Potentiation of in Vivo Model Murine Tumor Destruction by Combined Immunoradiotherapy

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ABSTRACT

The effects of tumor-specific immunotherapy (TSI), with the use of irradiated tumor cells given separately or as adjuvant to local radiation therapy, were studied on asynchronous EL 4 tumors growing exponentially in the peritoneal cavities of syngeneic C57BL/6J mice. Therapeutic efficacy was assessed in terms of tumor rejection rates, host survival times, and tumor cytokinetic age distribution parameters. Administration of TSI accelerated the clearance of i.p.-challenged 125I-labeled EL 4 cells, approximately 2-fold compared to that of untreated controls 7 days after injection, and slightly but significantly (p = 0.05) prolonged the mean survival time of 20 to 25% of animals challenged i.p. with 0.5 x 10^3 tumor cells from 17.5 days for controls to 25.4 days for experimental mice. Minimal or no cytokinetic effects were detected in the TSI-treated tumor populations as evidenced by sequential flow cytofluorometric DNA distribution analysis. Local 250-rad radiation therapy given in situ to EL 4 tumors induced cell cycle age distribution perturbations, characterized by an early S-phase block that reached a maximum magnitude of 25% of the tumor population at 90 to 100 min after irradiation and release of the block at 120 min posttreatment. A G2-associated block that persisted for approximately 3.5 hr was also evident in the irradiated EL 4 tumors. Combined immunoradiotherapy with the use of a similar TSI and radiation dose regimen significantly prolonged the survival time of mice challenged with 5 x 10^3 cells i.p. compared to single-modality therapy. Flow cytophotometric DNA distribution analysis indicated that this combined immunoradiotherapy treatment induced a dramatic cytokinetic therapeutic effect, suggestive of synergistic or additive immune destruction of sublethally radiation-damaged S-phase EL 4 cells. The cytokinetic recovery time of the surviving EL 4 cells in the immunoradiotherapy-treated tumors was prolonged (compared to radiation-treated only) by a period approximately equal to one EL 4 tumor cell cycle generation time of 12 to 13 hr. The data indicated that rapid cytotoxic destruction of sublethally radiation-damaged S-phase EL 4 cells occurred in immunoradiotherapy-treated tumors, suggestive of radiation-potentiated tumor killing by TSI-stimulated, activated macrophages.

INTRODUCTION

Therapeutic use of ionizing radiation is based upon preferential killing of tumor cells relative to associated normal tissues (16, 40). Several characteristics of both normal and tumor tissues are known to accentuate this preferential killing of neoplastic cells. Normal cells, for example, usually repair sublethal radiation damage more effectively than do tumor cells (40, 47). Tumor tissues commonly exhibit a more undifferentiated state with higher mitotic indices (3, 17), and in some tumors the G2 phase tends to be longer for a given cell cycle time (40). Metabolic differences exist in certain tumors that potentiate or accelerate the expression of radiation damage (6, 27), and tumor cells have tumor-specific antigens important in the host immune recognition of these abnormal cells (21, 62).

Failure of modern radiation therapy to control some cancers reflects the relatively low level of the preferential killing effect, i.e., therapeutic ratio (16), with a high proportion of failures attributed to regrowth of a small number of residual clonogenic and perhaps hypoxic tumor cells in the irradiation area (13, 16). A better understanding of the interrelationships of tumor cytokinetic responses to therapy and the radiobiological and immunological mechanisms of tumor killing might provide the basis for more effective therapeutic sterilization of certain primary and disseminated cancers. Properly scheduled adjuvant tumor-specific immunotherapy TSI2 and radiation therapy potentially could be used to achieve synergistic or additive therapeutic effects, to reduce or completely eliminate the residual clonogenic tumor burden, and thereby to decrease the probability of radiation failures (8, 50, 61). Presently, it is feasible to elicit therapeutically a tumor-specific immune response by 3 major pathways, i.e., macrophage, immunocompetent T-lymphocyte, and immunocompetent B-lymphocyte, together with their related pathways. The immunotherapeutic response against tumor antigens would also be expected to be dependent on host immune competence, therapeutic effectiveness of modality used, tumor antigenicity, and the various feedback controls, e.g., T-lymphocyte-derived macrophage activation factor, macrophage-inhibitory factor, B-lymphocyte helper function, and blastogenic factor(s).

Since our understanding of the immune process is presently quite limited and the immune reactions against autologous or syngeneic tumor are weak, TSI for gross tumor has had limited therapeutic success (9, 50, 63). Adjuvant TSI directed against a small number of tumor cells or residual clonogenic populations of tumor still holds promise (9, 50, 61). In addition, irradiation of a cancerous mass may facilitate immunological tumor killing (19, 36, 48, 59); therefore, a strong rationale exists for combining primary radiation treatment of appropriate tumors with TSI to achieve more effective therapy. To date, successful TSI has

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1 Supported by funds from the Veterans Administration Hospital, Miami, Fla. Received April 21, 1977; accepted January 18, 1978.

