

INDIRECT INDUCTION OF RADIATION LYMPHOMAS IN MICE

Evidence for a Novel, Transmissible Leukemogen

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In a series of elegant experiments, the late Henry S. Kaplan demonstrated that the lymphoid cells of a nonirradiated mouse thymus may undergo neoplastic transformation through mere residence within an irradiated host environment (1-3). These results were later confirmed in other laboratories (4, 5). It was thereby established unequivocally that radiation lymphomagenesis in mice proceeds through an indirect mechanism of induction. Soon thereafter, a leukemogenic retrovirus, designated radiation leukemia virus (RadLV),¹ was isolated from tumor tissue of C57BL/Ka mice with radiation-induced lymphomas (6), and by inference, the role of mediator was attributed to this virus. It was postulated that RadLV, activated in the host by ionizing radiation, is released and transported to the thymus, where lymphoblasts, generated during the postradiation recovery phase, constitute an optimal target cell population for both replication of and eventual transformation by virus (6-8). When it became apparent that RadLV is found in only 5-10% of radiation-induced lymphomas (9), the hypothesis required modification, and the concept of RadLV-0 was introduced. In this formulation, RadLV is initially activated in a replication-defective form (RadLV-0), in which only the oncogenic potential of the virus is expressed (10). Other investigators invoked the idea of somatic mutation to explain radiation lymphomagenesis, stipulating that radiation initiates neoplastic transformation of target cells in the bone marrow through a direct mutagenic effect (11-13). The radiation-damaged thymus then provides a suitable environment in which preleukemic bone marrow cells evolve to full neoplasia with no necessity for viral mediation. However, since Kaplan's thymus graft experiments are not consistent with such direct transformation of bone marrow cells, and since RadLV-0 has yet to be identified, new hypotheses may be considered. We undertook the present study to investigate the possibility that radiation lymphomagenesis is mediated by a transmissible agent altogether different from RadLV.

To assess further the relative contributions of bone marrow and thymic cells, as well as the role of RadLV in the disease process, we reexamined the fate of thymic grafts in irradiated hosts, using C57BL/Ka mice (Thy-1.2) and a Thy-1.1

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¹ *Abbreviations used in this paper:* RadLV, radiation leukemia virus; RTase, reverse transcriptase.

congenic substrain for this purpose. We also examined the lymphomagenic potential of adoptively transferred bone marrow cells from irradiated mice to evaluate their role as target cells for transformation, versus that of transmitters of a lymphomagenic agent to thymic target cells. Our results demonstrate that bone marrow is the site of the initial transforming event, but that T cell lymphomas originate in the thymus. In most instances, the signal transmitted by bone marrow is not identifiable as RadLV. The nature of this signal is the subject of ongoing investigation. In addition to thymic lymphomas, radiation induces a significant number of apparently Thy-1⁻ lymphomas, most of which express markers characteristic of the B cell lineage.

Materials and Methods

Animals

Mice of strain C57BL/Ka, expressing the Thy-1.2 determinant, and of the Thy-1 congenic substrain C57BL/Ka/Thy-1.1 (hereafter designated BL/1.1) were bred in our colony. One strain served as donor, the other as recipient in experiments involving thymic grafts and transfers of bone marrow or thymic cells. C57BL/6-Ly-5.2 mice were obtained through the courtesy of Dr. E. A. Boyse (Memorial Sloan-Kettering Cancer Center, New York).

Lymphoma Induction

Graft Experiments. Recipient mice were thymectomized at age 4 wk and started 1 wk later on a leukemogenic course of four weekly, whole-body exposures to x radiation of 1.75 Gy each. One thymic lobe from neonatal donor mice was placed under the kidney capsule of the recipient, either 1 wk before the start of irradiation or within 24 h after its completion.

Cell Transfer Experiments. One group of mice was thymectomized and irradiated as described; the other was not thymectomized. On days 1, 10, 30, and 60 after the last exposure, bone marrow suspensions were prepared from the leg bones of each mouse. Recipient mice aged 5–6 wk were exposed to a single dose of x radiation of 5 Gy (which is not by itself lymphomagenic) and, on the same day, injected intravenously with $1-2 \times 10^7$ donor cells, one donor to one recipient. Thymocyte suspensions were prepared at the 10-d interval only. Irradiated recipients were injected intrathymically with $5-10 \times 10^6$ cells.

