Further Studies on the Mechanism of Radiation Induced Thymic Lymphoma Prevention by Bone Marrow Transplantation in C57 BL Mice

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Whole body fractionated irradiation induces thymic lymphomas in C57BL/Ka mice after a latent period during which intrathymic lymphopoiesis is modified; thymocyte numbers are subnormal and the epithelial component of thymic nurse cells (TNCs) is altered as estimated by the number of TNCs in vivo and by its ability to interact with immature thymocytes in vitro. A graft of normal bone marrow cells immediately after the last irradiation prevents the development of lymphomas; but when such a graft is performed 1 month later, it does not inhibit the emergence of tumors. In both cases the grafted precursors home and repopulate the thymus. However, the delayed graft does not exert any effect upon the altered epithelial component of TNCs, whereas the early one restores the numbers of TNCs and the function of their epithelial component. The results thus demonstrate that lymphoid thymic repopulation by a bone marrow graft is not sufficient to prevent the development of lymphomas and that there is an intimate relationship between tumor development and alterations of nurse cells microenvironment.

INTRODUCTION

IN C57BL/KA mice, whole body fractionated irradiation (4 × 1.75Gy) induces thymic lymphomas in more than 90% of the animals after a latency period of 6–12 months (1). During this latency, called "preleukemic period," potentially neoplastic (preleukemic) cells are first detected in the thymus (as early as 2 days after the last dose of x-rays) and later in the bone marrow. By contrast, with the lymphoma cells, whose growth is autonomous, the preleukemic cells require thymic microenvironment for their survival and their full neoplastic transformation (1–5).

The factors involved are still unknown. Several alterations of thymic lymphopoiesis have been identified during the preleukemic period, but their contribution to the oncogenic process has not yet been defined. Let us mention the decrease of prothymocyte activity in bone marrow (4, 6–8), the modifications of thymocyte subpopulations (9; Rongy et al., in preparation), and the alterations of some functions of thymic epithelium (5, 10). A reduction of natural killer (NK) cell activity in the spleen has also been described (11–13).

In order to define which of these parameters, if any, is important for the induction of lymphomas, we have studied mice that received a graft of normal bone marrow cells within hours after the last dose of the lymphomagenic x-ray regimen ("early bone marrow graft").

Indeed, under these circumstances, preleukemic cells are still induced, but they disappear from the thymus and the bone marrow after a short time (5).

Consequently, the mice do not develop lymphomas (14). Thus, early bone marrow grafting obviously prevents the promotion of preleukemic cells towards lymphoma growth. One can ask whether this prevention is due to a direct cytotoxic effect of grafted marrow derived cells on the preleukemic cells or, alternatively, to the suppression of factors which usually contribute to the aforementioned progression. Several effects of the early bone marrow graft have already been documented, such as restoration of bone marrow prothymocyte activity (6), improvement of thymus repopulation (15), recovery of thymic epithelium (5, 10), and spleen NK cell activity (11–13); but their role in lymphoma prevention is still unknown.

Interestingly, when the bone marrow graft is given several weeks after completion of the irradiation regimen ("delayed bone marrow graft"), there is no inhibition of radiation induced lymphomagenesis (16). Comparing the effects of a delayed bone marrow graft with those of an early graft might provide some clues on the mechanism involved in lymphoma prevention.

In the present work, we report our observations on the capacities of grafted marrow cells to migrate into the thymus and to repopulate it, as well as their effects on thymic epithelium in 4 × 1.75 Gy irradiated mice. We have compared the results obtained after an early graft with those of a delayed bone marrow graft.

We have shown that the delayed bone marrow grafted cells can home in the thymus and repopulate it as well as early grafted cells. However, they do not display any effect upon the thymic epithelial cells. These results strongly suggest that the restoration of thymic epithelium by early marrow grafting might be the critical event for the prevention of radiation induced thymic lymphomas.

MATERIALS AND METHODS

Mice. One- to two-month-old C57BL/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.

