Cure of an Established Nonimmunogenic Tumor, SCC VII, With a Novel Interleukin 12–Based Immunotherapy Regimen in C3H Mice

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**Objective:** To develop a murine model of effective treatment with immunotherapy for established head and neck squamous cell carcinoma.

**Design:** Prospective animal study.

**Subjects:** Female C3H mice, 8 to 12 weeks old.

**Interventions:** A subcutaneous inoculation of $2 \times 10^5$ SCC VII cells in C3H mice was established for 7 to 12 days. Tests for concomitant immunity were performed, with and without interleukin 12 modification. Tumors were also tested for responsiveness to interleukin 12 (5 mice) and to cyclophosphamide followed by interleukin 12 (5 mice). SCC VII tumors in 24 mice were treated with interleukin 12 followed by cyclophosphamide and interleukin 12. Five mice with tumors treated with isotonic sodium chloride solution served as controls. Tumors were measured 3 to 4 times weekly, and cure was defined as complete regression of the tumor for at least 60 days. Cured mice were rechallenged with $2 \times 10^5$ SCC VII cells to verify antitumor immunity. Immunohistochemistry of regressing tumors was performed for CD4+ and CD8+ T cells.

**Results:** Tumor-bearing mice easily developed second tumors when challenged with $2 \times 10^5$ tumor cells in the opposite flank. However, interleukin 12 treatment provided immunity to second tumors in 8 (100%) of 8 mice when started at day 4 and in 2 (40%) of 5 when treated from day 7. SCC VII did not respond to standard interleukin 12 or cyclophosphamide plus interleukin 12 therapy. Seventy-five percent of animals (18/24) treated with interleukin 12 followed by cyclophosphamide plus interleukin 12 were successfully cured, and all cured mice resisted subsequent challenge with SCC VII. Immunohistochemistry of regressed tumors showed an intense CD4+ and CD8+ infiltrate that was absent in the untreated and nonresponding tumors.

**Conclusions:** Nonimmunogenic SCC VII is a nonimmunogenic tumor that can be converted into an immunogenic tumor with interleukin 12 treatment. Additional treatment with cyclophosphamide plus interleukin 12 leads to complete regression in 75% of mice.

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**Clinical Interest** in immunotherapy for head and neck cancer has had a recent resurgence. Since advances in surgical technique, radiation modalities, and chemotherapeutic agents have not had significant effects on long-term survival of patients with head and neck squamous cell carcinoma (HNSCC), novel treatments have been explored. Patients with HNSCC have been well documented to exhibit local immunosuppression, with depressed T-cell–mediated responses as well as depressed natural killer cell and antibody-dependent cell cytotoxicity. The absence of B7 protein or class II major histocompatibility complex antigen expression on human HNSCC may contribute to the failure of tumors to induce T-cell proliferation in vitro. Natural killer cell function and T-cell activation have increased with peritumoral injections of interleukin 2. Clinical effectiveness of peritumoral interleukin 2 injections in patients with advanced HNSCC, however, has been poor.

The use of various cytokines in cancer treatment has demonstrated profound effectiveness in immunogenic tumors, but not in nonimmunogenic tumors. Among these, interleukin 12 has been shown to be the most effective antitumor cytokine. The exact mechanism by which it achieves this antitumor effect has been difficult to determine because of its varied effects. Interleukin 12 was identified as the natural killer cell stimulating factor and is known to enhance cytolytic function of natural killer cells. Its function on T cells is in antigen presentation, proliferation of activated T cells, and dif-
ferentiation of T cells into a Th1 subset, thus enhancing cytolytic activity. In our laboratory, using a methylcholanthrene-induced sarcoma (MCA 207) tumor model, we showed that interleukin 12 induces a Th1-directed, macrophage-mediated delayed-type hypersensitivity (DTH) response that results in regression of those tumors. The addition of cyclophosphamide, a DTH-potentiating agent, has enabled treatment of large MCA 207 tumors (>20 mm), and cyclophosphamide plus interleukin 12 has been effective against other immunogenic tumors as well.

