

and CM07302, CA70095, and CA01709 (National Cancer Institute) from the National Institutes of Health, Department of Health and Human Services.

We thank Mary Duerr and Beth Hoker for data collection, Lisa Hurowitz, Hong Su, and Shirley George for the analysis of plasma samples, Terri Simanek for the preparation of the manuscript, the medical and nursing staffs at The Johns Hopkins Oncology Center and the General Clinical Research Center at The Johns Hopkins Hospital who provided care and support for the patients in this study, and Sharyn Baker for comments on an earlier draft of this manuscript.

Presented in part at the 30th Annual Meeting of the American Society of Clinical Oncology, Dallas, TX, May 14-17, 1994; and at the 8th National Cancer Institute-European Organization for Research and Treatment of Cancer Symposium on New Drugs in Cancer Therapy, Amsterdam, The Netherlands, March 15-18, 1994.

Manuscript received November 7, 1995; revised February 27, 1996; accepted March 19, 1996.

Prevention of Murine Radiogenic Thymic Lymphomas by Tumor Necrosis Factor or by Marrow Grafting

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Background: Split-dose irradiation (1.75 Gy given weekly for 4 weeks) of C57BL/Ka mice induces the emergence of preleukemic cells (PLCs). These cells develop into leukemic cells after a latency period of 3-6 months. The survival and transformation of PLCs are dependent on radiation-induced alterations of the thymic epithelium and of resident lymphocyte (i.e., thymocyte) subpopulations in the thymus. PLCs can be eliminated, concomitantly with the restoration of the thymus, by grafting bone marrow cells immediately after the last irradiation. Our hypothesis was that any agent able to restore the thymus after leukemogenic irradiation would exert the same effects as a bone marrow graft. Tumor necrosis factor- α (TNF- α) is one such possible agent, since it has been shown to modulate some functions of the thymic epithelium and thymocyte subpopulations. **Purpose:** The goal of this study was to assess the ability of repeated intraperitoneal injections of TNF- α to functionally replace bone marrow transplantation in the restoration of normal intrathymic lymphopoiesis and in the prevention of thymic lymphomas in split-dose-irradiated mice. **Methods:** We replaced the bone marrow graft with repeated injections of TNF- α (25 000 U/injection) in the split-dose-irradiated (4×1.75 Gy) C57BL/Ka mouse model. We analyzed the expression of the cell differentiation markers CD4 and CD8 on thymocytes by flow cytometry. We also studied the thymic environment by isolating thymic nurse cells, the bone marrow prothymocyte activity by analyzing thymic repopulation, and the evolution

of PLCs by an in vivo transplantation assay. Local production of TNF- α after bone marrow grafting was examined by in situ hybridization. Injections of anti-TNF- α antibodies were given to split-dose-irradiated mice to test the effect of neutralizing TNF- α in vivo. One-way analysis of variance and Newman-Keuls two-tailed tests were used to test statistical significance. **Results:** Multiple injections of TNF- α into split-dose-irradiated mice did not influence bone marrow prothymocyte activity but restored thymocyte subpopulations and thymic epithelium, induced the disappearance of PLCs, and prevented the development of lymphomas. Moreover, a bone marrow graft significantly stimulated intrathymic production of TNF- α messenger RNA ($P < .01$), and anti-TNF- α antibodies partially inhibited the antilymphomatous effects of bone marrow graft in split-dose-irradiated mice ($P < .05$). **Conclusion:** These data strongly suggest that TNF- α is a mediator that is involved in the mechanisms by which bone marrow transplantation functions to prevent thymic lymphomas in split-dose-irradiated mice. **Implications:** Cytokines might be used in some biological systems, particularly in the hemopoietic system, as a therapeutic agent for the secondary prevention of cancer. [J Natl Cancer Inst 1996;88:824-31]

Fractionated whole-body x irradiation induces a 90% incidence of thymic lymphomas in C57BL/Ka mice. Tumors are preceded by a 3-9 month preleukemic period (1,2), during which preleukemic cells (PLCs) appear (3). These cells require the thymus for their neoplastic transformation (4-6). As a result of the radiation treatment, thymic lymphopoiesis is profoundly altered, the capacity of bone marrow prothymocytes to repopulate the thymus is markedly reduced (3,7,8), the

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balance of thymocyte subpopulations is disturbed (9,10), and some functions of the thymic epithelium are modified (6).

When mice receive bone marrow grafts immediately after irradiation, the development of radiation-induced lymphomas is prevented (3,8,11). This treatment does not inhibit the emergence of PLCs but rather induces their disappearance 1-2 months later (6). The grafted lymphoid precursors actively repopulate the thymus (1,3,8), giving rise to normal thymocyte subpopulations (10) and bringing about the restoration of thymic stromal cells (6).

