

SYNERGISTIC ANTITUMOR ACTIVITY OF TUMOR-INFILTRATING LYMPHOCYTES, INTERLEUKIN 2, AND LOCAL TUMOR IRRADIATION

Studies on the Mechanism of Action

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IL-2 given either alone or in combination with the administration of lymphokine-activated killer (LAK)¹ cells has been shown to mediate the regression of established metastatic tumor both in mice and man (1-3). Furthermore, adoptive transfer of tumor-infiltrating lymphocytes (TIL) concomitant with the administration of IL-2 can mediate antitumor effects that are 50-100 times more potent in murine metastatic tumor models than those observed with LAK cells (4, 5). In early human trials using TIL, regression of established metastatic disease has been observed in selected patients (6, 7).

In 6- and 14-d murine metastatic models, however, successful TIL therapy is dependent on the use of either cyclophosphamide (CP) or whole body irradiation (WBX) (4). The reason for this requirement remains unclear, but reports have demonstrated that mice bearing immunogenic tumors develop suppressor cells capable of inhibiting the antitumor activity of adoptively transferred Lyt-2⁺ cytotoxic lymphocytes (8, 9).

To assess the possible role of immunosuppression, suppressor cells, and direct antitumor activity of CP and WBX in the augmentation of the antitumor activity mediated by Lyt-2⁺ TIL and IL-2, we first compared WBX with local tumor radiation (LTX) in the treatment of established 7-d liver metastases. We also assessed the requirement for CP in B mice that were devoid of mature T cells and, therefore, any Ts cell activity. When taken together, our results strongly suggest that the effects of CP and WBX that combine with Lyt-2⁺ TIL to mediate antitumor activity against established 7-d hepatic metastases do not involve immunosuppression or the elimination of suppressor cells, but rather a direct antitumor activity.

Materials and Methods

Mice. Female C57BL/6 mice were used in all experiments. Mice were 8 wk of age when undergoing thymectomy. All other mice were at least 12 wk of age when used in experiments, and were obtained from the Small Animal Section, Veterinary Resources Branch, Division of Research Services, National Institutes of Health, Bethesda, MD.

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¹ *Abbreviations used in this paper:* CM, complete medium; CP, cyclophosphamide; LAK, lymphokine-activated killer cells; LTX, local tumor irradiation; MLR, mixed lymphocyte reaction; MST, median survival time; TIL, tumor-infiltrating lymphocytes; WBC, white blood cells; WBX, whole body irradiation.

Tumors. MC-38 is a weakly immunogenic murine adenocarcinoma induced by the subcutaneous injection of dimethylhydrazine and passaged subcutaneously in mice. Single cell suspensions were prepared as previously described (2). Briefly, growing subcutaneous tumors were harvested and digested with a triple enzyme solution containing 0.1% collagenase, type IV; 0.01% hyaluronidase, type V; and 0.002% DNase, type I (Sigma Chemical Co., St. Louis, MO). The resulting single cell suspensions were washed in HBSS (Biofluids, Rockville, MD), counted, and diluted to a concentration of 5×10^5 cells/ml for induction of experimental liver metastases.

Medium. In vitro culture of TIL was performed in complete medium consisting of RPMI 1640 medium (Biofluids) supplemented with 10% heat-inactivated FCS (Biofluids), 0.03% fresh glutamine (NIH Media Unit), 0.1 mM nonessential amino acids (M. A. Bioproducts, Walkersville, MD), 1 mM sodium pyruvate (Biofluids), 5×10^{-5} M 2-ME (Aldrich Chemical Co., Milwaukee, WI), 100 U/ml penicillin (NIH Media Unit), 100 μ g/ml streptomycin (NIH Media Unit), 50 μ g/ml gentamicin (Schering, Kenilworth, NJ), and 0.5 μ g/ml amphotericin B (Flow Laboratories, McLean, VA).

Lymphokines. Human rIL-2 was kindly supplied by Cetus Corp., Emeryville, CA. The specific activity was 3.0×10^6 Cetus U/mg (8×10^6 Biological Response Modifiers Program U/ μ g), and the endotoxin level was <0.024 ng/ml, as measured by limulus amoebocyte lysate assay.

TIL. Thy-1.2⁺, Lyt-2⁺, tumor-specific cytotoxic TIL were grown from subcutaneous MC-38 tumors using a method described previously (5). In some experiments, however, a method slightly modified from that described earlier was used. Briefly, in these experiments, subcutaneous tumors were digested with triple enzymes as described above. Thy-1.2⁺ lymphocytes were isolated from the resulting single cell suspensions by incubating 10^8 tumor cells/ml with 10^8 Dynabeads M-450 immunomagnetic beads/ml (Dynal Inc., Great Neck, NY) previously coated with anti-Thy-1.2 antibody (New England Nuclear, Boston, MA) for 45 min at 4°C. The lymphocyte/bead complexes were then extracted with a magnet and cultured at 37°C in complete medium (CM) containing 10 U/ml of IL-2. After 24 h, lymphocytes that had fallen off the beads were harvested and placed into culture at 10^5 cells/ml in CM containing 10 U/ml of IL-2. Initially, 5×10^5 splenocytes/ml irradiated with 3,000 cGy were added as feeders, and 10^5 tumor cells/ml irradiated with 10,000–30,000 cGy were added as specific stimulator cells. Subsequently, growing lymphocytes were diluted to a concentration of 10^5 cells/ml and supplemented every 7–10 d with $2-4 \times 10^5$ splenocytes/ml and $1-2 \times 10^5$ tumor cells/ml. Both splenocytes and tumor cells were irradiated with 3,000 cGy before addition to TIL cultures. These TIL, when free of tumor cells, were used in therapy experiments, and were identical in both phenotype and specificity to those derived from the previously described method (10).

