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In Situ Tumor Vaccination with Interleukin-12-encapsulated Biodegradable Microspheres: Induction of Tumor Regression and Potent Antitumor Immunity[1]

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ABSTRACT

An alternative technology for the local and sustained delivery of cytokines to tumors for cancer immunotherapy was evaluated and shown here to induce tumor regression, suppression of metastasis, and development of systemic antitumor immunity. Treatment of tumor-bearing BALB/c mice with a single intratumoral injection of biodegradable polylactic acid microspheres loaded with recombinant interleukin-12 (IL-12) promoted complete regression of the primary tumor and prevented the metastatic spread to the lung. Mice that experienced tumor regression after being treated rejected a subsequent challenge with live tumor cells, which indicated the development of systemic antitumor immunity. *In situ* tumor vaccination, *i.e.*, injection of IL-12 microspheres into existing tumors, was superior to vaccination of mice with mixtures of tumor cells (live or irradiated) and IL-12 microspheres in inducing systemic antitumor immunity. The sustained release of IL-12 from the microspheres was superior to bolus injection of free IL-12, and intratumoral delivery of microspheres was more effective than other routes of administration. These studies establish the utility of biodegradable polymer microspheres as a clinically feasible alternative to systemic cytokine therapy and cytokine gene-modified cell vaccines for the treatment of neoplastic disease.

INTRODUCTION

The ability of cytokines and cytokine gene-modified tumor cell vaccines to induce effective antitumor immunity in syngeneic murine tumor models is well established (1, 2). On the basis of the successful results obtained in preclinical models, numerous Phase I and II clinical trials have been initiated in cancer patients (3). Systemic bolus cytokine therapy has been associated with low efficacy and severe side effects in the clinic (4). Although some encouraging results have been reported with cytokine gene-modified tumor cell vaccines (5), it has also become increasingly clear that with the possible exception of melanomas, the current gene transfer technologies lack the simplicity and the versatility required for universal clinical application (1, 6). The development of clinically more feasible and less expensive alternative technologies for the local and sustained delivery of cytokines to tumors can significantly enhance the clinical implementation of cytokine-based cancer immunotherapies.

The attraction of gene-modification lies mainly in the fact that the cytokine of choice can be delivered to the tumor microenvironment in a paracrine manner, circumventing the severe side effects associated with systemic cytokine immunotherapy (1, 2, 6). Local and sustained delivery of therapeutic agents can also be achieved with biodegradable controlled-release polymers (7). Biodegradable polymer microspheres have been used in humans for *in vivo* drug delivery (7), cancer chemotherapy (8), and vaccination with antigenic peptides (9). Although these delivery systems were initially developed for the sustained delivery of low-molecular-weight therapeutics (7, 8), recent advances in encapsulation technologies and protein stabilization have led to the successful encapsulation of a number of bioactive macromolecules including immunostimulatory cytokines (10). In two *in vivo* murine studies, stimulation of antitumor responses with GM-CSF[3] and IL-1α-loaded microsphere formulations have been reported (11, 12).

Recently, we described a novel technology (PIN) for highly efficient encapsulation of biologically active molecules into polymer microspheres (13). This spontaneous process does not require vigorous stirring/sonication during the formation of emulsions, and labile proteins are efficiently encapsulated without denaturation or losses to aqueous nonsolvent baths. In preliminary studies, we demonstrated that recombinant human IL-2-loaded PLA microspheres, prepared by PIN, release physiologically relevant quantities of bioactive IL-2 for extended periods and that the *in vivo* release of IL-2 from the PLA microspheres provokes a mouse NK cell-mediated suppression of human tumor xenografts in SCID mice (14). On the basis of these initial observations, we have further investigated the clinical potential of PIN/PLA microspheres loaded with three different cytokines for cancer immunotherapy in a weakly immunogenic syngeneic murine tumor model. We report here that intratumoral injection of IL-12-loaded PIN/PLA microspheres, but not IL-2 nor GM-CSF-loaded microspheres, induces the regression of established tumors, prevents spontaneous metastasis and promotes the development of tumor-specific immunity.

