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Abnormal thymocyte subpopulations in split dose irradiated C57BL/Ka mice before the onset of lymphomas

Effects of bone marrow grafting

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Summary. Fractionated whole-body X irradiation (4 × 1.75 Gy at weekly intervals) induces a high percentage of thymic lymphomas in C57BL/Ka mice. The present work reports the phenotypic alterations of thymocyte subpopulations during the preleukemic period: there were a decrease of CD4+CD8+ cells and an increase of CD4−CD8− and CD4−CD8+ cells. Marrow grafting early after irradiation that prevents lymphoma development restores the thymocyte subpopulations. In many instances, transplantation of 'preleukemic thymocyte inoculate' gives rise to an active and long lasting repopulation of recipient thymuses. However in all cases, donor lymphomas can develop after inoculation of 'preleukemic thymocyte inoculate'.

Introduction

In the C57BL/Ka strain of mice, thymic lymphoblastic lymphomas are experimentally induced by exposure to four weekly whole-body X ray irradiations of 1.75 Gy or by inoculation of Radiation Leukemia Virus (RadLV) [1,2,3].

In both cases, the incidence of tumours reaches more than 90% and neoplasms appear after a 'preleukemic period' lasting 3 to 12 months. During this latency, the target cells for the leukemogenic agent transform into potentially neoplastic cells, designated as 'preleukemic cells' [4,5,6]. These cells are dependent upon host factors and thymic microenvironment for their further evolution into thymic lymphoma [1,2,6,7,8,9]. They are found in mice that do not

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express any evidence of thymic lymphoma and are detected by an in vivo transplantation assay. After transfer into thymus bearing histocompatible mice, they give rise to lymphomas after a long latency period. However, they do not grow when they are injected into thymectomized animals [6,7]. The preleukemic cells differ from the autonomous leukemic cells found in the lymphomas which result into tumour development after transplantation into thymus bearing or thymectomized mice after a short latency period.

During the preleukemic period preceding the onset of radiation-induced tumours, several components of thymic lymphopoiesis are altered. In the bone marrow, the pool of prothymocytes is drastically reduced, as assessed by the decreased capacity of bone marrow cells to repopulate the thymus of sublethally irradiated mice [4,10]. In the thymus, the stromal cells are damaged: thymic nurse cells which are lymphoepithelial complexes involved in T lymphocyte differentiation disappear progressively [5] whereas the function of their epithelial component is altered [5].

In this model, tumour development can be totally inhibited by grafting normal bone marrow immediately after the leukemogenic split dose irradiation [4,11,12]. This graft does not prevent the emergence of preleukemic cells, nor the modifications of the microenvironment [5]. However, these alterations are reversible: preleukemic cells disappear and thymic nurse cells are restored during the second month following the treatment.

Radiation-induced injury to the thymocyte precursors or/and to thymic microenvironment could be responsible for alterations of thymocyte subpopulations. Indeed Chazan and Haran-Ghera [13] found a relative enrichment of thymocytes bearing low level of Thy-1 antigen and high level of H2 after a split dose leukemogenic irradiation. Similar observations were made in other models, such as in AKR mice during the late preleukemic period [14]. After injection of a mink cell focus MCF viral isolate into AKR mice, a novel population emerged 6 weeks post-injection and had the phenotype of small cortical thymocytes (Thy-1, Lyt-1, Lyt-2, PNA positive) [15].

Here, we report on the phenotypic alterations of thymocyte subpopulations during the preleukemic period in mice which had been treated by fractionated irradiation. Furthermore, we analysed also mice which had received a bone marrow graft after irradiation, in order to prevent lymphoma development. Finally, we analysed the fate and phenotype of preleukemic thymocytes after inoculation into normal hosts.

Materials and methods

Mice

C57BL/Ka mice and their congenic BL/1.1 mice were produced in our animal colony. C57BL/Ka mice bear the Thy 1.2 alleles and BL/1.1 mice the Thy 1.1 alleles.

Irradiation

For the induction of thymic lymphomas, C57BL/Ka mice were irradiated with 4 weekly whole-body doses of 1.75 Gy, starting at 4–5 weeks of age [16]. X irradiation was delivered under the following conditions: Stabilivolt Siemens, 190 KV, 18 mA, filter 0.5mm Cu, focal distance 35 cm and dose rate 1.6 Gy/minute.

For thymus repopulation experiments and for preleukemic cell detection, recipient animals were given a simple whole body irradiation of 4 Gy within 4 hours before all inoculation.

Bone marrow grafting

Bone marrow cell suspensions were prepared by flushing the contents of femurs of normal 1 to 2 months old BL/1.1 mice into PBS added with 5% foetal calf serum (FCS). After washing, 10^7 cells were injected intraveinously (i.v.) into 4×1.75 Gy treated C57BL/Ka mice within one or two hours following the last X ray exposure.

In vivo assay for the detection of preleukemic cells

The method described by Boniver et al. [17] and modified by Goffinet et al. [9] was used. Briefly, thymuses of irradiated 4×1.75 Gy Ka mice were removed at several time intervals after the completion of the irradiation. Five millions cell aliquots of thymus cell suspensions in 100 μl of RPMI 1640 were injected intrathymically (i.t.) to a 4 Gy whole-body irradiated congenic BL 1.1 recipient mice.

The animals were kept until they showed evidence of disease. They were sacrificed when moribund. The difference in Thy-1 antigens allowed the identification of the donor or host origin of the tumours, when present. Lymphoma cells derived from Thy-1.2 donor type assessed the presence of preleukemic cells into the cell inoculate.

Monoclonal antibodies and reagents

The fluorescein isothiocyanate (FITC) conjugate of anti-Thy-1.2 monoclonal antibody (mAb) (clone 30H12) [18] was purchased from Becton Dickinson (Sunnyvale, Ca., U.S.A.). Anti-Thy-1.1 mAb (clone HO-22-1) [19] was used with a FITC conjugated goat anti-mouse IgM serum (Nordic, Leuven, Bel-

gium) as second step antibody. CD4 was recognized by a biotinylated anti-L3T4 clone (H129.19) [20], which was revealed with a Texas Red conjugated Avidin (Tago, Burlingame, Ca., U.S.A.). Anti-Lyt-2 mAb from the rat hybridoma 53.6.72 [21] was used as a directly fluoresceinated reagent in order to label the CD8 antigen. Anti-Pgp-1 mAb (IM7.8.1) [22] kindly provided by J. Lesley, anti-IL-2 receptor (mAb) (7D4) [23] and anti-Transferrin receptor (mAb) (R17 217.13) [24] were used unconjugated. They were employed in conjunction with FITC conjugated $F(ab')^2$ fragments of goat anti-rat Ig immunoglobulins (Nordic, Leuven, Belgium).