Local Irradiation. Mice treated with local liver irradiation were first anesthetized with 0.2–0.225 ml of a 7% pentobarbital solution (Somnifer; Richmond Veterinary Supply Co., Richmond, VA) and placed into a lead holder. Appropriate lead shields were used to cover nothing (WBX), the liver, the entire body except the liver, or in some experiments, the entire body except one-half of the liver. Mice were then irradiated with an X-ray machine (RT 250; Philips Electronic Instruments, Inc., Mahwah, NJ) operating at 235 kV and 15 mamps (0.25-mm Cu and 0.55-mm Al filtration) delivering 63 cGy/min. After irradiation, animals within each treatment group were allowed to recover from anesthesia, randomized, and distributed to cages.

B Mice. B mice were generated using 8-wk-old C57BL/6 mice as previously described (11). Briefly, mice were anesthetized with 0.25 ml of a 7% pentobarbital solution, and a thymectomy was performed. Mice were then allowed to recover for a period of 2 wk. Subsequently, the mice were lethally irradiated using a ¹³⁷Cs irradiator (Gammacell 40) delivering 109 cGy/min (Atomic Energy of Canada Ltd., Kanata, Ontario, Canada), and immediately reconstituted with 5×10^6 T-depleted bone marrow cells. Normal C57BL/6 bone marrow cells were depleted of T lymphocytes by incubating 10^8 bone marrow cells/ml in rabbit anti-mouse brain antibody (kindly provided by Dr. David Sachs, Immunology Branch, NCI, Bethesda, MD) diluted 1:50 in Ca²⁺, Mg²⁺-free HBSS (Biofluids) for 45 min at 4°C, followed by 30 min of incubation with guinea pig complement (Gibco Laboratories, Grand Island, NY) diluted 1:3 in Ca²⁺, Mg²⁺-free HBSS. Mice were then allowed to recover for an additional 2 wk before use in therapy experiments.

Mixed Lymphocyte Response Assay. Splenocytes harvested from B mice were assayed for responsiveness to allogeneic antigens as a measure of T cell function. Spleens from three randomly selected B mice were sterilely harvested and crushed. The splenocytes were then filtered through 112- μ m nylon mesh (Nitex; Lawshe Industrial Co., Bethesda, MD). The resulting single cell suspension was washed three times after lysing the RBC with ACK lysing buffer (NIH Media Unit). After resuspending the cells in complete media, 10^5 cells in 0.1 ml were plated into 96-well flat-bottomed plates. Three spleens were harvested in an identical fashion from normal animals, and a single cell suspension of normal splenocytes was prepared as a positive control. Stimulator cells were prepared from normal C57BL/6 and DBA splenocytes and irradiated to 3,000 cGy. After resuspension in complete media, 10^5 of these splenocytes in 0.1 ml were plated into triplicate wells containing the responding cells, as well as three control wells containing media alone. CM was added to three wells containing responding cells as additional controls. The plates were then incubated at 37°C and 5% CO₂ for 2 or 4 d. On day 2 or 4, 1 mci of [³H]thymidine (New England Nuclear, Boston, MA) was added to each well. After 6 h of additional incubation, the cells were harvested on a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA), 3 ml of scintillation fluid was added, and the radioactivity was measured on a β liquid scintillation counter (LKB Instruments, Inc., Gaithersburg, MD). The uptake of [³H]thymidine in cpm was then used as a measure of stimulation.

Generation of Liver Metastases. Multiple liver metastases were induced by the intrasplenic injection of single cell tumor preparations, as previously described (12). Briefly, C57BL/6 mice were anesthetized with 0.2–0.225 ml of a 7% Somnifer solution and then immobilized. A left subcostal incision (5–7 mm) was then made and the spleen externalized and isolated with a sterile 2 × 2-inch gauze. A 25-gauge needle (American Hospital Supply, Chicago, IL) was used to puncture the splenic capsule and inject 5×10^5 tumor cells in 1.0 ml directly into the spleen. Gentle pressure was applied for a period of 1 min to prevent hemorrhage and tumor cell extravasation. The splenic pedicle was then clamped with a medium hemoclip (Edward Weck & Company, Inc., Research Triangle Park, NC) and the spleen removed. The skin and abdominal wall were closed in one layer with 9-mm Autoclip wound clips (Clay Adams, Parsippany, NJ). The mice were allowed to recover and then were randomized before being distributed to isolator cages. At the conclusion (day 16 or 17) of experiments designed to determine the number of liver metastases, mice were randomized and ear tagged. 0.75–1.0 ml of a 15% India ink solution was then injected via the lateral tail vein, after which 0.1 ml of 100% pentobarbital was injected intraperitoneally to kill the mice. The stained livers were collected and placed into Fekete's solution for at least 1 h to bleach the metastases, which stand out as white nodules against a black background of normal liver parenchyma. The livers were divided into right-sided lobes (three) and left-sided lobes (four) of approximately equal volume. The tumor nodules were then blindly counted and recorded.

Clonogenic Assay. Single cell suspensions were prepared by triple enzyme digestion, as described above, from livers bearing MC-38 metastases. The cells were washed three times and resuspended in CM. Triplicate samples were plated into six-well plates from each of five serial 1:10 dilutions prepared from a suspension containing 10^7 cells/ml. The cells were thoroughly mixed in the wells and allowed to incubate for 14 d at 37°C in 5% CO₂. After 2 wk, the adherent cells were fixed with 25% methanol and stained with 5% crystal violet in a 10% ethanol solution (NIH Media Unit). The number of colonies was then blindly counted and the number of colonies per 10^6 original cells determined.

Immunotherapy Model. Multiple liver metastases were induced on day 0 as described above. After 6 d, when metastatic nodules could be identified on the surface of the liver, mice were treated with LTX, WBX, or WBX with liver shielding. On day 7, some mice were given a single injection of TIL intravenously, and then IL-2 was administered intraperitoneally thrice daily for 5 d. After treatment, mice were followed for survival or killed on day 17 for enumeration of liver metastases. In survival experiments, all dead animals were autopsied for evidence of tumor.

Statistical Analysis. In experiments enumerating the number of liver metastases, comparisons were made using the Kaplan-Meier method and Mantel-Haenszel test for significance within groups. Survival analyses were performed using the Kruskal-Wallis test. All *p* values are two tailed.

