Cooperativity of \textit{Staphylococcus aureus} Enterotoxin B Superantigen, Major Histocompatibility Complex Class II, and CD80 for Immunotherapy of Advanced Spontaneous Metastases in a Clinically Relevant Postoperative Mouse Breast Cancer Model

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INTRODUCTION

As a result of recent discoveries and advances in immunology and molecular cloning, many novel immunotherapeutic strategies for the treatment of cancer are being developed (1, 2). Most of these strategies are focused on eliminating primary tumors, which are frequently successfully treated by conventional methods, such as surgery. In contrast, few immunotherapeutic approaches are targeting disseminated metastatic disease, for which conventional therapies frequently have limited success. Development of therapies for the treatment of metastatic disease is complicated by the shortage of animal models for spontaneously metastatic cancers. We recently described a novel cell-based vaccine for the therapy of metastatic disease and tested it using the mouse 4T1 mammary carcinoma model. The 4T1 tumor shares many characteristics with its human counterpart (3), making it an excellent animal model.

In most clinical situations, primary mammary tumors are cured by surgery, yet approximately 33% of women successfully treated for primary tumors die subsequently from spontaneous metastatic disease.

MATERIALS AND METHODS

cDNA Expression Vectors. The expression vectors pH\(\beta\)-Apr-1-neo containing MHC class II (I-A\(\alpha\)) and C80 (B7.1) costimulatory molecules genes were designed to enhance activation of tumor-specific CD4\(^+\) T lymphocytes via improved presentation of tumor-encoded class II-restricted epitopes. Although CD8\(^+\) T lymphocytes have been traditionally the focus of immunotherapy approaches, accumulating results have demonstrated that CD4\(^+\) T lymphocytes also play a critical role in effective antitumor immunity (5–9). Whereas our previous vaccines showed significant reduction of established, spontaneous metastatic tumor, the antitumor response was limited to small burdens of metastatic cells and did not completely eliminate metastases (3). In addition, we did not assess the effects of immunotherapy on survival. Furthermore, the vaccine was tested in mice with metastatic disease and carrying intact primary tumor so the model did not mimic the clinical situation in which primary tumor would have been surgically removed before initiation of immunotherapy. We now report a second-generation cell-based vaccine that is significantly more effective than the original vaccine for the treatment of spontaneous 4T1 metastatic mammary cancer and that is tested in a postsurgical model. The new vaccine incorporates a gene encoding the bacterial toxin SEB (3). SEB is a sAg that when complexed with MHC class II molecules on APCs is a potent polyclonal activator of CD4\(^+\) T lymphocytes (10, 11). Although CD4\(^+\) T-cell activation by SEB is not antigen specific, we reasoned that the addition of SEB to the MHC class II/C80 vaccine will provide additional activation signals to the CD4\(^+\) T cells that have been activated in an antigen-specific fashion by the MHC class II C80\(^+\) vaccinating cells.

Received 11/15/99; accepted 3/17/00.

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1 Supported by grants from the U.S. Army Research and Development Command (DAMD17-94-J-432) and the NIH (ROI CA52527). B. P. is supported by a postdoctoral fellowship from the U.S. Army Research and Development Command (DAMD17-97-1-7152).

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3 The abbreviations used are: SEB, \textit{Staphylococcus aureus} enterotoxin B; sAg, superantigen; APC, antigen-presenting cell; TD, mean tumor diameter.
by Dr. Fred R. Miller (Michigan Cancer Foundation, Detroit, MI) and grown in culture as described previously (3). Transfectants were made to express MHC class II and CD80, or SEB by using lipofectin (Life Technologies, Inc.) according to manufacturer’s instructions. Cells were selected with 400 μg/ml G-418 (Life Technologies, Inc.) or 200 μg/ml zeocin (Invitrogen), cloned by limiting dilution, stained for surface antigen expression, and analyzed by flow cytometry, as described previously (3).

**SEB Assay.** Naive BALB/c spleen cells (5–10 x 10⁶) were cultured in serial dilutions of transfectants’ supernatants or purified SEB (Sigma Chemical Co., St. Louis, MO), as indicated. To demonstrate specific SEB activity, a polyclonal rabbit antibody against SEB (Sigma Chemical Co.) was added to cultures, as indicated. After 3 days in culture, spleen cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent, as described previously (14).

**Surgery.** All surgical supplies and surgical equipment were purchased from Henry Schein Veterinary & Medical Supply Catalogue (Melville, NY) and Roboz (Rockville, MD), respectively, unless indicated otherwise. Before surgery, animals were weighed and anesthetized with i.p. injections (0.02 ml/g body weight) of 2.5% recrystalized avertin (2,2,2tribromomethanol; Sigma Chemical Co.). Once the animals were unconscious, the tumor-bearing abdominal area was prepared and sterilized for surgery by shaving with Oster Finisher Trimmer, followed by 2 washes each with diluted Novolvan surgical scrub (chlorhexidine) and isopropanol. Tumors were resected with sterilized surgical instruments, removing the smallest amount of skin tissue as possible. Wounds were closed with either Nexaband liquid or stainless steel 9-mm wound clamps with a Mikon autoclamp applier, as necessary. Wound clamps were removed 10 days after surgery with a Mikon autoclamp remover. Mice were monitored for survival, and those that died from surgery (within 1–4 days after surgery, survival rate of 67–80%) were not included in the experiment. All mice were autopsied at the time of death to confirm the presence of lung metastases as well as recurrence of the primary tumor.