Antibodies and Cell Surface Staining

A panel of mAbs was used to identify cell surface determinants on thymocytes in repopulated grafts and on tumor cells. Fluorescein-conjugated anti-Thy-1.2 mAb was purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). The hybridoma cell line 19VE12, secreting mouse anti-Thy-1.1 mAb, was kindly provided by Dr. R. Nowinski (Hutchinson Cancer Research Center, Seattle, WA). Rat mAb RA3-6B2, specific for the B220 antigen expressed on mouse cells of the B lineage, including pre-B cells (14) was a gift from Dr. R. Coffman (DNAX Research Institute, Palo Alto, CA), as was rat mAb RA3-8C5, specific for mouse granulocytes. Additional rat mAbs used were 11B5 (15), which binds mouse IgM, and M1/70 (16), specific for the mouse macrophage antigen Mac-1. The unlabeled mAbs were concentrated by ammonium sulfate precipitation from hybridoma culture fluids. Second-stage antibodies were fluorescein-conjugated goat anti-mouse Ig (Tago, Inc., Burlingame, CA) and anti-rat Ig (Cappel Laboratories, Cochranville, PA). Controls were treated with second-stage antibody alone. Preparation of cell suspensions and staining were carried out as previously described (17). The stained cells were mounted in Cunningham chambers and examined with a Zeiss fluorescence microscope under epi-illumination.

Detection of MuLV in Lymphoma Cells

Cell suspensions were prepared from lymphomatous tissue (thymus or thymic graft when possible) and processed as described (18) to detect the presence of retroviral antigens in the cytoplasm of cells by immunofluorescence. Briefly, cell suspensions were placed on the wells of toxoplasmosis slides (Bellco Glass, Vineland NJ), air-dried, fixed in acetone, and stained with a polyvalent anti-RadLV rat regressor serum (19) and fluorescein-conjugated second-stage anti-rat Ig. The preparation was counterstained with Evans Blue stain to render nonfluorescent cells visible. By this method, a single fluorescent cell among $1-2 \times 10^4$ negative cells can be readily identified. However, unambiguous identification of cells exhibiting low levels of fluorescence (i.e., containing low concentrations of viral antigens) is difficult. Therefore an *in vitro* infectious center assay was also used. Radiation-killed (100 Gy) lymphoma cells were cocultivated with cells of the lymphoma line BL/RL12-NP (20), which does not produce retrovirus but is highly susceptible to infection by RadLV, and consequently becomes immunofluorescence-positive. Medium from these cultures was used for assays of reverse transcriptase (RTase) activity (21); in all tests, immunofluorescence and RTase results coincided. In some instances, an *in vivo* infectious center assay was carried out as well, in which radiation-killed tumor cells were injected intrathymically in mice to test lymphomagenic activity. Where lymphomas developed, they were in turn analyzed for expression of cell surface determinants (both to distinguish host vs. donor origin, and to define the cell type transformed) and of RadLV.

Lymphoma Cell Cultures

To establish lymphomas *in vitro*, small pieces of tumor, together with tumor cells in suspension were seeded in Iscove's medium supplemented with 10% FCS, 5×10^{-5} M 2-ME, and antibiotics. Partial medium changes were carried out once or twice weekly until the cultures were well established. Thereafter, the cultures were divided 1:10 at 3-4-d intervals.