Immunofluorescence staining

For phenotypic studies, thymocytes or lymphoma cells were suspended in RPMI 1640 plus 5% FCS. 10^6 cell aliquots in 50 μl were incubated in 1 ml glass tubes with appropriate amounts of specific mAbs at optimal dilutions at 4°C for 30 minutes. Cells were washed in FCS and resuspended in PBS plus 1% paraformaldehyde for fixation, when FITC conjugated mAbs were used.

For indirect immunofluorescence, when unconjugated mAbs were used, cells were first incubated with the specific mAbs and washed as above, then incubated for 30 minutes at 4°C with a second step FITC conjugated antibody or for biotinylated antibodies, with Avidin-Texas Red (Tago, Burlingame, Ca., USA). Then cells were washed in FCS and fixed in PBS containing 1% paraformaldehyde.

Flow cytometry and data analysis

All analyses were performed on a FACS IV (Becton Dickinson, Sunnyvale, Ca., USA) equiped with a dual laser optical bench. The lasers used were a model 164-05 Argon ion laser and a model 164-05 Krypton ion laser (Spectra Physics, Mountain View, Ca., USA). The 488 nm line of the argon ion laser operating at 400 mW was used to excite FITC (green fluorescence). The 568 nm line of the krypton ion laser operating at 200 mW was used to excite Texas Red (red fluorescence) [25]. The two laser beams were focused to spots approximately 250 μm apart so that the emissions generated at these two points were separated temporally, spatially and spectrally. The green fluorescence was collected through a band pass 530/30 filter (Becton Dickinson # 19-62774-00). The red fluorescence was collected through a band pass 625/35 filter (Becton Dickinson # 19-62774-06). The sheat line pressure was adjusted to obtain a rate analysis of 800–1200 cells/second.

For each sample, 10000 cells were analyzed. The data were collected as a four

parameter list (forward and right scattersd, green and red fluorescences) on a Consort 30 data management system (Becton Dickinson). Damaged cells were excluded from the analysis by gating based upon forward light scatter [25]. Statistics and graphics were displayed by the program LYSYS (Becton Dickinson) as contour plots or as isometric two parameter frequency histograms. Fluorescence intensities were displayed on a 3-decade-LOG scale. The relative cell number is displayed as LOG density (contour plots) or LIN density (isometric).

Cell depletion by antibody and complement cytotoxicity

Purification of Thy-1.2 low lymphocytes was performed by treating the cells with monoclonal anti-Thy-1.2 antibody and rabbit complement following the protocol described by Hyman [26]. Briefly 10^8 cells/ml were incubated for 45 minutes at 4°C with a 1/10000 dilution of anti-Thy-1.2 (New England Nuclear, Liège, Belgium). The cells were washed once and were resuspended in a 1/10 dilution of low -tox rabbit complement (Cederlane Laboratories, Ontario, Canada). After incubation for 45 minutes at 37°C, cells were washed twice before isolation on Ficoll-Hypaque gradients.

Results

Thymic lymphomas

In a representative experiment, the first thymic lymphoma was observed 60 days after the last X ray exposure and 90% of treated mice developed neoplasia within 8 months. The phenotype of 15 lymphomas is shown in Table 1.

All lymphomas were Thy-1 positive. They displayed variable expression of CD4 and CD8 antigens. In most of them, the percentages of CD4+ CD8+ cells were decreased and those of single positive CD4+ or CD8+ cells were increased, as compared with normal thymocyte populations. There was no lymphoma with a phenotype corresponding to a well defined normal thymocyte subpopulation (Fig. 1). Staining for other markers such as Pgp-1, IL-2 receptor or Transferrin receptor was also heterogeneous. The percentages of IL-2 receptor positive cells were often increased and most of lymphomas expressed Transferrin receptors.

Thymocyte subpopulations after 4×1.75 Gy irradiations

Mice were irradiated with 4 doses of 1.75 Gy at weekly intervals. Within 1 to 2 hours after the last irradiation, half of the mice were inoculated with 10^7

Table 1. Phenotype of radiation – induced thymic lymphomas

Total number	Percent of cells							
	Thy-1	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^-CD8^-$	Pgp-1	Il-2R	Trf-R
1	98	76,6	2	20	1,4	8,6	34	10
2	97	28,5	14,6	55,5	1,4	26	46	23,2
3	97	84	1,6	11,6	2	27,2	8,8	42,8
4	96,9	25.5	5	68,7	0,8	5	2	62
5	98	47,9	3,9	44,8	3,4	17,9	29,9	39,5
6	98	31,6	13,3	51,4	3,7	8	14	18
7	98	4,8	59	18,9	17,3	3,7	39,2	63,1
8	97,7	18,5	31,3	45,7	4,5	9	41	54
9	98	22,6	36,2	37,5	3,7	3	4	50
10	97,4	18,5	16,4	62,8	2,3	11	15	8
11	97,8	70,1	9,1	17,8	3	ND	ND	87,4
12	98	10,7	48	39,2	2	ND	ND	66,4
13	98	37,3	2,1	5,9	1,5	7,6	51,6	72,9
14	98	14,3	26,1	54,8	4,8	7	27,1	51,3
15	98,5	56,2	5,7	35,9	2,2	93,7	0,7	41
Normal young adult thymus	99	83	8,9	5	3,1	10,1	1,2	2,3