2 The abbreviations used are: TSI, tumor-specific immunotherapy; FCP, flow cyt fluorometry; PBS, phosphate-buffered saline.
been accomplished in autologous and syngeneic animal models with the use of injections of irradiated, replication-blocked tumor cells (8, 49, 50). More recently, TSI has been attempted clinically with the use of multiple therapeutic injections of irradiated tumor cells. However, the short follow-up periods to date preclude any definite assessment of the therapeutic benefit (60, 61, 67). This report describes some therapeutic effects of TSI with the use of irradiated tumor cells and radiation therapy on the tumor growth, rejection, and cytokinetic distribution parameters of a model murine syngeneic tumor system.

MATERIALS AND METHODS

Animals and Tumor Cells. Six- to 8-week-old female C57BL/6J (H-2b) and DBA/2J mice from The Jackson Laboratory, Bar Harbor, Maine, were used in these studies. C57BL/6J animals were given injections of 10⁷ syngeneic EL 4 leukemia cells (H-2b), and the ascites fluid from the peritoneal cavities of these carrier mice was collected weekly. Ascites fluid samples containing EL 4 cells were routinely washed 3 times in PBS prior to transplantation. Cell concentrations were determined by use of a Coulter Model ZH, with Channelizer and cell viabilities checked by dye exclusion. Ascites P-815-X2 mastocytoma cells carried as i.p. transplants in DBA/2J mice with the use of the same injection and harvesting procedures were used as reference tumor populations for light scatter and DNA distribution analysis.

Normal Spleen and Lymph Node Cells. Normal spleens and retroperitoneal lymph nodes were removed from control, unchallenged C57BL/6J mice and from animals 0 to 7 days after i.p. 5 × 10⁷ EL 4 challenge. The spleens and nodes were separately minced, syringed with 19-gauge needles in ice-cold PBS, and then filtered through rayon. Following 2 PBS washings and 1200-rpm centrifugations, cell concentrations were determined, 0.5 × 10⁶ cells/ml were analyzed directly for light scatter (5) profiles, and replicate samples were stained with propidium iodide (34, 35) for FCP DNA distribution analysis (35, 39, 41).

Quantitation of ¹²⁵I-labeled EL 4 Tumor Clearance from C57BL/6J Mice. Asynchronous EL 4 leukemia cells growing exponentially in the peritoneal cavities of C57BL/6J carrier mice were labeled with the radioactive thymidine analog [¹²⁵I]iododeoxyuridine (New England Nuclear, Boston, Mass.) as 1 μCi/ml PBS with the use of the method of Porteous and Munro (49).³ Cell concentrations of freshly harvested [¹²⁵I]-labeled EL 4 cells were determined by Coulter analysis, and the [¹²⁵I] dpm per 10⁷ tumor cells were quantitated by triplicate sample counts of 10⁷ labeled tumor cells/ml PBS with the use of a Packard Auto-Gamma spectrometer. The same samples were then counted for comparison on a Packard ARMAC liquid crystal 4 π geometry spectrometer that was subsequently used for the total-body detection of [¹²⁵I] activity in these studies. Counting efficiencies for [¹²⁵I]

³ Carrier C57BL/6J animals used for [¹²⁵I]-labeled EL-4 tagging, and all recipient mice subsequently included in the challenge experiments were maintained ad libitum on 0.1% potassium iodide drinking water from 3 days prior to and for the duration of [¹²³I] clearance experiments. This minimized [¹²³I] uptake into the thyroid tissue and thus kept the total-body, non-EL 4 tumor-associated [¹²³I] activity to a low level.

For each 5 replicate experiments, time-course curves for [¹²³I]-labeled EL 4 clearance were defined for 3 different experimental groups with the use of a minimum of 8 to 10 animals per group per run. Group 1 C57BL/6J controls were given injections i.p. of 10⁷ [¹²³I]-labeled EL 4 cells at Day 0 but no therapy; Group 2 C57BL/6J mice were challenged similarly to Group 1 and then received TSI i.p. (10⁷ radiation-blocked EL 4 cells on Days 1, 2, and 3); and Group 3 allogeneic DBA/2J animals were given a comparable i.p. challenge dose of 10⁷ viable [¹²³I]-labeled EL 4 cells on Day 0 and received no therapy. The total-body [¹²³I] activity per animal was then measured by total-body counting in the ARMAC unit, for each group, from Days 0 to 13 after tumor challenge, and the data were expressed as the relative percentage of [¹²³I] activity remaining following [¹²³I]-labeled EL 4 challenge. FORTRAN-computerized statistical t test analyses of means, differences between means, and S.D.’s were then carried out on each experimental day (0 to 14).

