Administration of Interleukin 12 With Pulse Interleukin 2 and the Rapid and Complete Eradication of Murine Renal Carcinoma

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Background: Interleukin 2 (IL-2) and interleukin 12 (IL-12) are potent immunoregulatory cytokines that exhibit antitumor activity. Preliminary evidence suggests that combined administration of IL-2 and IL-12 may yield greater antitumor activity than that observed with either agent alone. Purpose: We evaluated the ability of combination regimens of IL-2 and IL-12 to induce regression of established primary and metastatic murine renal carcinoma (Renca) tumors. Methods: BALB/c mice were given either subcutaneous or intrarenal injections of 10⁵ Renca cells; tumor cell injections were given to 10-12 mice for each treatment group. Mice bearing subcutaneous primary tumors were treated with chronic IL-2 (300,000 IU given on a daily basis) or pulse IL-2 (300,000 IU given twice daily one day per week) alone, IL-12 alone (0.5 µg given on a daily basis), or IL-12 in combination with either chronic or pulse IL-2. Mice with metastatic tumors (arising from intrarenal implants; animals were nephrectomized to remove the primary tumors) were treated with IL-12 plus or minus pulse IL-2; in these experiments, IL-12 was given at doses of either 0.5 or 1.0 µg. In most experiments, treatment was continued for at least 3 weeks. Two-sided statistical tests were used to evaluate the data. Results: Most mice with subcutaneous Renca tumors treated with the combination of IL-12 and chronic IL-2 died of treatment-related toxic effects within 7-14 days. In contrast, treatment with IL-12 plus pulse IL-2 was well tolerated, and six of 10 mice experienced complete tumor regression; none of the mice treated with either IL-12 alone or pulse IL-2 alone experienced a curative response. Seven of eight and nine of nine mice with metastatic tumors experienced complete tumor regression after treatment with 0.5 µg IL-12 plus pulse IL-2 or 1.0 µg IL-12 plus pulse IL-2, respectively; two of 12 mice treated with pulse IL-2 alone and 10% or less of mice treated with IL-12 alone were cured of metastatic tumors (with 0.5 µg IL-12, none of 10 mice; with 1.0 µg IL-12, one of 10 mice). Five of 10 mice with metastatic tumors treated with a short-course regimen of IL-12 and pulse IL-2 (two pulses of IL-2 flanking 5 days of 0.5 µg IL-12) experienced complete tumor regression, while only one of the 12 mice treated with IL-2 alone and none of the mice treated with IL-12 alone experienced complete tumor regression. Virtually all curative response frequencies obtained with IL-12 and pulse IL-2 combination regimens differed significantly (P<.05) from those obtained with corresponding single-agent treatments. Conclusions: IL-12 administered in combination with pulse IL-2 induced rapid and complete regression of primary and metastatic Renca tumors and displayed greater antitumor activity than that observed with either IL-12 or IL-2 alone. [J Natl Cancer Inst 1996;88:38-43]

Interleukin 12 (IL-12) is a recently discovered immunoregulatory cytokine that may provide an important link between nonspecific immune surveillance mechanisms and the initiation and development of a specific T-cell-mediated immune response (1-4). In particular, IL-12 may enhance proliferation, cytokine production, and cytotoxic activity of T lymphocytes and natural killer (NK) cells (1-4), and it may influence the development of a T-helper type 1 (Th1) (i.e., IL-2 and interferon gamma [IFN γ]) versus a T-helper type 2 (i.e., IL-4 and IL-10) pattern of cytokine production during the evolution of an immune response (5,6). In mice infected with Leishmania major, promotion of a Th1 response via an IFN γ-dependent mechanism appears to account for the ability of IL-12 to successfully induce clearance of the infection (7,8). Given the ability of IL-12 to modulate T-cell-mediated immune responses, its therapeutic potential has been evaluated in several preclinical models of infectious (7,9-11) and malignant (12,13) disease. IL-12 has exhibited antitumor activity in a variety of murine cancer models (including renal cancer, B16 melanoma, M5076 reticulum cell sarcoma, and C26 colon carcinoma) (12-14), thus making it an attractive candidate for combination therapy with IL-2, another potent immunoregulatory cytokine with antitumor activity (15-18).

