IN VITRO GENERATION OF CYTOTOXIC LYMPHOCYTES AGAINST RADIATION- AND RADIATION LEUKEMIA VIRUS-INDUCED TUMORS

III. Suppression of Anti-Tumor Immunity In Vitro by Lymphocytes of Mice Undergoing Radiation Leukemia Virus-induced Leukemogenesis*

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T cell leukemias may be induced by exposing adult C57BL/6 mice to fractionated x radiation (four 170-rad doses) or by intrathymic inoculation of the radiation leukemia virus (RadLV),[1] originally isolated from a radiation-induced leukemia (1). As early as 10 d after termination of the leukemogenic treatment, preleukemic cells appear in the bone marrow and in the thymus (2), and 10–18 wk later, 80–100% of the animals develop overt lymphomas (3, 4).

In a previous study (5) we investigated the in vitro induction of cell-mediated immunity against radiation- and RadLV-induced leukemias and found that coculti-vation of normal syngeneic splenocytes with inactivated tumor cells resulted in a considerable proliferative response, although cytotoxicity was not generated. To generate anti-tumor cytotoxic T lymphocytes (CTL) in vitro, it was essential to preimmunize (prime) the spleen cell donor with the corresponding stimulator tumor cells. The cytotoxicity was induced by, and directed at, virally associated antigens expressed on RadLV-induced, but absent in radiation-induced, primary thymomas (5–7).

Although mice that received a leukemogenic treatment (and, therefore, are expected to eventually develop tumors) are deprived of a demonstrable anti-tumor immunity, we explored the possibility that their lymphocytes are primed and thus may become cytotoxic upon in vitro stimulation with the corresponding tumor cell. Although we failed to demonstrate anti-tumor cytotoxicity under such circumstances, we noticed that splenocytes from RadLV-injected, but not irradiated, mice could suppress the

* Supported by contract N01-CB-64050 from the National Institutes of Health, Bethesda, Md. and by the Lautenberg Endowment Fund, Jerusalem.

‡ Recipient of a fellowship from the Cancer Research Institute Inc., New York.

[1] *Abbreviations used in this paper:* 136.5/BL, C57BL/6 mice inoculated subcutaneously in both flanks with 3×10^6 heavily irradiated (9,000 rad) 136.5 cells; C′, complement; ConA, concanavalin A; CTL, cytotoxic T lymphocyte(s); FCS, fetal calf serum; LU, lytic unit(s); MLC, mixed lymphocyte culture(s); MLTC, mixed lymphocyte tumor culture(s); RadLV, radiation leukemia virus; RadLV/BL, C57BL/6 mice inoculated intrathymically with 20 μl of a 24-h 136.5 supernate; SI, stimulation index; x-ray/BL, C57BL/6 mice exposed to whole-body irradiation in four weekly doses of 170 rad.

generation of anti-tumor CTL in a syngeneic mixed lymphocyte tumor culture (MLTC) with splenocytes derived from preimmunized (primed) donors.

The data presented here suggest that the suppression is specific, is mediated by T cells, and is not apparent in mice exposed to fractionated radiation.

Materials and Methods

Mice and Tumors. Female C57BL/6 and BALB/c mice aged 5–6 wk of age at the start of the experiments were employed. Cell line 136.5 was established from a RadLV-induced C57BL/6 thymoma and was generously provided by Dr. M. Haas from The Weizmann Institute of Science, Rehovot, Israel. The 136.5 cells release high titers of leukemogenic RadLV into the culture supernate (8) and express virus-related antigenicity on their surface membrane (5, 9). The line was maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Biolab, Jerusalem, Israel) and antibiotics.

Leukemogenic Treatment. RadLV leukemogenesis was induced by intrathymic inoculation of C57BL/6 mice with 20 μl of a 24-h-old 136.5 supernate as described elsewhere (1). X-ray leukemogenesis was induced by exposing C57BL/6 mice to whole-body irradiation in four weekly doses of 170 rad as described elsewhere (1). The leukemogenic animals were designated RadLV/BL and x-ray/BL, respectively.

