Intrasplenic Administration of Interleukin-2 to Potentiate Specific Chemoimmunotherapy in Tumor-bearing Mice

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ABSTRACT

Augmentation of specific chemoimmunotherapy by daily, intrasplenic injection of interleukin-2 (IL-2) was assessed in a methylcholanthrene (MCA)-induced fibrosarcoma model in C3H/HeJ mice. Daily access to the spleen was achieved by relocating the organ into the subcutis while leaving its blood supply intact. Following intrasplenic injection of 80 units of human IL-2 into MCA-F tumor-bearing mice for 6 days, spleen cells tested in the local adoptive transfer assay showed specific neutralization of MCA-F, but not the antigenically different MCA-D, tumor. Depletion of the spleen cell population with monoclonal antibodies and complement showed that the responding cell bore the surface markers Thy 1.2 and Lyt 2. Mice bearing established MCA-F tumors underwent a variety of chemoimmunotherapy regimens, including 1 µg of 1-butanol, extracted isoelectrophoretically purified tumor-specific transplantation antigen, a single i.p. dose of cyclophosphamide (20 mg/kg), and/or either i.p. or intrasplenic injection of 80 units of IL-2. Specific triple chemoimmunotherapy including daily intrasplenic IL-2, but not i.p., administration was superior in the degree of tumor neutralization to all single or double therapy protocols. Furthermore, the combined triple modality inhibited spontaneous lung colonization by clone 9-4, a highly metastatic variant of MCA-F; both the numbers of lung colonies (median, 17; range, 2 to 55, versus median, 3, range, 0 to 42; P < 0.005) and the incidence were decreased. The combined treatment group displayed 35% of hosts free of lung metastasis, while 100% of the control animals had lung colonies (P < 0.02). Thus antitumor immunity was augmented in vivo using IL-2 delivered by intrasplenic, but not i.p., injection. Furthermore, chemoimmunotherapy including intrasplenic IL-2 injection potentiated the antitumor immunity achieved with combined tumor-specific transplantation antigen and cyclophosphamide.

INTRODUCTION

TSTA on the surface of MCA-induced fibrosarcomas are extracted with 3 M KC1 or single-phase solutions of 1-butanol (1–5). TSTA extracts induce a primary, prophylactic, immune response producing host resistance against supralethal tumor challenge (2, 4). On the other hand, therapeutic administration of extracted TSTA in tumor-bearing hosts may actually decrease antitumor immunity due to stimulation of suppressor T-cells (6, 7). Chemoimmunotherapy combining CY, known to decrease the induction of murine suppressor T-cells, and TSTA decreases local tumor recurrence (8) and spontaneous lung colonization (7) following resection of a primary tumor. Recently, highly purified IL-2 has been shown to induce proliferation of cytotoxic T-lymphocytes in vivo or in vitro following antigen stimulation (9–14). Therefore, the experiments presented herein test the hypothesis that specific chemoimmunotherapy combining exogenous IL-2 injection as a third agent augments antitumor immunity. Since in vivo administration of IL-2 sometimes fails to potentiate host resistance because of its rapid degradation (13, 15) or due to the presence of an IL-2 inhibitor in the serum (16), the cytokine was introduced directly into its splenic, target lymphoid organ and compared to the same dose of IL-2 administered systemically. The spleen was translocated, leaving the splenic artery and vein intact, and relocated to the subcutis.

MATERIALS AND METHODS

Animals and Tumors. Two established non-cross-reactive sarcomas (MCA-F and MCA-D) were induced in female inbred C3H/HeJ mice (17). The spontaneously metastasizing subline of MCA-F, clone 9-4, was obtained from the sixth in vivo passage as described previously (7) using the selection procedure of Lata (18). Tumor cell extraction with 1-butanol was performed as previously described (8). All experiments are performed with low passage tumor cell preparations wherein parental tumor and sublines are used within the first seven transplant generations.

Splenic Translocation. After mice had been anesthetized with i.m. injection of a cocktail, containing ketamine (10 mg/kg), xylazine (2 mg/kg), and acepromazine (1 mg/kg), a paramedian incision was used to relocate the spleen into the lateral subcutis, after cauterizing the superior short gastric vessels without compromising the splenic artery and vein. Peritoneum was closed with an aperture for the splenic vessels; the muscular layers and skin were approximated in two layers with continuous 5-0 silk suture. After 14 days of convalescence, mice were randomized for the therapeutic protocols.