Results

Lymphomas in Mice Bearing Thymic Grafts. Thymectomized, irradiated mice that had received a thymic graft from Thy-1 congenic, neonatal donors either before the start of the radiation regimen or on its completion were divided into two groups, one to be used for examination of the grafts at 6 wk after their implantation, the other to await lymphoma development. The grafts removed at 6 wk appeared normal and were repopulated by lymphoid cells. The genetic origin of these cells was ascertained by their Thy-1 phenotype. When 85% or more of cells in the population were of a given phenotype, that phenotype was assigned to the entire population. When the proportion was less, the population was deemed to be of mixed origin. By this criterion, all grafts implanted before irradiation and most of those implanted thereafter were reconstituted by cells originating in the irradiated host (Table I). Many of the grafted mice became leukemic. In these animals, the graft was invariably enlarged, and its normal thymic architecture was obliterated by tumor cells. The adjacent kidney, spleen, liver, and lymph nodes were usually involved as well. In lymphomatous tissue, the tumor cells were all of a single Thy-1 phenotype, without admixture of the opposite type. In the group grafted before irradiation, most lymphomas (76%) were of host origin (Table I). In contrast, tumors in mice bearing postradiation grafts were predominantly (77%) of donor phenotype. The frequency of lymphomas was substantially reduced in this group (28 vs. 60%). RadLV-expressing lymphomas did not exceed 10% of the total in either group of leukemic mice (data not shown).

TABLE I
*Lymphocyte Derivation in Repopulated Thymic Grafts and
 in Graft Lymphomas*

Phenotype of lymphocytes	Host treatment [†]	
	Graft → X ray (%)	X ray → Graft (%)
Repopulated grafts*		
Graft donor	0/12 (0)	4/59 (7)
Host	12/12 (100)	49/59 (83)
Mixed	0/12 (0)	6/59 (10)
Graft lymphomas		
Graft donor	6/25 (24)	17/22 (77)
Host	19/25 (76)	5/22 (23)
Mixed	0/25 (0)	0/22 (0)
Tumor incidence [‡]	25/42 (60)	22/78 (28)

* Thymectomized, irradiated (1.75 Gy, four doses) hosts and graft donors differ at Thy-1. Repopulated grafts were examined 6 wk after implantation.

[†] Number of grafts showing indicated phenotype per number tested.

[‡] Number of leukemic mice per total.

Lymphomas in Recipients of Bone Marrow and Thymus Cells. In the preceding experiment, the appearance of lymphomas in unirradiated grafts residing in irradiated hosts could be explained by Kaplan's assumption that a leukemogenic agent is transmitted from the host to thymic target cells. Transmission could proceed via bone marrow cells migrating to the thymus, which transplantation or irradiation has depleted of lymphocytes (7). On the other hand, development of tumors derived from the host in mice grafted before irradiation lends support to Haran-Ghera's view that bone marrow cells from irradiated mice may themselves undergo neoplastic transformation. It was therefore necessary to examine further the role of bone marrow in radiation leukemogenesis. C57BL/Ka mice, either intact or thymectomized, were exposed to the leukemogenic regimen of radiation and used as bone marrow donors. The cells were collected at intervals after radiation, and injected into congenic BL/1.1 recipients as described. Thymocytes, harvested 10 d after the last x-ray exposure, were similarly injected. Starting at ~3 mo after cell transfer, lymphomas developed in 9 of 19 mice that had received donor thymocytes (47%), and in 79 of 118 bone marrow recipients (67%) (Table II). Characterization of the tumors with respect to cell surface determinants and RadLV expression yielded clear results: the T cell (Thy-1⁺) lymphomas elicited by bone marrow injection were exclusively of recipient origin. In contrast, lymphomas developing in thymocyte-injected mice were mainly of donor origin. Neither time interval to bone marrow transfer nor donor thymectomy significantly influenced tumor incidence or origin. Lymphomas that were Thy-1⁻ by the assay used occurred with remarkably high frequency in bone marrow recipients (25 vs. 41% Thy-1⁺ tumors), but were not observed in thymocyte recipients. Infectious RadLV was detected in 9% of Thy-1⁺ lymphomas and in 30% of Thy-1⁻ tumors.