Results

The Effect of LTX on Immunotherapy with TIL and IL-2. To assess the impact of LTX on the effectiveness of immunotherapy with TIL and IL-2, LTX was directly compared with our previously published regimen using WBX (4). Mice bearing multiple 6-d hepatic metastases were treated with 750-cGy X-ray irradiation to the whole body, to the liver alone, or to the whole body, shielding the liver. An additional group of mice received no radiation therapy. All mice receiving 750-cGy WBX were immediately reconstituted with $1-1.3 \times 10^7$ normal bone marrow cells. 1 d after irradiation, groups of six mice from each radiation group were randomly placed into one of three treatment regimens: HBSS (0.5 ml i.p.) thrice daily for 5 d, IL-2 (50,000 U i.p.) thrice daily for 5 d, or $1-2.0 \times 10^7$ TIL intravenously on day 7 followed by IL-2 (50,000 U i.p.) thrice daily for 5 d. After completion of therapy, the mice were followed for survival. Fig. 1 represents data compiled from four identical experiments. As shown in the top frame, there was no efficacy to treatment with TIL and IL-2 if no radiation was given (median survival times [MST] = 11.5, 17.0, and 15.1 d with HBSS, IL-2, and IL-2 plus TIL, respectively).

With 750-cGy WBX, a small, albeit statistically significant, survival advantage was observed due to the direct effects of radiation therapy (MST = 21.0 d with WBX vs. 11.5 d without radiation in control mice; $p < 0.0001$). No survival benefit was seen with the addition of IL-2 (MST = 21.0 d), however, in mice given both TIL and IL-2 after 750-cGy WBX, a substantial prolongation of survival was noted when compared with mice receiving WBX alone or WBX with IL-2 (MST of >120 d and 75%

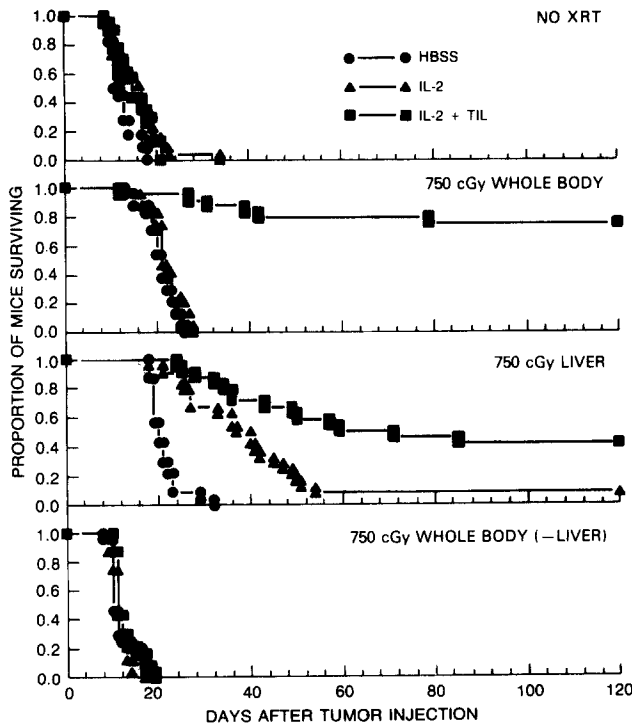


FIGURE 1. Comparison of LTX with WBX. The data shown are from four combined experiments with a total of 22-24 mice per treatment group. Mice bearing 6-d MC-38 hepatic metastases were given no radiation (controls), 750-cGy WBX, 750-cGy liver irradiation, or 750-cGy WBX with shielding of the liver. Mice receiving WBX were immediately reconstituted with 10^7 normal bone marrow cells. After 24 h (day 7), immunotherapy was begun with HBSS, IL-2 (50,000 U i.p.) thrice daily for 5 d, or IL-2 (50,000 U i.p.) thrice daily for 5 d combined with one dose of $1-2 \times 10^7$ TIL given on day 7. All mice were then followed for survival, and all dead mice were autopsied for the presence of tumor.

long-term survival compared with an MST of 21.0 d and 0% long-term survival with both WBX alone and WBX followed by IL-2 therapy; $p < 0.0001$). These findings are consistent with our earlier publication (4).

If 750-cGy LTX was delivered to the liver alone, prolongation of survival was again noted in control mice due to a direct antitumor effect (MST = 20.0 d vs. 11.5 d in mice treated with HBSS alone; $p < 0.0001$), and in mice treated with the combination of LTX, IL-2, and TIL (MST = 65.0 d and 41.7% long-term survival vs. 20.0 d and 0% long-term survival with LTX alone; $p < 0.0001$). These findings are similar to those noted above with WBX, although TIL therapy was slightly less successful with LTX, possibly reflecting tumor that was missed by the LTX port. However, unlike WBX, the addition of IL-2 to LTX produced a substantial increase in the MST (MST = 38.5 vs. 20.0 d in mice treated with LTX alone; $p < 0.0001$). This antitumor activity mediated by the combination of LTX and IL-2 represents a component of host immunity that was recruited by IL-2 administration after LTX but not after WBX.

Finally, when WBX was used while shielding the liver (WBX/S), no therapeutic advantage was noted in any of the treatment groups, even when compared with mice receiving no radiation therapy (MST = 10.0, 11.0, and 11.0 vs. 11.5 d for WBX/S + HBSS, WBX/S + IL-2, and WBX/S + IL-2 + TIL vs. HBSS alone, respectively). Thus, irradiation of the tumor-bearing organ rather than total body lymphoid suppression appeared necessary for the therapeutic effect of TIL. Furthermore, when total body immunosuppression was avoided (as with LTX), a potent antitumor effect mediated by IL-2 administration alone became evident.