If, however, the mice do not receive bone marrow grafts until 1 month after irradiation, the development of lymphomas is not prevented (11). Under these conditions, grafted precursors actively repopulate the thymus, but the thymic epithelium and thymocyte subsets do not become normal again (12).

From these observations, it appears that the long-term persistence and neoplastic transformation of the PLCs are associated with irreversible alterations of the thymic lymphopoiesis.

A possible mechanism to explain the effects of bone marrow transplantation might be that precursors, after seeding the thymus, induce a local production of cytokines that can then act on thymic lymphopoiesis and PLCs. This hypothesis is supported by previous observations (13) that intraperitoneal injections of exogenous interferon gamma (IFN γ) or tumor necrosis factor- α (TNF- α) into split-dose-irradiated mice inhibited development of thymic lymphomas, mimicking the effects of bone marrow grafting.

In this study, we have extended our research regarding the ability of TNF- α to inhibit the development of lymphomas in split-dose-irradiated mice. We have examined the evolution of PLCs and the different thymocyte subpopulations after TNF- α treatment. The TNF- α production has been quantified in the thymuses of irradiated mice that received bone marrow grafts. The effect of repeated injections of anti-TNF- α antibodies on the lymphoma development was also studied.

Materials and Methods

Mice

One- to 2-month-old male and female C57BL/Ka mice and congenic Thy-1.1 C57BL/Ka mice (called

BL/1.1 for convenience) were used, which were developed by and obtained from M. Lieberman (Stanford University, CA). They were raised in our animal colony. C57BL/Ka and BL/1.1 mice bear the Thy-1.2 and the Thy-1.1 alleles, respectively. Animal care was provided in accordance with procedures outlined in the "Law for Care and Use of Laboratory Animals" (Arrêté Royal-November 14, 1993, Belgium).

Irradiation

For lymphoma induction, mice received whole-body irradiation of 1.75 Gy once each week for 4 weeks. The irradiation was delivered by an x-ray apparatus (Stabilivolt; 190 kV, 18 mA, HVL of 0.5 mm Cu, and focal distance of 35 cm; Siemens, Berlin, Federal Republic of Germany) at a dose rate of 1.6 Gy/minute.

For PLC detection, recipient animals were given a single whole-body irradiation of 4 Gy at 0-4 hours before cell inoculation.

Treatment With TNF- α

Human recombinant TNF- α (rTNF- α) (10^7 U/mg) and recombinant murine rTNF- α (rMuTNF- α) (1.2×10^7 U/mg), produced by Genentec, Inc. (South San Francisco, CA) and provided by Boehringer Ingelheim International (Brussels, Belgium), were diluted in RPMI-1640 medium supplemented with 10% fetal calf serum. During a 6-week period, mice were given an intraperitoneal injection three times per week of 2.5×10^5 U of human rTNF- α in 200 μ L of RPMI-1640 medium or 2.5×10^4 U of murine rTNF- α in 200 μ L of RPMI-1640 medium, with the first injection being administered 1 hour after the last 1.75-Gy irradiation.

Treatment With Anti-TNF- α Antibodies

Rabbit anti-TNF- α antibodies (14) were produced and provided by D. G. Grau (World Health Organization, Immunology Research and Training Center, Department of Pathology, University of Geneva, Switzerland). Ten days before the last irradiation of 1.75 Gy and the first injection of antibodies, the mice were intraperitoneally injected with 2.5 mg of rabbit immunoglobulins (Igs) (Jackson Immunoresearch Laboratories, West Grove, PA) diluted in 200 μ L of RPMI-1640 medium. During a 9-week period, mice were given an intravenous injection once per week of 0.5 mg of anti-TNF- α antibodies that had been diluted in 100 μ L of RPMI-1640 medium, with the first injection being administered 1 hour after the last irradiation. Control mice were injected with the same dose of rabbit Igs, but the anti-TNF- α antibodies were replaced by RPMI-1640 medium alone.

Immunofluorescence Staining

We used fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibody (MAb) (clone 53-6-72) (Becton Dickinson, Sunnyvale, CA), R-phycoerythrin (PE)-conjugated anti-CD4 MAb (Becton Dickinson), FITC-conjugated anti-Thy-1.2 MAb (clone 30H12; Becton Dickinson), and anti-Thy-1.1 MAb (clone HO-22-1 obtained from the American Type Culture Collection, Rock-

ville, MD) (15) followed with an FITC-conjugated goat anti-mouse immunoglobulin M serum (Nordic, Leuven, Belgium). Immunofluorescence staining was performed as previously described (10). Cell suspensions were analyzed on a fluorescence-activated cell sorter (FACS IV, Becton Dickinson).

Assay of Bone Marrow Prothymocyte Activity

Aliquots (200 μ L) of RPMI-1640 medium containing 5×10^6 bone marrow cells collected from BL/1.1 mice (Thy-1.1) were intravenously injected into C57BL/Ka mice (Thy-1.2) within 2 hours following a single 4-Gy exposure. Twenty, 30, 45, and 60 days later, three recipients per group were killed, and thymus cell suspensions were incubated with monoclonal anti-Thy-1.1 and anti-Thy-1.2 antibodies, followed by an FITC-labeled second-stage antibody.