TABLE II
Characterization of Lymphomas Induced by Transfer of Cells from Irradiated Mice

Cell donors			Lymphomas in cell recipients				
Cells transferred	Treatment*	Time to cell transfer	Incidence	Thy-1 ⁺ tumors host/donor origin	RadLV ⁺	Thy-1 ⁻ tumors	RadLV ⁺
<i>d</i>							
Bone marrow							
	Thymectomy	1	7/12	5/0	0	2	0
	—	1	7/13	5/0	0	2	0
	Thymectomy	10	11/15	8/0	0	3	2
	—	10	10/13	6/0	1	4	2
	Thymectomy	30	14/20	7/0	1	6	2
	—	30	13/19	5/0	1	8	2
	Thymectomy	60	9/11	4/0	0	5	1
	—	60	8/15	8/0	0	0	0
Thymocytes							
	—	10	9/19	1/8	2	0	0
Nonviable cells [‡]							
	Bone marrow	10	4/22	4/0	0	0	0
	Thymocytes	10	6/23	6/0	1	0	0

* All donors were irradiated with 1.75 Gy of four doses. Bone marrow donors were either intact or had been thymectomized before irradiation. At the indicated intervals after the last exposure, donor cells were injected into Thy-1 congenic, irradiated (5 Gy) recipients.

[‡] Cells were lethally irradiated (100 Gy) before injection.

Transmissible Leukemogen in Lethally Irradiated Cells. The host origin of lymphomas induced by transferred bone marrow suggests the presence of a subcellular, transmissible leukemogen(s). Because a fragile agent might be destroyed in the course of preparing cell-free extracts, intact, lethally irradiated (100 Gy) thymus and marrow cells, collected 10 d after the last x-ray exposure were injected intrathymically in congenic mice. Lymphomas of recipient phenotype were induced (Table II), more effectively by thymocytes than by bone marrow; all but one were free of detectable RadLV. If RadLV were nonetheless the etiologic agent in the virus-negative lymphomas, but present at levels too low to be detected, an additional round of infection should permit sufficient amplification of the virus to attain detectable levels. To evaluate this possibility, several RadLV⁻, Thy-1⁺, and Thy-1⁻ lymphomas, as well as a small number of RadLV⁺ tumors were lethally irradiated and injected into congenic mice. Approximately half the lymphomas tested were productive and induced tumors (Table III). Most were T cell lymphomas of recipient phenotype, others were Thy-1⁻. Only 14% of Thy-1⁺ and 22% of Thy-1⁻ lymphomas expressed RadLV. Although the number of RadLV⁺ lymphomas tested in this manner is very small, it is of interest that only one of three tumors induced was virus positive. Moreover, tumors induced by any one lymphoma did not necessarily match each other or the

TABLE III
Lymphomagenic Agent in Marrow of Irradiated Mice: Further Evidence of Transmissibility

Primary lymphomas*			Host lymphomas						
Pheno- type	RadLV expres- sion ^{†‡}	Productive tumors/ number tested [§]	Incidence	Phenotypes and RadLV expression					
				T		B		0	T/B
				-	+	-	+	-	-
T	-	14/17	18/47	10	2	1	1	3	1
B	-	5/12	9/19	7	1	1	0	0	0
B	+	2/3	3/5	1	0	0	1	1	0

* Lymphomas induced by transferred bone marrow cells from irradiated mice. Phenotypes were Thy-1 (T) or B220 (B).

† RadLV expression is shown as + or -.

‡ Number of primary lymphomas which, on transfer of lethally irradiated cells, induced lymphomas in the hosts, per number of lymphomas tested.

§ Phenotypes and RadLV expression as in primary lymphomas, with the addition of tumors containing both Thy-1 and B220-expressing cells (T/B), and tumors expressing neither determinant (0). Numbers shown specify individual lymphomas with the indicated characteristics.

parent tumor with respect to phenotype and expression of RadLV.