Immunotherapy with the Use of Radiation-blocked Tumor Cells (TSI). Freshly harvested 7-day EL 4 leukemia cells were pipetted into plastic Petri dishes (60 x 15 mm; Falcon No. 1007) at 10⁶ cells in 10 ml PBS. Tumor cells were then irradiated with 6 MeV photons from a clinic linear accelerator through 1.3 cm of tissue-equivalent density built-up material at 100 cm source-to-skin distance. The half-value layer of the beam was determined to be 12 mm lead and a dose rate of 250 rads/min, with the total dose of 2000 rads delivered to achieve replication blocking, based on previous data (26).

Three separate series of TSI experiments were carried out with the use of radiation-blocked tumor cells. The first series of experiments was designed to measure the effect of immune stimulation on the clearance rate of i.p.-challenged [¹²³I]-labeled EL 4 cells from C57BL/6J syngeneic animals. The second set of experiments assessed the efficacy of TSI on the survival times of syngeneic animals challenged with low doses (5 × 10⁷) of EL 4 tumor. A total of 282 C57BL/6J mice, with 6 groups (40 to 50/group), were used in this series. Group 1 animals received no immunotherapy; Group 2 animals were given immunotherapy consisting of 3 i.p. injections of 10⁷ glutaraldehyde-fixed and radiation-blocked EL 4 cells (1:1) on Days 1, 2, and 3 after tumor challenge. Group 3 animals received 3 i.p. injections of 10⁷ glutaraldehyde-fixed EL 4 cells on Days 1, 2, and 3 after 5 × 10³ i.p. tumor challenge. Group 4 mice received 3 i.p. injections of 10⁷ radiation-blocked EL 4 cells on Days 1, 2, and 3 after tumor challenge. Group 5 animals received local radiation therapy of 250 rads photons on Day 2. Group 6 mice were given combined immunotherapy similar to that for Group 4 animals and radiation therapy similar to that for Group 5 animals. The survival rate for each of the 6 groups was compared for means, differences between means, and S.D.’s with the use of FORTRAN-computerized statistical t-test analysis.

The third series of immunotherapy experiments dealt with the effects of TSI on the FCP cytokinetic DNA distribution patterns of EL 4 leukemia cells growing in the peritoneal cavities of C57BL/6J animals. This series included 4 groups of mice (28 to 30/group), with all animals given i.p. 0.5 × 10⁷
EL 4 tumor challenge on Day 0. Group A control tumor-bearing animals received 0.5 ml PBS i.p. at 4 and 24 hr, respectively, after primary tumor challenge. Group B mice were given TSI consisting of 10⁷ irradiated EL 4 cells at 4 and 24 hr, respectively, after initial tumor challenge. Group C animals received 250 rads in situ radiation therapy to the peritoneal cavities on Day 2, while Group D mice each received combined therapy consisting of TSI similar to that for Group B plus irradiation similar to that for Group C. The cytokinetic FCP DNA distributions of freshly harvested and propidium iodide-stained tumor samples were then and analyzed sequentially for Groups A, B, C, and D from Days 2 to 3 after transplantation for assessment of possible therapeutic effects on tumor cell cycle progression and age distribution patterns. Additional samples were also assayed from Groups A and D at 28 to 36 hr after irradiation to obtain a better estimate for the duration of recovery time.

**Radiation Therapy in Situ.** A Clinac linear accelerator operated at 6 MeV in the photon mode was used for irradiation of EL 4 tumors in situ. C57BL/6J mice challenged i.p. on Day 0 with 0.5 × 10⁷ EL 4 leukosis cells were placed in polyethylene restrainer tubes on Day 2, just prior to irradiation. Control animals were sham-irradiated, while experimental mice were irradiated anteriorly to posteriorly at 100 cm surface-to-skin distance to the peritoneal cavity area with the use of 1 cm of tissue-equivalent density built-up material. A therapeutic dose of 250 rads was delivered at 250 rads/min based on previous studies (26).