Realizing that squamous cell carcinoma is classically described as nonimmunogenic, we wished to determine the effect of our novel cyclophosphamide plus interleukin 12 therapy on squamous cell carcinoma. A preclinical model for squamous cell carcinoma has been described that uses the SCC VII tumor cell line in C3H mice. SCC VII in C3H mice is an immunocompetent syngeneic model of squamous cell carcinoma with features similar to those of HNSCC. Like HNSCC, SCC VII has been shown to lack major histocompatibility complex class II and B7 costimulatory molecules. In mice, it has been successfully implanted in the floor of the mouth to mimic oral cavity cancer. While models of immunotherapy with SCC VII have shown poor results with interleukin 2 and inhibition of growth with interleukin 12, no model has shown a long-lasting complete regression of an established SCC VII tumor. Using a unique combination of immune modulation and immunotherapy, we present our model of a durable complete regression of SCC VII tumor with immunotherapy.

**METHODS**

**ANIMALS AND TUMOR CELL LINE**

Female C3H mice aged 8 to 12 weeks were obtained commercially (The Jackson Laboratory, Bar Harbor, Maine). SCC VII is a spontaneously arising cell line in C3H mice. The cells were maintained free of Mycoplasma contamination. Tumor cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM glutamine, 100-mg/mL streptomycin, 100-IU/mL penicillin, and 5 x 10^-3 M 2-mercaptoethanol. Prior approval from the Institutional Animal Care and Use Committee was obtained for all subsequent experiments.

**TEST FOR CONCOMITANT IMMUNITY**

Concomitant immunity tests were performed by inoculating bilateral flanks on separate days to evaluate for tumor growth. As shown in Figure 1, in 15 mice 2 x 10^6 cultured tumor cells were subcutaneously inoculated on day 0 in the right flank, and on day 4, 7, or 13, the contralateral flank was inoculated with 2 x 10^5 cultured tumor cells. The presence of tumor growth and size were monitored. In 2 concurrent groups, 13 mice were inoculated with 2 x 10^6 cultured tumor cells in the right flank on day 0. On day 4 (8 mice) and on day 7 (5 mice), mice were treated with interleukin 12, 200 ng every other day for 3 doses, and then inoculated with 2 x 10^5 cultured tumor cells in the contralateral flank on day 10 or 13, respectively. Again, tumor growth and size were monitored. Naïve mice were injected with 2 x 10^5 cultured tumor cells at the time of second inoculations to ensure tumor cell viability.

**TUMOR TREATMENT**

Tumors were established by subcutaneous inoculation of 2 x 10^6 cultured tumor cells in 0.1 mL of isotonic sodium chloride solution into the right flanks of 39 C3H mice. Size was measured in 2 dimensions with microcalipers. Mice were observed for toxicity and killed when tumor size exceeded 20 mm in any one dimension. Mice were considered cured when no recurrent growth was noted 60 days after initial complete regression of tumor. Tumors were grown for the days or size indicated in the text and figures. Animals were treated with a murine interleukin 12 (Genetics Institute, Inc, Andover, Mass) at a dose of 200 ng via intraperitoneal injection and/or cyclophosphamide (Sigma-Aldrich Corp, St Louis, Mo) at a dose of 3 mg per mouse (100-125 mg/kg) in 0.5 mL of isotonic sodium chloride solution intraperitoneally, according to 1 of 3 experimental protocols: (1) interleukin 12, 200 ng every other day for 5 doses (5 mice); (2) cyclophosphamide, 3 mg once followed 3 days later by interleukin 12, 200 ng/d for 5 doses (5 mice); or (3) interleukin 12, 200 ng every other day for 5 doses followed 2 days later by cyclophosphamide plus interleukin 12 (24 mice) (Figure 2). In the first experiment to determine whether SCC VII is a responder to interleukin 12 or cyclophosphamide plus interleukin 12 therapy, mice were treated with interleukin 12 or cyclophosphamide plus interleukin 12. Subsequent experiments studied cyclophosphamide plus interleukin 12, and interleukin 12 followed by cyclophosphamide plus interleukin 12. Five mice treated with isotonic sodium chlo-
and the sections were then counterstained with hematoxylin, (Vector Red; Vector Laboratories) was used to develop color, followed by the avidin-biotinylated enzyme complex (ABC) system. Secondary antibody was a biotinylated goat anti–rat IgG (5 µg/mL, Pharmingen, San Diego, Calif) and was incubated for 20 minutes. The antibodies used were purified monoclonal rat anti–mouse CD8 (15 µg/mL, clone H129.19) and purified monoclonal rat anti–mouse CD4 (15 µg/mL, clone 53-6.7).