MATERIALS AND METHODS

Mice and Cell Lines. Male or female BALB/c mice at 6–8 weeks of age were obtained from Taconic Laboratories (Germantown, NY). CB-17 scid/scid mice were obtained from our breeding colony. All of the mice were maintained in microisolation cages (Laboratory Products, Federalsburg, MA) under pathogen-free conditions. Animals of both sexes were used in the studies at 8–12 weeks of age. Line-1 (a BALB/c lung alveolar carcinoma cell line) was a gift from Dr. John G. Frelinger (University of Rochester, School of Medicine and Dentistry, Rochester, NY). CB.17 SCID mice were depleted of NK cells by a single i.p. injection of the monoclonal antibody TM-β1 1 day before the tumor inoculations (a generous gift of Dr. T. Tanaka, Tokyo Metropolitan Institute of Medical Science, Japan), which has been shown to effectively deplete murine NK cells for up to 5 weeks (15).

Cytokines. Recombinant human PEG-IL-2 (6×10^6 units/mg) was a gift from Chiron, Inc. (Emeryville, CA). Recombinant murine IL-12 (2.7×10^6 units/mg) was donated by Genetics Institute, Inc. (Andover, MA) and recombinant murine GM-CSF (7.2×10^7 units/mg) was donated by Immunex, Inc. (Seattle, WA).

Microspheres. A PIN technique was used for encapsulation of cytokines as described previously (13). Briefly, BSA (RIA grade, Sigma Chemical Co., St. Louis, MO), PLA (M_r 24,000 and M_r 2,000; 1:1 (w/w)], Birmingham Poly-

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[3] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; PIN, phase inversion nanoencapsulation; PLA, polylactic acid; NK, natural killer; SCID, severe combined immunodeficient; PEG, polyethylene glycol.

mers, Inc, Birmingham, AL), and recombinant cytokine in methylene chloride (Fisher, Pittsburgh, PA) was rapidly poured into petroleum ether (Fisher) for formation of microspheres. Microspheres were filtered and lyophilized overnight for complete removal of solvent. Four formulations containing 1% BSA (w/w) were produced: (*a*) control (no cytokines); (*b*) human PEG-IL-2 [~10 μg (60,000 IU)/mg PLA); (*c*) murine IL-12 [~10 μg (270,000 units)/mg PLA]; and (*d*) murine GM-CSF [~10 μg (7.2×10^5 units)/mg PLA). Scanning electron micrographs demonstrated that the microspheres were 1–5 μm in diameter and were easily injectable with a 28.5-gauge needle. The encapsulation efficiencies for the cytokines were extrapolated from the measurements of total protein encapsulated into the microspheres as described (16).

Cytokine Release and Bioactivity Assays. The assay for the quantitation of *in vitro* cytokine release from the microspheres has been described previously (14). Briefly, 3 mg of particles in 200 μl of tissue culture medium (DMEM + 10% FCS) were incubated in the wells of a 96-well culture plate in triplicate at 37°C. The medium was changed daily for 12–16 consecutive days, and the aliquots were stored at 4°C. The quantity of cytokine in the medium was determined either by ELISA (R&D Systems, Minneapolis, MN), or in the case of PEG-IL-2, by a bioactivity assay using an IL-2-dependent murine T-cell line proliferation assay (14). The bioactivity assay for recombinant murine IL-12 was performed using a murine splenocyte proliferation assay as described previously (17).

RESULTS

Cytokines Are Efficiently Encapsulated into and Released from the PLA Microspheres. The encapsulation efficiencies and *in vitro* release patterns of three different recombinant cytokines were evaluated. Encapsulation efficiency into PLA microspheres was determined to be 67 ± 1% for murine heterodimeric IL-12 (M_r 70,000), 95 ± 6% for murine GM-CSF (M_r 23,000), and 65 ± 6% for human PEG-IL-2 (M_r 15,000–94,000). The *in vitro* release patterns of IL-12, GM-CSF, and PEG-IL-2 from the microspheres are shown in Fig. 1. The initial release of cytokines is followed by a rapid decline with an eventual stabilization of the release kinetics after day 7. Both PEG-IL-2 and IL-12, which were released from the cytokines were shown to be bioactive *in vitro* (Fig. 1). The results indicate that significant quantities of cytokine can be released from the microspheres for at least 12 days, but that the absolute quantities and the release rates vary, depending on the particular cytokine that is encapsulated.