Although IL-2 has shown some efficacy in the treatment of metastatic renal carcinoma and melanoma in humans, the frequency of complete responses is low.
its use at high doses has been limited by the occurrence of cardiovascular and/or renal toxic effects. In this regard, IL-12 displays substantial preclinical antitumor activity in vivo at lower, potentially less toxic doses than those necessary for the optimal induction of antitumor immune responses with IL-2. At these lower doses, IL-12 substantially prolongs the survival of mice bearing well-established tumors, and a portion of the mice are cured of their tumors.

Preliminary in vitro evidence suggests that the combined administration of IL-12 and IL-2 may produce additive or even synergistic immunomodulatory activity and may yield greater antitumor activity than that exhibited with either agent alone. IL-12 and IL-2 use parallel intracellular signaling pathways (19), and they complement each other in promoting the proliferation, IFN γ production, and cytotoxic activity of T and/or NK cells (20-28). IL-2 also enhances the expression of IL-12 receptors on T and NK cells (29). Furthermore, human peripheral blood mononuclear cells (PBMC) treated with IL-12 and IL-2 kill neuroblastoma target cells in vitro more effectively than PBMC treated with either cytokine alone (30). In view of these findings, we evaluated the ability of various regimens of IL-2 administered in combination with IL-12 to induce the regression of established primary and/or metastatic tumors.

Materials and Methods

Mice and Tumor Cells

BALB/c mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center. They were maintained in a dedicated pathogen-free environment and used between 8 and 10 weeks of age. Renca, a BALB/c (mouse) renal adenocarcinoma of spontaneous origin (17), was the tumor cell source for these experiments. The Renca cell line was maintained in BALB/c mice by serial intraperitoneal passage. Animal care was provided in accordance with procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH Publ. No. 86-23, 1985).

Reagents

Recombinant murine IL-12 (specific activity, 7 x 10^6 U/mg) was provided by Hoffmann-La Roche, Inc. (Nutley, NJ). Stock solutions prepared with Dulbecco's phosphate-buffered saline (PBS) were stored at -70 °C until use. For in vivo administration, the stock solutions were diluted as necessary with PBS containing 0.1% (vol:vol) sterile-filtered BALB/c mouse serum and used within 48 hours. Highly purified, recombinant human IL-2 (from Escherichia coli) was provided by Hoffmann-La Roche, Inc., or by Chiron Corporation (Emeryville, CA) (22,33). After reconstitution with sterile water, IL-2 was diluted with Hank's balanced salt solution (HBSS) containing 0.1% BALB/c mouse serum for in vivo administration.

Tumor Models

For the established, subcutaneous primary tumor model, mice were given midflank injections of 1 x 10^5 Renca cells (in 0.2 mL HBSS). For the metastatic tumor model, mice were anesthetized with metofane (given to effect in line with oxygen) in a Plexiglas box in a vented hood. After inducing level III, stage 2-3 anesthesia, a left flank incision was made in the left flank of each mouse. With pressure, the proximal kidney was pushed against the intact peritoneal membrane, making it fully visible. With the aid of a 1-mL syringe equipped with a 27-gauge needle, 1 x 10^6 Renca cells (in 0.1 mL HBSS) were injected into the kidney capsule. The margins of the incision were then approximated and closed with Clay Adams 9 millimeter autolips. Twelve days after tumor cell implantation, the mice were nephrectomized to remove the primary tumors. In brief, the mice were anesthetized as described above, the autolips were removed, and the initial incisions were reopened and extended slightly. In each mouse, the peritoneum was incised, and the tumor-bearing kidney was exposed with the use of gentle pressure. After identification and ligation of the renal vasculature, the tumor-bearing kidney was resected, and the incision was closed again with Clay Adams 9 millimeter autolips. After an appropriate period of time for recovery from anesthesia and surgery, therapy was initiated as described below.

In the metastatic Renca model, a small percentage of mice experience rear-leg paralysis as a surgical complication. These mice are killed with CO2 and excluded from treatment. Our long-standing experience with this model has demonstrated that intra-abdominal seeding and/or distant metastases (lung and liver) occur uniformly in the interval between tumor implantation and nephrectomy, thereby allowing evaluation of the efficacy of treatment against established, widespread metastatic disease.