In vivo priming. 3×10^6 heavily irradiated (9,000 rad) 136.5 cells were inoculated subcutaneously into both flanks of C57BL/6 mice (designated 136.5/BL). Primed lymphocytes were prepared from spleens of 136.5/BL animals 10–14 d after 136.5 inoculation.

Unidirectional Mixed Lymphocyte Culture (MLC) and MLTC. Single-cell suspensions of normal C57BL/6, 136.5/BL, RadLV/BL, and x-ray/BL splenocytes or mixtures thereof were cocultivated in microplates or in tubes with heavily irradiated 136.5 cells (9,000 rad) or BALB/c splenocytes (3,000 rad) under conditions described elsewhere (10). In some experiments, RadLV/BL lymphocytes were fractionated on a nylon wool column or treated with anti-Thy-1.2 antibodies plus complement (C′) as described elsewhere (11, 12), and subsequently added to MLTC of 136.5/BL responding splenocytes and 136.5 stimulator cells. The MLTC were incubated for 3 d (in microplates) and for 6 d (in tubes) in a humidified 5% CO_2 atmosphere at 37°C.

Concanavalin A (ConA)-induced Stimulation. Normal C57BL/6, 136.5/BL, RadLV/BL, and x-ray/BL splenocytes or mixtures thereof were incubated in microplates with 10% FCS-RPMI-1640 medium that contained 2.5 μg/ml ConA (type III; Sigma Chemical Co., St. Louis, Mo.) for 3 d as described above.

DNA Synthesis Measurement. Microplate cultures were pulsed with 1 μCi [^{3}H]thymidine for the last 16 h and subsequently precipitated on fiberblass filters with a multiple sample harvester (Titertek; Skatron A.S., Norway). The filters were air-dried and their radioactivity was measured by scintillation counting. The DNA stimulation index (SI) was calculated by dividing the counts per minute of test cultures by the counts per minute in control cultures without stimulating cells.

Lymphocyte-mediated Cytotoxicity Assay. After a 6-d incubation, cells cultured in tubes were harvested and their cytotoxic activity was determined using the ^{51}Cr-release assay as described elsewhere (12), with 6 h incubation. The percent specific ^{51}Cr release was calculated according to the formula:

$$\text{percent specific release} = \left[\frac{\text{release in test} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}}\right] \times 100.$$

Cytotoxic activity was also expressed in lytic units (LU)/culture, calculated from the dose-response curve according to the method of Cerottini and Brunner (13). 1 LU was defined arbitrarily as the number of viable effector cells required to lyse 30% of 10^4 ^{51}Cr-labeled target cells for 6 h at 37°C.

Results

Splenocytes derived from 136.5/BL mice, but not from normal C57BL/6 mice, turn cytotoxic after in vitro stimulation with 136.5 cells. This cytotoxicity is, apparently,

induced by and directed at RadLV-associated antigens expressed on the tumor cell membrane (5). However, no generation of CTL was observed among lymphocytes derived from RadLV/BL mice (4 and 8 wk after virus inoculation), after in vitro stimulation with 136.5 cells (Fig. 1). In fact, the cytotoxicity levels with such lymphocytes were much below the background cytotoxicity conferred by normal lymphocytes sensitized with 136.5 cells.

When splenocytes from RadLV/BL were added to MLTC comprised of 136.5/BL splenocytes and inactivated 136.5 cells, a marked suppression of anti-136.5 CTL generation was observed (Fig. 2). The suppressive effect was most efficient with a 1:1 RadLV/BL:136.5/BL ratio (~80% suppression), but was also evident with a 1:5 ratio (65% suppression) and a 1:10 ratio (40% suppression). Equal numbers of normal C57BL/6 splenocytes did not inhibit the cytotoxic response. Suppression was equally efficient when mediated by RadLV/BL, 4, 6 or 8 wk after virus inoculation. The suppressive effect was already evident in splenocytes of RadLV/BL 5 d after virus inoculation, and after ~2 wk, it reached the maximal level of suppression which remained constant during the entire leukemogenic process (Fig. 3).