Irradiation. For the induction of lymphomas mice were given four whole body irradiations of 1.75 Gy applied at weekly intervals. The irradiation was delivered by an x-ray apparatus (Stabilivolt Siemens, 190 KV, 18 mA, HVL : 0.5 mm Cu) at a dose rate of 1.60 Gy/min.

Bone Marrow Cell Suspensions and Grafting. Bone marrow cells from normal 1-month-old mice were suspended in phosphate buffered saline (PBS) supplemented with 5% fetal calf serum (FCS). Aliquots (200 μl) containing 10^7 cells were injected intravenously within 3 hr after the last irradiation for the "early" graft, and 1 month after the last irradiation for the "delayed" graft.

In Vivo Thymus Homing Assay. The bone marrow cells were incubated for 20 min at 37°C in a solution of 30 μg/ml of fluorescein isothiocyanate (FITC) in PBS (17, 18). They were then washed in FCS and PBS before being injected. Aliquots of 10^7 cells were injected

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intravenously within 2 hr following x-ray exposure; 24–72 hr later, recipients were killed, and the percentage of FITC labeled thymocytes was determined in cell suspensions treated with propidium iodide to exclude death cells. To evaluate the number of migrants, the number of total thymocytes per thymus was also calculated.

The Thymus Repopulation Assay. As previously described (19), aliquots of 10^7 bone marrow cells collected in BL/1.1 mice (Thy 1.1) were injected intravenously into C57BL/Ka mice (Thy 1.2). Recipients were killed 15, 20, 30, and 56 days later. Thymus cell suspensions were treated with monoclonal anti-Thy 1.1 clone HO-22-1 (20) and anti-Thy 1.2 clone HO-13-49 (21) antibodies, followed by a FITC labeled second stage antibody (goat anti-mouse IgM, Nordic, Leuven, Belgium).

Fluorescence Analyses. Cell suspensions were analyzed on a fluorescence activated cell sorter (FACS IV, Becton Dickinson, Sunnyvale, California). Only the fluorescence of viable cells (discriminated on the basis of labeling with propidium iodide) was considered.

Thymus Dissociation and Thymic Nurse Cells (TNCs) Isolation. In these experiments, TNCs were isolated from 10 thymuses. The tissues 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 (Boehringer Mannheim, Brussels, Belgium). TNCs were isolated from the resulting suspension by successive runs of 1-g sedimentation by using a slight modification (22) of the method originally described by Wekerle and Ketelsen (23). 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.

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 Nakayama and Wekerle (personal communication) and adapted by the authors (10). Isolated TNCs were suspended in RPMI 1640 culture medium (Gibco Bioculture Ltd., Belgium), supplemented with 10% of heat inactivated FCS, 2 mM L-glutamine, 1% of nonessential amino acids, 1 mM of sodium pyruvate, 150 U/ml of penicillin, and 75 μg/ml of streptomycin. After a 24-hr incubation in Petri dishes (Lux Scientific Corporation, The Netherlands) at 37°C in a 5% CO_2 atmosphere, the epithelial cells were adherent to the plastic surface; the lymphocytes which had been released from TNCs were discarded. The remaining epithelial cells were incubated for an additional period of 2 days.

After this time interval, they were treated twice with trypsin (0.25% in PBS) for 20 min at 37°C and resuspended in culture medium supplemented with 10% of heat inactivated FCS. The epithelial cells were then mixed with thymocytes obtained from thymuses recovered from 16–17-day-old embryos in a ratio of one epithelial cell to 10 thymocytes; 20 μl aliquots of this cell suspension were incubated for 6 hr in an inverted Terasaki plate. The percentage of epithelial cells forming lymphoepithelial complexes in such experimental conditions was then estimated.

RESULTS

All the observations were done in the following four groups of mice:

4 × 1.75 Gy irradiated.