Treatment Regimens

Cohorts of 10-12 mice were used for all treatment groups. We initially examined the activity of IL-2 regimens (chronic versus pulse) administered alone or in combination with IL-12 to treat established primary, subcutaneous Renca tumors. On the basis of evidence that IL-2 treatment may increase IL-12 receptor expression on T and/or NK cells (29), we chose, as an initial approach, to structure our treatment regimens so that IL-2 administration preceded IL-12 administration. Briefly, treatment with IL-2 (or the HBSS plus mouse serum vehicle alone) was initiated 7 days after tumor cell implantation. Mice receiving the chronic IL-2 regimen were given daily intraperitoneal injections of IL-2 (300 000 IU) on days 7-11 and 14-18. Mice receiving the pulse regimen were given 300 000 IU IL-2 intraperitoneally twice daily on days 7, 14, 1, and 28. IL-12 (0.5 μg) (or PBS plus mouse serum vehicle alone) was administered by daily intraperitoneal injection on days 14-18, 21-25, and 28-32. The antitumor activity of the IL-12 plus pulse IL-2 combination was also evaluated in the more therapeutically challenging metastatic Renca model. As indicated above, mice were nephrectomized 12 days after tumor implantation. A pulse IL-2 regimen (or vehicle alone) similar to that described above was initiated on the day of nephrectomy and included treatments on days 19, 26, and 33. IL-12 (or vehicle alone) was administered at a dose of either 0.5 or 1.0 μg as described above on days 13-16, 19-23, and 26-30.

Last, we evaluated the antitumor activity of a short-course IL-12 plus pulse IL-2 regimen in the same metastatic Renca model. Again, mice were nephrectomized 12 days after tumor implantation. Pulse IL-2 (or vehicle alone) was administered only on the day of nephrectomy and on day 19. IL-12 (or vehicle alone) was administered at a dose of 0.5 μg on days 12-16.

Statistical Analysis

The proportions of mice experiencing curative responses in the various treatment groups were compared using Fisher's exact test. Tumor volumes were estimated by measuring the smallest and largest diameters of each tumor and calculating the product of the square of the smallest dimension multiplied by the largest dimension. Calculated tumor volumes were compared between groups using the nonparametric Wilcoxon rank sum test. The Jonckheere test for trend was used to evaluate the trend in tumor volumes when comparing control mice with mice receiving single-agent therapy (IL-2 or IL-12) and with mice receiving combination therapy (IL-2 and IL-12). For some groups, survival duration was compared by use of the Kaplan–Meier method. All P values were obtained from two-tailed tests of statistical significance and were considered significant when P<0.05.

Results

In initial experiments, IL-12 was administered daily, alone or in combination with IL-2 that was given either daily (chronic regimen) or weekly (pulse regimen), to mice bearing established subcutaneous Renca tumors. At the doses used, chronic IL-2 administered with IL-12 appeared to be extremely toxic; 80% of the treated mice died between day 14 and day 21 after tumor cell implantation (7-14 days after treatment initiation, where the IL-2 and IL-12 treatments overlapped), compared with no deaths among the untreated controls by as late as day 20 (data not shown). Some modest improvements in survival were noted among mice treated with chronic IL-2 or with IL-12 alone compared with untreated control mice (data not shown).
Curative responses were seen in three of 10 mice treated with chronic IL-2 compared with none of 10 mice treated with IL-12 alone and one of two mice surviving the drug-related toxicity seen with the combination of IL-12 and IL-2.

In contrast, daily administration of IL-12 in combination with weekly pulses of IL-2 was well tolerated, with no apparent drug-related deaths. In addition, this treatment regimen produced considerably improved antitumor activity, with 60% of the mice (i.e., six of 10) treated with IL-12 plus pulse IL-2 achieving a curative response, while no curative responses were noted among mice treated with either agent alone (P = .011, IL-12 plus pulse IL-2 versus IL-12 alone; P = .011, IL-12 plus pulse IL-2 versus pulse IL-2 alone) (Fig. 1). A substantial reduction in median tumor volume was noted after completion of only two pulses of IL-2 and 5 days of IL-12 (21 days after tumor implantation) (Fig. 2). This reduction in tumor volume was statistically significant in mice treated with the combination of IL-12 and pulse IL-2 compared with untreated controls (P = .003), with marginally significant reductions among mice treated with pulse IL-2 (P = .04) or IL-12 (P = .052) alone compared with controls. When tumor volumes of mice treated with either IL-2 or IL-12 alone were compared with one another or with the tumor volumes of mice treated with the combination of IL-12 and pulse IL-2, they were not significantly different (P = 1.0, pulse IL-2 versus IL-12; P = .31, pulse IL-2 versus IL-12 plus pulse IL-2; and P = .13, IL-12 versus IL-12 plus pulse IL-2). Further analysis, however, indicated a highly significant (P = .0026) trend toward reduction in tumor volume in mice receiving the combination of IL-12 and pulse IL-2 versus those receiving only IL-12 or IL-2.