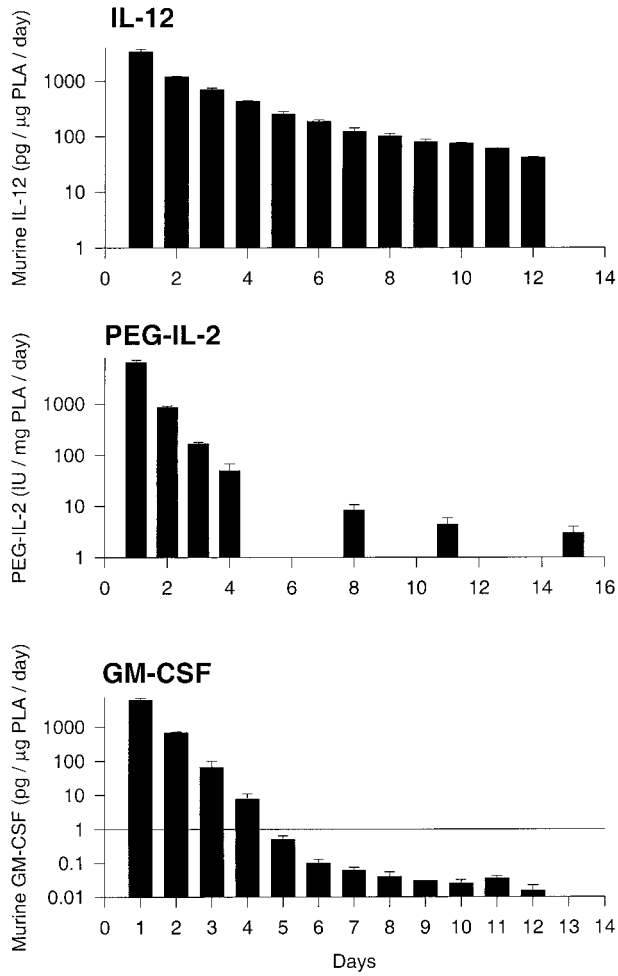

Fig. 1. *In vitro* release of cytokines from the microspheres. Release of recombinant human PEG-IL-2, murine IL-12, and murine GM-CSF were measured as described in "Materials and Methods." The bioactivity of IL-12 that was released from the microspheres was determined to be 2.2×10^5 units/mg using a murine splenocyte proliferation assay (see "Materials and Methods"). Bioactivity of GM-CSF was not assayed. Each data point was measured in triplicate. *Bars*, SD.

Coinjection of Cytokine-loaded Microspheres with a Single-Cell Suspension of Live Tumor Cells Suppresses Tumor Engraftment. The *in vivo* immunotherapeutic potential of the cytokine-loaded microspheres was initially tested by coinjecting the microspheres with live Line-1 tumor cells s.c. into BALB/c mice. Line-1 is a lung alveolar cell carcinoma that arose spontaneously in a female BALB/c mouse (18). This poorly immunogenic tumor grows rapidly and progressively in the s.c. site and ultimately metastasizes to the lungs of the inoculated mice (18). Mice were injected with Line-1 cells mixed with either control (BSA) or cytokine-loaded microspheres, and tumor growth was monitored weekly. The results are shown in Fig. 2*A*. At the tumor cell dose used, all of the mice in the control group (BSA microspheres) developed palpable tumors by day 3, with tumors reaching a diameter of ~5 mm within 7–8 days. In contrast, all of the mice that were treated with the IL-12-loaded microspheres remained tumor-free for at least 6 weeks. Mice that were treated with GM-CSF- or PEG-IL-2-loaded microspheres experienced a significant, albeit less dramatic, inhibition or delay in tumor growth. Two of five mice that received the GM-CSF microspheres remained tumor-free for 6 weeks, whereas all of the mice that were treated with PEG-IL-2-loaded microspheres developed tumors, although tumor growth in these mice was delayed compared with the controls. The antitumor effect observed with GM-CSF was surprising. This cytokine induces potent antitumor immunity when used in a prophylactic vaccine setting; however, it has not been shown to suppress tumor growth directly (1). Interestingly, IL-2, which has been shown to induce tumor suppression in numerous murine tumor models, had only a weak antitumor effect here. The observed effects (or lack thereof) could be related to the dose and the release pattern of the particular cytokine delivered by the microspheres. Regardless of the relative antitumor efficacy of individual cytokines, the above results establish that the cytokines released from the microspheres are biologically active *in vivo*, and that tumor growth can be completely arrested when IL-12-loaded microspheres are injected at the same time that tumors are inoculated into mice.