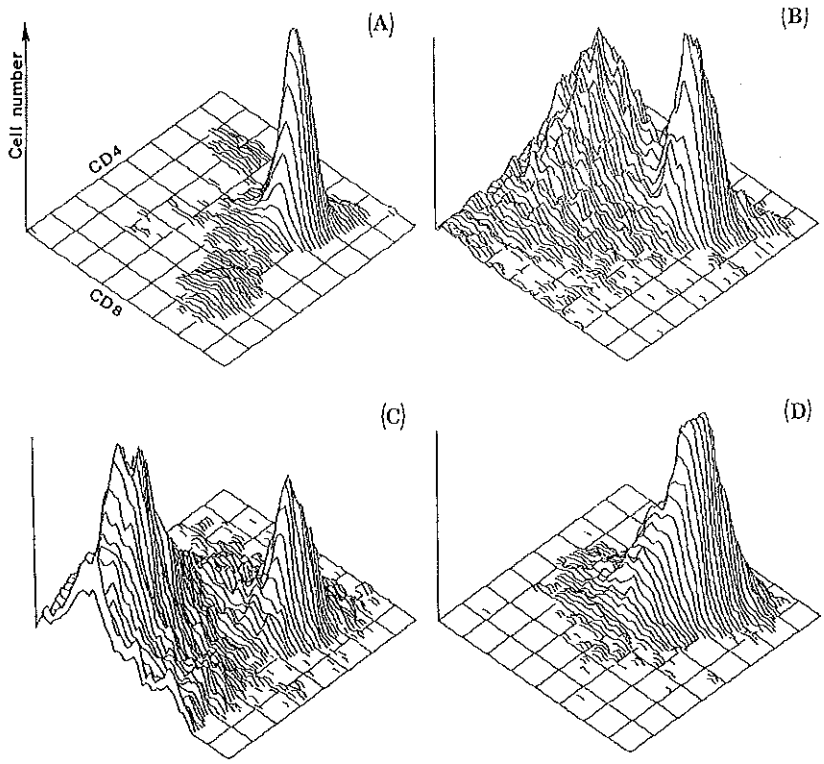


Fig. 1. Two parameters frequency histograms illustrating cell surface CD4 (red) and CD8 (green) expression. Normal thymocytes (A) and 3 radiation – induced thymic lymphomas (B, C, D). Thymocytes were stained with FITC conjugated anti-Lyt-2 mAb and biotinylated anti-L3T4 mAb followed by Avidin-Texas Red.

normal bone marrow cells. They were sacrificed on day 15, 30, 45, 60, 75, 90 and 105 after the irradiation. Normal mice of the same strain and age were used as controls. Five experiments were performed. In each of them, 3–4 mice were used for every experimental point. Since the time course of thymus modification, although generally similar, was not synchronous in all the experiments, the results of a representative experiment are shown (Figs. 2–5).

As compared with controls, the weight of 4×1.75 Gy irradiated thymuses was lower during the whole preleukemic period. The increase observed on day 105 is related to the development of the first lymphomas (Fig. 2).

The most striking phenotypic modifications occurred generally 30 to 45 days after the last irradiation. There was a decrease of the CD4+ CD8+ thymocytes, concomitantly with an increase of the CD4− CD8− cells. A slight increase of the percentage of CD4− CD8+ single positive cells was also observed. At some time intervals, the IL-2 receptor positive subpopulation

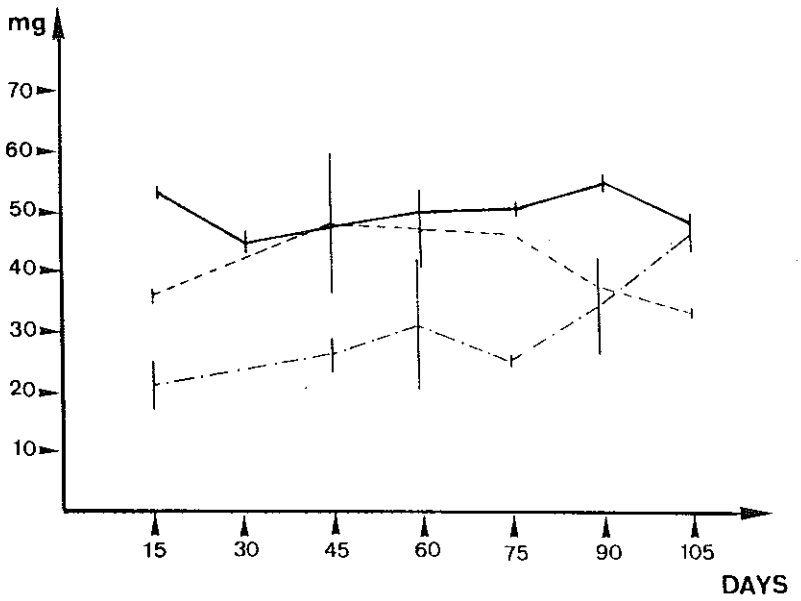


Fig. 2. Evolution of thymic weight in normal mice (——), 4×1.75 Gy irradiated mice (—·—·—) or 4×1.75 Gy irradiated mice grafted with 10^7 bone marrow cells (---). (4 mice per experimental point; mean +/− standard error of the mean (s.e.)).

increased (data not shown). There were some variations from one experiment to another (Figs. 3 and 4B show the data of a representative experiment).

In irradiated and marrow grafted animals, thymic weight returned to normal values by the end of the first month after the treatment (Fig. 2). This restoration was correlated with the active thymic repopulation by cells derived from the grafted marrow as assessed by the time course of the Thy-1.1 donor type positive cells (Fig. 5). This repopulation by marrow derived cells was very fast. Indeed, on day 15, donor type cells represented about 70% of thymocytes and reached 90% on day 30. As shown in Figs. 3 and 4C, only few phenotypical alterations of thymocyte subpopulations were observed during the fourteen weeks of this study, as compared with control thymocytes.

Fate of preleukemic thymocytes after transplantation into congenic recipients

Five 10^6 aliquots of thymocytes from 4×1.75 Gy irradiated C57BL/Ka (Thy-1.2) mice, which were sacrificed at various time intervals after the last irradiation, were inoculated intrathymically into congenic BL/1.1 (Thy-1.1) mice ('Preleukemic thymocyte inoculate'). Recipient thymuses were removed at various time intervals thereafter and phenotypically analyzed. Two quite different behaviours were observed, as shown in Fig. 6.