Dose-dependent Effects of LTX. A dose titration of LTX was performed to ascertain the level of radiation for the augmentation of TIL-mediated antitumor effects. Mice bearing 6-d MC-38 hepatic metastases received 0-, 250-, 500-, or 1,000-cGy LTX. 1 d later, mice from each radiation group were given immunotherapy with HBSS, IL-2 (25,000 U i.p.) thrice daily for 5 d, or a single dose of 1.3×10^7 TIL intravenously on day 7 followed by IL-2 (25,000 U i.p.) thrice daily for 5 d. Upon completion of the immunotherapy regimen, the mice were followed for survival. All dead animals were autopsied for the presence of tumor. The results, illustrated in Fig. 2, demonstrated that doses of at least 500 cGy are necessary to impact on survival. Treatment with LTX in the absence of immunotherapy (HBSS-treated groups) exhibited a dose-dependent prolongation in survival (MST = 12, 13.5, 18, and 25 d in the 0-, 250-, 500-, and 1,000-cGy groups, respectively). With 500 and 1,000 cGy (as with 750 cGy in the previous experiments), a survival benefit was seen in mice given IL-2 alone when compared with mice treated with LTX alone ($p < 0.02$). Furthermore, the addition of TIL to the immunotherapy regimen enhanced the survival benefit over that produced by IL-2 alone ($p < 0.05$). The effects in both IL-2 alone and IL-2 combined with TIL were much more pronounced at the highest dose of LTX.

Lymphoid Effects of LTX Therapy. To further address the role of immunosuppression in the treatment of established MC-38 macrometastases with TIL and IL-2, groups of three normal mice were treated with the same radiation protocols as used in the previous experiments: (a) no radiation; (b) 750-cGy WBX with bone marrow reconstitution; (c) 750-cGy LTX; and (d) 750-cGy WBX excluding the liver. After 1, 3, and 5 d, blood was collected for determination of peripheral white blood cell

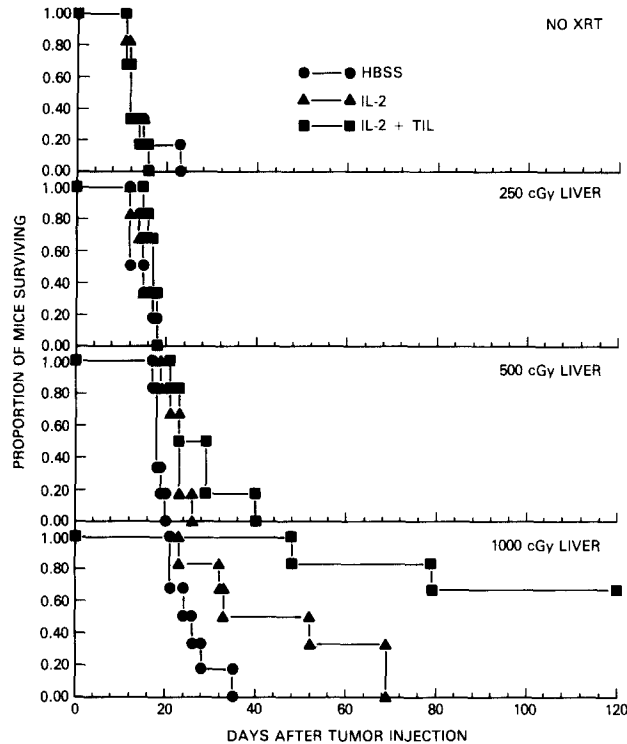


FIGURE 2. Dose-dependent effect of LTX. Mice bearing 6-d MC-38 hepatic metastases were irradiated with 0-, 250-, 500-, or 1,000-cGy local liver irradiation. After 24 h (day 7), groups of six mice from each dose level were treated with HBSS, IL-2 (25,000 U i.p.) thrice daily for 5 d, or 1.3×10^7 TIL given as a single intravenous dose combined with IL-2 (25,000 U i.p.) thrice daily for 5 d. After completion of immunotherapy, mice were followed for survival and autopsied upon death for the presence of tumor.

(WBC) counts. Subsequently, the mice were killed, and their spleens, femurs, and tibias were individually harvested to ascertain bone marrow and splenic cellularity. The results from two repeated experiments were similar. Data from one representative experiment shows that all three radiation regimens significantly reduced the peripheral WBC on day 1 (312 ± 30 , 440 ± 17 , and 468 ± 23.5 in WBX, LTX, and WBX/S vs. $2,404 \pm 207$ in the no radiation group, respectively; $p < 0.05$). In the two groups that included liver radiation (WBX and LTX), the counts remained significantly depressed on days 3 and 5 ($p < 0.05$), although by day 5 the counts in all groups had begun to recover.

In a similar manner, splenic cellularity was decreased on day 1 in all radiation groups, compared with HBSS-injected controls (1.2 ± 0.6 , 5.8 ± 2.6 , 11.3 ± 1.8 , and $52.0 \pm 11.0 \times 10^6$ cells/spleen in WBX, LTX, WBX/S, and normal control groups, respectively; $p < 0.05$). By day 5, however, all groups had begun to recover. Bone marrow cellularity was also significantly depressed after WBX with or without liver shielding (0.4 ± 0.12 , 0.7 ± 0.07 , and $9.0 \pm 1.0 \times 10^6$ cells/femur in WBX, WBX/S, and no radiation control groups, respectively; $p < 0.05$), and the counts in groups receiving radiation to the long bones and pelvis (WBX and WBX/S) continued to be substantially depressed on days 3 and 5 ($p < 0.05$). While the femur cellularity of mice treated with LTX was found to be intermediate but statistically decreased on day 1 (5.0 ± 0.4 vs. $9.0 \pm 1.0 \times 10^6$ cells/femur in LTX vs. no irradiation; $p < 0.05$), it was not different from control mice on days 3 or 5. Thus,

the immunosuppression observed in these experiments correlated well with the organs irradiated. That is, the bone marrow was suppressed most in animals receiving radiation to the marrow-containing long bones (WBX and WBX/S), and the splenic cellularity was most suppressed when the liver and spleen were included in the radiation field (WBX and LTX).