Thymus Dissociation and Preparation of Thymic Nurse Cells (TNCs)

TNCs were isolated from five thymuses. The tissues were minced with scissors and washed for 15 minutes in phosphate-buffered saline at room temperature. The fragments were dissociated by repeated incubations in the presence of dispase, collagenase, and deoxyribonuclease I (Boehringer Mannheim, Brussels, Belgium) at 37 °C. TNCs were isolated from the resulting suspensions by sedimentation at 1 g on ice. Each step yielded a supernatant and a pellet. The cell pellets in each case were resuspended and resedimented until a satisfactory enrichment with TNC was obtained (i.e., four or five cycles) (16). After the isolation procedure, the cell number in each fraction was scored with a Malassez's plate and the number of TNCs per thymus was then calculated.

In Vivo Assay for Detection of PLCs

Briefly, thymuses of 4×1.75 -Gy-irradiated mice were removed at several time intervals after the last irradiation. Aliquots of 5×10^6 thymocytes in 100 μ L RPMI-1640 medium were injected intrathymically to ether-anesthetized irradiated (i.e., 4 Gy to the whole body) congenic recipient mice (6).

TNF- α Probe

The PSTI-PSTI fragment from the complementary DNA clone pGEM-1mTNF contains all of the coding sequences for murine TNF- α . This vector was provided by W. Fiers (Gent University, Gent, Belgium). Linearized plasmids were used as templates for the in vitro synthesis of RNA probes (1644 base pair for the coding sequence) complementary to TNF- α messenger RNA (mRNA) (antisense probe). RNA was also transcribed from the opposite direction (sense probe) and used as a negative control. These probes were labeled by random priming using SP6 or T7 RNA polymerases and 35 S-labeled uridine triphosphate according to the manufacturer's recommendations (Boehringer Mannheim, Mannheim, Federal Republic of Germany). Approximately 10^8 cpm were incorporated into RNA per microgram of DNA template.

In Situ Hybridization

Cryosections of thymuses (5-6 μm) were placed on glass slides that had been coated with poly-L-lysine (Sigma, Deisenhofen, Federal Republic of Germany). The tissue sections were then fixed in paraformaldehyde (4% in phosphate-buffered saline) containing 20-mM vanadyl ribonucleosides complexes (Sigma) and 5 mM MgCl_2 for 15 minutes at room temperature. After treatment with 0.25% Triton X-100 in water (Sigma), sections were incubated with the TNF- α antisense riboprobe as previously described (15). As negative controls, slides were pretreated in a solution containing 20 $\mu\text{g}/\text{mL}$ ribonuclease A (Boehringer Mannheim) or the antisense probe was replaced by the sense probe. After washing and hybridization, the slides were autoradiographed and stained with hematoxylin-eosin (UCB, Leuven, Belgium). We considered as positive cells (i.e., cells expressing TNF- α mRNA) those cells that had more than eight silver grains per

cell. At different stages, three thymuses were analyzed per group.

Statistical Analysis

One-way analysis of variance and Newman-Keuls two-tailed tests were performed with the Instat Mac software (Graph Pad software, San Diego, CA).

Results

PLCs and Lymphoma Incidence

To determine whether TNF- α can induce the disappearance of PLCs, we compared the evolution of PLCs and the incidence of lymphomas in split-dose-irradiated mice that were either treated or not treated with TNF- α .

PLCs were detected in all irradiated animals (Table 1). Their leukemogenic potential increased with time: five (41.7%) of the 12 animals receiving injections of PLCs collected 9 days after a leukemogenic irradiation developed a thymic lymphoma, whereas this percentage reached 85% (i.e., 17 of 20 animals receiving injections of PLCs) when the preleukemic suspensions were derived from mice that were irradiated 60 days before ($P < .0001$).

After TNF- α injections in irradiated mice, PLCs were detected in all animals on days 9, 16, 20, and 27 (Table 1). Their leukemogenic potential did not increase significantly ($P > .1$) with time. PLCs from