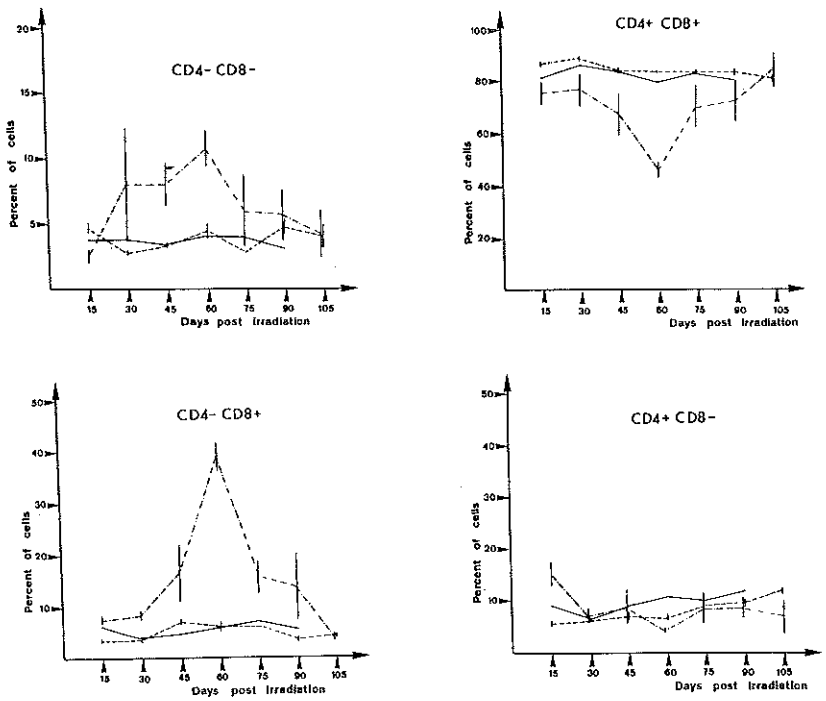
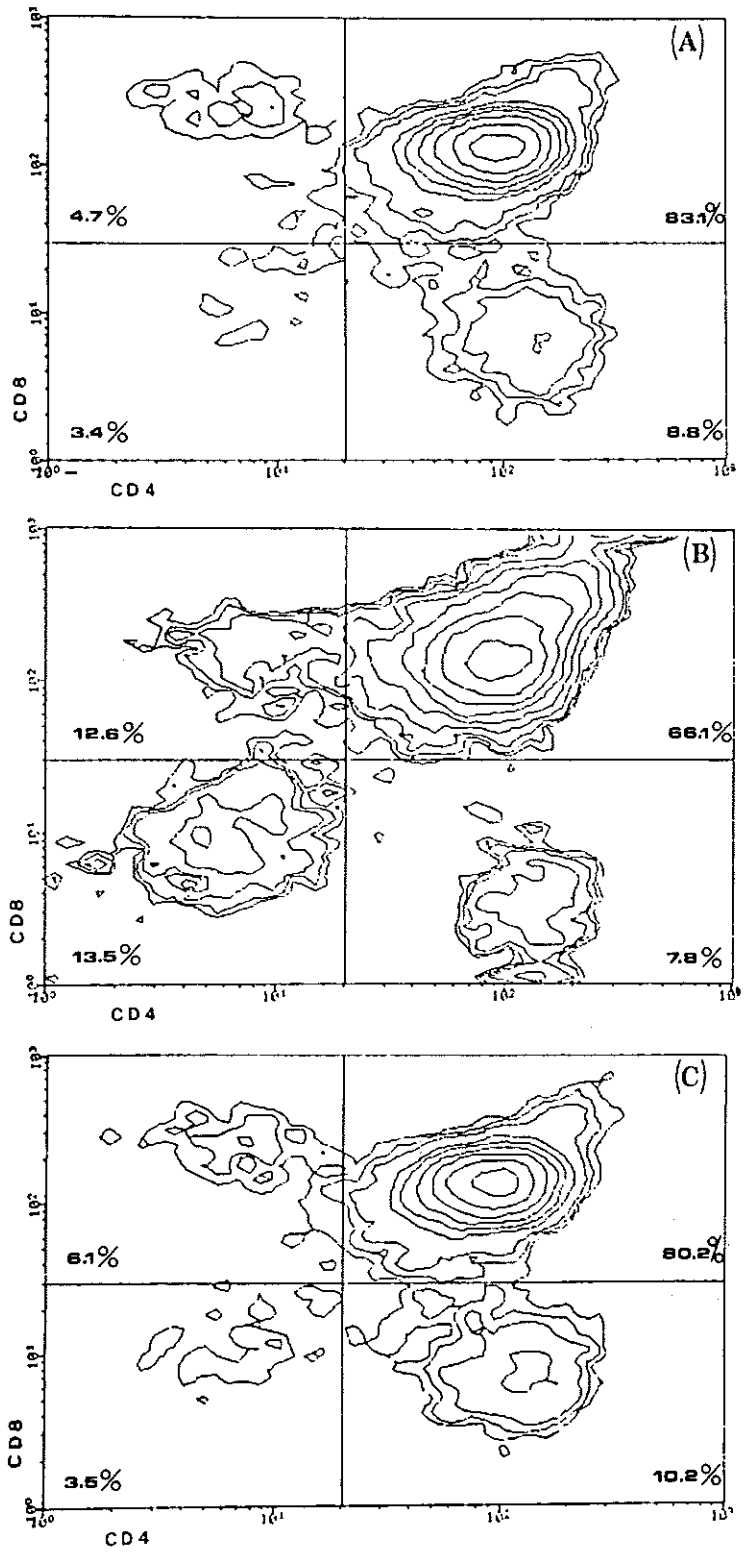


Fig. 3. Time course evolution of the four thymocyte subpopulations defined by the expression of the CD4 and CD8 markers: normal mice (——), 4×1.75 Gy irradiated mice (—·—·—) and 4×1.75 Gy irradiated mice grafted with 10^7 bone marrow cells (----). (4 mice per experimental point; mean +/− s.e.).

In some instances (4 out of 9 experiments), the frequencies of donor type thymocytes in the recipient thymuses were low, except during a short transient period of about 30 days, when they reached 10% of the whole thymocyte population as shown in Fig. 6, with the 5 days and 30 days inoculates. This time course was similar to that observed in recipients of normal thymocytes. There were no significant alterations in the phenotype of thymocyte subpopulations (data not shown). However, these cell inoculates, although obviously uncapable of thymocyte repopulation, gave rise to donor type thymic lymphomas (Table 2).