Given the potent antitumor activity of the combination of IL-12 and pulse IL-2, we next evaluated it in a more therapeutically challenging model designed to assess activity against established, widespread metastatic disease. In an attempt to further optimize the antitumor activity of the cytokine combination, we also compared use of IL-12 at the original dose of 0.5 μg with use at a dose of 1.0 μg, administered alone or in combination with pulse IL-2. In these experiments, we observed an even more striking effect with the combination of IL-12 plus pulse IL-2 (Fig. 3). IL-12 given alone at doses of 0.5 or 1 μg prolonged median survival (in comparison with controls) by 8 days (P = .2; not significant) and 17 days (P = .035), respectively, although most mice died as a consequence of tumor progression after the discontinuation of therapy. However, curative responses were seen in 100% and 88% of mice treated with pulse IL-2 and the 1.0- or the 0.5-μg dose of...
IL-12 (nine of nine mice versus seven of eight mice), respectively. Only 17% of the mice (two of 12) treated with pulse IL-2 alone and 10% (one of 10) and 0% (none of 10) of the mice treated with the 1.0- and 0.5-μg doses of IL-12 alone, respectively, experienced curative responses \( (P = .00012, 1.0 \, \mu g \, IL-12 \) plus pulse IL-2 versus 1.0 μg IL-12; \( P = .00022, 1.0 \, \mu g \, IL-12 \) plus pulse IL-2 versus pulse IL-2; \( P = .00025, 0.5 \, \mu g \, IL-12 \) plus pulse IL-2 versus 0.5 μg IL-12; and \( P = .0045, 0.5 \, \mu g \, IL-12 \) plus pulse IL-2 versus pulse IL-2). Among mice cured of their original tumors, five of seven (71%) mice treated with the 0.5-μg dose of IL-12 plus pulse IL-2 and four of nine (44%) mice treated with the 1.0-μg dose of IL-12 plus pulse IL-2 rejected a rechallenge with \( 1.0 \times 10^5 \) Renca cells that were administered subcutaneously; none of eight (0%) naive control mice were resistant.

Given the rapid and complete regression of tumors induced by IL-12 plus pulse IL-2, we initiated an examination of the antitumor activity of a short course of this regimen in the metastatic Renca model. Approximately 60 days after tumor implantation, we have found that seven (70%) of 10 mice treated with short-course 0.5-μg IL-12 plus pulse IL-2 are alive and five (50%) are tumor free (Fig. 4). In contrast, three (25%) of 12 mice treated with IL-2 alone are alive, with only one of these remaining tumor free \( (P = .056 \) for complete tumor regression, IL-12 plus pulse IL-2 versus pulse IL-2); among mice treated with IL-12 alone, only one (10%) of 10 has survived, and this mouse is harboring progressive, residual tumor \( (P = .033 \) for complete tumor regression, IL-12 plus pulse IL-2 versus IL-12).

Discussion

IL-2 has been used successfully to treat some patients with advanced cancer \( (15-18) \), but its use has been limited by the occurrence of severe toxic effects, especially in some high-dose regimens. It is clear that optimal expansion and activation of cytotoxic effector cells in vivo probably require an array of appropriately timed signals and cellular interactions. As a consequence, efforts have been directed toward the development of treatment regimens using combinations of biological response modifiers in an attempt to optimize antitumor immune responses. Given evidence that IL-12 and IL-2 may interact favorably in the induction of an immune response \( (19-30) \), we have begun to evaluate the antitumor activity of combinations of these cytokines in vivo.

Treatment of well-established primary tumors and/or metastatic disease is the most relevant setting for preclinical evaluation of immunotherapy regimens. In models using rapidly growing murine tumors, such evaluation is often complicated by the relatively short time interval...
between tumor implantation and the development of a lethal tumor burden. However, it has been reported that IL-12 alone possesses substantial therapeutic activity against a variety of established murine tumors, including Renca [13,14]. We have confirmed this observation and have developed a combination regimen consisting of treatment with IL-12 and IL-2 (currently a Food and Drug Administration-approved agent) that is not only well tolerated, but also exhibits dramatic activity against established Renca tumors. Although injection of 1 × 10^5 Renca cells is uniformly fatal to untreated mice by day 35 to day 45 after implantation, we have shown that the combination regimen (IL-12 plus pulse IL-2) may be initiated after tumors are well established and/or metastatic disease has developed, yet it can still induce complete regression of primary and/or metastatic disease in up to 88%-100% of treated mice.