LATA. Spleen cells (2 x 10⁶) harvested from experimental groups of mice were admixed with 10⁶ cultured MCA-F or MCA-D cells (effectortarget ratio, 200:1) in 0.2 ml of Hanks' balanced salt solution. Each mixture was administered s.c. into the flanks of ten naive mice; tumor outgrowth was measured serially by calipers. Tumor size was expressed as the mean of two perpendicular diameters.

Monoclonal Antibodies. A direct cytotoxicity method was used to deplete all, or specific subpopulations of, T-cells. After spleen cells from MCA-F tumor-bearing mice which had been treated with intrasplenic injection of IL-2 were suspended in Hanks' balanced salt solution at a concentration of 6 x 10⁶/ml, 0.1 µg of anti-Lyt 2 or anti-Lyt 1 (Becton-Dickinson, Mountain View, CA) per 3 x 10⁶ cells or 0.12 µg of anti-Thy 1.2 antibody per 10⁶ cells was added. Following incubation at 4°C for 45 min, the cells were washed twice with PBS and incubated with a 1:20 dilution of rabbit complement (Low-Tox-M rabbit complement; Cedarlane Laboratories, Limited, Hornby, Ontario, Canada) at 37°C for 45 min. After three washes with PBS, the selected cells were admixed with MCA-F cells (effectortarget ratio, 200:1) for LATA.

Immunotherapy Model. Two wk after splenic relocation, mice were given injections s.c. into the right flank with 1 x 10⁶ MCA-F cells. Groups of mice were treated with weekly s.c. injection of 1 µg of isoelectrophoretically purified TSTA, a single i.p. injection of 20 mg of CY per kg, and/or daily injection of IL-2 (human interleukin 2; Genzyme, Boston, MA). Tumor outgrowth was measured serially by calipers and expressed as the mean of the two perpendicular diameters.

For the spontaneous lung metastasis model, 1 x 10⁶ clone 9-4 cells were injected into the hind footpad of mice. Eighteen days later, the tumor-bearing leg was amputated, and combination therapy was initiated. IL-2 therapy was performed for 14 days. Mice were sacrificed on the day after the final IL-2 treatment. Lung colonies were microscopically counted. All in vivo experiments were performed in accordance with the animal welfare regulations of the University of Texas Health Science Center at Houston.

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2The abbreviations used are: TSTA, tumor-specific transplantation antigen(s); CTL, cytotoxic T-lymphocyte(s); CY, cyclophosphamide; IL-2, interleukin-2; LATA, local adoptive transfer assay; MCA, methylcholanthrene; PBS, phosphate-buffered saline.

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RESULTS

Dose Response of Intrasplenic or Intrapertoneally Injected IL-2 in Tumor-bearing Hosts. To determine the optimal in vivo amount of IL-2, serial doses were injected intrasplenic or i.p. into tumor-bearing mice treated with a combination of TSTA and CY. Groups of spleen-relocated mice bearing 10-day MCA-F tumors (approximately 4 mm in diameter) were treated with IL-2 (1 to 640 units intrasplenically or i.p.) for 9 days (Fig. 1). There was no effect observed in mice at doses less than or equal to 40 units/day with both intrasplenic and i.p. injections. Intrasplenic administration of IL-2 (80 units/day) inhibited tumor outgrowth by 58% (17.5 ± 1.8 mm versus 7.4 ± 1.4 mm; P < 0.001) while 320 units/day did not inhibit tumor growth (14.5 ± 2.1 mm; not significant), IL-2 administration i.p. significantly retarded tumor growth at doses of 80 units or more per day (80 units, 10.7 ± 1.2 mm, P < 0.01; 160 units, 11.4 ± 1.2 mm, P < 0.025; 320 units, 8.2 ± 1.3 mm, P < 0.001; 640 units, 9.1 ± 1.2 mm, P < 0.005). However, the LATA using the spleen cells of tumor-bearing mice treated with IL-2 injection alone showed that higher doses of IL-2 (>160 units/day of intrasplenic or >320 units/day of i.p. injection) induced tumor-enhancing activity rather than inhibition of tumor-neutralizing activity (data not shown). Therefore, IL-2 alone in vivo may promote the emergence of suppressor/regulatory mechanisms, especially when IL-2 was injected locally, such as intrasplenically in tumor-bearing hosts.

Specific Cytotoxicity of Spleen Cells Treated with IL-2 in Tumor-bearing Mice. Specificity of the tumor neutralization induced in the spleen by IL-2 treatment was assessed by LATA comparing MCA-F and the antigenically different MCA-D cells as target cells (Fig. 2). Groups of 10 mice bearing 8-mm MCA-F tumors were treated with daily injections of 80 units of IL-2.