IL-12- but not PEG-IL-2- or GM-CSF-loaded Microspheres Induce Complete Regression of Established and Progressively Growing Tumors after a Single Intratumoral Injection. The ability to prevent tumor engraftment is a useful initial screen for evaluating the potential of an anticancer therapy. However, a more clinically relevant approach involves treating established tumors to determine whether or not the local and sustained release of cytokines from the microspheres is able to induce tumor remission and not simply prevent its engraftment. To this end, mice were inoculated with Line-1 cells s.c., and the tumors were allowed to grow to ~4 mm in diameter prior to treatment. These tumors were then injected with cytokine-loaded microspheres, and tumor growth was monitored weekly. In these experiments, the dose of microspheres was increased

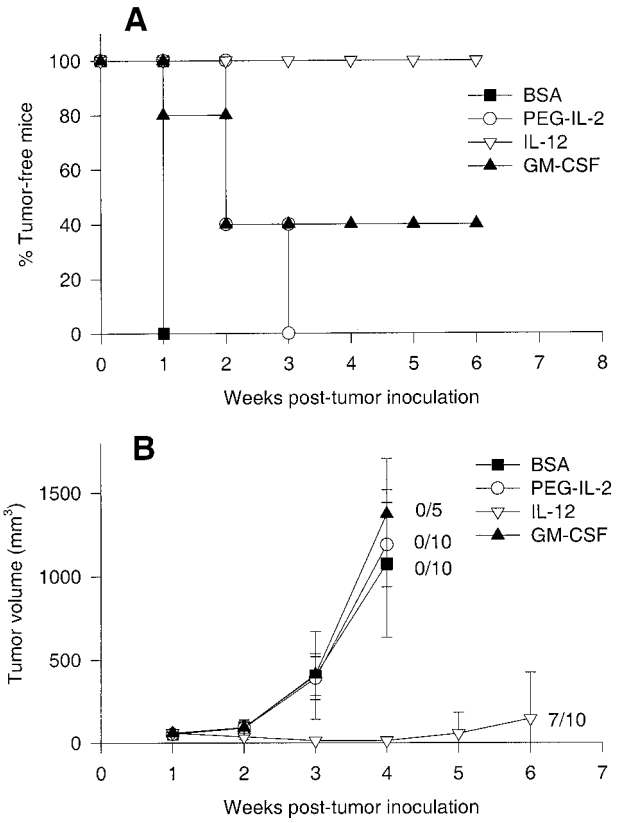

Fig. 2. Effect of IL-12 microspheres on Line-1 tumor engraftment and growth of established tumors in BALB/c mice. *A*, Line-1 tumor cells (1×10^6) and microspheres (50 μg) were mixed and injected s.c. in 100 μl of DMEM into BALB/c mice. Mice were scored as tumor-positive when the diameter of the tumor was >3 mm ($n = 5$). *B*, mice were injected with 1×10^6 Line-1 cells s.c., and tumors were allowed to grow to ~4 mm in diameter. Tumors were then injected directly with 2 mg of microspheres in 50 μl of DMEM using a 28.5-gauge needle, and tumor growth was monitored weekly. Tumor volume was calculated based on the formula $a^2 \times b/2$ where a and b are the shortest and the longest dimensions of the tumor, respectively ($n = 10$ for BSA, PEG-IL-2, and IL-12 groups; and 5 for the GM-CSF group). The differences between the BSA, PEG-IL-2, and GM-CSF-treated groups were not significant at any time point ($P > 0.22$); whereas the differences between the IL-12 group and the other groups were significant at weeks 3 and 4 ($P < 0.002$) in *B*. *Bars*, SD.

significantly as compared with that used in the coengraftment studies (2 mg as opposed to 50 μg per injection) because the number of tumor cells within the established tumors is greater and established tumors are more difficult to suppress and eradicate. The results are shown in Fig. 2*B*. There was no significant difference between the growth patterns of tumors treated with control (BSA-loaded) microspheres and PEG-IL-2 or GM-CSF-loaded microspheres, with which tumors grew progressively. However, a single intratumoral injection of IL-12-loaded microspheres promoted complete tumor regression in 7 of 10 mice, and tumor growth was suppressed in the 3 remaining mice. These results demonstrate that the sustained release of IL-12 from the microspheres can induce potent antitumor activity in a clinically relevant setting.