4 × 1.75 Gy irradiated and grafted with normal bone marrow cells within the first 3 hr following the last irradiation (early bone marrow graft).

4 × 175 Gy irradiated and grafted with normal bone marrow cells 1 month after the last dose of 1.75 Gy (delayed bone marrow graft).

4 × 1.75 Gy irradiated and 1 month later irradiated with 4 Gy and grafted with normal bone marrow cells (4 Gy irradiated and delayed bone marrow graft). This last group was designed because it could be expected that the thymus, 1 month after the last 1.75 Gy irradiation, would be refractory to the grafted bone marrow cells homing.

Survival and Lymphoma Incidence. As shown in Figure 1, the survival of the mice which received an early bone marrow transplantation after irradiation was similar to that observed in control nonirradiated mice. At the opposite, the survival curve of mice which were grafted with bone marrow cells 1 month after the end of the irradiation regimen (with or without a further 4 Gy irradiation) was very close to that seen in the ungrafted irradiated animals. The dead mice have developed a thymic lymphoma. The tumors were of the recipient phenotype (data not shown).

Thus, the data confirm previous studies (16) that a delayed bone marrow graft after fractionated irradiation, whether the mice receive an additional 4 Gy dose or not, does not prevent the development of 4 × 1.75 Gy induced thymic lymphomas.

Thymus Homing. The next series of experiments were performed to test whether the inability of a delayed bone marrow graft to prevent radiation induced lymphomas, was correlated with insufficient thymus seeding by grafted marrow. FITC labeled normal bone marrow cells were intravenously injected into 4 × 1.75 Gy irradiated mice, either immediately after the last irradiation (early graft) or 1 month later (delayed graft). The numbers of migrants in the thymus were determined 24, 48, or 72 hr thereafter. The results were compared with those observed in unirradiated 3-month-old mice that received an intravenous injection of FITC labeled marrow cells and in mice that had been 4 or 9 Gy irradiated immediately before FITC stained marrow inoculation. These two latter groups of mice were used as positive controls (24).

The relative and absolute numbers of thymus homing cells were rather similar in the three groups of mice which had been grafted immediately after irradiation (Table 1). They were higher than in grafted unirradiated control mice. The number of migrants were 10–20-fold lower in the mice which were inoculated with bone marrow cells 1 month after the last 1.75 Gy irradiation, suggesting that the 30-day preleukemic thymus was partially nonreceptive to the immigration of bone marrow cells. As shown in Table 1, this limited susceptibility to homing was radiosensitive since the frequency of thymus migrants was restored to normal values when a 4 Gy irradiation was given just before marrow grafting in those 30-day preleukemic mice.

These data indicate the lack of correlation between lymphoma prevention and the rate of thymus homing by grafted bone marrow cells.

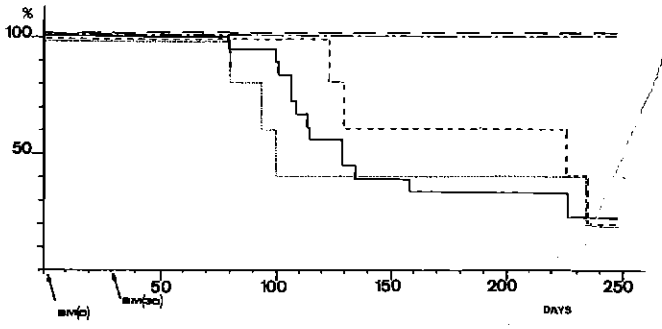

Figure 1. Effects of an early—or a delayed—bone marrow graft on the survival of C57BL/Ka mice after a split dose leukemogenic irradiation. Controls (— —); 4 × 1.75 Gy (———); 4 × 1.75 Gy + early bone marrow (BMO) graft (—— -); 4 × 1.75 Gy + 1 month delayed bone marrow (BM 30) graft (·····); 4 × 1.75 Gy + one month delayed bone marrow (BM 30) graft with a prior 4 Gy irradiation (- - - - -).