Treatment of RadLV/BL splenocytes with anti-Thy-1.2 antibodies and C′ com-

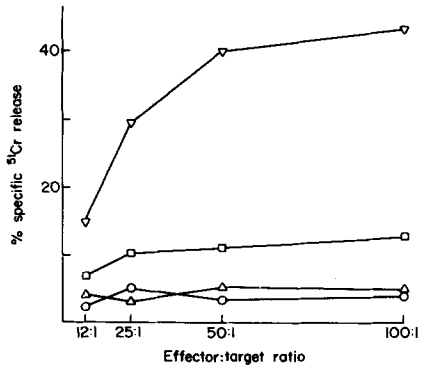

FIG. 1. Cytotoxic activity of splenocytes stimulated in vitro with 136.5 cells. Target cells: 136.5. (□) Normal C57BL/6; (▽) 136.5/BL; and RadLV/BL 4 (△) and 6 (○) wk after virus inoculation.

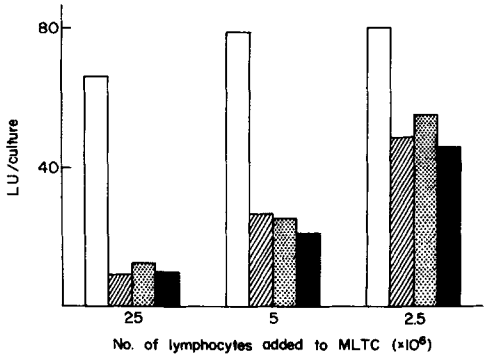

FIG. 2. RadLV/BL splenocytes suppress generation of syngeneic anti-136.5 CTL. 25 × 10^6 136.5/BL lymphocytes were mixed with increasing numbers of normal C57BL/6 (open bars) and RadLV/BL splenocytes 4 (hatched bars), 6 (dotted bars) and 8 (dark bars) wk after viral inoculation. The mixtures were cocultivated with 2.5 × 10^6 inactivated 136.5 cells for 6 d and assayed for cytotoxicity with ^{51}Cr-labeled 136.5 target cells.

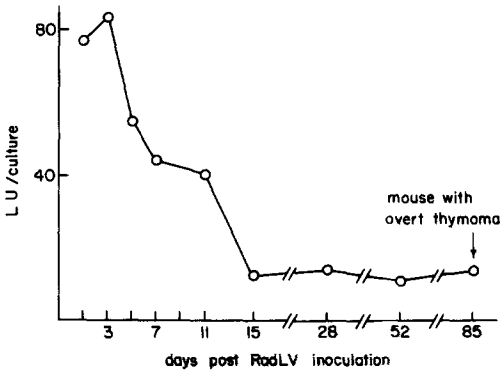

FIG. 3. Kinetics of appearance of suppressor splenocytes in RadLV/BL. 25 × 10^6 136.5/BL splenocytes were mixed with 25 × 10^6 RadLV/BL splenocytes taken at various times after virus inoculation. The mixtures were cocultivated with 2.5 × 10^6 inactivated 136.5 cells for 6 d and assayed for cytotoxicity with ^{51}Cr-labeled target cells.

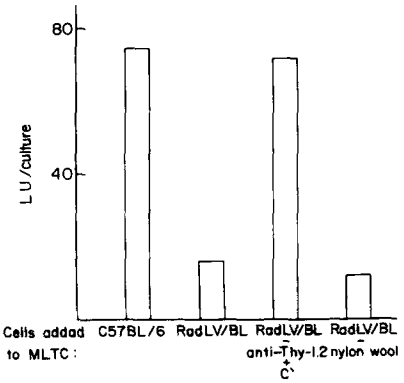

FIG. 4. Effect of fractionation on a nylon wool column and treatment with anti-Thy-1.2 plus C' on suppressive activity of RadLV/BL splenocytes. 136.5/BL splenocytes were mixed with equal number of normal C57BL, RadLV/BL, nylon wool-fractionated RadLV/BL, and RadLV/BL treated with anti-Thy 1.2 and C'. The mixtures were cocultivated with inactivated 136.5 cells for 6 d and their cytotoxic activity toward ^{51}Cr-labeled 136.5 targets was measured.

pletely abolished their capacity to suppress the cytotoxic stimulation of 136.5/BL splenocytes by 136.5 cells (Fig. 4). On the other hand, fractionation on a nylon wool column did not alter the suppressive effect.