**FCP Methodology and Instrumentation.** The application of FCP has led to improvements in rapid kinetic analysis, particularly in measurements of the distribution of the cellular DNA content in experimental and clinical tumor samples (39, 41). Cellular kinetic analysis by FCP is based on the assumption that the cellular DNA content described the age distribution of cells in a population and that dynamic information about the effects of therapy on the specific stages of the cell cycle can be obtained by sequential, quantitative measurements of the fluorescence of dyes, which specifically bind to or intercalate with cellular DNA. In this investigation, EL 4 tumors were harvested from syngeneic C57BL/6J mice 2 to 3 days after 0.5 × 10⁷ i.p. primary tumor challenge from control Group A animals, from Group B (TSI)-treated mice, from Group C radiation therapy-treated animals, and from Group D mice treated with a combination of TSI and radiation therapy. Freshly harvested tumor cells were washed in ice-cold PBS and centrifuged at 1200 rpm at 0° for 10 min; then the pellet was resuspended in ice-cold PBS. Cell concentrations were measured by Coulter analysis, and 0.5 × 10⁶ cells/ml were stained in triplicate for 1 hr (on ice, in the dark) with the use of the direct propidium iodide method of Krishan (34, 35). The TPS was equipped with a 35-milliwatt argon ion laser for fluorescent excitation at 4800 Å of DNA-intercalated propidium iodide, and the flow rate during FCP measurements was maintained at approximately 8 × 10³ nuclei/min. DNA distribution data for each sample were initially displayed in the TPS scope for the purpose of optimizing the photomultiplier tube, and, if needed, the photomultiplier tube was adjusted for the purpose of positioning the tumor G₁ peak in Channels 39 to 40. Time-sequential DNA distributions were studied for each of the 4 groups (Control A plus experimental B, C, and D), and samples were analyzed by a computerized mathematical program for quantitating FCP age distribution data.

**RESULTS**

**Immunotherapeutic Effect on Tumor Clearance and Animal Survival.** The clearance rate of i.p.-challenged ¹²⁵I-labeled EL 4 tumor cells from C57BL/6J syngeneic recipient mice was accelerated by TSI with the use of radiation-blocked EL 4 cells. Untreated syngeneic animals required 11 to 12 days after initial 10⁷ i.p.-labeled tumor cell challenge for ¹²⁵I radioactivity to reach 10% of initial levels, while TSI-treated syngeneic mice and allogeneic DBA/2J untreated animals reached this same 10% level in only 5 days (Chart 1). The syngeneic immunotherapy-treated and untreated allogeneic animal ¹²⁵I-labeled EL 4 clearance curves were statistically similar to control Group 1 data (p = 0.05) for Days 1 to 4, and then they both became significantly different from controls by Day 5 (p = 0.05). However, the allogeneic animals appeared to reject the ¹²⁵I-labeled EL 4 transplants more effectively than did TSI-treated C57BL/6J mice from the 8- to 14-day period (Chart 1). Those untreated C57BL/6J animals (45 of 45) given i.p. 10⁷ ¹²⁵I-labeled EL 4 cells died by 14 days after challenge (Chart 1, _bottom_) as did 19 of 19 laboratory control mice given a comparable challenge dose of unlabeled EL 4 cells (not plotted). Immunotherapy, treated syngeneic animals (55 of 55) survived 14 days post-tumor challenge, then some mice died at Day 15, and 0 of 55 survived past Day 22 (Chart 1). Allogeneic recipients (40 of 40) survived indefinitely.

The results of a separate series of tumor challenge and survival time experiments carried out to test the immunotherapeutic efficacy of multiple i.p. TSI injections are shown in Chart 2. In these investigations, a lower tumor challenge dose of 5 × 10⁶ EL 4 cells was used in an attempt to favor expression of an immunotherapeutic effect. The mean survival times (the animal mean survival times, statistical variability, and statistical differences between groups were determined by computerized FORTRAN statistical comparison of the differences and ranges between means) for animals maintained without immunotherapy was 17.5 days, and none of the 50 control animals survived longer than 19 days after i.p. 5 × 10³ EL 4 transplantation. The mean survival time for TSI-treated mice with the use of irradiated cells only (Group 4) was bimodal; _i.e.,_ 75 to 80% of the animals did not respond to therapy (mean survival time was 18.3 days and was not significantly different from that of controls at p = 0.05), and 20 to 25% of the animals showed a weak but significant response (mean survival time was 25.4 days and was different from that of controls at p = 0.05). Immunotherapy, with the same number of injections, quantity of transplanted cells, and time course (3 i.p. injections of 10⁷ cells per injection on Days 1, 2, and 3), appeared to be less effective when the tumor cell inoculum comprised glutaraldehyde-fixed cells plus radiation-blocked cells (1:1) or glutaraldehyde-fixed cells only as shown in Chart 1. The survival times for both of these modified im-

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* T. S. Johnson and A. Brunsting. DNA Distribution Analysis and Biological Interpretation, manuscript in preparation.
Chart 1. Time-course-total body $^{125}$I activity curves for C57BL/6J mice showing the effect of TSI on the clearance rate of i.p.-challenged $10^7$ $^{125}$I-labeled EL4 tumor cells given on Day 0. Group 1 syngeneic C57BL/6J mice (n = 50) received no therapy; Group 2 C57BL/6J animals (n = 50) were treated with TSI (3 i.p. injections of $10^7$ irradiated EL 4 cells given at 1, 2, and 3 days after tumor challenge); and Group 3 allogeneic DBA/2J control mice (n = 40) were challenged in a manner similar to that for Group 1 and Group 2 animals and received no therapy. The animal survival data for Group 1, Group 2, and Group 3 mice are plotted below the $^{125}$I-activity clearance curves to illustrate the effect of TSI on prolonged survival times observed during the first 14 days after tumor challenge. Laboratory control mice (19 of 19) challenged with $10^7$ unlabeled EL 4 cells died by 14 days postchallenge (not plotted).