IMMUNOHISTOCHEMISTRY

Tumors were resected and snap frozen. Slides were prepared by making 6-µm cryostat sections fixed in cold acetone dried at −20°C and then stored at −70°C. The sections were dried at room temperature before staining. After rehydration with phosphate-buffered saline, sections were blocked with 1% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline, sections were blocked with 1% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline, sections were blocked with 1% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline, sections were blocked with 1% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline, sections were blocked with 1% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline, sections were blocked with 1% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline, sections were blocked with 1% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline. The antibodies used were pu-

Our laboratory previously studied the effects of interleukin 12 and cyclophosphamide to determine which algorithm best achieves a cure in MCA 207 tumors. Small tumors (4-8 mm) are easily cured with interleukin 12, 200 ng given every other day for 3 doses. The optimal therapy of 1 dose of 3 mg of cyclophosphamide followed by interleukin 12, 200 ng for 5 days, is effective for curing large (15-20 mm) MCA 207 tumors. We used these 2 findings as the basis of SCC VII tumor therapy.

An inoculation of 2 × 10^5 tumor cells was given in the right flank of 15 mice and allowed to become established for 12 days to reach 4 to 8 mm in diameter. Mice were treated with interleukin 12 (5 mice), cyclophosphamide plus interleukin 12 (5 mice), or isotonic sodium chloride solution (5 mice), as shown in Figure 2. Figure 3 shows that untreated mice had a fairly rapid growth curve mimicked by growth after interleukin 12 treatment. Mice treated with cyclophosphamide plus interleukin 12 had a slight regression followed by rapid recurrent growth. These growth curves show that SCC VII (REPRINTED) ARCH OTOLARYNGOL HEAD NECK SURG/ VOL 129, JULY 2003 WWW.ARCHOTO.COM

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Table 1. Establishment of Tumor by Time of Inoculation and Interleukin 12 Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>1st Tumor</th>
<th>2nd Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days 0 + 4 SCC cells</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Days 0 + 10 SCC cells</td>
<td>5/5 (100)</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Days 0 + 13 SCC cells/ day 4 interleukin 12</td>
<td>5/8 (62.5)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Days 0 + 13 SCC cells/ day 7 interleukin 12</td>
<td>5/5 (100)</td>
<td>3/5 (60)</td>
</tr>
</tbody>
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Abbreviation: SCC, squamous cell carcinoma.

Figure 3. Results of standard interleukin 12 and cyclophosphamide plus interleukin 12 treatment. Interleukin 12 alone had no effect on the growth curve of SCC VII; cyclophosphamide plus interleukin 12 had a transient effect on tumor size but then growth resumed.

RESULTS

DEMONSTRATION OF SCC VII AS A NONIMMUNOGENIC TUMOR THAT ELICITS IMMUNOGENICITY WITH INTERLEUKIN 12 TREATMENT

To confirm that SCC VII is a nonimmunogenic tumor, a test of classic concomitant immunity was performed. Although immunogenic tumors can induce an antitumor response on establishment, nonimmunogenic tumors do not. Concomitant immunity is the ability of an immunogenic tumor to develop a T-cell–mediated response that, while not powerful enough to reject the primary tumor, is able to reject a secondary homologous tumor. Figure 1 shows the time line for inoculation of tumor, and Table 1 shows the numbers and percentages of mice in which tumors were established. In all mice not treated with interleukin 12, both the primary and secondary tumors were established easily, showing that SCC VII does not induce an immune response to demonstrate concomitant immunity.

When mice were treated with interleukin 12 between tumor inoculations of SCC VII, signs of immune modulation were present. Mice treated early (day 4) had complete inhibition of the second tumor, and 40% of mice treated on day 7 had inhibition of the second tumor (Table 1). This finding suggests the induction of an immune response to SCC VII by interleukin 12. This effect was more pronounced in the few days after inoculation and diminished by day 7. Early treatment with interleukin 12 also prevented establishment of SCC VII tumors.

LACK OF RESPONSE OF SCC VII TO INTERLEUKIN 12 AND CYCLOPHOSPHAMIDE PLUS INTERLEUKIN 12 TREATMENT
TUMOR REGRESSION IN MICE TREATED WITH INTERLEUKIN 12/CYCLOPHOSPHAMIDE PLUS INTERLEUKIN 12 PROTOCOL