Table 1. Incidence of lymphomas and preleukemic cells (PLCs) in mice after a split-dose irradiation, followed or not by repeated injections of rMuTNF- α

| Experiment No. | Incidence of lymphomas | Time interval (d) after split-dose irradiation | Individual thymocyte suspension | PLC activity in cell suspensions collected at various time intervals after last irradiation | | No. of donor mice with PLCs/No. of donor mice |
|---|-----------------------------|--|---------------------------------|---|--|---|
| | | | | Incidence* | Lymphomas No. of lymphomas/ No. of injected recipients Percentage | |
| <i>Split-dose irradiated mice</i> | | | | | | |
| 1 | 14/15 | Day 9 | A | 2/4 | 5/12 | 41.7 |
| 2 | 14/15 | | B | 2/5 | | |
| 3 | 13/15 | | C | 1/3 | | |
| 4 | 10/10 | Day 16 | A | 3/4 | 6/10 | 60.0 |
| 5 | 9/10 | | B | 2/3 | | |
| 6 | 9/10 | | C | 1/3 | | |
| Total | 69/75 (91.9% \pm 4.5%) | Day 20 | A | 3/4 | 15/18 | 83.3 |
| | | B | 4/5 | | | |
| | | C | 3/4 | | | |
| | | Day 27 | D | 5/5 | 15/21 | 71.4 |
| | | | A | 5/5 | | |
| | | | B | 3/5 | | |
| | | Day 60 | C | 3/5 | 17/20 | 85.0 |
| | | | D | 4/6 | | |
| | | | A | 6/6 | | |
| | | | B | 3/4 | | |
| | | | C | 5/6 | | |
| | | | D | 3/4 | | |
| <i>Split-dose-irradiated mice that were treated with rMuTNF-α</i> | | | | | | |
| 1 | Not tested | Day 9 | A | 2/5 | 4/11 | 36.4 |
| 2 | Not tested | | B | 1/2 | | |
| 3 | 2/15 | | C | 1/4 | | |
| 4 | 3/10 | Day 16 | A | 2/3 | 5/9 | 55.6 |
| 5 | 1/10 | | B | 1/2 | | |
| 6 | 1/10 | | C | 2/4 | | |
| Total | 6/45 (13.3% + 9.5%) | Day 20 | A | 3/4 | 9/17 | 52.9 |
| | | B | 1/4 | | | |
| | | C | 4/4 | | | |
| | | Day 27 | D | 1/5 | 3/13 | 23.1 |
| | | | A | 1/3 | | |
| | | | B | 1/6 | | |
| | | Day 60 | C | 1/4 | 2/19 | 10.5 |
| | | | A | 0/4 | | |
| | | | B | 0/5 | | |
| | | | C | 2/4 | | |
| | | | D | 0/6 | | |

*Number of lymphomas/number of recipients given injections.

mice that had been irradiated 27 days earlier and treated with TNF- α were observed to induce lymphomas in three (23.1%) of the 13 recipient animals, whereas PLCs from mice that had only been irradiated were found to induce tumors in 15 (71.4%) of the 21 recipient animals ($P < .01$). Later, PLCs disappeared from most of the TNF- α -injected mice, on day 60 after the irradiation. Only one (25%) of the four TNF- α -treated donor mice contained PLCs in the thymus (thymic lymphomas were induced by these PLCs in two [10.5%] of the 19 recipient animals); by contrast, all four untreated donor mice contained PLCs in the thymus (thymic lymphomas were induced by these PLCs in 17 [85%] of the 20 recipient animals).

As expected, there was a significant decrease ($P < .0001$) in the incidence of lymphomas after TNF- α treatment (mean incidence \pm standard deviation: 13% \pm 9.5%) compared with control mice (mean incidence \pm standard deviation: 92% \pm 4.5%).

As was found in irradiated mice that had been treated by bone marrow transplantation, TNF- α injections did not inhibit the appearance of PLCs but induced their disappearance.

Thymocyte Subpopulations

Since the balance between different thymocyte subpopulations, as defined by the expression of CD4 and CD8 molecules, is a reflection of normal intrathymic differentiation, we compared the CD4 and CD8 phenotypes of thymocytes from normal and preleukemic thymuses.

Irradiated mice, treated or not by the administration of rMuTNF- α , were killed on days 10, 15, 30, 45, 60, and 90 after the last irradiation. For the three experiments performed, three to five mice were used for each time point. The results of a representative experiment are shown in Fig. 1.

The most striking phenotypic modifications generally occurred 30 days after the last irradiation. There was a decrease of the CD4+/CD8+ thymocytes concomitantly with an increase of CD4-/CD8- cells. The proportion of CD4-/CD8+ cells also increased, but to a lesser extent. In most of the animals, these alterations persisted during the preleukemic period.

These alterations were also detected in irradiated mice treated with rMuTNF- α .