Chart 2. The effects of single-modality immunotherapy and radiation therapy (Rad. Ther.) versus combined immunoradiotherapy on the survival of C57BL/6J mice challenged i.p. with $5 \times 10^3$ syngeneic EL 4 tumor. Group 1 mice (n = 50) received no therapy. Group 2 animals (n = 50) were given immunotherapy consisting of 3 i.p. injections of $10^7$ glutaraldehyde-fixed plus irradiated EL 4 cells (1:1) given on Days 1, 2, and 3; Group 3 mice (n = 50) received immunotherapy consisting of 3 i.p. injections of $10^7$ glutaraldehyde-fixed EL 4 cells on Days 1, 2, and 3; Group 4 animals (n = 50) received TSI by 3 i.p. injections of $10^7$ irradiated EL 4 cells on Days 1, 2, and 3. Group 5 mice (n = 42) were treated with 250 rads local radiation therapy on Day 2; Group 6 animals (n = 40) were given TSI like Group 4 plus irradiation similar to that for Group 5.

Immunotherapy regimens, i.e., 18.8 and 17.5 days for Groups 2 and 3, respectively, as illustrated in Chart 2, were not significantly different at $p = 0.05$ from control Group 1 survival, although they showed broader statistical spreading of survival data distributions than did controls. These modified immunotherapy regimen groups also lacked the bimodal distributions evident in the TSI-treated animals given irradiated cells. The mean survival time of 18.1 days of the radiation therapy-treated animal (Group 5) was similar to that of controls at $p = 0.05$, while 25 to 30% of mice given combined immunoradiotherapy (TSI similar to Group 4 animals plus irradiation similar to that of Group 5) showed the best therapeutic response (mean survival time, 28.8 days). Two animals (2 of 40) given combined therapy survived indefinitely.

Flow-through Light Scatter Profiles and FCP DNA Distri-
butions of Host Somatic Cells and EL 4 Tumors. Chart 3 illustrates typical Coulter TPS light scatter profiles obtained for normal C57BL/6J RBC, retroperitoneal lymph node cells, and 7-day EL 4 tumor cells. The differential light scatter profiles allowed light scatter-gated, electronic cell sorting of host somatic versus EL 4 tumor cells from harvested mouse peritoneal exudate, heterogeneous samples. The sorted somatic and tumor cells were then stained with propidium iodide (34) and were used as reference populations in the subsequent DNA distribution experiments. Chart 4 shows the time dependency for DNA distributions obtained for C57BL/6J mouse retroperitoneal lymph node cells and tumor harvested from the peritoneal cavities of i.p. EL 4-challenged syngeneic animals. Lymph node populations from unchallenged control animals had age distributions as follows: G1-G0 = 96 to 97%; S = 1 to 2%; and G2 + M = 1 to 3%, as compared to blastogenic-responding lymph node populations of tumor-challenged mice (G1-G0 = 78 to 79%, S = 7 to 8%, and G2 + M = 10 to 11%) at 7 days following i.p. 0.5 \times 10^7 EL 4 tumor challenge. Concomitantly, the cell cycle DNA distributions obtained for EL 4 tumors harvested from the peritoneal cavities of the same groups of animals used for the lymph node samples showed age distributions indicative of asynchronous exponentially growing populations at 1 to 2 days posttransplantation, i.e., G1-G0 = 41 to 42%, S = 50 to 52%, and G2 + M = 6.5 to 7.5%, with a progressive change to G1-G0 = 54 to 55%, S = 35 to 36%, and G2 + M = 8 to 9% at Day 7.

Sequential DNA distribution analysis of C57BL/6J mouse peritoneal exudate samples indicated that quantitatively only a relatively small proportion of somatic cells were present in the tumor samples from challenge Day 0 to 7 days postchallenge (Chart 4). The somatic G1-G0 DNA distribution peak characteristic for C57BL/6J normal diploid spleen cells, lymphocytes, macrophages, etc., always occurred at the lower portion of the fluorescent histogram, at one-half the DNA content per nucleus measurable for the EL 4 tumor cell G1-G0 peak (Chart 4). If a large number of immune or unimmune somatic cells had been present in these samples, a somatic G1-G0 peak would have been readily apparent in the DNA distributions for 0- to 7-day samples. Eight days after the initial tumor challenge, a definite G1-G0 somatic peak was evident, and a broadening occurred in the tumor G1-G0 peak (Chart 5).