Tumor Regression Is Accompanied by the Development of Protective Antitumor Immunity, the Potency of Which Is Dependent on the Method of Vaccination. The ultimate goal of immunotherapy is to promote the development of long-term systemic antitumor immunity to prevent recurrence of tumors, which cannot be achieved with conventional treatments such as chemotherapy and radiation. Cytokine gene-modified tumor cell vaccines, especially those involving the cytokines IL-12 and GM-CSF, have been shown to provoke the development of effective antitumor immunity in mice (19, 20). To test whether IL-12 delivered by microspheres directly into existing tumors is able to promote similar protective antitumor immunity, mice that were able to reject established s.c. tumors after treatment with IL-12-loaded microspheres were challenged with live tumor cells at a different site 5–6 weeks after the original tumor had completely regressed. The results of this experiment are shown in Table 1. Of the 15 vaccinated mice that were challenged, 12 (80%) rejected the tumor, which suggests the development of potent protective antitumor immunity in these mice.

In parallel experiments, the antitumor efficacy of different vaccination strategies with mixtures of IL-12-loaded microspheres and single-cell suspensions of tumor cells (live or irradiated) were compared with direct intratumoral (*in situ*) treatments of progressively growing tumors. As shown in Table 1, vaccination of mice with mixtures of IL-12 microspheres and live Line-1 cells provided less protection from a subsequent tumor challenge than did *in situ* vaccination (57 *versus* 80%). Only 10% of the mice were protected from tumor challenge with an irradiated-cell/IL-12 microsphere vaccine, which was identical to that obtained with irradiated cells alone. In the control nonvaccinated group, none of the mice were able to reject tumor challenge.

To determine whether the immunity provoked by the cytokine-loaded microspheres was tumor-specific, mice that rejected s.c. Line-1 tumors after vaccination *in situ* were challenged either with Line-1 or Colon 26 (an unrelated colon tumor cell line derived from BALB/c mice) cells and tumor growth was monitored. Whereas six of six mice vaccinated with Line-1 rejected the Line-1challenge, only one of six vaccinated mice rejected a challenge with Colon 26 tumor cells (Table 2). Nonvaccinated control mice did not reject challenges with either tumor cell line. These results demonstrate that the systemic antitumor immunity induced by the IL-12-loaded microspheres was tumor-specific.

IL-12-loaded Microspheres Stimulate a NK Cell-dependent Delay in Tumor Growth But Fail to Induce Complete Tumor Regression in CB.17 SCID Mice. The antitumor activity of IL-12 has been shown to be mediated in part by T-lymphocytes and NK cells through an IFN-γ-dependent mechanism (21, 22). To determine whether the microsphere-mediated tumor regression observed here was induced by a similar mechanism, microsphere vaccination experiments were repeated in CB.17 SCID mice, which lack functional B- and T-lymphocytes. Mice with established s.c. tumors were treated with intratumoral injections of IL-12-loaded microspheres, and tumor

Table 1 *The potency of the protective antitumor immunity induced by the IL-12-loaded microspheres is dependent on the vaccination method*

Method of vaccination	% tumor rejection after challenge[a]
Established tumor + IL-12 microspheres[b]	80 (12 of 15)
Live Line-1 cells + IL-12 microspheres[c]	57 (8 of 14)
Irradiated Line-1 cells + IL-12 microspheres[d]	10 (1 of 10)
Irradiated Line-1 cells alone	10 (1 of 10)
No treatment	0 (0 of 5)

[a] Mice challenged with 1×10^4 tumor cells injected s.c.
[b] Tumor was injected with 2 mg of microspheres.
[c] 1×10^6 tumor cells + 50 μg of microspheres.
[d] 2×10^6 tumor cells + 50 μg microspheres.