The Effects of Partial LTX on Immunotherapy with IL-2 and TIL. To detect the possible existence of tumor-associated Ts cells that may be eliminated with WBX, LTX, and as previously reported, with CP, mice bearing 6-d MC-38 hepatic macrometastases were treated with: (a) no radiation; (b) WBX with bone marrow reconstitution; and (c) radiation to the right side of the liver, shielding the left side (LTX_{1/2}). The following day, immunotherapy was begun in each radiation group with HBSS (0.5 ml i.p.) thrice daily for 5 d, IL-2 (50,000 U i.p.) thrice daily for 5 d, or 1.2×10^7 TIL given intravenously on day 7 followed by IL-2 (50,000 U i.p.) thrice daily for 5 d. On day 17, the mice were killed and the livers harvested. The number of liver metastases on the left and right sides of the liver were individually counted in a blinded, coded fashion. The experiment was repeated five times, and the results from one representative experiment are listed in Table I. As with the previous survival experiments, no substantial effect was noted on either side of the liver in the absence of any radiation therapy (mean number of metastases in the HBSS, IL-2, and IL-2 + TIL groups were [left/right] 500/500, 500/500, and $432 \pm 16.7/451 \pm 12.1$, respectively). With WBX, a marked reduction in the number of metastases on both sides was observed in animals receiving IL-2 + TIL ($14.4 \pm 20.8/3.2 \pm 3.4$ metastases vs. 500/500

TABLE I
Treatment of Hepatic Macrometastases with Limited LTX and TIL

Treatment*			Left side [†]		Right side [‡]	
XRT [§]	IL-2	TIL	Metastases	Mean \pm SEM	Metastases	Mean \pm SEM
-	-	-	500,500,500	500	500,500,500	500
-	+	-	500,500,500,500,500,500	500	500,500,500,500,500,500	500
-	+	+	500,500,500,500,500,92	432 ± 16.7	500,500,500,500,500,204	451 ± 12.1
WBX	-	-	500,500,500,500	500	500,500,500,500	500
WBX	+	-	500,500,500	500	500,500,500	500
WBX	+	+	0,3,8,10,51	$14.4 \pm 20.8^{\ddagger}$	8,5,3,0,0	$3.2 \pm 3.4^{\ddagger}$
LTX**	-	-	500,500,500,500,500	500	500,500,500,500,136	427 ± 16.3
LTX	+	-	500,500,500,500,500,500	500	500,500,500,500,500,207	451 ± 21.8
LTX	+	+	500,500,152,101,77,15	$224 \pm 21.8^{\ddagger\ddagger}$	500,2,0,0,0,0	$83.7 \pm 20.4^{\ddagger\ddagger}$

* No radiation, 750-cGy WBX, or 750-cGy LTX was given on day 6 after tumor injection. On day 7, immunotherapy was begun with HBSS alone, IL-2 (50,000 U i.p.) thrice daily for 5 d, or a single dose of 10^7 TIL on day 7 followed by IL-2 (50,000 U i.p.) thrice daily for 5 d.

[†] The liver was divided into left and right sides based on lobes. The right side consisted of one large lobe and two intermediate-sized lobes, while the left side consisted of one large lobe, one intermediate-sized lobe, and one small lobe. The total volumes of each side were approximately equal.

[§] All radiation was delivered using an X-ray machine (Philips Electronic Instruments, Inc.) operating at 235 kV and 15 mamps and delivering 63 cGy/min.

^{||} Metastases were identified as white nodules on a dark, India ink-stained background. The number of metastases was counted to a maximum of 500, and if the metastases were too numerous to count, a value of 500 was assigned. All experiments were counted in a blinded, coded fashion.

[‡] $p < 0.02$ compared with radiation alone and radiation combined with IL-2.

** Mice treated with LTX received radiation only to the right side of the liver with shielding of the left side.

^{‡‡} $p < 0.05$ compared with radiation alone and radiation combined with IL-2.

^{‡‡‡} $p < 0.01$ compared with radiation alone and radiation combined with IL-2.

in both the WBX + HBSS and WBX + IL-2 groups; $p < 0.02$). Finally, metastases on either side of the liver were not significantly reduced in animals receiving LTX_{1/2} and either HBSS or IL-2 ($500/427 \pm 16.3$ and $500/451 \pm 21.8$, respectively). However, when LTX_{1/2} was combined with IL-2 and TIL, a highly significant reduction in the number of right-sided metastases was seen (83.7 ± 20.4 ; $p < 0.01$). In addition, the number of metastases on the left side of the livers was also moderately reduced (224 ± 218 vs. 500 ± 0 in both the HBSS- and IL-2-treated groups; $p < 0.02$). A photograph of the gross pathologic appearance of a liver treated with LTX_{1/2}, IL-2, and TIL is provided in Fig. 3. Therefore, despite the presence of unirradiated tumor cells and theoretically suppressor cells in the same organ, no suppression in the antitumor effects of IL-2 and TIL was apparent. The slight reduction observed in the left-sided metastases suggests that the antitumor effects of IL-2 and TIL may extend to the unirradiated side as well, although partial irradiation of the