Effects of Immunotherapy and Radiation Therapy on Tumor Population Cytokinetic Age Distributions. The DNA distributions for EL 4 tumors harvested from C57BL/6J animals challenged i.p. with 0.5 \times 10^7 EL 4 cells and receiving either no therapy (Group A, control tumors), TSI (Group B), radiation therapy (Group C), or combined immunoradiotherapy (Group D, TSI + radiation therapy) are shown in Chart 6. Invariably, the tumor cytokinetic age distributions obtained from untreated Group A animals at 2
to 3 days after i.p. tumor challenge were indicative of unperturbed, asynchronous exponentially growing populations ($G_1-G_2 = 40$ to $42\%$, $S = 50$ to $52\%$, and $G_2 + M = 6.5$ to $7.5\%$) (Chart 6A). Age distributions of tumors analyzed during this same period for Group B immunotherapy-treated animals were similar to those for Group A untreated tumors (Chart 6, A and B). Tumors given 250 rads of local radiation therapy on Day 2 and analyzed sequentially from 15 min to 24 hr after irradiation displayed radiation-induced cell cycle progression delays consisting of a prominent early S-phase delay and what was considered as a $G_2$ block (Chart 6C).

The early S-phase transition block was evident as a shoulder 15 min postirradiation and then developed progressively into a discrete perturbed component peak (designated $S_p$) persisting for 90 to 100 min, compared to 3- to 4-hr duration for the $G_2$ block. The $S_p$, the early S-phase perturbed component peak, reached a maximum at 1.5 to 1.75 hr after irradiation and 25% of the total tumor age distribution curve and then was unresolvable as a discrete component by 2 hr posttherapy (Table 1). By 24 hr postradiation therapy, the age distribution of EL 4 tumors was very similar to that for controls, except for a 4 to 5% higher cycling cell index ($S + G_2 + M$), implying that the major cytokinetic recovery had occurred and that a slight compensatory "overshoot" was operative (Chart 6C).

Those C57BL/6J tumor-bearing animals treated with combined immunoradiotherapy showed dramatic effects on the tumor population cytokinetic age distributions. Chart 6 (Group D) graphically illustrates this cytokinetic therapeutic effect of combined TSI plus 250 rads local irradiation given on Day 2. In contrast to those tumors treated with radiation therapy (Chart 6C), the age distributions of EL 4 tumor populations that received combined immunoradiotherapy were devoid of both the early S-phase, $S_p$ perturbed peak, and $G_2$ block. Tumor populations from combined-therapy-treated animals consistently showed DNA distribution patterns suggestive of preferential loss of radiation-damaged S-phase tumor cells and delayed kinetic recovery (Chart 6D).

**DISCUSSION**

More effective radiation sterilization of certain primary...
and recurrent tumors may be possible by use of adjuvant immunotherapy (8, 50, 61). This report describes some observed effects of in situ radiation therapy given separately and in combination with TSI on the growth, total-body clearance rate, and cytokinetic DNA distribution patterns of a murine model tumor system. Evidence was obtained that indicated that TSI elicits in C57BL/6J mice accelerated rejection of syngeneic EL 4 peritoneal ascites tumor cells and slightly prolonged survival times, without apparent growth-inhibitory effects on the cycling tumor cells. When TSI was used as adjuvant to local radiation therapy, animal survival times were prolonged significantly compared to single-modality TSI (p = 0.05) or radiation therapy (p = 0.01). FCP DNA distribution data suggested that, in animals treated with combined immunoradiotherapy, a radiation potentiation effect existed within the first few hr after in situ irradiation of EL 4 cells for immune destruction of sublethally radiation-damaged, repairing, and/or recovering S-phase tumor cells. Such a radiation potentiation effect, if operative in human tumor systems, might be exploited in fractionated regimens effectively to increase the therapeutic ratio and to decrease the probability of residual, clonalogenic tumor cells in the treatment field without additional complications due to tissue tolerance.

In this study, active TSI, with the use of multiple i.p. injections of radiation-blocked EL 4 cells, caused a minimal yet significant therapeutic effect on EL 4 leukemia transplants growing in the peritoneal cavity of syngeneic C57BL/6J mice. The short-term (0 to 7 days post-tumor challenge and TSI) clearance rate of 125I-labeled EL 4 tumor was increased in TSI-treated animals to a rate comparable to that for untreated allogeneic recipients (Chart 1), and the survival times of these syngeneic hosts were prolonged by immunotherapy (Charts 1 and 2).