The Thy-1⁻ Lymphomas. In mice with Thy-1⁻ lymphomas, the lymphoid organs were generally enlarged, but the thymus was normal-sized or atrophic, and there was frequently a blood-tinged, cellular pleural effusion. The host vs. donor origin of these tumors could not be ascertained in the absence of the Thy-1 marker. Subsequently C57BL/6-Ly-5.2 mice were used as recipients of bone marrow from BL/1.1 (Ly-5.1) irradiated donors, to take advantage of the presence of the Ly-5 marker on both T and B lymphocytes (22). Preliminary data (three of four lymphomas tested) suggest that the Thy-1⁻ tumors are probably of donor origin. In membrane immunofluorescence assays using mAbs to cell surface antigens, only antibody RA3-6B2, specific for the B220 antigen expressed on cells of the B lineage, was found to bind. Because mAb 11B5 did not bind, surface IgM is presumably not available on the cells, and we assume that they are at the pre-B stage. The relative abundance of B220⁺ cells varied with individual tumors, ranging from ~20 to nearly 100% of the population. Four B220⁺ lymphomas were established in culture, but their phenotype proved unstable. Three lines lost B220 expression soon after becoming established and became Thy-1⁺; the fourth line did so after 2-3 mo in culture. To determine whether the Thy-1⁺ cells in this line were clonally derived from B220⁺ cells, their immunoglobulin μ arrangement was analyzed. The Thy-1⁺ cells retain one germline allele that is rearranged in the B220⁺ cells (data not shown), ruling out progression of a B220⁺ phenotype to a Thy-1⁺ phenotype. In all cases, when the cells became Thy-1⁺, the recipient allotype was expressed.

Discussion

Although the induction of thymic lymphomas in mice by ionizing radiation has been the subject of extensive study, the site of origin of the neoplastic process and the role of viral mediation are still incompletely understood. In the present

experiments, we have more fully evaluated these issues, using C57BL/Ka mice congenic at the Thy-1 locus. The results of this study both confirm previous observations and force a reinterpretation of those observations. As in the past, thymic cells are demonstrated unequivocally to be the target of transformation by an entity carried within, and transmitted by, irradiated bone marrow cells. It is now equally clear, however, that in the majority of cases, the transmissible entity is not RadLV.

Thymic Cells Are the Target of Neoplastic Transformation by a Lymphomagenic Agent(s) Induced in and Transmitted by Bone Marrow. As first shown by Kaplan et al. (3), lymphomas appearing in thymectomized mice grafted with a neonatal thymus after completion of a leukemogenic course of x radiation are predominantly of graft donor origin. This genesis was previously explained by assuming the transmission of RadLV from the irradiated host to target cells in the unirradiated thymic grafts (7). Our present results indicate that the source of the leukemogenic agent (probably not RadLV) is the bone marrow, and that it is transported to the thymus by homing bone marrow cells after being activated by radiation. The target cells for neoplastic transformation are the progeny of host-derived marrow cells in grafts implanted before irradiation. In grafts implanted after irradiation, the incoming, irradiated marrow cells presumably do not constitute an appropriate target cell population, and surviving cells of graft origin are the target. The transmission of a lymphomagenic agent from the irradiated marrow to the unirradiated thymus is further demonstrated by the induction of recipient lymphomas on transfer of bone marrow cells from irradiated donors to congenic mice. In contrast, the transfer of thymocytes induces lymphomas of donor origin under similar circumstances, demonstrating that these cells can be transformed by the agent. Thymectomy does not prevent activation of the agent in the marrow of irradiated mice, and, once activated, the agent appears to persist for at least 2 mo. Our results are at variance with those of Haran-Ghera (11, 12) who observed lymphomas of donor origin on transfer of bone marrow cells from irradiated C57BL/6 mice to (BALB/c × C57BL/6)_F₁ hybrids. Possible reasons for the difference include reduced susceptibility of hybrid recipients to C57BL-derived agents and the evaluation of tumor origin on the basis of transplantability. The presence of Thy-1 was not determined, and the results would therefore include both Thy-1⁺ and Thy-1⁻ tumors, while our data only reveal the origin of T cell lymphomas.

Radiation Also Induces Pre-B Cell Lymphomas. C57BL/Ka mice exposed to fractionated, whole-body x radiation develop a high incidence (90–100%) of thymic lymphomas (23), but virtually no other malignancy. It was therefore surprising to note the relatively high frequency of pre-B cell lymphomas in mice injected with marrow from irradiated donors. Incomplete evidence indicates that these tumors are of donor origin. They are presumably derived from bone marrow progenitor cells in which the agent was activated, or from susceptible cells in the marrow to which it was transmitted. Since lethally irradiated T and pre-B lymphoma cells induce lymphomas of either class in recipient mice, it appears that the same agent(s) is involved in both T and pre-B lymphomagenesis. RadLV in contrast is known to induce T cell lymphomas only. The loss of B220 expression and the appearance of Thy-1 on cultured lymphoma cells demon-

strates that both classes of tumor may coexist in the animal; pre-B tumors may predominate in vivo, but are gradually replaced by the T-cell component in vitro.