Table 1. Thymus Homing: FITC Labeled Cells in the Thymus

Experimental Groups	Time Intervals after Bone Marrow Graft		
	24 hr	48 hr	72 hr
Nonirradiated + graft	0.67×10^{-4} (10.291×10^3)	0.52×10^{-4} (14.951×10^3)	0.34×10^{-4} (8.604×10^3)
4 × 1.75 Gy + early graft	29.3×10^{-4} (23.273×10^3)	29.1×10^{-4} (22.497×10^3)	19.2×10^{-4} (20.174×10^3)
4 × 1.75 Gy + delayed graft	0.3×10^{-4} (2.851×10^3)	0.25×10^{-4} (1.12×10^3)	0.22×10^{-4} (1.581×10^3)
4 × 1.75 Gy + (4 Gy + delayed graft)	25.9×10^{-4} (27.434×10^3)	24.8×10^{-4} (132.14×10^3)	11.2×10^{-4} (10.956×10^3)
4 Gy + early graft	28.7×10^{-4} (20.664×10^3)	32.4×10^{-4} (49.532×10^3)	15.7×10^{-4} (19.97×10^3)
9 Gy + early graft	35.1×10^{-4} (27.389×10^3)	32.9×10^{-4} (42.597×10^3)	32.7×10^{-4} (39.275×10^3)

The results shown are the numbers of fluorescent cells per 10^4 thymocytes at various time intervals after intravenous grafting of 10^7 FITC-labeled bone marrow cells in several experimental groups; the numbers in parentheses represent the absolute numbers of labeled thymocytes in each condition. "Early" refers to a bone marrow transplantation within 3 hr after irradiation, whereas "delayed" means a graft performed 30 days after x-rays.

Thymus Repopulation. Next, we looked for the thymus repopulating capacities of marrow cells to see whether they were transplanted early or late after completion of the split dose irradiation regimen. The percentages of marrow derived donor type thymocytes were measured at various time intervals after graft.

In all cases, the thymus was actively repopulated, but with a delay of several weeks when the graft was performed 1 month after irradiation, without 4 Gy irradiation.

Indeed, the percentages of donor type cells reached about 80%, respectively, on day 16 after an early marrow graft (and thus after the fourth 1.75 Gy irradiation), on day 55 after the delayed graft (i.e., 85 days after the last 1.75 Gy irradiation), and on day 20 in mice which received a late graft preceded by a 4 Gy irradiation (and thus 50 days after the fourth 1.75 Gy irradiation) (Fig. 2).

This experiment did not show any parallelism between thymic repopulation by bone marrow cells (Fig. 2) and lymphoma development inhibition (Fig. 1).

Thymic Nurse Cells. Since neither differences in thymus homing properties or in thymic repopulation activity could explain why a delayed bone marrow graft did not protect mice against lymphoma development, we investigated whether such a graft could act upon thymic microenvironment in the same way as an early graft.

The numbers of TNCs per thymus were evaluated in the experimental groups and in controls. As shown in Figure 3, the time course of TNCs number was similar in 4 × 1.75 Gy irradiated mice and in those one which further received a delayed bone marrow graft preceded or not by a 4 Gy irradiation. Indeed, TNCs became scarcer with time and reach very low values after 2 months. At the opposite, in mice which were inoculated with bone marrow cells within the 3 hr after irradiation, there was a progressive restoration of the number of TNCs which even displayed an overshoot after 2 months.

Thus, by contrast with the early marrow graft, the delayed one was not capable to restore TNCs numbers after 4 × 1.75 Gy irradiation.

The capacity of epithelial nurse cells to establish interaction with immature thymocytes in vitro was tested. Epithelial cells were prepared from TNCs of the four experimental groups and controls. The animals were killed on day 30 after the bone marrow graft. The capacity of epithelial cells to build up new nurse cells in vitro after contact with immature thymocytes is shown in Figure 4. Again, a delayed marrow graft, preceded or not by a 4 Gy irradiation, by contrast with an early graft, did not restore the capacity of thymic epithelial cells to reconstitute lymphoepithelial complexes in vitro.