The tumor-specific antigenicity of EL 4 leukemia cells is considered low and ordinarily does not elicit an effective immune response in the C57BL/6J host (1, 26, 28, 38). Therapeutically, the weak response observed in this study was consistent with previously reported data, indicating that only a very limited immune capacity to respond to immunotherapy exists (18, 50, 58, 63). This apparent low level of immune exploitable activity may prove to be a major limiting factor for successful clinical use of adjuvant TSI. In addition, immune competence tends to decrease with tumor progression (29, 32, 55, 58) and stimulation of antitumor cell-mediated immunity can concomitantly increase the number of suppressor T-lymphocytes (42). The relatively weak therapeutic survival response achieved in this study with the use of TSI appeared to be a bimodal response, where the majority of animals (75 to 80%) failed to respond, while 20 to 25% of the animals did respond. This is suggestive of biological variability for C57BL/6J host immune responsiveness to the active TSI method used. A comparable 27% therapeutic response has been reported for C57BL/6J mouse i.p.-challenged murine lymphoid leukemia with the use of Bacillus Calmette-Guérin immunotherapy by Olsson and Mathé (46).

The TSI method used in this study preferentially evokes an initial activated macrophage response of short duration in the C57BL/6J animals and tumor-specific cell-mediated cytotoxic lymphocyte activity after 8 to 10 days, apparently without enhancement (26). This would suggest that involvement of macrophage phagocytic activity or possibly macrophage-derived cytotoxic serum factors accounted for the short-term therapeutic effects of TSI on 125I-labeled EL 4 clearance syngeneic animals and contributed to the increased survival times. Other investigators assaying in vivo 125I-labeled tumor destruction and clearance from experimental mouse tumor systems have reported similar short-term effects attributed to phagocytic activity (22, 48), with at least 1 study demonstrating augmentation of in vivo destruction by immune stimulation with the use of radiation-blocked tumor cells (48). Numerous other studies have substantiated the relatively rapid, short-term recruitment of macrophages (14, 64), macrophage-mediated cytotoxic killing of tumor cells (14, 31, 45), and increased macrophage activity in response to immunotherapy (15, 37, 54). The 2 most obvious macrophage activation pathways that TSI...
might influence and thereby account for the short-term therapeutic effects observed against EL 4 tumors are direct activation of phagocytic cells in response to immune stimulation and indirect recruitment of macrophages via macrophage activation or macrophage-inhibitory factors from lymphocytes. Whatever the mechanism, TSI is most effective as multiple “booster” doses of irradiated tumor cells, and the timing and quantity of inoculum is critical.

The therapeutic effects obtainable with TSI were too weak to cure effectively even tumor challenge doses as low as 5 × 10⁶ EL 4 cells, but they were thought to be of use as adjuvant therapy to irradiation. It was reasoned that, if TSI were combined appropriately with radiation therapy, the recognition phase and/or tumor target destruction phase of activated macrophages or even T-lymphocytes might be potentiated if the tumor cells were radiation-damaged cells undergoing DNA repair or kinetic recovery. In effect, I hypothesized that sublethally radiation-damaged, perturbed tumor cells, ordinarily destined to repair, recover, and proliferate, should be more susceptible to cytotoxic destruction than are undamaged tumor cells. Experimentally, combined immunoradiotherapy significantly increased the mean survival times of EL 4 i.p.-challenged mice compared to animals treated with single-modality radiation therapy (p = 0.01) or TSI immunoradiotherapy (p = 0.05). Sequential DNA distribution cytokinetic analysis of EL 4 tumor populations from animals given single versus combined modality therapy indicated that immunoradiotherapy induced a dramatic cytokinetic effect on EL 4 tumors not observed for either TSI or irradiation given separately. Irradiated tumors always showed predictable time-dose-dependent cell cycle age distributions, characterized by an early S-phase and G₂-phase-associated progression delays (Chart 6, Group C). Immunotherapy-treated animals had tumor populations with DNA distributions similar to tumors from untreated animals during the periods studied (Chart 6B). In contrast, tumors from combined immunoradiotherapy-treated mice showed dramatic cytokinetic age distribution perturbations (Chart 6D). These cell cycle age distribution perturbations were indicative of preferential S-phase and possible G₂-phase loss of radiation-damaged EL 4 cells from the combined therapy-treated tumor populations. The evidence pointing to a preferential loss of S-phase radiation-damaged tumor cells was the precipitous decrease in percentage of S-phase cells and absence of any early S₀- or G₀-associated radiation perturbed component in 20 of 21 tumor DNA distributions analyzed from animals given combined therapy and assayed from 0.5 to 4 hr after irradiation. The duration for kinetic recovery was prolonged in combination therapy-treated animal tumors by approximately 1 EL 4 generation cycle time of 12 to 13 hr (24 hr for irradiated only versus approximately 36-hr recovery time for immunoradiotherapy treated). The argument to support this as a biological phenomenon and not a misinterpreted artifact is based on the consistency of the DNA distribution data together with previously reported and unpublished morphological, Coulter volume analysis, and radioiodinated tumor clearance data for i.p.-injected syngeneic murine tumor cells (2, 26).