In other cases (5 out of 9 experiments), the 'preleukemic thymocyte inoculates' induced a very fast and active thymocyte repopulation in the recipients. An example of this behaviour is illustrated in Fig. 6 with the 15 days inoculate. These thymuses however remained macroscopically safe of tumours for 3 months.

By contrast with the observations in thymuses which were not actively restored by the 'preleukemic thymocyte inoculates', these repopulated thymuses


(A)
4.7%
83.1%
3.4%
8.8%
CD8
CD4
(B)
12.6%
66.1%
13.5%
7.8%
CD8
CD4
(C)
6.1%
80.2%
3.5%
10.2%
CD8
CD4

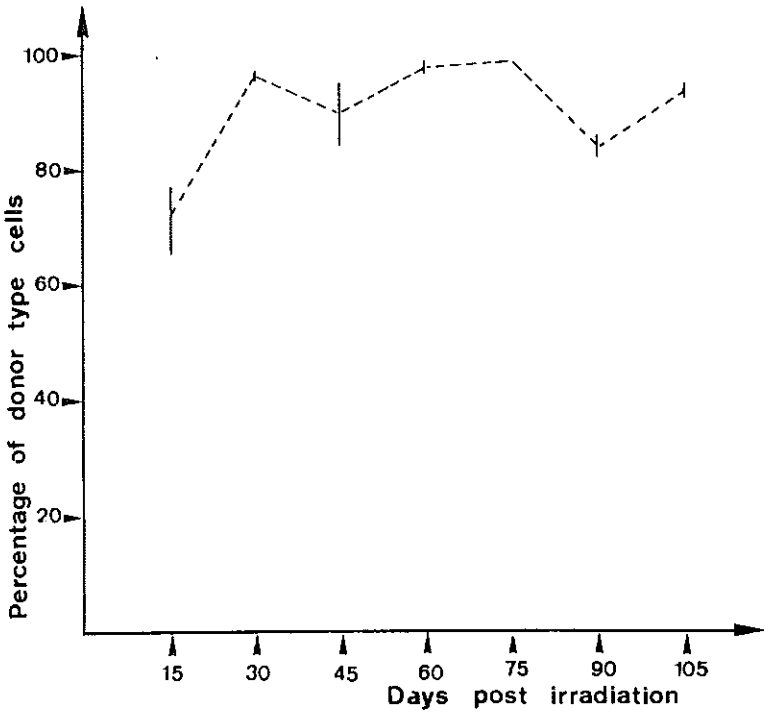


Fig. 5. Time course of thymus repopulation by donor (Thy-1.1) cells after grafting BL/1.1 bone marrow cells to 4×1.75 Gy irradiated C57BL/Ka mice (Thy-1.2). (4 mice per experimental point; mean +/− s.e.).

displayed strong phenotypic alterations (Figs. 7 and 8). There was a drop of the CD4+ CD8+ cells, together with an increase of CD4− CD8− and CD4− CD8+ cells. These phenotypical changes were in fact similar to those observed in preleukemic 4×1.75 Gy treated mice (Figs. 3 and 4). Eventually, these thymuses became lymphomatous (Table 2). The phenotype of the lymphomas was similar to that observed in donor mice (data not shown).

The fact that the 'preleukemic thymocyte inoculate' induced or not an active thymus repopulation in the recipient was not related with the time when it was collected after irradiation.

←

Fig. 4. Examples of contour plots of two color analysis of CD4 and CD8 cell surface CD4 and CD8 expression in thymocytes from a normal mouse (A), or a mouse sacrificed 60 days after 4×1.75 Gy irradiation (B) or a mouse sacrificed 60 days after 4×1.75 Gy irradiation and bone marrow graft (c); cells were stained with FITC conjugated anti-Lyt-2 mAb and biotinylated anti-L3T4 followed by Avidin-Texas Red.

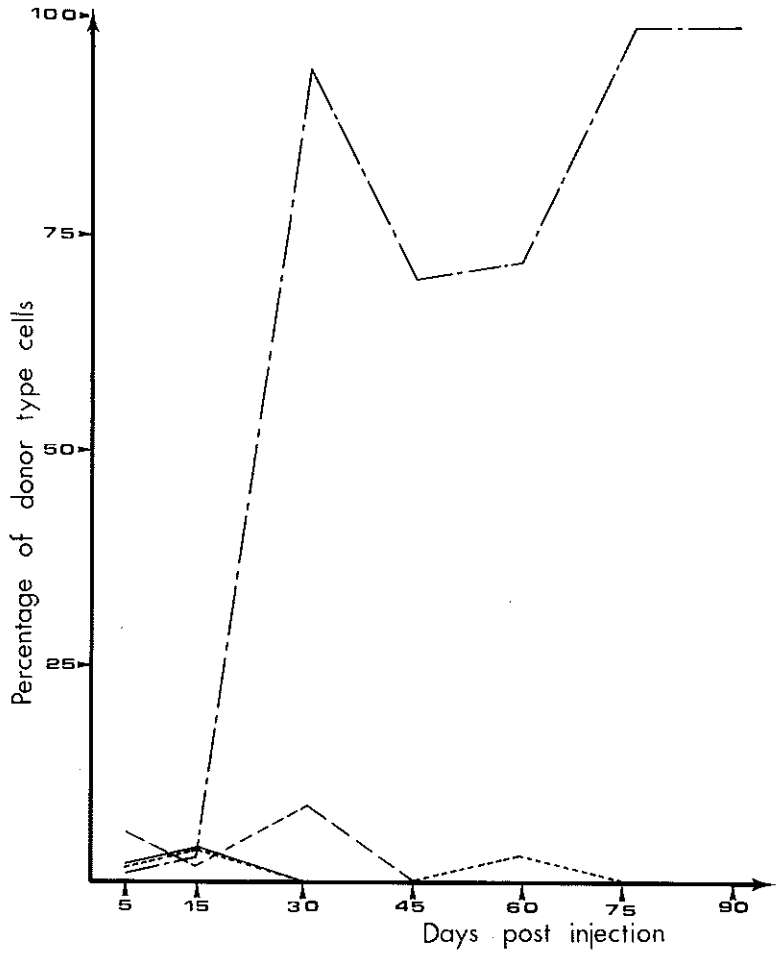


Fig. 6. Time course of donor type cells (Thy-1.2) in the thymus after injection of a 'preleukemic thymocyte inoculate'. Four Gy irradiated BL/1.1 recipients were given an intrathymic injection of 5.10^6 normal (-) or 5.10^6 irradiated thymocytes removed at various time intervals after the completion of the leukemogenic irradiation: 5 days (····), 15 days (—·—·—·—), 30 days (----). 3 recipient mice per experimental point. In the illustrated experiment, only the 15 days inoculate gave rise to an active repopulation.