Table 2 *The antitumor immunity that results from vaccination with IL-12 microspheres is tumor-specific*

Method of vaccination	Tumor challenge	% tumor rejection
Established Line-1 tumors + IL-12 microspheres[a]	Line-1	100 (6 of 6)
Established Line-1 tumors + IL-12 microspheres[a]	Colon 26	17 (1 of 6)
No treatment	Line-1	0 (0 of 5)
No treatment	Colon 26	0 (0 of 5)

[a] Treated with 2 mg of microspheres, challenged with 1×10^4 live cells.

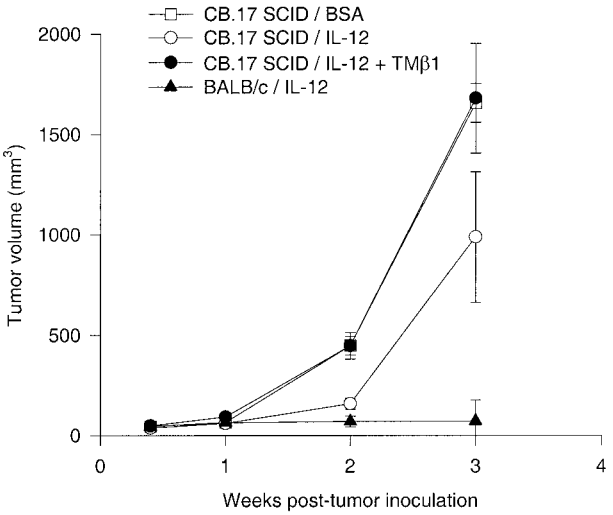

Fig. 3. Effect of IL-12 microspheres on the growth of established Line-1 tumors in CB.17 SCID mice. Established Line-1 tumors (~4 mm in diameter) in CB.17 SCID or BALB/c mice were injected either with BSA- or IL-12-loaded microspheres (2 mg/tumor in 50 μl of DMEM), and tumor growth was monitored weekly ($n = 5$). The differences between the IL-12-treated SCID mice and the IL-12- + TMβ1-treated SCID mice are highly significant at weeks 2 and 3 ($P < 0.007$). *Bars*, SD.

growth was monitored. The results are shown in Fig. 3. Treatment with IL-12-loaded microspheres delayed tumor growth by 1 week in the CB.17 SCID mice but failed to promote tumor regression. The limited antitumor response observed in the CB.17 SCID mice was shown to be NK-cell-dependent because the depletion of the mouse NK cells with the monoclonal antibody TMβ1 resulted in the loss of the tumor-suppressive activity. In contrast, a significant tumor suppression was observed in the immunocompetent BALB/c mice with tumors regressing completely in three of five mice.

Intratumoral Administration of Microspheres Is Critical to Tumor Eradication and Treatment with IL-12-loaded Microspheres Is Superior to Bolus Injections of Free IL-12. Repeated local or systemic administration of free IL-12 has been shown to promote tumor regression in several murine tumor models (22–24). To determine whether local release of IL-12 from the microspheres to the tumor microenvironment was necessary, mice were inoculated with IL-12-loaded microspheres either intratumorally or on the contralateral side of tumor-bearing mice, and tumor growth was monitored. The results are shown in Table 3. In this experiment, 53% of the tumors regressed completely after intratumoral delivery, whereas none of the tumors regressed when the microspheres were injected on the contralateral flank of tumor-bearing mice. Moreover, a single intratumoral injection of free IL-12 at a dose equal to that delivered by the microspheres resulted in the regression of tumors in only 20% of the animals; whereas i.p. delivery of free IL-12 did not promote any tumor regression. These results demonstrate that local and sustained delivery of IL-12 to tumors is superior to local or systemic bolus delivery.