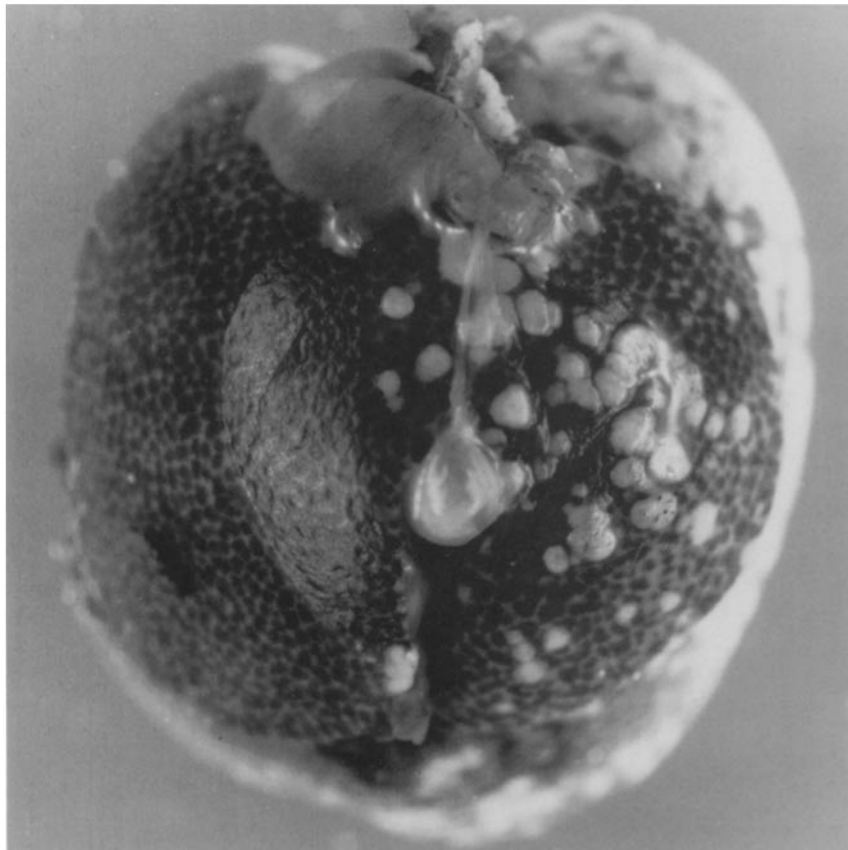


FIGURE 3. Partial LTX. Mice bearing 6-d MC-38 hepatic metastases received no radiation therapy, 750-cGy WBX, or 750-cGy X-ray irradiation to the right side of the liver with the left side shielded. 1 d later (day 7), immunotherapy was begun with HBSS, IL-2 (2,000 U i.p.) thrice daily for 5 d, or one intravenous dose of 1.3×10^7 TIL followed by IL-2 (2,000 U i.p.) thrice daily for 5 d. On day 17 after tumor injection, the mice were killed and the number of metastases on each side of the liver was counted. The data shown are representative of five experiments.

left side of the liver could also explain this finding. These results, however, are not consistent with the existence of suppressor cells, since a substantial antitumor effect was seen in the right side of the liver in the presence of extensive tumor in the left side of the liver.

The Antitumor Effects of IL-2 and TIL in B Mice. To assess the postulated existence of suppressor cells in a different model, 8-wk-old C57BL/6 mice underwent thymectomy followed 2 wk later by lethal γ irradiation and bone marrow reconstitution using T-depleted bone marrow. Before using B mice in immunotherapy experiments, splenocytes from these animals were tested for allogeneic responsiveness as measured by an anti-DBA mixed lymphocyte reaction (MLR). Normal C57BL/6 splenocytes were compared with those obtained from B mice. In response to DBA allogeneic antigens, normal splenocytes proliferated by day 2, and on day 4, the response had increased, as measured by [^3H]thymidine incorporation (net cpm = 68,959, compared with normal splenocytes stimulated with syngeneic cells). The B mouse splenocytes did not respond at either time point (net cpm = -4,325 compared with B splenocytes stimulated with syngeneic cells). In other experiments, mice were injected intravenously with tumor cell suspensions to induce pulmonary metastases. After 14 d, splenocytes from these animals were also assayed for T cell activity in an anti-DBA MLR. The results from these experiments showed that these mice continued to lack allogeneic responsiveness at the time immunotherapy was instituted (data not shown). Since splenectomy was performed after the induction of multiple liver metastases, these animals were not tested after the injection of tumor.

After demonstrating that B mice produced in this manner were devoid of T cell responsiveness both before and after tumor injection, 6-d multiple hepatic macro-metastases were generated in both normal and B mice. Some mice from each group were then treated with CP (100 mg/kg), followed by HBSS, IL-2, or IL-2 combined with TIL. These data are depicted in Fig. 4. CP was chosen since the B mice had already received >1,000 cGY and generally do not tolerate more radiation, and since CP and WBX both augment TIL antitumor activity in a similar manner. As in the radiation experiments, no effect was seen in either normal or B mice in the absence of CP. In normal mice receiving HBSS, IL-2, and IL-2 in combination with TIL, the MSTs were 18.0, 14.0, and 20.0 d, respectively, while in B mice, the same treatments resulted in MSTs of 25.0, 22.0, and 18.5 d. If CP was administered in normal mice, a significant survival advantage was noted (MST = 34.5 d with CP and HBSS vs. 18.0 d with HBSS alone). Similarly, CP administration in B mice prolonged the survival of HBSS-treated mice, albeit to a lesser degree (MST = 30.5 vs. 25.0 d in HBSS control mice). When IL-2 was added to both normal and B mice, an additional prolongation in survival was noted (45.5 d in normal and 48.0 d in B mice). This is similar to previously reported synergy between CP and IL-2 (13). When TIL were then added to the immunotherapy regimen, all normal and B mice survived tumor free for >120 d ($p < 0.0008$ vs. HBSS- and IL-2-treated mice). Thus, in both normal mice and mice depleted of T cells, the therapeutic efficacy of TIL depended on the use of CP. These data, along with the results from the LTX_{1/2} experiments, suggest that the mechanism of radiation augmentation of TIL antitumor activity is not dependent on the elimination of mature T lymphocyte suppressor cells.

The Direct Antitumor Effect of CP and Radiation Therapy. To investigate alternate mechanisms of interaction between CP/WBX and TIL immunotherapy, we measured

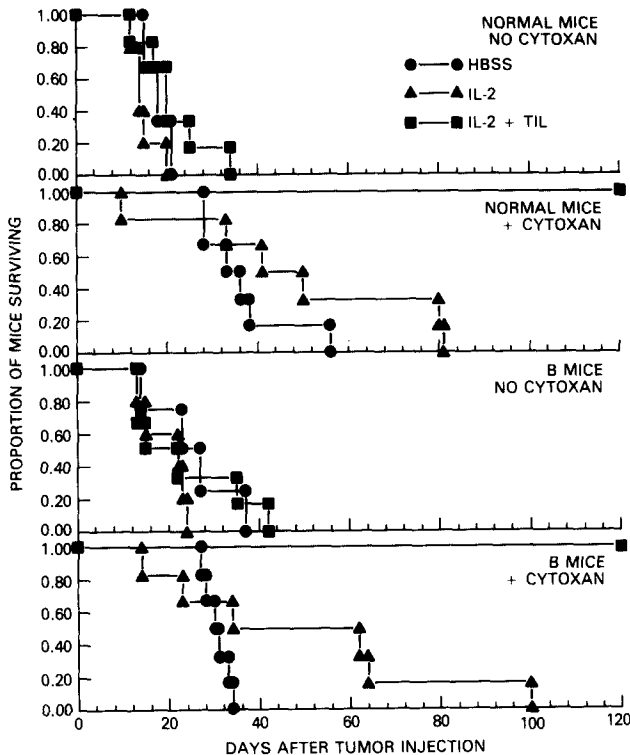


FIGURE 4. Immunotherapy of B mice. Both normal and B mice bearing 10-d MC-38 hepatic metastases were given either no injection or a single intravenous injection of 2 mg of cyclophosphamide (100 mg/kg). 24 h later, groups of six mice each were then treated with HBSS, IL-2 (7,500 U i.p.) thrice daily for 5 d, or a single dose of 5×10^6 TIL intravenously combined with IL-2 (7,500 U i.p.) thrice daily for 5 d. After completion of immunotherapy, the mice were followed for survival. Upon death, all mice were autopsied to confirm the presence of tumor.