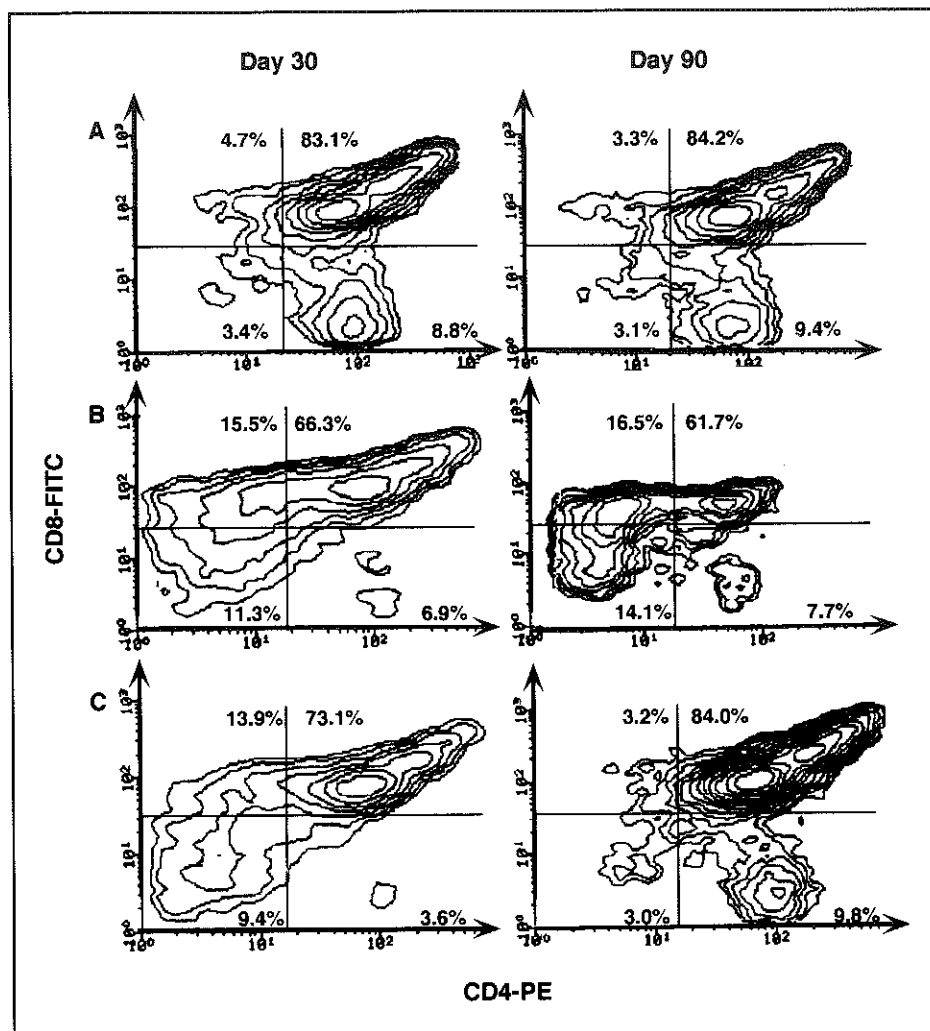


Fig. 1. Example of contour plots of two-color analysis of CD4 and CD8 expression in thymocytes from a normal mouse (A), a split-dose-irradiated mouse (B), or a split-dose-irradiated mouse treated with rMuTNF- α (C). The mice were killed 30 or 90 days after 4×1.75 -Gy irradiation. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibody and with phycoerythrin (PE)-conjugated anti-CD4 monoclonal antibody. Quadrants of each plot are defined as follows: lower left = CD4-/CD8-, lower right = CD4+/CD8-, upper left = CD4-/CD8+, and upper right = CD4+/CD8+.

They were, however, reversible in most of the animals (85% \pm 5% [17 of the 20 TNF- α -treated animals] for the three experiments): the depleted CD4+/CD8+ thymocyte population started to increase again by day 45 and reached values identical to those of control animals on day 90. Similarly, the other thymocyte populations started to be restored on day 45 and reached control values on days 60 and 90.

It therefore appears that TNF- α injections can restore a normal pattern of intrathymic differentiation in split-dose-irradiated mice.

Prothymocyte Activity in TNF- α -Treated Irradiated Mice

Experiments were performed to test whether the restoration of thymocyte sub-

populations in split-dose-irradiated, TNF- α -treated mice was due to the recovery of bone marrow prothymocyte activity. C57BL/Ka mice that had received a 4-Gy radiation dose were injected with bone marrow precursors from BL/1.1 mice. The bone marrow cells were collected from nonirradiated mice or from split-dose-irradiated mice, treated or not treated with TNF- α at 20, 42, and 60 days after the last irradiation. Another group of recipients was injected with bone marrow cells collected from normal mice that had received injections of TNF- α . The percentage of donor thymocytes (Thy-1.1) was scored at several time intervals after the bone marrow graft (three independent experiments and three mice per group in each experiment). The percentage of cells

derived from the bone marrow of control mice reached 90% ($\pm 2.9\%$) from day 30 after the irradiation (Fig. 2). The bone marrow cells from normal mice treated with TNF- α had the same capacity of thymus repopulation ($P = .7$).

The bone marrow prothymocyte activity was drastically reduced in split-dose-irradiated mice: the percentage of donor thymocytes reached less than 20% on day 60 after the injection ($P < .001$). The prothymocyte activity was not restored after treatment with rMuTNF- α : the percentage of donor cells in the thymus never reached more than 40%.