These experiments indicate that the development of lymphomas is always preceded by irreversible functional alterations of epithelial TNCs, as estimated by the number of TNCs in vivo and by the ability of their epithelial component to establish interactions with immature thymocytes in vitro.

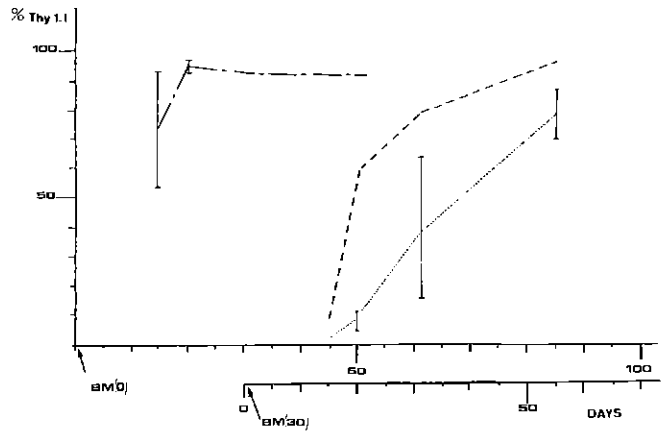

Figure 2. Thymic repopulation by grafted marrow cells. Evolution of the percentage of donor type Thy-1.1 thymocytes in Thy-1.2 mice irradiated at 4 × 1.75 Gy and grafted with normal bone marrow cells from (Thy-1.1) BL/1.1 mice: early bone marrow (BMO) graft (— - — - —); 1 month delayed bone marrow (BM30) graft (·····); 1 month delayed bone marrow (BM30) graft with a prior 4 Gy irradiation (- - - - - -).

DISCUSSION

The experiments reported in this paper were performed in an attempt to understand the mechanisms by which a bone marrow graft inhibits the development of radiation induced thymic lymphomas. For that purpose, several parameters related to thymic lymphopoiesis were compared after an early bone marrow graft (which prevents the onset of lymphomas) and a delayed graft (which does not display any inhibitory effect on lymphoma development) (1).

We first looked for thymus homing and thymus repopulating activity of bone marrow grafted cells. We observed a clear correlation between these two parameters: bone marrow cells home to the thymus and repopulate it as well when they are grafted immediately after the last 1.75 Gy irradiation or when they are injected 1 month thereafter, providing that a 4 Gy irradiation was given before the graft. In contrast, the frequency of thymus homing cells was low, and the speed of thymus repopulation slow in mice which received a delayed bone marrow graft without any 4 Gy irradiation. This phenomenon is most likely due to a "space" competition within the thymus; in fact, when the bone marrow graft is realized within the first hours after a whole body irradiation of either 1.75 Gy or 4 Gy, there is an immediate, very intense lymphocyte depletion in the thymus, due to the cytotoxic effects of ionizing radiation