To determine the effects of a novel treatment of interleukin 12 followed by cyclophosphamide plus interleukin 12 on tumor regression, we inoculated $2 \times 10^5$ tumor cells in the right flank of 24 mice and allowed establishment of tumors. Mice were treated with cyclophosphamide plus interleukin 12; interleukin 12 followed by cyclophosphamide plus interleukin 12; or isotonic sodium chloride solution, as shown in Figure 2. Mice treated with isotonic sodium chloride solution showed growth similar to that in Figure 3 (data not shown). Mice treated with cyclophosphamide plus interleukin 12 had moderate regression (days 15-25) and resumed growth (Figure 4). Mice that were treated with interleukin 12 followed by cyclophosphamide plus interleukin 12 had significant regression; most (18 of 24) had complete regression, but some (6/24) had resumption of growth. The overall cure rate for treatment with interleukin 12 followed by cyclophosphamide plus interleukin 12 was 75% (Table 2), compared with cure rates of 0% for treatment with interleukin 12, cyclophosphamide plus interleukin 12, or isotonic sodium chloride solution. These 18 cured mice resisted a rechallenge with $2 \times 10^5$ SCC VII tumor cells 100% of the time. Mice were rechallenged on the contralateral flank 8 to 12 weeks after complete regression of the initial tumor.

CD4+ AND CD8+ CELLS ON IMMUNOHISTOCHEMISTRY OF REGRESSING TUMORS

Animals with SCC VII tumors treated with interleukin 12 followed by cyclophosphamide plus interleukin 12 that showed marked regression were killed and their tumors were harvested, frozen, and prepared on slides for immunohistochemistry. Untreated day 12 tumors and day 31 tumors treated with interleukin 12 or cyclophosphamide plus interleukin 12 were also examined. Staining with monoclonal anti-CD4 and anti-CD8 antibodies showed that untreated tumors had no CD4+ or CD8+ cellular infiltrate, whereas treatment with interleukin 12 induced a presence of CD4+ and CD8+ infiltrate (Figure 5). The infiltrate increased as the regressing tumor was farther along its course, as is demonstrated by increased staining of CD4+ and CD8+ cells in these specimens (Figure 6).

**COMMENT**

SCC VII in C3H mice is a tumor model that has been shown to mimic HNSCC. An intraoral model described by O’Malley et al23 in 1997 has the advantage of demonstrating local tissue invasion and regional and distant metastases similar to that of oral cavity squamous cell carcinoma. Khurana et al20 recently showed that SCC VII is an aggressive tumor and poorly immunogenic in their intradermal and pulmonary metastases model. In vitro cytotoxicity assays using tumor-draining lymph nodes as the effector cells did not show any significant tumor cell lysis. Adoptive immunotherapy of the tumor-draining lymph nodes with or without interleukin 2 showed no decrease in the number of pulmonary metastases, suggestive of the high resistance to treatment of SCC VII with immunotherapy.21 Meyers et al22 also showed that if SCC VII is transfected with a retrovirus that produces interleukin 12 (SCC VII–interleukin 12), the local production of interleukin 12 inhibits establishment of the tumors and a subsequent challenge with the wild-type cell line results in growth inhibition. These studies demonstrate the utility of SCC VII in C3H mice as a model for HNSCC.

Our results are consistent with the nonimmunogenicity of the tumor cell line. Our group has previously shown that other established immunogenic tumors are responders to cyclophosphamide plus interleukin 12 treatment, but that nonimmunogenic tumors such as B16 melanoma or Lewis lung carcinoma are nonresponders to cyclophosphamide plus interleukin 12 treatment.19 SCC VII also does not respond to standard cyclophospha-
mide plus interleukin 12 therapy, adding additional proof of its immune status. It does not demonstrate concomitant immunity, and the lack of CD4+ and CD8+ cellular infiltrate in the untreated tumor specimen also shows that alone it does not promote an immune cell infiltrate. Mice treated with cyclophosphamide plus interleukin 12 did not demonstrate a cellular infiltrate either. However, treatment with interleukin 12 resulted in an alteration of the

Figure 5. Immunohistochemistry of SCC VII. Untreated tumors and tumors treated with cyclophosphamide plus interleukin 12 showed no infiltration of CD4+ and CD8+ cells, whereas tumors treated with interleukin 12 followed by cyclophosphamide plus interleukin 12 had infiltration of both CD4+ and CD8+ cells (original magnification ×100).

Figure 6. Comparison by immunohistochemistry of CD4+ and CD8+ infiltration in tumors treated with interleukin 12 followed by cyclophosphamide plus interleukin 12. These tumors showed an increase in CD4+ and CD8+ infiltrate as the tumors continued to regress (original magnification ×100).
immune recognition of SCC VII. Mice treated by day 4 were able to reject second tumors completely, while treatment by day 7 still conferred some immunity. With immunohistochemistry, we saw that treatment with interleukin 12 alone resulted in infiltration of some CD4+ cells. We hypothesize that the first interleukin 12 treatment is responsible for this change in immunity and propose the mechanism to be enhanced antigen presentation.