Treatment of Established s.c. Tumors with IL-12-loaded Microspheres Suppresses Both the Growth of s.c. Tumors and the Distant Metastatic Lesions. Line-1 cells, when injected s.c., metastasize spontaneously to the lungs of the BALB/c mice (18). To determine whether treatment of established s.c. tumors with IL-12-loaded microspheres could also promote the suppression of metastasis, mice with established large (~7–8 mm) s.c. tumors were treated with IL-12-loaded microspheres, and their lungs were analyzed 2 weeks after treatment. The results are shown in Fig. 4. Treatment with IL-12-loaded microspheres induced significant suppression of tumor growth compared with treatment with BSA-loaded microspheres (Fig. 4*A*). Although the primary s.c. tumors were suppressed, treatment here did not result in complete regression because of the larger tumor inoculum and the greater size of the tumors at the time of treatment compared with previous experiments. More interestingly however, the examination of the lungs 2 weeks after treatment revealed significant suppression of lung metastasis in the IL-12-treated animals as compared with the controls (Fig. 4*B*). These results demonstrate that the local treatment of primary tumors with IL-12-loaded microspheres can suppress both the growth of the primary tumor and metastasis to distant sites. Whether the antimetastatic effect observed here was attributable to the systemic presence of the cytokine released by the microspheres or to the development of systemic antitumor immunity that resulted from a release of the cytokine into the tumor microenvironment was not determined. The results shown in Tables 1 and 2 establish that intratumoral delivery of IL-12 microspheres induces the development of a potent tumor-specific systemic immunity. Moreover, the results summarized in Table 3 demonstrate that when the IL-12 microspheres are injected contralateral to tumors, tumor regression is not induced. Together, these data support the notion that the suppression of lung metastasis observed here is most likely mediated by the development of a systemic antitumor immunity and is not

Table 3 *Local and sustained delivery of IL-12 is critical to cure of established tumors in the Line-1/BALB/c model*

Method of delivery	Location	% tumor cure[a]
Microspheres[b]	Intratumoral	53 (8/15)
Microspheres[b]	Contralateral	0 (0/5)
Free cytokine[c]	Intratumoral	20 (1/5)
Free cytokine[c]	Intraperitoneal	0 (0/5)

[a] Defined as complete regression with no evidence of recurrence for at least 6 weeks.
[b] 2 mg of microspheres (~29,000 units of IL-12).
[c] 1 μg of free IL-12 (~27,000 units of IL-12).

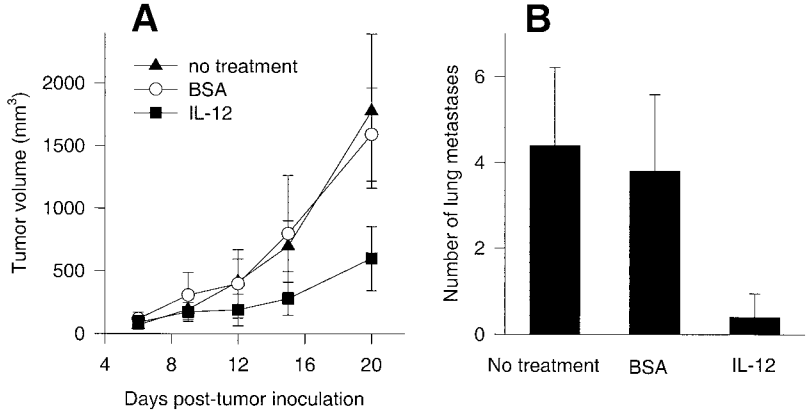

Fig. 4. Effect of IL-12-loaded microspheres on the growth of spontaneous lung metastases in BALB/c mice. BALB/c mice were given injections of Line-1 cells (5×10^7) in 200 μl of DMEM s.c. in the ventral caudal midline on day 0. Tumors were allowed to reach a diameter of 7–8 mm and were treated with a single intratumoral injection of either BSA- or IL-12-loaded microspheres (1 mg/mouse in 100 μl). *A*, growth of established tumors was monitored every 3 days. The differences between the BSA-treated and the IL-12-treated mice were significant on days 15 and 20 ($P = 0.04$ and 0.001, respectively). *B*, mice were killed 14 days after treatment, and the lungs were examined for tumor nodules under a dissecting microscope ($n = 5$). The differences between the no-treatment/BSA-treated groups and the IL-12-treated groups were highly significant ($P = 0.0036$ and 0.0015, respectively). *Bars*, SD.

simply attributable to systemic release of IL-12 from the microspheres.

