* [28] for testing lymphoid cell migration. Two-month old C57 BL/Ka mice were irradiated with 400 rads, grafted with 15.10^6 FITC labelled bone marrow cells, and sacrificed after 6, 9, 16, 20, 24, 40, or 48 h. The percentage of fluorescent cells was estimated in the whole thymus. As seen in Table 1, the number of fluorescent cells increased gradually and reached a maximum between 16 and 20 h after the injection. The bone marrow cells settling in the thymus were not found in TNCs. Indeed, thymuses collected from mice sacrificed 18 and 48 h after bone marrow grafting were processed following the method for TNC isolation; no TNCs were recovered from these thymuses. Since bone marrow derived cells were already present in the thymus at that time, the data indicate that the presence of TNCs as such is not required for homing of marrow cells into the thymus early after sublethal irradiation.

The following experiments were designed to define whether TNCs were involved in the early proliferation of marrow derived cells after homing the irradiated thymus. As shown in the above described experiments, this proliferation cannot be detected until day 8 or 9 after irradiation and marrow grafting. C57BL/Ka mice were irradiated with 400 rads and immediately reconstituted with 5.10^6 congenic BL/1.1 bone marrow cells. At various time intervals thereafter, the percentages of 'donor' type cells were measured in the whole thymocyte population and in the intra-TNC lymphoid cell population. Immunofluorescence staining was performed on individual permeabilized TNCs. However, for quantitation, the membrane immunofluorescence assay was made on thymocytes released from TNCs after a short term *in vitro* incubation. The ratio of the percentage of donor type lymphoid

Table 1. Migration of FITC labelled bone marrow cells in 400 rads treated thymuses

Delay after bone marrow grafting (hours)	Frequency of FITC labelled cells in the thymus	Number of TNCs per thymus
6	$0.86/10^4$	nd
9	1.6	nd
16	1.8	nd
18	nd	0
20	1.85	nd
24	1.07	nd
40	1.33	nd
48	1.5	0

Table 2. Localization of proliferating bone marrow derived cells within TNCs

Days post irradiation	Percentage of donor type cells in the thymus	Percentage of donor type cells in the TNCs	Enrichment[a] for donor type cells in the TNC fraction
9	0.005	0.44	88
11	0.25	1.6	6.4
12	1.5	2.8	1.8
13	2.8	3.3	1.1
15	11	13	1.18

[a] $\text{Enrichment} = \dfrac{\text{Percentage of donor type cells in the TNC fraction}}{\text{Percentage of donor type cells in the thymus}}$

cells in TNCs to that found in the whole thymocyte population gave an estimate of the enrichment for donor type cells in TNCs. In the experiment reported in Table 2, the donor type cells began to be detected in the thymus on day 9; they represented 1 in 20 000 thymocytes. At the same time, the intra-TNC lymphoid cell population was 85 times enriched for donor cells. Later on, the percentage of donor cells increased in the thymus and reached 11% on day 15; during the same period of observation, the percentages of donor lymphocytes in TNCs became progressively similar to those observed in the whole thymus. In another experiment, BL/1.1 mice were irradiated with 400 rads and reconstituted with bone marrow cells from C57BL/Ka mice depleted of T cells by treatment with monoclonal anti-Thy-1.2 antibody plus C: the results obtained were similar, indicating that the first proliferating cells derived from the grafted marrow are preferentially associated with TNCs.

Discussion

The present work provides evidence on the involvement of Thymic Nurse Cells (TNCs) in thymus repopulation after sublethal whole-body irradiation. Early after the treatment, followed, or not, by bone marrow reconstitution, the thymus undergoes a first involution during which no TNCs were detected by the usual methods of investigation. Owing to the fact that the epithelial cells are resistant to the X-ray dose range used in the present experiments, the disappearance of TNCs clearly indicates that TNCs-associated lymphocytes, or at least most of them, are radiosensitive. Their susceptibility to cortisone had been previously demonstrated [29]. As established with great accuracy by our study, the pattern of TNCs restoration after sublethal irradiation was closely parallel to that of the thymocyte population. This suggests that the intra-TNC regenerating lymphoid cells belong to the progeny of both intrathymic radioresistant and marrow derived T cell precursors. Indeed, these cells are responsible, respectively, for the primary and the secondary phases of thymic regeneration [21–27]. The close relation of bone marrow derived precursors to TNCs restoration was further supported by our observations

in marrow radiochimeras. Indeed the proliferation of donor marrow derived thymocytes prevented the secondary disappearance of TNCs as well as the secondary thymic atrophy, which were observed in non-marrow grafted irradiated mice. In our experiments, the first donor-type lymphoid cells which were detected in the thymus were preferentially located within TNCs.

The sequence of events occurring between bone marrow grafting and proliferation of donor type thymocytes is still unclear. Indeed, our homing experiments demonstrated that bone marrow cells enter the thymus during the first 24 h after irradiation. The presence of TNCs as such, i.e. as intact lymphoepithelial complexes, is not required for intrathymic settlement of bone marrow cells. These cells acquire rapidly (within 3 h) the Thy-1 antigens [19]. However, no active proliferation of donor-type cells was detected until day 8 or 9. It is not known whether the regenerating thymocytes derive from these cells which homed the thymus early after irradiation (see above) or from cells which penetrated later on. The signals triggering the proliferation of donor type thymocytes have not been defined yet. It was recently shown that these cells were first associated with 1-A negative macrophages in the central thymus, and after a short delay, within TNC and medullary dendritic cells [3]. The authors speculated that the immature thymocytes associated with these three types of stromal cells might belong to a common precursor stage. Interactions with the respective stromal cells might induce their differentiation into different lineages [3]. In any case, the present data demonstrate that TNCs are involved in regeneration of the radiosensitive thymocyte population. The pattern of TNCs restoration as well as the preferential association of donor type cells within TNCs that we have observed indicates that immature thymocytes-epithelial cell interactions within TNCs are critical for this process.

Acknowledgements

We are grateful to Drs. H.S. Kaplan and M. Lieberman for providing us with the mice used in this work. We thank Mrs. E. Franssen, Mrs. J. Niessen and Mr. T. Dessart for their excellent technical assistance, Mr. D. Bourguignon for iconography and Mrs. A.M. Valkenborgh for typing the manuscript. We thank Prof. E.H. Betz for reviewing the manuscript. This work was supported in part by grants from the Fonds de la Recherche Scientifique Medicale (Belgium) and the Centre Anticancéreux près l'Université de Liège.

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