After 9 days of treatment, both i.p. and intrasplenic injection of IL-2 retarded the outgrowth of MCA-F tumors (1.1 ± 1.1 mm versus 9.7 ± 2.8 mm; P < 0.01), but did not affect the outgrowth of MCA-D tumors (14.6 ± 1.6 mm versus 17.0 ± 2.3 mm; not significant). Thus treatment with intrasplenic injection of IL-2 specifically activated spleen cells from tumor-bearing mice to be cytotoxic to homotypic MCA-F cells.

Phenotype of the Responding Spleen Cell Populations in IL-2-treated Tumor Bearers. To determine the phenotype of the responding cells, spleen cells augmented by intrasplenic injection of IL-2 were pretreated with monoclonal antibodies and complement prior to transfer in LATA (Fig. 3). Hosts bearing tumor burdens of about 4-mm diameter were treated with daily intrasplenic injections of 80 units of IL-2 for 6 days. The active element in the spleen cell population was assessed by depletion of Thy 1.2-, Lyt 1-, or Lyt 2-positive cells using monoclonal antibodies and complement prior to admixture with MCA-F.
target cells for s.c. injection for LATA in naive mice. After 28 days the mean tumor size in mice receiving spleen cells incubated with anti-Thy 1.2 was 12.3 ± 0.8 mm (P < 0.05), with anti-Lyt 1, it was 10.7 ± 1.0 mm (not significant) or with anti-Lyt 2, it was 14.7 ± 1.2 mm (P < 0.005). These data suggest that the responding spleen cell populations in IL-2-treated tumor bearers are of the Thy 1.2* and Lyt 2* phenotype.

Combined Triple Modality Therapy Including TSTA, CY, and IL-2. The effect of combination therapy with TSTA, CY, and IL-2 against tumor progression was assessed using groups of mice bearing 10-day MCA-F tumors of about 3.5-mm diameter (Fig. 4). The hosts were treated with weekly s.c. injections of 1 μg of isoelectrophoretically purified TSTA, a single i.p. dose of CY (20 mg/kg), and/or daily intrasplenic injection of 80 units of IL-2. There was no significant difference in mean tumor size among the groups at the time of initiation of therapy. After 9 days of treatment the mean tumor size in mice treated with the triple modality therapy (9.1 ± 0.4 mm) was significantly reduced compared with the CY plus IL-2 group (10.6 ± 0.3 mm; P < 0.01) or the CY plus TSTA group (11.3 ± 0.6 mm; P < 0.01) as well as all single therapy arms. Control mice displayed a mean tumor diameter of 13.9 ± 0.6 mm. Further, to compare the effect of intrasplenic injection with i.p. IL-2, bearing 10-day MCA-F tumors were treated with the triple modality therapy including either intrasplenic or i.p. injection of IL-2 of 80 units of IL-2. Fig. 5 shows intrasplenic injection of IL-2 to be superior to i.p. administration of the cytokine (8.2 ± 0.4 mm versus 10.8 ± 0.8 mm; P < 0.05). Thus, the chemoimmunotherapeutic effect of specific TSTA/CY therapy was augmented by intrasplenic injection of IL-2. While triple modality therapy, including intrasplenic IL-2 injection, was superior to any single or double agent therapy, i.p. administration of IL-2 did not augment the effect.

Combined Triple Modality Therapy with TSTA, CY, and IL-2 against Postsurgical Lung Metastasis. The effect of combined therapy with MCA-F TSTA, CY, and IL-2 upon spontaneous lung colonization was examined following amputation of the footpad bearing the highly metastatic MCA-F variant, clone 9-4 (Fig. 6). One million clone 9-4 cells were injected into the hind footpad 18 days prior to amputation of the tumor-bearing limb (mean tumor size, 17 mm). Triple modality therapy was initiated on the day of surgery for a 14-day treatment course, whereafter the mice were sacrificed, and lung colonies were enumerated. The therapeutic efficacy of the TSTA, CY, intrasplenic IL-2 injection regimen was superior to all other therapies. The number of lung colonies was greatest in the control mice and greatly reduced in triple regimens of the TSTA/CY and CY plus intrasplenic IL-2 injections. The therapeutic efficacy of combined chemoimmunotherapy using i.p. or intrasplenic administration of IL-2 to tumor-bearing mice. Ten days after s.c. inoculation of 1 x 10^4 MCA-F cells, groups of 9 spleen translocated mice were treated weekly with 1 μg of TSTA, a single i.p. administration of CY (20 mg/kg), and/or 80 units of IL-2 i.p. or intrasplaneously. All groups of mice were given injections i.p. and intrasplaneously with IL-2 or PBS. The tumor sizes were serially measured, and the mean tumor diameters of each group of mice were compared on Day 0 (the initiation day of therapy), Day 3 (D), Day 6 (D), or Day 9 (D). CNT, control; AC, TSTA plus CY; AS, TSTA plus intrasplenic injection of IL-2; CS, CY plus intrasplenic injection of IL-2; II, intrasplenic injection of IL-2; ACS, TSTA, CY, plus intrasplenic injection of IL-2; AP, TSTA plus i.p. injection of IL-2; CP, CY plus i.p. injection of IL-2; ACP, TSTA, CY, plus i.p. injection of IL-2. On Day 9 the statistical differences in mean tumor size between the groups were calculated by Student’s t test. Probability values are as follows: ACS versus S, P < 0.05; ACS versus CS, AS, AC, or CNT, P < 0.001; ACP versus AS or CNT; P < 0.01.