In our tumor model, while SCC VII did not respond to cyclophosphamide plus interleukin 12, we were able to achieve a 75% cure of a nonimmunogenic tumor SCC VII with interleukin 12 followed by cyclophosphamide plus interleukin 12 treatment. No other treatment yielded any curative results. It is unclear, however, what trigger differentiates regressing tumors that achieve cure from those that resume growth. Size appears to be a determinant and perhaps should be examined closely in future experiments. However, since mice are randomly selected for treatment, the effect of size should be negligible. In the MCA 207 large tumor model treated with cyclophosphamide plus interleukin 12, some tumors have required weekly interleukin 12 injections to enhance the curative effect. By a similar mechanism, our model may require weekly interleukin 12 to maintain the Th1-directed response.

The effect of cyclophosphamide has been shown in other models to potentiate the DTH response. 17 Cyclophosphamide has been shown to decrease the tumor burden, which may allow for interleukin 12 to initiate the Th1 response. Although this mechanism may be responsible for the tumor regression seen in animals treated with cyclophosphamide plus interleukin 12 alone (Figures 3 and 4), it is unlikely to be the effect for the tumors treated with interleukin 12 followed by cyclophosphamide plus interleukin 12. If it were, tumors treated with interleukin 12 alone should have been effectively treated. Other potential roles of cyclophosphamide are in removing suppressor cells or affecting antigen presentation that results in T-cell activation. 24, 25 The most likely mechanism is increased antigen presentation of tumor cells increasing T-cell activation, which is then modulated by interleukin 12 therapy.

Meyers et al 22 showed that SCC VII transfected with a retrovirus producing interleukin 12 when inoculated in C3H mice causes local interleukin 12 production that results in inhibition of tumor establishment in 94.5% of mice. Increasing the inoculant to 106 cells resulted in inhibition in 88.0% of mice. Mice then challenged with wild-type SCC VII failed to grow tumors, suggesting that SCC VII-interleukin 12 acted as a vaccine. 22 This same interleukin 12 mechanism could explain why 3 tumors treated at day 4 with interleukin 12 failed to become established. In our case, the interleukin 12 was exogenously delivered. Similarly, when mice were inoculated with a second tumor after interleukin 12 treatment at day 4, the tumor failed to become established. It seems that the presence of interleukin 12 soon after inoculation is critical in developing the immunity responsible for rejecting further challenges. Mice treated at day 7 with the same course of interleukin 12 were not as successful in developing resistance to rechallenge. We hypothesize that the mechanism of effective treatment of SCC VII with interleukin 12 followed by cyclophosphamide plus interleukin 12 is a Th1-directed DTH response. The first treatment of interleukin 12 results in antigen presentation and priming of T cells, in this case against SCC VII. The first treatment is responsible for developing a repertoire of T cells against SCC VII. Immunohistochemistry supports this hypothesis, as CD4+ cells are seen in tumors treated with interleukin 12 alone and interleukin 12 followed by cyclophosphamide plus interleukin 12. Once the tumor-specific T cells are present, the addition of cyclophosphamide, a potentiator of the DTH response, facilitates the interleukin 12–dependent Th1 response. As in our other models of cyclophosphamide plus interleukin 12–mediated regression, we hypothesize that, although T cells are required for this immune regression, they are unlikely to be the effector cells. We have found that, in other models, this regression has been shown to be mediated by either CD4+ or CD8+ subsets of T cells in mice with either antibody depletion or specific gene knockout conditions. 29 We also have some evidence that macrophages are the effector cells in this T-cell–dependent rejection of tumors. 26 Therefore, while T cells are important in establishment of an immune response, classic cytotoxic T-lymphocyte activity is not likely to be the effector mechanism of this tumor regression mediated by treatment with interleukin 12 followed by cyclophosphamide plus interleukin 12.

In conclusion, we have shown that SCC VII is a nonimmunogenic tumor as demonstrated by lack of concomitant immunity, lack of response to cyclophosphamide plus interleukin 12 treatment, and results of immunohistochemistry. Despite this nonimmunogenic state, an immunogenic response can be elicited by induction treatment with interleukin 12, leading to an invigorated T-cell response. This elicited response effectively changes the immunogenicity and thus the immunoatherapeutic response of SCC VII such that cure can be achieved in 75% of mice.

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