CV = \frac{\text{peak channel width at half-maximal}}{\text{peak channel} \times 2.35}

compared to the untreated tumor DNA distribution G₁-G₃ peaks, consistent with selective removal of tumor cells from the population, as opposed to broadening of the G₁-G₃ peak component (and increased coefficient of variation) that occurs when damaged and dying G₁-G₃ cells are present in the population. In addition, activated macrophages elicited by the TSI regimen used would not be expected to be significantly affected functionally by the single 250-rad irradiation dose used, inasmuch as 99 to 100% of these cells in the peritoneal cavity are kinetically noncycling and only moderately radiosensitive (7, 65, 66).

These results are consistent with the hypothesis that some rapidly responding immune process, i.e., probably activated macrophages, destroyed the sublethally radiation-damaged (cell cycle progression blocked early-S-phase EL 4 cells) plus other radiation-damaged S-phase tumor cells and possible G₂-blocked cells. Numerous possible explanations may be advanced to account for this additive or synergistic therapeutic effect. Some of these explanations are increased recognition of tumor targets by the effector cells caused by radiation disruption and slowing of cycling EL 4 cells (19, 40, 63), alteration of the tumor-specific membrane components (10, 43, 51), or transient radiation-induced changes in the tumor membrane structural and biophysical charge characteristics recognizable by the effector cells (50, 52, 57, 65). The destruction phase of cell-mediated cytotoxicity could have been potentiated simply because these sublethally damaged tumor cells were more susceptible to killing in the radiation-damaged state (19, 20, 59).

Whatever the mechanism, it is known that the monocyte-macrophage system of phagocyte cells can not only recognize tumor-specific tumor antigens for autologous and syngeneic tumors (12, 14, 30, 31, 33) but can also recognize cell surface features of damaged cells, abnormal cells, cell debris, and dead cells (65, 66). Following recognition and subsequent macrophage-target cell-to-cell interaction, as well as macrophage-mediated cytotoxic activity, the cytotoxic process commences rapidly. This antitumor cytotoxic activity is especially prominent for specifically activated phagocytic cells, e.g., the active TSI used in this study. It is this inherent ability of the monocyte-macrophage system to respond rapidly to and to destroy undamaged and damaged tumor cells that is the basis of macrophage-mediated im-

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munotherapy. This study suggests that sublethally radiation-damaged EL 4 tumor cells, especially early S-phase cells initiating DNA synthesis at the time of irradiation, may be more susceptible to activated macrophage-mediated immune destruction. Experiments are in progress to test directly the tumor cell cycle dependency for activated macrophage cytotoxic killing of tumor target cells in relation to single TSI and irradiation treatment regimens versus combined immunoradiotherapy.

The cause and effect relationships for the observed radiation potentiation phenomena remain to be defined. This study, however, together with other available experimental data cited in this report, indicates that the successful consequences of combined TSI and radiation therapy are very dependent on the therapeutic time-dose fractionation schedule used and cell kinetic and radiobiological state of the tumor (the relatively long S-phase duration and short G0 of most ascites tumor cells like the EL 4 leukemia cells used in this study) would tend to favor expression of an S-phase radiobiological effect and would be expected to be less dramatic, yet significant, in slower-growing long-normal age distribution tumor populations, together with the effectiveness of immune stimulation. In retrospect, it has been known for many years that host factors are important determinants of tumor radiation response (8, 9, 19, 23), and it is becoming increasingly apparent that adjuvant immunotherapy can facilitate destruction of small numbers of viable tumor cells remaining in the treatment area after irradiation. Experimental data are now available showing that immune stimulation can, in some tumors, facilitate killing of postmitotic, noncycling tumor cells, with little effect on the kinetics of cycling cells (25); cause preferential destruction of G2-G or early S-phase tumor cells (46) and G2 cells (24); inhibit tumor DNA synthesis (33); or shorten tumor S-phase duration and mean cell cycle times (4). Evidence is also available that suggests that a cell cycle phase dependency exists for expression of tumor antigenicity (11), and irradiated tumor cells are in some instances more likely to be destroyed cytotoxicly than are unirradiated tumor cells (20, 36, 53, 59). Unfortunately, the basic biological mechanisms and related radiobiological, radiotherapeutic implications of these tumor cell kinetic phenomena and of the radiation potentiation effect of TSI and irradiation described in this report are not well understood. They do, however, hold some promise for increasing the therapeutic ratio for certain primary and disseminated cancers by use of combined immunoradiotherapy by decreasing the probability of residual clonogenic tumor cells locally in the treatment field, as well as systematically.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Everett V. Sugarbaker, Dr. Albert Brunsting, Dr. Jerry T. Thortonwaite, and Dr. Howard G. Gratzner for their advice and constructive discussions. JoAnne McDermott and Paul Kizakevich provided excellent computer statistical analysis assistance.

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Potentiation of in Vivo Model Murine Tumor Destruction by Combined Immunoradiotherapy

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