the direct antitumor activity of both CP and WBX, as well as that of adriamycin (ADR). Mice were injected with MC-38 adenocarcinoma intrasplenically to induce multiple hepatic metastases in an identical manner to that previously described. After 10 d, groups of three mice were given one of five therapeutic treatments: HBSS, 500-rad WBX, 1,000-rad WBX, CP (200 mg/kg), and ADR (10 mg/kg). After 24 h, the mice were killed, and the livers were sterilely harvested. Subsequently, the livers were digested with triple enzyme solution, and the resulting tumor cell/hepatocyte suspension from each treatment group was washed three times and placed into a clonogenic assay. Five 1:10 serial dilutions of each treated cell type (10^6 - 10^2) were plated into triplicate wells of six-well plates, as described in Materials and Methods. After 14 d, the colonies were stained and counted. The results of two similar experiments are shown in Table II. With no treatment (HBSS), $\sim 51\%$ and $\sim 37\%$ of the cells plated in Exp. 1 and 2, respectively, grew into colonies with the histologic appearance of MC-38 adenocarcinoma, indicating that one-third to one-half of the liver had been replaced by tumor. Rarely, small fibroblast colonies were noted, however, these were not counted. WBX reduced the number of colonies formed per 10^6 cells plated in a dose-dependent manner. Irradiation with 500 cGy lowered the ability of the tumor cells to form colonies by 92.8 and 55.2 times and 1,000 cGy by 1,159 and 507 times. CP had a similar potent antitumor effect, lowering the clonogenic potential by 662 and 1,276 times but ADR, which does not synergize with IL-2, failed to decrease the clonogenic potential >68.9 times in either experiment. This

TABLE II
The Direct Antitumor Effects of Radiation and Chemotherapy

Treatment*	Exp. 1		Exp. 2	
	Mean no. CFU/10 ⁶ cells [‡]	Fold reduction [§]	Mean no. CFU/10 ⁶ cells [‡]	Fold reduction [§]
HBSS	510,000 ± 51,000	-	370,000 ± 107,000	-
500 cGy	5,500 ± 450	92.8	6,700 ± 250	55.2
1,000 cGy	440 ± 54	1,159	730 ± 60	507
CP	770 ± 90	662	290 ± 30	1,276
ADR	7,400 ± 350	68.9	21,000 ± 3,100	17.6

* Mice bearing multiple 10-d MC-38 hepatic (Exp. 1) or pulmonary (Exp. 2) metastases were treated with HBSS intraperitoneally, 500-cGy WBX, 1,000-cGy WBX, CP (200 mg/kg i.v.), or ADR (10 mg/kg i.v.), and after 24 h, the livers were harvested, digested, and the resulting cells plated into clonogenic assays.

[‡] The mean number of colonies formed in triplicate wells is expressed per 10⁶ cells plated.

[§] The reduction in the mean no. CFU/10⁶ cells is compared with the HBSS-treated controls.

^{||} All groups are statistically significantly different from each other by student's *t* test (*p* < 0.05).

suggests that agents that are known to synergize with IL-2 and TIL are those that have direct antitumor effects against the MC-38 adenocarcinoma.

Discussion

The adoptive transfer of TIL in combination with the administration of IL-2 has been shown to mediate the regression of established tumor in mice as well as man (5-7). In murine tumor models, successful immunotherapy of established 6- and 14-d metastatic disease with TIL and IL-2 requires the administration of CP or WBX. This requirement has been attributed to one of the following: (a) the need to eliminate endogenous systemic suppressor cells that, in at least one model, have been shown to depress active T cell-mediated antitumor activity (8, 9); (b) a direct antitumor action mediated through either a direct cytotoxic mechanism or through increased tumor susceptibility to TIL cytotoxicity; (c) the creation of "lymphoid" space either in the tumor or lymph organs that TIL require in order to exert their antitumor activity; or (d) to facilitate increased cell trafficking to the tumor.

We have shown that LTX, in a MC-38 murine hepatic metastatic model, augments the antitumor effects of TIL in a manner similar to WBX and CP. Furthermore, LTX significantly enhances the antitumor activity of IL-2 in a manner similar to that previously shown for CP administration (13). WBX, however, does not increase the effectiveness of immunotherapy with IL-2, but rather, in the absence of adoptively transferred cells, it abrogates any activity of systemic IL-2 administration. In the experiments depicted in Figs. 1 and 2, no antitumor effects were observed with the adoptive transfer of TIL unless the tumor itself was irradiated with either LTX or WBX. Even if WBX excluding the liver was given, which produces immunosuppression not unlike WBX and LTX, no antitumor effects were seen. These findings indicate that the primary action of WBX and LTX occurs at the site of the tumor itself and not on the extratumoral immune cells, including any existing systemic suppressor cells (all mice have undergone splenectomy, and therefore, the majority of immune cells are found outside of the radiation field). These experi-

ments, however, do not eliminate the possible existence of intratumoral Ts cells or any of the other explanations for the synergistic interaction between CP/WBX and TIL discussed above.