The antitumor response induced by this combination of cytokines may be initiated very rapidly. Mice bearing subcutaneous Renca tumors that had received as little as 5 days of IL-12 treatment and two pulses of IL-2 exhibited significantly smaller tumor volumes compared with those of untreated mice. Furthermore, a highly significant trend toward smaller tumors was observed in mice treated with the combination compared with mice receiving either cytokine alone. In addition, in the metastatic Renca model, treatment with a total of only two pulses of IL-2 flanking 5 days of IL-12 (the short-course regimen) induced complete tumor regression in 50% of the treated mice. Thus, the combination regimen not only induces a substantial delay in tumor growth that is manifest early in therapy, but it also possesses a potent ability to engage antitumor mechanisms sufficient to achieve complete and sustained eradication of tumors, even with a very short course of application. To further limit potential toxicity, we are attempting to identify even less dose-intensive schedules of this cytokine combination that retain comparable antitumor activity.

Mice cured of Renca tumors by immunotherapy consisting of flavone acetic acid plus IL-2 are resistant to rechallenge [34]. Thus, although most of the mice cured of tumors after treatment with IL-12 plus pulse IL-2 resisted tumor rechallenge, it is surprising, given the striking antitumor activity of the cytokine combination and the potent effects of both IL-2 and IL-12 on T cells, that a substantial proportion of cured mice failed to resist rechallenge. This finding suggests that T-cell-independent mechanisms may also play an important role in the antitumor activity of the IL-12 plus IL-2 combination. One could speculate that NK cells and monocyte and macrophage cell populations and their biochemical products (e.g., nitric oxide), the influence of IL-12 and/or IL-2 on vascularization of the tumor bed, and/or the direct effects of IL-12 and IL-2 on tumor cells themselves might also contribute to the overall antitumor effects. For example, it has been reported that macrophage-derived nitric oxide can induce tumor apoptosis in vitro [35], and we have shown recently that not only does IL-12 induce marked production of nitric oxide in vivo, but it also primes peritoneal macrophages for nitric oxide production upon subsequent treatment with IL-2 ex vivo (Wigginton JM, Kuhrs DB, Back TC, Brunda MJ, Wiltrout RH, Cox GW: unpublished results). In addition, IL-12 alone has recently been shown to inhibit angiogenesis in a murine corneal neovascularization model [36].

A broad range of immune deficits has been described in tumor-bearing mice, including recent descriptions of alterations in the T-cell signal transduction apparatus (NFκB/Rel proteins) in mice bearing Renca tumors [37,38]. Given the ability of IL-12 plus pulse IL-2 to overcome these deficits and rapidly induce tumor eradication, a unique combination of mechanisms may well account for the potent antitumor activity observed. Ongoing investigation in our laboratory has been directed toward elucidating these mechanisms, evaluating the activity of this cytokine combination in other tumor models, and determining the impact of variations in treatment schedule and dose on the efficacy of this regimen.

References
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(18) The repository of the Biological Response Modifiers Program (BRMP), Division of Cancer Treatment (DCT), NCI, NIH, announces the availability of recombinant human lymphokines IL-1α, IL-1β, and IL-2; the monoclonal antibody 11B.11 against mouse IL-4; and the monoclonal antibody 3ZD against human IL-1β.

Use of these materials is limited solely to in vivo and in vitro basic research studies and is not intended for administration to humans. The lymphokine materials are aliquoted in 100 μg amounts (≥10⁶ units) and are available to investigators with peer-reviewed support. However, manufacturers' restrictions prohibit distribution of these materials to for-profit institutions or commercial establishments.

The monoclonal antibodies are available to peer-reviewed investigators, for-profit institutions or commercial establishments. The 11B.11 antibody is available in either 3 or 20 mg vials. The 3ZD antibody is available in 5 or 20 mg amounts.

Investigators wishing to obtain any of these materials should send requests to:

Dr. Craig W. Reynolds
Biological Response Modifiers Program
NCI-FCRDC
Building 1052, Room 253
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FAX: 301-846-5429

All requests should be accompanied by:
(1) A brief paragraph outlining the purpose for which materials are to be used, (2) the amount desired, (3) description of investigator's peer-reviewed support. Recipients will be required to sign a Materials Transfer Agreement and to pay shipping and handling costs. Please allow 4 to 6 weeks for delivery.

Notes
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