The restoration of thymocyte subpopulations in split-dose-irradiated mice treated by TNF- α is not due to a restoration of prothymocyte activity but rather to a direct effect of TNF- α on the thymus.

Thymic Nurse Cells

TNCs are lymphoepithelial complexes involved in thymocyte differentiation. The number of TNCs isolated from a thymus reflects the capacity of epithelial cells to establish functional interactions with thymocytes (6,16). After a leukemogenic irradiation, epithelial cells lose this capacity that was restored by a bone marrow graft (6). We then tested whether TNF- α also has an effect on TNCs. TNCs were isolated from control and irradiated mice treated or not treated with rMuTNF- α . In split-dose-irradiated mice, numbers of TNCs were very low during the pre-leukemic period. After TNF- α treatment, the number of TNCs increased 45-60 days after the last irradiation but never reached the level observed in the control mice (Table 2).

Increase of Intrathymic TNF- α mRNA Transcripts After Leukemogenic Irradiation and Bone Marrow Graft

Since TNF- α and bone marrow graft exert similar effects in split-dose-irradiated mice, we tested for the presence of TNF- α mRNA in the thymus after bone marrow grafting in two independent experiments by *in situ* hybridization.

In the thymus of normal and split-dose-irradiated adult mice, very few cells contained TNF- α transcripts. After marrow grafting, there was an increase ($P < .01$) in the number of TNF- α positive cells 15 or 30 days after treatment (Table 2).

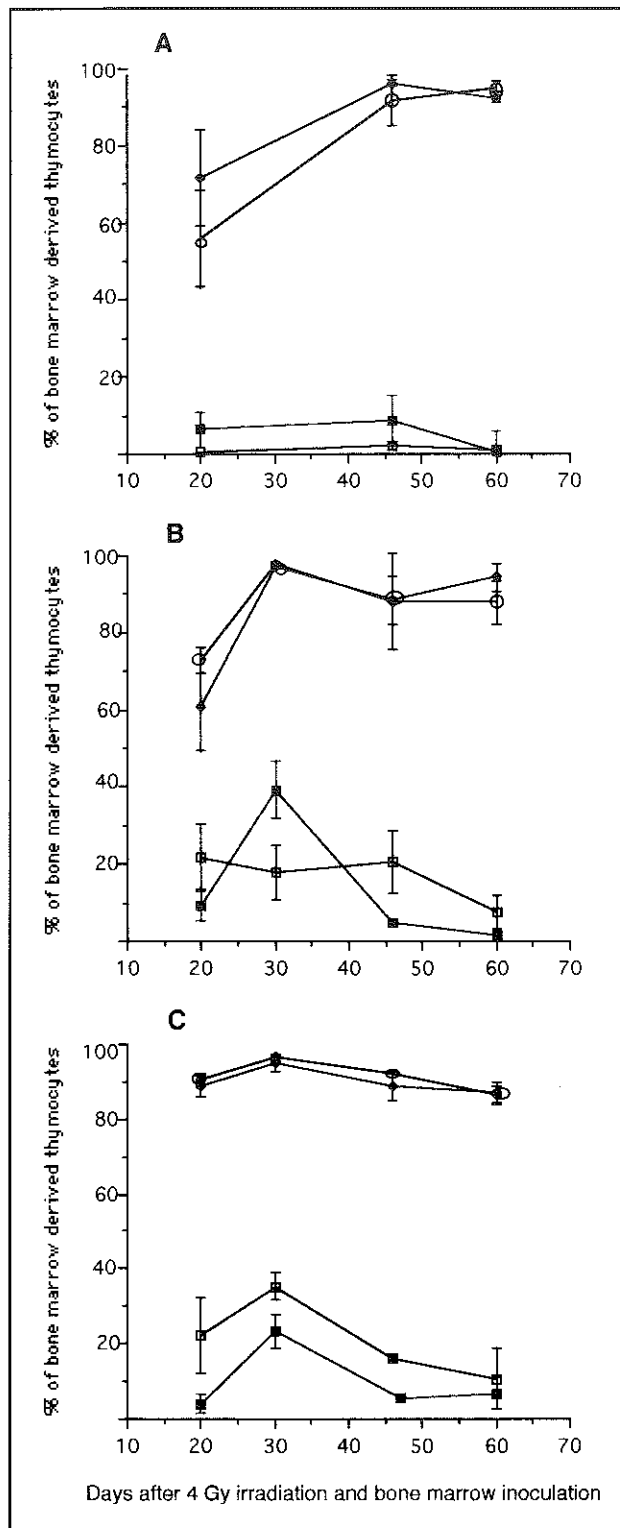


Fig. 2. Prothymocyte activity in bone marrow of tumor necrosis factor- α -treated mice. Evolution of donor type thymocytes in the 4-Gy irradiated mice after injection of donor bone marrow cells collected from split-dose-irradiated mice 20 (A), 42 (B), and 60 (C) days before (open squares) and from rMuTNF- α -treated split-dose-irradiated mice (black squares). The prothymocyte activities in bone marrow are also presented for nonirradiated mice treated (black circles) or not (open circles) with rMuTNF- α .