In contrast with their unresponsiveness to stimulation with syngeneic 136.5 cells, RadLV/BL splenocytes could be readily sensitized by allogeneic (BALB/c) lymphocytes (Fig. 5). Such splenocytes had no suppressive effect on generation of allocytotoxic T cells in MLC of C57BL/6 lymphocytes responding to BALB/c stimulating cells (Fig. 5).

Splenocytes taken from x-ray/BL mice 4 wk after termination of irradiation did not generate cytotoxicity after cocultivation with 136.5 cells (Fig. 6). However, 136.5 priming of x-ray/BL mice and subsequent in vitro sensitization of their splenocytes with 136.5 cells, led to generation of anti-136.5 CTL, almost as efficiently as with splenocytes from 136.5/BL controls (Fig. 6). In contrast, RadLV/BL splenocytes did not turn cytotoxic after MLTC with 136.5 stimulating cells, even after in vivo priming with the tumor cells.

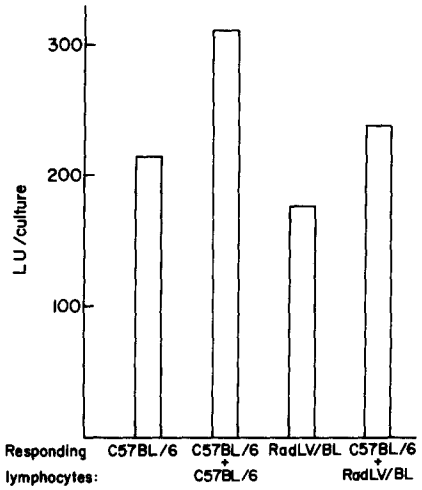

FIG. 5. RadLV/BL splenocytes do not suppress generation of allocytotoxic cells. C57BL/6 and RadLV/BL splenocytes were mixed in equal numbers and cocultivated with inactivated BALB/c lymphocytes. Cytotoxicity was measured after 6 d with ^{51}Cr-labeled ConA-induced BALB/c lymphoblasts.

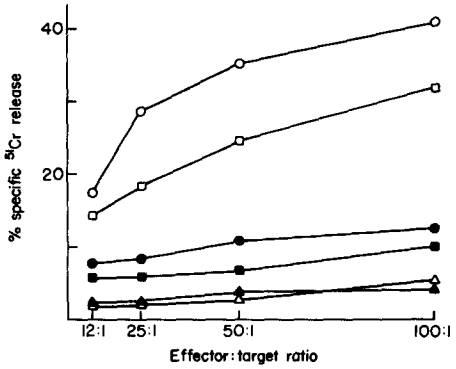

FIG. 6. Cytotoxic activity of lymphocytes from unprimed C57BL/6 and 136.5/BL, RadLV/BL, and x-ray/BL mice after in vitro stimulation with 136.5 cells. Target cells: 136.5. (●) Normal C57BL/6; (○) 136.5/BL; (▲) RadLV/BL (4 wk after virus inoculation); (△) RadLV/BL primed with 136.5 (4 wk after virus inoculation); (■) x-ray/BL (4 wk after last irradiation dose); and (□) x-ray/BL primed with 136.5 cells (4 wk after last irradiation dose).

The addition of x-ray/BL effector splenocytes to MLTC comprised of 136.5/BL responding lymphocytes and inactivated 136.5 stimulating cells did not abolish CTL generation in 1:10, 1:5, and even 1:1 x-ray/BL:136.5/BL ratios (Fig. 7). Under such circumstances, splenocytes from RadLV/BL strongly suppressed CTL generation (Fig. 2).