To further clarify the role of Ts cells and, in particular, to evaluate the role of intrahepatic suppressor cells in the immunotherapy of MC-38 hepatic macrometastases, the right half of the liver was irradiated while shielding the left lobes to determine the antitumor effects of TIL in the presence of unirradiated tumor and, presumably, Ts cells in the same organ. The results shown in Table I clearly indicate that the TIL remain effective in reducing the number of hepatic metastases on the irradiated right side. No suppression of their effectiveness was noted, and, as depicted in Fig. 3, a distinct line demarcating the irradiated and unirradiated liver was evident. These findings, while not constituting absolute evidence, are highly suggestive that irradiation does not eliminate suppressor cells, since the efficacy of TIL on the right side of the liver was not affected by the possible presence of suppressor cells on the opposite side.

To further test the possible role of Ts cells, we investigated the effect of TIL in B mice bearing macrometastatic tumor. These mice were shown to be devoid of T cell responsiveness as measured by an anti-DBA MLR. If Ts cells were responsible for the inhibition of TIL, immunotherapy with TIL in B mice should not require CP or radiation therapy; as shown in Fig. 4, this is not the case. Even in B mice, CP was required for the therapeutic efficacy of adoptively transferred TIL. In addition, CP also incremented the effect of IL-2 alone in a manner similar to that seen in normal animals, possibly through the *in vivo* induction of LAK cells. Thus, in mice lacking detectable functional mature T cells, CP was still required for successful immunotherapy with TIL and IL-2, indicating that CP (and probably WBX and LTX as well) did not eliminate intratumoral Ts cells as its primary mechanism of action. Creation of lymphoid space also is not consistent with these findings since adult T lymphocytes are not present in B mice.

Since CP, WBX, and LTX did not appear to synergize with TIL and IL-2 by eliminating Ts cells, we investigated the direct antitumor activity against MC-38 as a possible mechanism of action. As shown by the clonogenic assay in Table II, WBX at either 500 or 1,000 cGy, as well as CP (100 mg/kg), had potent direct antitumor activity. ADR, however, which does not synergize with IL-2 in the treatment of weakly immunogenic murine sarcomas, as shown in previous studies (13), only produces a mild reduction in clonogenic potential at similar published therapeutic doses (10 mg/kg). These data indicate that agents that possess strong antitumor activity against the treated tumor are most likely to augment the antitumor effects of TIL. Furthermore, in several experiments designed to evaluate the lysability of 10-d MC-38 liver and lung metastases after treatment with radiation, CP, or WBX, we were unable to demonstrate consistent changes in tumor lysability by TIL (data not shown). Therefore, the most likely action of radiation and CP remains a direct antitumor activity, although alterations in cell trafficking due to either increased local inflammation or some other unknown factor cannot be excluded. The need for direct antitumor agents in addition to therapy with TIL and IL-2 may be due to: (a) a need to decrease tumor bulk so that TIL are required to lyse fewer cells before they themselves die; (b) a need to slow the growth of the tumor so that the rate of tumor cell lysis by TIL exceeds that of tumor growth; or (c) a need to

inhibit the tumor production of suppressive factors, such as TGF- β , that have been shown to inhibit the function of immune cells (14-16). Experiments to differentiate these possibilities represent important areas of future research.

The most significant clinically useful data derived from these experiments, however, is the interaction between LTX and TIL. To date, LTX has not been administered before immunotherapy, since WBX had been shown in many instances to inhibit the immune system. However, the present studies show that LTX can be successfully combined with both IL-2 and TIL in the immunotherapy of a murine adenocarcinoma. The usefulness of this combination in man remains to be determined, and we have begun clinical trials designed to answer this question.

Summary

The adoptive transfer of tumor-infiltrating lymphocytes (TIL) with the concomitant administration of IL-2 has been shown to mediate the regression of established 6- and 14-d murine hepatic and pulmonary metastases. For successful immunotherapy with TIL, however, pretreatment with either cyclophosphamide (CP) or whole body irradiation (WBX) was required. The exact mechanism of CP and WBX augmentation of TIL antitumor activity remains unknown, but the elimination of Ts cells has been frequently invoked as an explanation. To address this possibility and to determine if local tumor irradiation (LTX) could synergize with TIL as well as WBX, we investigated the effect of LTX on the therapeutic efficacy of TIL and IL-2 in the treatment of multiple 7-d murine hepatic metastases. Experiments studying the treatment of a weakly immunogenic murine adenocarcinoma, MC-38, showed prolonged survival of mice treated with the combination of IL-2, TIL, and either LTX or WBX, compared with treatment with radiation alone or radiation plus IL-2 controls ($p < 0.0001$). In addition, therapy with LTX and IL-2 prolonged survival, compared with LTX administration alone, whereas therapy with WBX combined with IL-2 did not alter survival. This augmentation of TIL-mediated antitumor activity was dependent on the dose of radiation used. To assess the possibility that tumor-associated Ts cells inhibit the function of adoptively transferred TIL in animals with 7-d metastatic tumor and are eliminated by WBX and LTX, we repeated the above experiments leaving some tumor unirradiated. Mice underwent either LTX or limited LTX, which included only the right side of the liver (LTX_{1/2}). The number of right- and left-sided metastases were then individually counted. These studies showed that the reduction in the number of right-sided metastases was identical between the two groups and that the presence of left-sided tumor in the LTX_{1/2} group did not suppress the observed antitumor activity of TIL against irradiated tumor. Additional evidence against the elimination of suppressor cells as an important mechanism in radiation-induced augmentation of TIL antitumor activity was provided by experiments studying the effectiveness of TIL in thymectomized, lethally irradiated, and reconstituted B mice. Unless CP was administered before the adoptive transfer of TIL, therapy with IL-2 and TIL in these B mice was ineffective in the absence of demonstrable T lymphocytes. Finally, clonogenic assays performed after treatment with CP, adriamycin, or WBX demonstrated that the degree of direct antitumor activity exhibited by these agents correlated well with their known ability to synergize with TIL and IL-2. Thus, these experiments not only provide evidence of a synergistic interaction between LTX and TIL in general, but they

also provide indirect data contradicting a critical role for suppressor cells in the radiation- and CP-mediated therapeutic augmentation of TIL and IL-2. In addition, the marked synergy between LTX and TIL provides a rational basis for clinical trials designed to determine the extent to which LTX and TIL interact in the treatment of human malignancies.

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