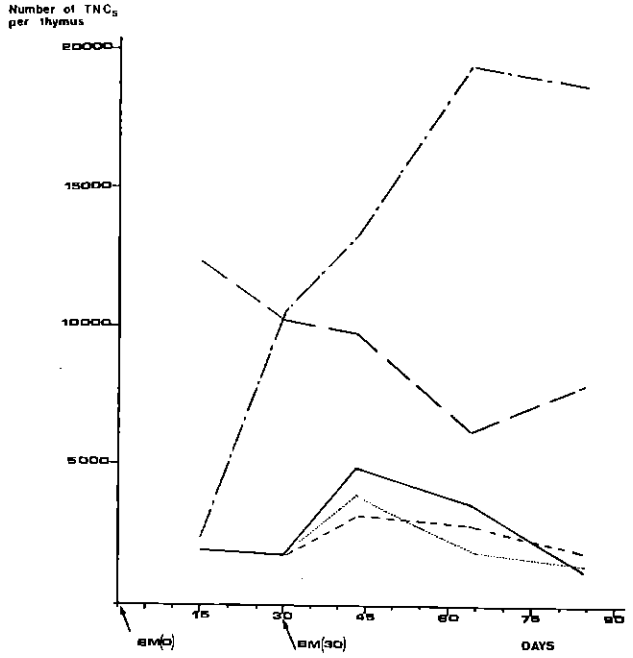

Figure 3. Effects of bone marrow grafting on TNCs after a split dose leukemogenic irradiation: the figure shows the number of TNCs, which were isolated from thymuses in the following experimental groups: controls (— —); 4 × 1.75 Gy (———); 4 × 1.75 Gy + early bone marrow (BMO) graft (—— -); 4 × 1.75 Gy + 1 month delayed bone marrow (BM30) graft (·····); 4 × 1.75 Gy + 1 month delayed bone marrow (BM30) graft with a prior 4 Gy irradiation (- - - -).

(25). However, the obstacle to thymus repopulation in late grafted animals was only transient since the frequency of donor cells in the thymus eventually reached the highest values (>80%) after 50–60 days.

By contrast, the rather efficient thymus seeding (as measured by absolute numbers of homing cells) in unirradiated mice grafted with normal bone marrow cells was not followed by the proliferation of the inoculated cells within the thymus.

In this case, however, no damage to the thymic populations,

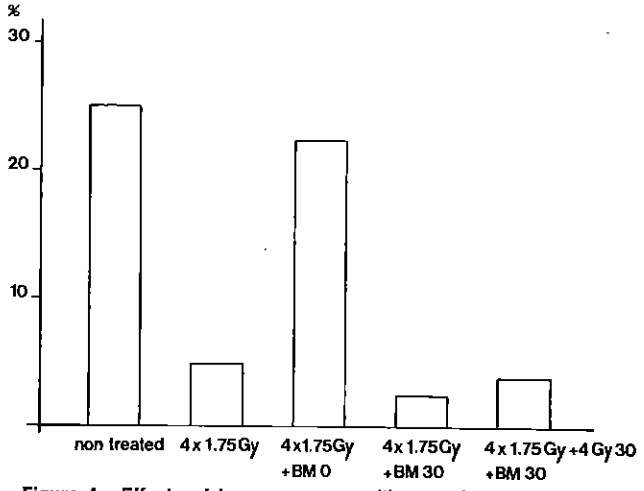

Figure 4. Effects of bone marrow grafting on the capacity of TNC derived epithelial cells to form complexes with immature thymocytes in vitro. The figure shows the results observed with epithelial cells prepared 1 month after 4 × 1.75 Gy or after an early (BMO)—or a delayed (BM30)—bone marrow grafting. The reported values are the percentages of cultured TNC derived epithelial cells, which reconstitute lymphoepithelial complexes with 17-day-old fetus thymocytes after a 4-hr incubation in vitro.

either lymphoid or stromal, had been induced; hence, no conditions for proliferation of grafted marrow cells were encountered. The active, though slow, repopulation observed in the 4 × 1.75 Gy irradiated mice, which were grafted 1 month later with normal marrow, might be explained by the deep alterations, at the quantitative and qualitative levels, of marrow prothymocytes and thymocyte subpopulations in the preleukemic irradiated mice, eventually allowing the proliferation of donor type cells. Taken together, the observed differences in thymus homing and repopulating capacities of the grafted marrow cells in the various experimental groups thus cannot explain the lack of lymphoma prevention by a delayed graft.