The proliferative responses of splenocytes derived from C57BL/6, 136.5/BL, RadLV/BL, and x-ray/BL mice, or mixtures thereof, to syngeneic, allogeneic, or mitogenic stimulation were also examined. As shown in Table I, all four lymphocyte populations responded vigorously to a BALB/c or ConA stimulus. Stimulation by 136.5 cells was considerably low, but evident among normal, x-ray/BL, and 136.5/BL splenocytes, with a higher SI in the latter. However, RadLV/BL splenocytes,

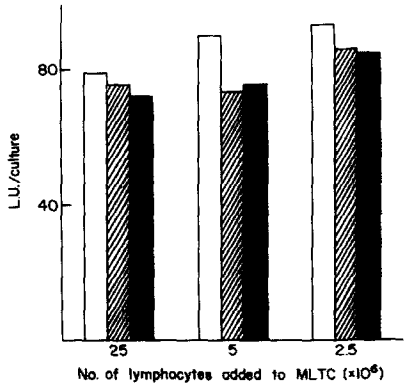

FIG. 7. X-ray/BL splenocytes do not suppress generation of syngeneic anti-136.5 CTL. 136.5/BL splenocytes were mixed with increasing numbers of splenocytes from normal C57BL/6 (open bars) or x-ray/BL mice 4 (hatched bars) and 6 (dark bars) wk after termination of the leukemogenic treatment. The mixtures were cocultivated 6 d with inactivated 136.5 and assayed for cytotoxicity toward ^{51}Cr-labeled 136.5 targets.

TABLE I

Effect of Splenocytes from RadLV/BL or X-ray/BL on DNA Synthesis in Individual and Mixed Cultures of Normal C57BL/6 and 136.5/BL Lymphocytes after Syngeneic, Allogeneic, and ConA Stimulation

Responder I (1.5×10^5 cells)	Responder II (1.5×10^5 cells)	Stimulator					
		136.5 (1.5×10^4 cells)		BALB/c (1.5×10^4 cells)		ConA (2.5 μg/ml)	
		cpm	*SI*	*cpm*	*SI*	*cpm*	*SI*
C57BL/6	—	5,706*	6.9	15,059	26.7	35,702	37.7
		(827)‡		(564)		(947)	
136.5/BL	—	9,301	9.2	14,169	27.3	45,859	34.9
		(1,011)		(519)		(1,314)	
RadLV/BL	—	1,171	1.2	16,195	24.1	26,198	31.3
		(976)		(672)		(837)	
X-ray/BL	—	4,782	5.7	13,298	22.2	28,724	31.6
		(839)		(599)		(909)	
C57BL/6	C57BL/6	10,731	7.3	22,113	31.5	51,910	42.1
		(1,470)		(702)		(1233)	
C57BL/6	RadLV/BL	1,330	1.4	18,094	26.3	35,307	36.4
		(950)		(688)		(970)	
C57BL/6	X-ray/BL	8,768	7.1	19,082	29.0	39,891	32.6
		(1,235)		(658)		(1224)	
136.5/BL	136.5/BL	8,177	7.4	20,959	30.2	44,533	38.0
		(1,106)		(694)		(1172)	
136.5/BL	RadLV/BL	1,079	1.4	19,034	26.4	29,058	33.4
		(771)		(721)		(870)	
136.5/BL	X-ray/BL	9,471	9.5	17,013	25.7	34,057	35.7
		(997)		(662)		(954)	

* Mean uptake of [^{3}H]thymidine at day 3 of culture.

‡ Background counts per minute in cultures without stimulator cells.

although stimulated strongly by BALB/c lymphocytes or ConA, did not respond to 136.5 stimulation.

When RadLV/BL lymphocytes were mixed in equal numbers with normal

C57BL/6 or 136.5/BL lymphocytes, DNA synthesis after stimulation with 136.5 cells was inhibited. Such inhibition was not observed in mixtures of C57BL/6 or 136.5/BL splenocytes with C57BL/6 or x-ray/BL splenocytes. However, stimulation by BALB/c cells or by ConA was not inhibited by x-ray/BL nor by RadLV/BL splenocytes.

Discussion

A large variety of experimental tumors possess tumor-specific transplantation antigens capable of evoking cell-mediated immune responses in vitro (13, 14). Yet, such tumors can grow progressively in vivo and kill their hosts, which, generally are devoid of effective anti-tumor immunity.