Fig. 4. Treatment of MCA-F tumor-bearing mice using TSTA, CY, and intrasplenic injection of IL-2. Ten days after s.c. inoculation of 1 x 10^4 MCA-F cells, groups of 9 spleen translocated mice bearing established tumors (approximately 3.5 mm in diameter) were treated with weekly injections of 1 μg of TSTA, single doses of 20 mg of CY, and/or daily intrasplenic injections of 80 units of IL-2. All groups of mice received daily intrasplenic injections of 0.1 ml of PBS or IL-2. The tumor sizes were serially measured in order to compare the mean tumor diameters of each group of mice on Day 0 (the initiation day of therapy), Day 3 (D), Day 6 (D), or Day 9 (D). CNT, control; 4, TSTA; 6, TSTA plus CY; C, CY; A, TSTA plus IL-2; 4, CY plus IL-2; II, TSTA, CY, plus IL-2. On Day 9 the statistical differences between the ACP group and other groups were calculated by Student’s t test (columns). Calculated P values are as follows: ACP versus AS, C, or II was P < 0.01; ACP versus CNT, A, II, or I was P < 0.001.

Fig. 5. Therapeutic efficacy of combined chemoimmunotherapy with i.p. or intrasplenic administration of IL-2 to tumor-bearing mice. Ten days after s.c. inoculation of 1 x 10^4 MCA-F cells, groups of 9 spleen translocated mice were treated weekly with 1 μg of TSTA, a single i.p. administration of CY (20 mg/kg), and/or 80 units of IL-2 i.p. or intrasplaneously. All groups of mice were given injections i.p. and intrasplaneously with IL-2 or PBS. The tumor sizes were serially measured, and the mean tumor diameters of each group of mice were compared on Day 0 (the initiation day of therapy), Day 3 (D), Day 6 (D), or Day 9 (D). CNT, control; AC, TSTA plus CY; AS, TSTA plus intrasplenic injection of IL-2; CS, CY plus intrasplenic injection of IL-2; II, intrasplenic injection of IL-2; ACS, TSTA, CY, plus intrasplenic injection of IL-2; AP, TSTA plus i.p. injection of IL-2; CP, CY plus i.p. injection of IL-2; ACP, TSTA, CY, plus i.p. injection of IL-2. On Day 9 the statistical differences in mean tumor size between the groups were calculated by Student’s t test. Probability values are as follows: ACS versus S, P < 0.05; ACS versus CS, AS, AC, or CNT, P < 0.001; ACP versus AS or CNT; P < 0.01.
mice had at least one lung colony, while 35% of mice treated with TSTA, CY, and intrasplenic IL-2 (7 of 20) were free of lung colonies (P < 0.02). Thus, combined triple modality therapy augments the antimetastatic effect of specific chemoimmunotherapy using TSTA and CY.

DISCUSSION

Intrasplenic injection of IL-2 (80 units/day) combined with specific chemoimmunotherapy using TSTA and CY augments tumor-neutralizing activity in tumor-bearing mouse spleen. Higher doses of IL-2 used alone induced tumor-enhancing cells. Depending on its concentration, IL-2 may induce tumor-neutralizing T-cells or suppressor T-cells. In vivo data (Fig. 1) and other investigations in vitro (21) indicate that high doses of murine, rat, or human IL-2 generate suppressor T-cells. The desired result may be obtained only when the dosage of IL-2 is carefully monitored. LATA showed that the tumor-neutralizing activity of the spleen cells prepared from intrasplenic IL-2-treated tumor bearers was antigen specific. Depletion experiments suggested that the responding spleen cell population bore the surface markers Thy 1.2+ and Lyt 2+. Combination triple modality therapy including TSTA, CY, and intrasplenic injection of IL-2 revealed that the combined therapy regimen was superior to any single or double agent therapy against tumor outgrowth or postoperative lung metastasis. Furthermore, even though IL-2 alone induces tumor-neutralizing cells (Fig. 2), these cells do not readily egress the spleen (Fig. 4) in sufficient numbers to dramatically suppress subcutaneous tumor growth. Thus the combined therapy protocol not only affords activation of tumor-neutralizing cells, but also may enable activated cells to better seek target cells.