In two accompanying experiments, the 'preleukemic thymocyte' suspensions were treated with anti-Thy-1.2 mAb and complement before i.t. inoculation into irradiated congenic recipients. Cell kill was about 90%. Owing the experiment, the phenotype of surviving cells was: 50–80% Thy-1+ (with a weak fluorescence), 30–50% CD8+, 30–50% CD4+, 40% IL2-R+, 35–60% Pgp-1+. After i.t. inoculation, the progeny in the recipient thymuses was compared with that of untreated inoculate. In the first experiment, the inoculate of anti-Thy-1+ C′ treated cells did not induce an active repopulation in the

Table 2. Incidence of thymic lymphomas in mice after injection of 'preleukemic thymocyte inoculates'[a]

Incidence[c]		Origin[d]		Latency period
		D	R	
PTI[b] 5 days	3/6	2	1	120 days
PTI 15 days	5/5	5	0	135 days
PTI 30 days	3/5	3	0	180 days
4×1.75 Gy	10/10			150 days

[a]The results shown here are those obtained in the same representative experiment as in Fig. 5.
[b]PTI = preleukemic thymocyte inoculate.
[c]Number of lymphomas/number of injected recipients.
[d]D = donor; R = recipient.

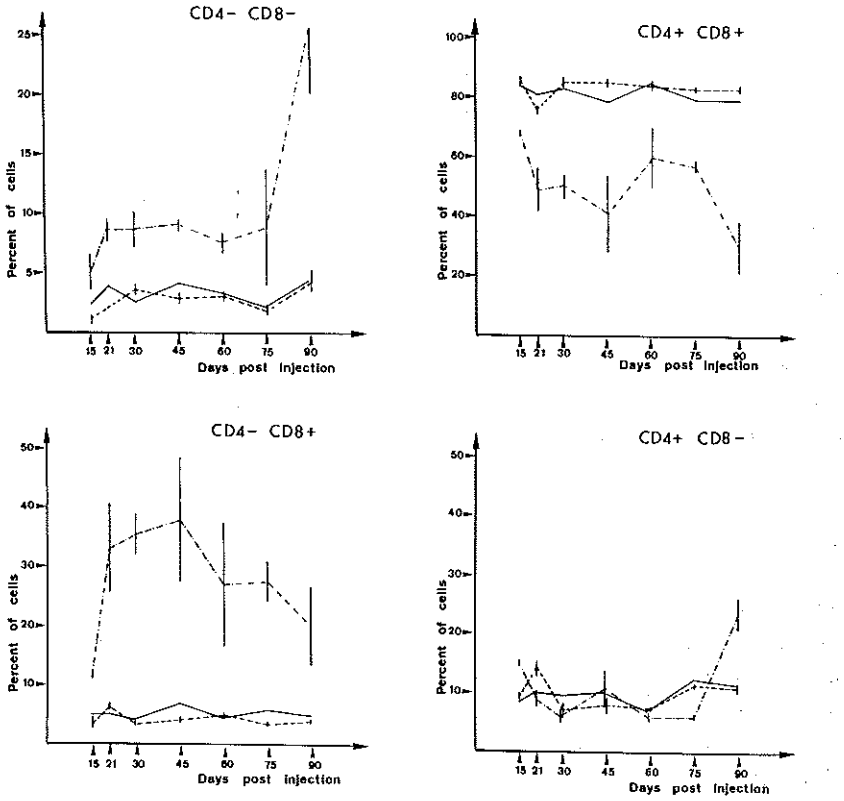


Fig. 7. Time course evolution of the four thymocyte subpopulations defined by the expression of the CD4 and CD8 markers: normal mice (——), mice injected with normal thymocytes (---) or mice injected with a 'preleukemic thymocyte inoculate' (—·—·—·—) which induced an active repopulation.

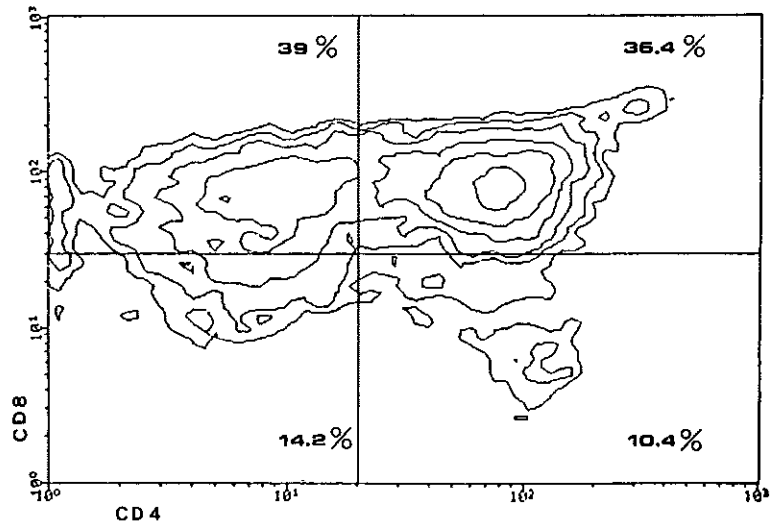


Fig. 8. Contour plots of two color analysis of cell surface expression of CD4 and CD8 expression in the thymus of mice which were injected with a 'preleukemic thymocyte inoculate' and sacrificed 45 days thereafter. Compare to Fig. 4A for controls.

thymus, although it gave rise to thymic lymphomas (data not shown). In the second experiment, as shown in Fig. 9, the 'preleukemic thymocyte' inoculate displayed a strong repopulation activity, which was inhibited by the prior anti-Thy-1 mAb +C′ treatment. However this cell population still displayed its neoplastic capacities, since the mAb treated cell inoculate also induced lymphomas. In fact, the untreated 'preleukemic cell' inoculate gave rise to 7 donor type lymphomas out of 7 injected animals, whereas the anti-Thy-1 +C′ treated cells induced lymphomas in 3 out of 5 injected mice.