Because various types of humoral and cellular immune reactivities have been shown to be regulated by suppressor cells (15, 16), it has been proposed that tumor rejection in vivo may be hampered by host-suppressor cells capable of interfering with a potential anti-tumor immunity (17).

Suppressor cells associated with animal and human malignancies have been characterized in many systems (18). Kirchner et al. (19) have described a suppressor macrophage in C57BL/6 mice that bore murine sarcoma virus-induced sarcomas, that could nonspecifically abrogate a variety of humoral and cell-mediated immune responses. Nonspecific immunosuppression mediated by T cells was found in C57BL/6 mice that bore 3LL Lewis lung carcinoma (20). Fujimoto et al. (21) and Takei et al. (22) have described a T cell population in mice that bore progressive tumors, which could specifically suppress anti-tumor cell-mediated immune reactivity.

Although the existence of suppressor cells in tumor-bearing individuals has been widely documented, little is known about their role during premalignant stages. It is therefore difficult to conclude whether suppressor cells are indeed a necessary component of the neoplastic process, promoting tumor cell proliferation, or if they merely represent a secondary consequence of the growing tumor (23).

Cegalowski and Friedman (24) have found that mice infected with Friend leukemia virus show a suppressed antibody response to sheep erythrocytes (SRBC), long before overt leukemia can be detected. Similarly, Peled and Haran-Ghera (25) have shown that intrathymic inoculation of RadLV into C57BL/6 animals markedly reduced their plaque-forming ability after immunization with SRBC, shortly after virus inoculation. However, cell-mediated immunity to allogeneic cells was not affected.

Because it was possible to generate anti-tumor cytotoxic responses during MLTC with 136.5/BL lymphocytes responding to the RadLV-infected 136.5 cells (5), we examined the possibility that anti-tumor cytotoxic manifestation may be demonstrated also in lymphocytes of RadLV-inoculated mice during the latency period. It was found that splenocytes of RadLV/BL mice neither lyse 136.5 cells directly nor after in vitro stimulation with the tumor. Moreover, even splenocytes of RadLV/BL animals that had been preimmunized with 136.5 cells did not respond with DNA synthesis, nor did they turn cytotoxic upon cocultivation with 136.5 cells.

The inability of RadLV/BL lymphocytes to respond in MLTC with 136.5 cells prompted us to explore the question whether it was a result of a general immune unresponsiveness of mice undergoing a leukemogenic process, or that activation of a suppressive mechanism in such mice inhibited a potential anti-tumor immune reactivity. Therefore, 136.5/BL lymphocytes, known to respond in syngeneic MLTC, were mixed with RadLV/BL splenocytes and cocultivated with inactivated 136.5 stimu-

lator cells. A strong suppression in generation of anti-136.5 cytotoxicity was observed under such circumstances. The suppressive effect of RadLV/BL splenocytes was specific to syngeneic anti-136.5 responsiveness, because RadLV/BL splenocytes could vigorously respond to BALB/c cells with extensive DNA synthesis followed by generation of allocytotoxic cells. Moreover, allosensitization of normal C57BL/6 lymphocytes was not inhibited, but was somehow augmented, by RadLV/BL splenocytes. The increase in both the proliferative and cytotoxic alloresponsiveness was probably a result of lymphocyte clones in the added RadLV/BL spleen cells that also responded to the allostimulus, as addition of equal number of normal C57BL/6 lymphocytes to the MLC also resulted with increased proliferation and cytotoxicity.

Similarily, RadLV/BL splenocytes strongly responded with DNA synthesis when stimulated with ConA and did not interfere with normal C57BL/6 lymphocytes responding to the mitogen.

The abrogation of the inhibitory effect in RadLV/BL splenocytes treated with anti-Thy-1.2 plus C′, and its retention after fractionation on a nylon wool column indicated that the suppression was mediated by a nonadherent T cell population. Such cells, taken from animals that bore progressively growing tumors were shown to inhibit anti-tumor cell-mediated immunity in a specific manner (18).