Lymphoma Incidence After Leukemogenic Irradiation, Bone Marrow Graft, and Injections of Anti-TNF- α Antibodies

To demonstrate a role for TNF- α in the prevention of radiogenic lymphomas after bone marrow grafting, we tested the incidence of thymic lymphomas in split-

dose-irradiated, bone marrow-grafted mice treated or not treated with anti-TNF- α antibodies in two independent experiments. Antibodies to TNF- α partially restored the leukemogenicity of PLCs in mice receiving marrow grafts; the incidence of lymphomas reached 35% ($\pm 7\%$) (seven of 20 treated mice) and was

Table 2. Comparison of the effects of tumor necrosis factor- α (TNF- α) treatment and bone marrow grafting in split-dose-irradiated mice*

| | Control mice | | Irradiated mice | | Irradiated mice treated with TNF- α | | Irradiated mice grafted with bone marrow | |
|---|-----------------|-----------------|-----------------|-----------------|--|--------------|--|------------------|
| | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 |
| <i>No. of thymic nurse cells</i> | | | | | | | | |
| Day 5 | 20 000 | 19 563 | 1900 | 1500 | 1800 | 1700 | nt | nt |
| Day 10 | 19 800 | 18 520 | 3600 | 2100 | 1800 | 1650 | nt | nt |
| Day 15 | 19 500 | 18 650 | 4685 | 2800 | 833 | 1100 | nt | nt |
| Day 30 | 14 480 | 16 420 | 2800 | 2500 | 1933 | 2100 | nt | nt |
| Day 45 | 18 400 | 17 000 | 1765 | 2100 | 2250 | 3980 | nt | nt |
| Day 60 | 17 000 | 16 230 | 2750 | 2160 | 8940 | 7980 | nt | nt |
| Day 90 | 16 900 | 16 500 | 1500 | 1800 | 9920 | 10 890 | nt | nt |
| <i>No. of TNF-α mRNA-positive cells/mm² (mean \pm standard deviation)</i> | | | | | | | | |
| Day 15 | 1.40 \pm 0.12 | 1.31 \pm 0.21 | 4.10 \pm 0.95 | 2.91 \pm 0.21 | nt | nt | 8.92 \pm 1.10† | 3.42 \pm 0.35 |
| Day 30 | 1.30 \pm 0.21 | 1.52 \pm 0.32 | 1.30 \pm 0.21 | 1.11 \pm 0.31 | nt | nt | 2.32 \pm 0.36 | 8.79 \pm 2.45† |
| Day 45 | 1.29 \pm 0.24 | 0.90 \pm 0.11 | 1.25 \pm 0.22 | 1.60 \pm 0.32 | nt | nt | 3.63 \pm 0.26 | 2.12 \pm 0.41 |
| Day 60 | 0.60 \pm 0.31 | nt | 0.70 \pm 0.21 | nt | nt | nt | 0.40 \pm 0.11 | nt |
| Day 75 | 0.75 \pm 0.31 | nt | 0.40 \pm 0.31 | nt | nt | nt | 0.40 \pm 0.12 | nt |
| Day 90 | 1.91 \pm 0.22 | 0.90 \pm 0.24 | 0.80 \pm 0.15 | 1.21 \pm 0.21 | nt | nt | 0.81 \pm 0.21 | 0.42 \pm 0.20 |

*nt = not tested.

†Significant difference with the control mice ($P < .01$).

significantly different ($P < .05$) from that observed in split-dose-irradiated, bone marrow-grafted mice (three [6.84% \pm 2.61%] of the 43 treated mice developed lymphomas). There was no significant difference ($P > 1$) in the tumor incidence observed in this group given leukemogenic irradiation and a bone marrow graft and the control group given leukemogenic irradiation, an injection of rabbit Igs, a bone marrow graft, and repeated injections of RPMI-1640 medium (one lymphoma [5.9% \pm 2.1%] for 17 treated mice). These experiments indicated that anti-TNF- α antibodies partially inhibited the antilymphomatous effects of bone marrow graft in split-dose-irradiated mice.

Discussion

In this report, we confirm that exogenous rTNF- α inhibits thymic lymphoma development in split-dose-irradiated C57BL/Ka mice (13).

After the last of four whole-body irradiation treatments, TNF- α was injected into the mice when PLCs were already present in the tissues (3-5). As was observed when irradiated mice were given bone marrow grafts (6), TNF- α induced the disappearance of PLCs. Two weeks after the completion of rMuTNF- α treatment, PLCs were no longer observed in most of the mice, indicating that exoge-

nous rMuTNF- α acted on the pre-leukemic stage of the disease.