Discussion

The present investigations were undertaken in order to analyze the relations of preleukemic thymocytes to the other thymocyte subpopulations during the latency period preceding the onset of lymphomas after a split dose lymphomagenic irradiation in C57BL/Ka mice.

We demonstrate strong phenotypc alterations of the thymocyte subpopulations in the irradiated animals: a decrease of CD4+ CD8+ cells and an increase of CD4− CD8− and CD4− CD8+ cells was observed. These alterations reflect a modification of the thymocyte differentiation pattern, which might be due to the emergence of an abnormal subset of thymocyte precursors. This view is supported by our results in mice, which were inoculated intra-

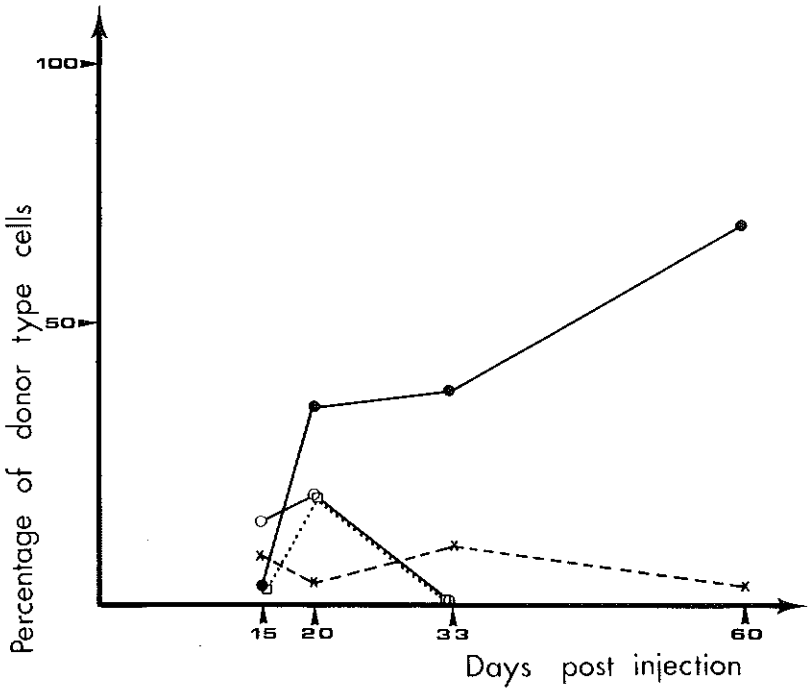


Fig. 9. Percentages of donor (Thy-1.2) cells in recipient (Thy-1.1) mice after intrathymic injection of 1.10^6 normal cells (—o—), 1.10^6 normal cells treated with anti-Thy-1.2 and C′ (· · · □ · · ·), 1.10^6 preleukemic cells (—●—) or 1.10^6 preleukemic cells treated with anti-Thy-1.2 and C′ (--x--). Results of one experiment, two recipient mice per experimental point.

thymically with 'preleukemic thymocytes'. Indeed, we observed that in many instances, the inoculation of thymus cells from mice sacrificed during the first 4 weeks after irradiation led to a very active and long lasting thymic repopulation in irradiated recipient mice. The donor type population displayed strong phenotypical alterations, similar to those described in the split dose irradiated donor mice. It is generally admitted that a normal thymus does not contain precursors with such a sustained proliferation potential; in fact, a normal thymocyte inoculate, even enriched for various types of CD4 – CD8 – precursors, only results in a very transient wave of thymocyte repopulation [27,28, 29,30]. Under normal conditions, the short-lived intrathymic thymocyte precursors are continually replaced by bone marrow derived prothymocytes [31].

The present study thus indicates that fractionated irradiation induces abnormal thymocyte precursors in most of treated animals. It is likely that similar cells emerge also in other experimental conditions. For example, it has been shown that thymic grafts in thymectomized 4×1.75 Gy irradiated mice are not

constantly repopulated by marrow cells from the recipients [32] as it is generally the case when thymic lobes are grafted in mice which have received a single sublethal irradiation [33,34,35]; in fact, they are most often composed of donor (graft) type cells, which have acquired some self renewing capacity since they persist in the graft until they eventually give rise to lymphomas [32]. More recently, Ezine [36] observed the occurrence of a self renewing population of precursors in the thymus of mice which had been injected with a 'stem cell enriched' marrow preparation. Further studies will have to demonstrate whether the abnormal populations are similar in all these experimental conditions.

Our phenotypic studies strongly indicate, that the newly formed abnormal precursors, i.e. with a sustained self renewal capacity, give rise to an abnormal progeny, with a drop of the CD4+ CD8+ thymocytes and an increase of CD4− CD8− and CD4− CD8+ cells. It is interesting to notice that this pattern resembles those observed in cultured thymic rudiments: under these conditions, there was always an rather low frequency of CD4+ CD8+ cells and a high frequency of CD4− CD8− and CD4− CD8+ cells [37,38]. A similar tendency towards an increased proportion of CD4− CD8+/CD4+ CD8− was also described in chimeras, in which the progeny of a single pre T cell clone was analyzed [39]. It was also observed in mice which had been treated at birth with an anti-Ia monoclonal antibody [40].