A more pronounced difference between the experimental groups concerned the thymic nurse cells; in fact, the late grafted mice, either treated with an additional 4 Gy irradiation or not, never reconstituted their TNC population; furthermore, the epithelial components of these TNCs were never capable to reconstitute lymphoepithelial complexes in vitro in the presence of fetal thymocytes. This observation provides a further argument for the role of thymic epithelial cells in the pathogenesis of thymic lymphomas. In fact, in the present experiments as in others, there was an intimate correlation between the modifications of the nurse cell epithelium and the development of lymphomas. This was observed also after inoculation of radiation leukemia virus (26) or treatment with methylnitrosourea (27).

These correlations lead us to ask how the damages to thymic epithelium are induced by x-rays, and conversely, how they are restored by an early bone marrow graft. Since the damage to epithelial cells can be reverted by an early bone marrow graft (5), it is probably not due to a direct effect of ionizing radiations on the thymic epithelium.

In fact, previous ultrastructural studies never mentioned obvious morphological alterations of these stromal cells (15, 28, 29). Fractionated irradiation might in fact destroy or inactivate other radiosensitive thymic cells which are normally required for inducing and/or maintaining the physiological functions of the thymic epithelium. This process is marrow dependent, as suggested by the effects of an early bone marrow graft.

However, the mechanisms involved cannot relate to a possible replacement of radiation-damaged thymic epithelial cells, and particularly the nurse cells, by grafted marrow cells. It has been widely established from embryological studies that thymic epithelium is derived from the endoderm, and perhaps partially from the ectoderm (30). Moreover, in radiochimeras, many thymic cell lineages, such as lymphocytes, macrophages, and dendritic cells, are bone marrow derived, whereas thymic epithelial cells are always host derived (31).

In our experimental model the restoration of thymic nurse cells in the early marrow grafted animals was thus necessarily due to an indirect mechanism; some marrow derived component may act in resident thymic epithelial cells so as to make them functionally normal again.

Several observations indicate indeed that marrow derived cells can act on thymus epithelium. As an example, a restored thymulin secretion, a property of thymic epithelium, has been induced by bone marrow transplantation in children with severe combined immunodeficiences (32). Furthermore, the expression of class II MHC antigen in the epithelium of thymic organ cultures is increased when marrow derived macrophages and dendritic cells are added. The effect can be inhibited by anti-interferon gamma (anti-IFN γ) antibodies (33).

A similar mechanism can be evoked about thymic nurse cells. Let us recall that epithelial cells from early bone marrow grafted animals can interact normally with immature thymocytes to reconstitute nurse cells in vitro. Furthermore, interferon gamma stimulates the capacity of epithelial cells from normal, or 4 × 1.75 Gy leukemic mice, to reconstitute nurse cells in vitro. (M. P. Defresne et al., in preparation). One may postulate that early bone marrow grafting after a leukemogenic split dose irradiation reconstitutes macrophages, dendritic cells, and thymocytes, leading to the restoration of intrathymic cytokine production and consequently of thymic epithelium functions. Our recent observations that inoculations of IFN-γ or tumor necrosis factor inhibit the development of radiation-induced lymphomas (34) strikingly argue in favor of this hypothesis.

Why does the delayed bone marrow graft not result in the restoration of thymic nurse cell epithelium? As mentioned above, there was an active and fast homing and repopulation of the thymus in mice transplanted with normal bone marrow 1 month after the split dose irradiation with an additional 4 Gy irradiation. Nevertheless, there was no restoration of the TNC population nor of their functional capacities. The observations suggest that the preleukemic epithelial "microenvironment" (one month after the split dose irradiation) is refractory to the inductive effects of marrow derived cells. This is presently under investigation.

The final question is to know why the delayed graft does not prevent the onset of lymphomas. The observed correlation between irreversible thymic epithelium alteration, with the susceptibility to lymphoma development, suggests but does not demonstrate that alterations to thymic epithelium are the most critical factor of promotion in this oncogenic system. Alternatively, the preleukemic cells, at 1 month after irradiation, might become insensitive to cytolytic factors produced by marrow derived cells, independently of thymic epithelial influences. Solving this question requires further studies.

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