Surgical procedures to relocate the spleen did not influence tumor growth. Surgically induced suppression (22) was avoided by allowing a 14-day interval before tumor cells were injected s.c. into the right flank. Three wk after s.c. injection of 1 × 10^5 MCA-F cells, the mean tumor size in spleen-removed mice (13.9 ± 0.7 mm) was similar to that of intact mice (12.4 ± 0.5 mm, not significant). Even 3 mo after surgery the spleen remained intact in the subcutis, except for the presence of surrounding fibrous tissue. Further, the relocated spleen size enlarged during tumor growth just as previously reported for mice with orthotopic spleens (23). Thus, the spleen in the subcutis remains intact and apparently functions normally.

Human IL-2 was used in this study, because adequate quantities were available for the in vivo injection. Human IL-2 differs from murine IL-2 in molecular weight and other biological properties. Nevertheless, mouse T-cells are responsive to human IL-2 (24). Systemic injection of IL-2 itself did not afford any therapeutic benefit, probably because of its short half-life in vivo (25). In other investigations, i.p. injection of IL-2 failed to retard tumor outgrowth or to extend survival time in mice bearing established MCA-induced tumors (13). Successful therapy was obtained if IL-2 was administered in a gelatin base to maintain adequate blood concentration (13), or locally into the tumor site (10, 25, 26). T-cell proliferation occurs primarily at the site of IL-2 exposure (4). Our results (Figs. 2 and 3) show that IL-2 injection directly into the spleen augments the capacity of splenocytes to mediate in vivo tumor neutralization.

IL-2 administration without antigen potentiates natural killer cells in vivo and in vitro (26–29). Coculture of in vivo immunized spleen cells with IL-2 and irradiated tumor cells induces specific CTL against the immunizing tumor (30) or selected members of the tumor cell line (25, 31). The experiments described herein showed that the effector cells augmented by IL-2 intrasplenic injection in tumor-bearing mice specifically neutralize the homotypic tumor in vivo and bear the Thy 1.2+, Lyt 2+ phenotype, a conclusion which confirms in vitro studies (31).

Tumor immunotherapy using IL-2 may be divided into two strategies: direct IL-2 injection in vivo or adoptive transfer of specific CTL expanded by cultivation in IL-2 following in vitro restimulation. In the former method, degradation was averted in vivo (13, 15) by injection of IL-2 in a gelatin base (13) or around the tumor itself (10). In the second method, adoptive immunotherapy of in vitro expanded cell populations prolongs the survival of tumor-bearing mice (14, 21, 32). Since only 56% of the transferred cells remain viable 28 h after i.v. injection (33), these cells must be capable of cell division in the host in order to be effective (34). In some experiments, IL-2-dependent, cultured cytotoxic cells must be administered in combination with exogenous IL-2 injection (13, 14, 35) in order to exceed the tumor-inhibitory threshold. Adoptive transfer of cultured CTL into humans carries the risk of contamination, a hazard that is completely obviated by direct in vivo administration of the cytokine. Intrasplenic injection of IL-2 is not only a successful alternative, but also may induce more efficient CTL. Intrasplenic inoculation of IL-2-induced CTL retarded tumor outgrowth and lung metastasis in the presence of specific chemoimmunotherapy. Our previous data showed that chemoimmunotherapy using TSTA and CY retarded postoperative lung colonization (32) or tumor outgrowth (36). The combined triple therapy induced additive antitumor effects. Presumably markers on the tumor induced IL-2 receptors on responder cells, and restimulation with extracted TSTA increased the IL-2 receptor number on the responder cells (9, 12, 35). CY administration decreases TSTA-induced suppressor T-cells (7) which abrogate IL-2 activity (2) or IL-2 inhibitor production (16). IL-2 rescues the CTL responses in CY-treated mice (36). Additionally, low doses of CY (15 mg/kg) induce immunopotentiating T-cells in the spleen of tumor-bearing mice (37). Thus, both TSTA and CY augment CTL induction by exogenous IL-2 injected into the spleen. The spleen, being the largest lymphoid organ, may be the appropriate site for systemic potentiation of immunity. In conclusion, augmentation of CTL by exogenous IL-2 was dramatic when it was delivered directly into the responding lymphoid organ in the presence of low doses of CY and TSTA.

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