The interpretation of all these observations is presently difficult because we still lack some knowledge on several aspects of thymic lymphopoiesis. The observed phenotypes might be related to the thymocyte precursors themselves, i.e., that only precursors for the observed subpopulations are present or might be due also to the microenvironment, where the precursors give rise to a progeny. With respect to this latter hypothesis, it is interesting to observed that in the above mentioned experimental systems [37,38,39,40], as in ours, the thymus microenvironment may have been altered to some extent, either by the culture condition (deoxyguanosine . . .), or by the in vivo irradiation, or by the treatment with anti-Ia monoclonal antibodies. In our system, it is well known that fractionated irradiation damages the thymic microenvironment, and particularly the epithelial component of thymic nurse cells [5,41]. The other possibility would relate the observed phenotypical alterations to the quality of the CD4−CD8− thymocyte precursors themselves. In fact, these cells constitute an heterogenous population in normal animals, with various phenotypes and several types of progeny [42,43,44,45]. It is therefore crucial to define more accurately the population of double negative cells in the preleukemic thymuses. Ongoing studies are now in progress, by using three color fluorescence and cell sorting, in order to analyze the expression of IL2-R, Pgp-1, CD3 and the rearrangements and expression of the various chains of TCR in the preleukemic CD4−CD8− cells. Similar studies on the CD4−CD8+ cells, which are more frequent in preleukemic thymuses, might also be of importance, since a small

class of thymocytes with this phenotype is immature and gives rise to the $\gamma\,\delta$ + peripheral cells. Thus, at the present time, one cannot conclude whether the abnormal thymocyte phenotypes observed in preleukemic irradiated mice are related to abnormal thymocyte precursors, or to altered microenvironment, or to both abnormalities simultaneously.

Our observations tend to indicate that these radiation-induced abnormal precursors are not identical to preleukemic cells. In fact, there is no absolute correlation between the sustained repopulation capacity and the lymphomagenic activity, as demonstrated by our observations on the fate of 'preleukemic thymocyte' inoculate in recipient thymuses. We showed that some inoculates repopulated the thymus actively, i.e. more than a normal one does, whereas others did not display such a repopulation capacity. Yet, all inoculates induced donor type lymphomas. Thus, the data suggest that fractionated irradiation induced two different abnormal populations in the thymus, the first one displaying lymphomagenic activity *without* repopulation capacities, the second one showing both abnormalous functions. One of our experiments of cell depletion by anti-Thy-1.2 mAb and complement suggests that the two populations differ by the level of Thy-1 expression: the repopulation activity is found among the cells which are susceptible to the mAb mediated cytotoxicity and thus can be considered as highly Thy-1 expressing cells [26]; by contrast the preleukemic cells can be found also among the cells which resist to the treatment by anti-Thy-1 + C', and thus express low levels, if some, of Thy-1 antigens. Preleukemic cells, or at least some of them might thus be more immatures than with repopulation capacities. However, it is certainly not possible at the present time to localize these two abnormal populations along the intrathymic T cell differentiation pathway. Further studies with more sophisticated methods, such as three color analysis or cell sorting, and markers such as CD3 and TCR chains are in progress for this purpose.

An other question is the possible relation between the two abnormalous cell populations. As above recalled, preleukemic activity is found in all cell inoculates, whereas abnormalous thymocyte precursors are observed in about half of cases. Does this mean that this latter population appears only seldomly whereas the former one is a constant effect of fractionated irradiation? Although this hypothesis is possible, we tend to believe that both activities are always present, but are not systematically detected for technical reasons. In fact, the abnormal phenotypical pattern observed in the progeny of the abnormal precursors, when they are detected by the repopulation experiments (Figs. 6–7), are always found in the 4×1.75 Gy irradiated mice (Fig. 3–5). Whether there is any filiation between the two populations is thus possible, but has to be demonstrated.

One has also to raise the question of the mechanisms by which irradiation induces the two abnormalous populations, that we have described. It is most

likely that they derive from radio-resistant thymocytes, probably of the intrathymic pool of T cell precursors. Their emergence might be facilitated by the extreme and long-lasting depletion of bone marrow prothymocytes, which has been widely described in 4×1.75 Gy irradiated preleukemic animals [4,10,12]. The surviving thymic T-cell precursors would then ensure thymic repopulation until more primitive stem cells regenerate in bone marrow and migrate to the thymus, what would explain that the phenotype of late preleukemic thymuses are ultimately the same as those observed in control animals. Whether this is sufficient to explain why the surviving thymic precursors display an apparent self renewal capacity is not certain. It is possible that these cells are damaged by irradiation either directly or, and this seems to us more likely, indirectly as the result of alterations of cell-to cell communications, as suggested by the damage to thymic epithelium that we have previously described [5].

We do not know much about the preleukemic cells, which are induced by the split dose regimen of irradiation. As previously discussed [46,47], these cells are not yet fully neoplastic, since they are strictly dependent upon thymus microenvironment for progression towards neoplastic growth. The mode of induction is still unknown, although it is well established that it can be indirect, as shown by the already mentioned experiments of thymic graft in 4×1.75 Gy irradiated thymectomized mice [48]. The present study suggest that they might belong to the most immature cell among thymocytes, since they are found in the low Thy-1 expressing population. It must be mentioned that in other closely related experimental systems, preleukemic cells have been ascribed to the bone marrow prothymocyte population [46,49]. This is of course compatible with the fact that all lymphomas express significant levels of Thy-1 antigens. Our data bring furthermore some informations on their kinetics properties: preleukemic cells obviously can persist for several months in so small numbers that they cannot be detected by flow cytometry. Whether they are in a dormant state or engaged in an asymmetrical pattern of division is still unknown.

The phenotypic alterations of thymocyte subpopulations are not observed in mice which had been grafted with normal bone marrow cells within a couple of hours after completion of the split dose irradiation. Thymuses were indeed rapidly repopulated by donor type cells, and displayed a normal distribution of thymocyte subpopulations. Interestingly, such a bone marrow graft restores also the functions of the epithelial component of thymic nurse cells [5,41]. Furthermore, although it does not prevent the induction of preleukemic cells by fractionated irradiation, it results in their disappearance, and hence inhibits the onset of lymphomas [5]. Thus, the abnormal radiation – induced thymocyte subpopulations, i.e. abnormal thymocyte precursors and thymus preleukemic cells, are overcome by the influx of normal prothymocytes and by their progeny. That quantitative factors are involved in this competition, is sug-

gested by the fact that low numbers of grafted marrow cells do not repopulate the thymus sufficiently and do not prevent the development of lymphomas (Boniver and Declève unpublished data; [12]). The mechanism by which the marrow graft inhibits tumours development is still poorly known and is the object of other investigations [50].

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