The Mediator in Radiation Lymphomagenesis Remains Undefined. Earlier studies (9, 24, 25) failed to reveal consistent evidence of RadLV etiology in radiation-induced lymphomas of C57BL/Ka mice. Recently, Defresne et al. found no RadLV-related antigens in T cell lymphomas produced by transfer of marrow or thymic cells from irradiated mice (26). In the present study, RadLV has been detected infrequently in either T or B cell lymphomas, which nevertheless were clearly induced by a transmissible, lymphomagenic agent present in the marrow of irradiated mice. The concept of RadLV-0 was introduced (10) to account for the scarcity of RadLV expression in radiation lymphomas and for its frequent de novo appearance on serial passage of tumor cells in vivo or in vitro. It postulates that radiation induces a replication-defective, transformation-competent subviral entity, RadLV-0, which initiates and maintains the transformed state in the absence of virus production, and is converted at a later stage to infectious RadLV. In the present experiments, RadLV-0 could conceivably be transmitted by cell-cell contact from marrow to thymic target cells. Because RadLV has been detected in tumors induced by lethally irradiated, RadLV⁻ lymphoma cells, this possibility cannot be discounted. The appearance of RadLV may however be a secondary phenomenon, resulting from increased opportunity for recombination among the nonpathogenic retroviruses endogenous to C57BL/Ka mice. We have previously shown that an otherwise nonthymotropic ecotropic retrovirus, BL/Ka(B), replicates actively in lymphoma cells (29), and furthermore, that lymphoma cells express elevated levels of xenotropic virus-related mRNA (28). Arguing against the likelihood of RadLV-0 etiology is the fact that the *env* recombination indicative of a leukemogenic retrovirus could not be demonstrated at the DNA or RNA level in radiation-induced lymphomas (28, 29). If the transmissible mediator of radiation lymphomagenesis is neither RadLV nor RadLV-0, it must be antigenically distinct as well from the common murine retroviruses, all of which are detected by the polyvalent rat regressor serum used in these studies. Moreover, since reverse transcriptase activity has consistently paralleled serological results, the agent may not be a retrovirus at all. Current attempts to reveal the hypothetical virus include transmission studies with subcellular tumor cell fractions, as well as cloning by screening cDNA libraries with a probe constructed by subtracting mRNA from tumors induced by cloned RadLV from primary radiation lymphoma cDNA. An alternative explanation of the results could be the production of a factor controlling growth regulation. Such a factor, induced in bone marrow cells but acting on thymic cells, might be akin to one described recently (30) in cultures of irradiated stromal bone marrow cells in vitro. To account for the present observations, however, the proposed growth factor would have to persist or induce its own production in the cells it transforms, and be capable of transmission. Such features blur the distinction between a growth factor-inducing element and an infectious, autonomously replicating virus.

Summary

The transmission of a lymphomagenic agent(s) from the bone marrow of irradiated mice to thymic target cells has been demonstrated by: (a) the induction of T cell lymphomas in nonirradiated thymic grafts implanted in irradiated, Thy-1-congenic mice, (b) the induction of T cell lymphomas of host origin in mice infused with bone marrow from irradiated, Thy-1-congenic donors. The latter procedure also yields an appreciable number of pre-B cell lymphomas of uncertain origin. The results confirm Kaplan's theory that radiation induces thymic lymphomas in mice by an indirect mechanism. However, the previously described radiation leukemia virus is clearly not involved in the majority of transferred lymphomas. We propose that the mediating agent in radiation lymphomagenesis is a novel, transmissible agent induced in the bone marrow, but exerting its transforming activity on cells in the thymus. The nature and mode of action of the agent are under investigation.

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