It was previously shown that in marrow-grafted irradiated mice, compared with irradiated animals, there was a restoration of several cellular compartments involved in thymic lymphopoiesis, such as the bone marrow prothymocyte activity (3,7,8), the balance between thymocyte subsets (10), the number of marrow-derived stromal cells (17), and the number as well as some in vitro functional properties of TNCs (6). Here, we showed that exogenous rMuTNF- α also restored the thymocyte differentiation and the TNC numbers but not the prothymocyte activity.

Two reports (18,19) have described the inhibitory effects of TNF- α on multipotential and committed hematopoietic precursors. Our observations indicate that TNF- α does not act on bone marrow-resistant precursors committed to the T-cell lineage. The restoration of thymocyte subpopulations after rMuTNF- α treatment appears then to be due to a direct effect on thymocytes. It is well established that TNF- α plays a role in thymic physiology. When combined with other cytokines, TNF- α induces in vitro immature thymocytes to proliferate and differentiate (20). In vitro, TNF- α is produced by thymocytes and thymic macrophages (21-24). We also found TNF- α mRNA transcripts in the normal

thymus in situ (15,25). Thus, the restoration of the balance between thymocyte subpopulations in TNF- α -treated mice observed in this study is concordant with the previously reported findings.

We observed an increase of TNC numbers in mice that had been irradiated and given injections of rMuTNF- α . The number of TNCs that can be isolated from the thymus depends on the interactions that immature thymocytes and epithelial cells are able to establish in vivo (16). In split-dose-irradiated animals, both cell partners are altered (3). The partial recovery of TNC numbers after TNF- α treatment is likely due to the induction of thymocyte restoration by TNF- α .

The similarity between the effects of TNF- α and those of a bone marrow graft in split-dose-irradiated animals suggests that TNF- α might be one of the mediators involved in the effect produced by the bone marrow graft. Compared with control mice, the number of TNF- α -expressing cells was shown to increase in irradiated marrow-grafted mice, thus supporting our hypothesis. Additional supporting evidence comes from our observation that anti-TNF- α antibodies partially inhibited the antilymphomatous effects of bone marrow grafts in split-dose-irradiated mice.

Exogenous IFN γ also eliminates PLCs, inhibits lymphoma development (13), and increases the number of TNCs (Humblet

C, Defresne MP: data not shown). However, neither TNF- α nor bone marrow transplantation was shown to induce IFN γ mRNA in the thymus (Humblet C: unpublished observations), indicating that TNF- α has a direct effect on either PLCs or the thymus rather than an effect via the induction of IFN γ . TNF- α appears to be crucial for the inhibition of lymphomas. Other cytokines might be involved; however, we did not observe an increase in the mRNAs encoding interleukin 1, interleukin 2, interleukin 4, or interleukin 6 in irradiated thymuses, whether or not the mice had been treated by a bone marrow graft (Humblet C: data not shown).

Two hypotheses could explain the mechanisms by which administration of exogenous TNF- α eliminate PLCs. TNF- α could exert a direct cytotoxic effect on PLCs, for example, by inducing apoptosis, as it does in some types of tumor cells (26,27). An indirect effect of TNF- α is also possible. As the PLCs evolve toward final neoplastic transformation, which appears to depend on altered intrathymic signals, one could postulate that the recovery of the thymic microenvironment and thymic lymphopoiesis might be incompatible with their persistence and/or transformation.

The nature of intrathymic TNF- α -producing cells remains unknown. Since bone marrow grafting yielded thymocyte precursors that actively repopulated the thymus (28) and generated precursors of some subsets of thymic stromal cells (29), some of these cells might produce TNF- α and contribute to the observed increases in numbers of TNF- α mRNA-expressing cells.

A previous study (17) showing an inverse relationship between the presence of dendritic cells and the incidence of lymphomas suggested that leukemogenesis is promoted by the deregulation of T-cell differentiation or a process that may be a consequence of damage inflicted on the thymic interdigitating cells by the leukemogenic irradiation. However, the fact that bone marrow cells of SCID mice fail to inhibit lymphoma development (30) suggests a role for T cells, presumably TCR+ cells, in the antilymphomatous activities of bone marrow cells.

This study demonstrates that administration of exogenous TNF- α inhibits the development of thymic lymphomas in

mice after leukemogenic irradiation by eliminating PLCs and restoring thymic lymphopoiesis. The similarity of these effects to those of a bone marrow graft, as well as the increased numbers of cells with TNF- α transcripts in the thymuses of marrow-grafted mice and the restoration of the leukemogenicity of PLCs by anti-TNF- α antibodies in bone marrow-grafted mice, suggest that TNF- α is involved in the antilymphomatous effect of bone marrow graft. This study indicates that cytokines can be used to inhibit the development of a specific hematopoietic cancer and that they might be proposed, at least in some biological systems, as therapeutic agents for the secondary prevention of cancers.

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