DISCUSSION

The work presented here establishes that biodegradable polymer microspheres prepared by the PIN technology can effectively deliver biologically active IL-12 to established tumors and thereby provoke a strong and lasting systemic antitumor immunity in several different embodiments of a weakly immunogenic syngeneic murine tumor model. This is the first report in which complete tumor regression, suppression of spontaneous metastasis, and the development of protective tumor-specific immunity is achieved using IL-12-loaded biodegradable microspheres, demonstrating the clinical potential of this technology. The overall applicability of this approach was also confirmed recently in another syngeneic tumor model,[4] in which complete regression of established Colon 26 tumors was achieved in four of five BALB/c mice after a single intratumoral injection of IL-12 microspheres. No tumor suppression was observed with control BSA-loaded microspheres in these experiments.

Our studies extend and confirm previous reports demonstrating the ability of IL-12 to induce tumor regression and the development of potent antitumor immunity in preclinical murine models (20–24). Additional studies have established that the potent antitumor effects of IL-12 are tempered by the dose- and schedule-dependent toxicity in mice (25) and in humans (26, 27) when administered systemically. Recent studies demonstrated that systemic administration of IL-12 also induces a transient generalized immunosuppression in mice (28, 29). The severe toxicity associated with systemic infusion of IL-12 in early clinical trials was partially alleviated by altering the schedule and dose of treatment, but the lack of significant antitumor efficacy in these trials has been disappointing (27, 30). Ineffectiveness of systemic IL-12 therapy in the clinic could be attributable to the inability of the cytokine to reach effective local concentrations in the tumor bed at maximum tolerated dose and/or the induction of a generalized suppression of T-cell responses.

The risk of toxicity and generalized immunosuppression is obviated when therapy is restricted to the intralesional treatment of a single tumor nodule (even in a patient with multiple tumor foci and/or distant metastases) rather than attempting to treat cancer patients systemically to deliver the cytokine to all of the tumor sites. The rationale for such an approach is clearly supported by the findings reported here that low doses of IL-12, released locally from the microspheres at a single tumor site in a sustained fashion, have a significant antitumor effect resulting in the disappearance of the primary tumor, reduction in distant metastases, and the development of systemic antitumor immunity. Although local and sustained delivery of cytokines to tumors can also be achieved by *ex vivo* or *in situ* cytokine gene-modification strategies resulting in a similar induction of antitumor immunity in mice (1–3, 20), these approaches involve complex and expensive protocols coupled with low and/or variable gene transfer efficiency that present difficulties in the clinical setting (1, 2, 6). In contrast, the approach described here with the PIN/PLA microspheres represents a simple, universal, and much less expensive alternative to gene therapy protocols for delivering low doses of cytokines to the tumor microenvironment in a sustained fashion.

Furthermore, our data establish that vaccination of tumor-bearing mice with IL-12-loaded microspheres *in situ* is superior to vaccination of mice with mixtures of live tumor cells and IL-12 microspheres, which in turn is more effective than irradiated tumor cell/microsphere mixtures in inducing protective antitumor immunity. This finding has obvious potential clinical relevance with respect to ultimately designing vaccination strategies for cancer patients. Others have shown that vaccination with live cytokine gene-modified tumor cells is more effective than vaccination with irradiated cytokine gene-modified tumor cells, and antigen dose has been suggested as a critical factor to explain these observations (6, 20). Regardless of the mechanism, these data are consistent with the notion that *in situ* tumor vaccination strategies should be preferred in the clinic when feasible, and microsphere-mediated delivery of therapeutics to tumors represents one such approach. The intralesional inoculation of a patient's tumor nodule with cytokine-loaded microspheres before surgical resection and/or chemotherapy would also allow for maximal stimulation of antitumor immunity without interfering with standard therapy. Accessibility of tumor would not be a major concern because stereotactic injections could be used for a large variety of lesions that are not directly accessible.

The ease of preparation, consistency, and long-term stability of cytokine-loaded microspheres (31) are also conducive to further manipulation of the vaccination protocols in the clinical setting to improve efficacy. Repeated booster vaccinations with mixtures of microspheres and irradiated tumor cell suspensions obtained from postsurgical specimens can be used to augment antitumor immunity after the initial vaccination and standard therapy. Other potential applications include more complex strategies involving multiple cytokines and/or combination therapy with other therapeutic agents that have been shown to be more effective than single cytokine approaches (32–34).

[4] N. K. Egilmez and R. B. Bankert, unpublished results.

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