RadLV leukemogenesis involves a latency period of several months (3). However, the suppressive effect of RadLV/BL splenocytes was evident very early in the leukemogenic process. It already reached its full capacity 2 wk after viral inoculation and remained constant during the entire preleukemic phase, as well as in mice developing overt lymphomas.

The emergence of overt lymphomas in C57BL/6 mice inoculated with RadLV is preceded by the induction of preleukemia cells shortly after virus injection (2). Interestingly, the appearance of the suppressor cell population in the spleens of such mice coincides with the development of preleukemic cells in the marrow and the thymus, which are detectable as soon as 10 d after virus inoculation (2). Further experimentation should be performed to find out whether the suppressive effect is induced by preleukemic cells or merely by the virus inoculum.

RadLV was originally isolated from a C57BL/6 thymoma induced by fractionated irradiation (26). Therefore it was believed for many years that x ray leukemogenesis involves activation of latent endogenous echotropic C-type virus, which in turn induces T cell leukemias (27, 28). This hypothesis was opposed by recent findings showing that, in contrast with the strong expression of C-type virus antigenicity on RadLV-induced tumors, the majority of x-ray-induced lymphomas are virus negative (7, 23). In a previous study (5), we reported that generation of cell-mediated immunity in vitro toward C57BL/6 T cell lymphomas was dependent on membrane-associated viral antigenic expression of the tumor cell. It was therefore interesting to know whether splenocytes of mice exposed to fractionated irradiation will also suppress syngeneic anti-136.5 cytotoxic responses. In contrast to RadLV/BL lymphocytes, splenocytes of x-ray/BL mice added to MLTC of 136.5/BL lymphocytes responding to 136.5 cells did not inhibit generation of anti-tumor cytotoxicity. Furthermore, 136.5 cells could stimulate DNA synthesis in x-ray-BL splenocytes, and lymphocytes from x-ray/BL animals that had been preimmunized with 136.5 cells could generate anti-tumor cytotoxicity when coculativated with 136.5 cells.

These results could be explained by the possibility that antigenically different

recombinant viruses may act in individual x-ray-induced leukemogenic processes (29). However, the lack of viral antigenicity and immunogenicity in primary radiogenic leukemias, as opposed to the virus expression and the efficient immunogenicity in virally induced primary tumors (5, 7, 30), does not favor involvement of viral etiology in x-ray leukemia induction. The difference demonstrated here between RadLV and x-ray-induced leukemogenesis with regard to the immunological pattern during the preleukemic latency further supports this notion.

Based on the findings reported herein, we hypothesize that RadLV-induced leukemogenesis is concomitant with expression of virus-related antigenicity capable of inducing specific immune suppression that sustains the proliferation of the RadLV-infected tumor cells in spite of a potential anti-tumor immunity. x-ray-induced leukemogenesis does not involve viral etiology and therefore is not immunogenic. Under such circumstances tumor progression occurs without immune stimulation of the host and, therefore, suppressor cells are not developed.

Studies were begun in our laboratory to test this hypothesis and to answer the question of whether the suppressor cells described herein play a significant role in the leukemogenic process and to what degree they are essential for tumor progression.

Summary

Adult C57BL/6 mice exposed to fractionated irradiation or inoculated with the radiation leukemia virus (RadLV), develop high incidence (80–100%) of lymphatic leukemias within 3–6 mo. RadLV-induced lymphomas can elicit cytotoxic responses in vitro in lymphocytes of preimmunized syngeneic mice, a reaction that is dependent on the expression of membrane-associated viral antigenicity. As soon as 5 d after RadLV inoculation, and during the entire leukemogenic process, suppressor T cells are detectable in the spleen that are capable of specifically abrogating generation of syngeneic anti-tumor cytotoxic cells in vitro. Mice exposed to fractionated x irradiation do not develop suppressor cells and their splenocytes may be stimulated in vitro to generate cytotoxicity toward RadLV-induced leukemias. These findings suggest that although RadLV has been isolated from radiation-induced leukemias, x-ray- and RadLV-induced leukemogenesis do not seem to involve a common viral etiology, and that induction of suppressor cells during RadLV leukemogenesis may be essential for tumor progression.

Received for publication 4 June 1980 and in revised form 21 July 1980.

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