**Tumor Challenges, Metastases Assays, and in Vivo Depletions.** Mice were challenged s.c. in the abdominal mammary gland with (7 x 10⁵/50 μl) parental 4T1 tumor cells. Primary tumor growth and spontaneous metastases were measured as described previously (3). Depletions of CD4⁺ and CD8⁺ T cells were performed as described previously (15). Splenocytes of all depleted mice were checked by immunofluorescence for depletion at the conclusion of the experiment. Mice depleted for CD4⁺ or CD8⁺ T cells had <4% or 7% of CD4⁺ T cells or CD8⁺ T cells, respectively.

**Statistical Analyses.** To determine the statistical significance of the data, the Tukey’s Honest Significant Difference Test was performed at a P set at 0.05. The Tukey’s test is a multicomparison test that determines the statistical significance of data sets of size 3 or greater and allows for unequal sample size (n) and sample variances (16). To determine the statistical significance of the effects of immunotherapy on primary tumor growth, the Student’s t test for unequal variances (Microsoft Excel, version 5.0) was performed.

## RESULTS

**Tumor Lethality Is Due to Early Metastases.** Our previous studies have demonstrated that the BALB/c-derived 4T1 mammary carcinoma is a poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes throughout the body in a pattern similar to human breast cancer (3). For example, primary 4T1 tumors that have been established for 2–3 weeks in BALB/c mice typically metastasize to the lymph nodes, lungs, and livers in 86%, 79%, and 20% of mice, respectively, and the numbers of micrometastatic cells found in these organs range between 2–57, 1–338, and 0–1, respectively. In addition, as the primary tumors age (i.e., by 4–5 weeks), the incidence of metastases in the lungs, livers, and now brains increases to 91%, 82%, and 36% of mice, respectively, and the range of metastatic cells for these organs is between 6–250,000, 7–7800, and 1–116, respectively (3).

As shown in Fig. 1, 4T1 is also similar to human mammary carcinoma in that morbidity is due to outgrowth of spontaneous micrometastatic tumor cells that migrate to distant organs relatively early (week 2) during primary tumor growth. Groups of female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7 x 10⁶ wild-type 4T1 tumor cells. Primary TDs were measured, and tumors were surgically resected at varying times after inoculation (weeks 2–5). Each point represents the survival time in days after primary tumor challenge for an individual mouse.

**POSTOPERATIVE TREATMENT OF MICE WITH TRANSFECTANTS EXPRESSING MHC CLASS II, CD80, AND SEB INCREASES SURVIVAL.** Previously, we have shown that therapy with transfectants expressing MHC class II or CD80 reduced metastatic disease in a model where the primary 4T1 tumor remained in situ and had been established for 9–14 days.
The success of this treatment, however, was limited to small tumor burdens and did not completely eliminate spontaneous metastases (3). A potential problem with this earlier therapy is that the transfectants did not coexpress MHC class II and CD80, and previous data using a mouse sarcoma showed that coexpression of these molecules is synergistic (15). We have, therefore, generated 4T1 transfectants that coexpress MHC class II and CD80 as detected by indirect immunofluorescence staining (data not shown) to test this hypothesis. To further increase the potency of the vaccine, we have combined the MHC class II/CD80+ double transfectants (4T1/A<sub>d</sub>/B7.1) with SEB<sup>+</sup> transfectants (4T1/SEB), reasoning that SEB may provide additional proliferation signals to the tumor-specific T cells activated via the MHC class II/CD80 interaction.

SEB expression was tested by coculturing supernatants of transfectants with naive BALB/c spleen cells and monitoring lymphocyte proliferation. To determine a relative amount of SEB secretion, splenocytes were also cultured with soluble SEB. As shown in Fig. 2, supernatants from two independent clones (4T1/SEB-12 and 4T1/SEB-14) stimulated splenocyte proliferation as efficiently as soluble supernatants from two independent clones (4T1/SEB-12 and 4T1/SEB-14) transfectants in the presence (open symbols) or absence (filled symbols) of antibody specific for SEB.

Fig. 2. Supernatants from 4T1/SEB transfectants stimulate proliferation of spleen cells. Naive BALB/c splenocytes were cocultured with soluble SEB starting at a concentration of 2 μg/ml (solid symbols) or supernatants from parental 4T1 (open symbols), 4T1/SEB-12 (●), or 4T1/SEB-14 (○) transfectants in the presence (open symbols) or absence (filled symbols) of antibody specific for SEB.

Vaccines such as the 4T1 transfectants are likely to be most useful for the treatment of disseminated spontaneous metastatic disease because primary tumors usually can be eliminated by surgery. Therefore, we have tested the combination vaccine in mice with established, disseminated spontaneous metastases following surgical removal of the primary tumor. Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7 × 10<sup>3</sup> wild-type 4T1 tumor cells. The tumors were allowed to grow and metastasize for 3 weeks, at which time the primary tumor burden was measured and surgically resected. The experiments in Fig. 1 and our previously published results (3) established that at this stage and size of primary tumor the mice have very extensive disseminated metastases. At the time of surgery (3 weeks after primary tumor challenge), the primary TD in each treatment group ranged between 3.5 mm and 5.7 mm. The extent of spontaneous metastatic cancer at this time is significantly more advanced than that tested in other immunotherapy experiments. Therapeutic injections of irradiated vaccine cells (1 × 10<sup>6</sup> total) were started 1 week after surgery (i.e., 4 weeks after initial tumor challenge) and were administered every 3–5 days for the duration of the experiment. Because we are interested in determining whether primary tumor size affects vaccine efficacy, the results in Fig. 3 are plotted as the survival time versus the size of the primary tumor at the time of surgery. A line denoting the average survival time of the 4T1-treated control group (45 days) is included to demonstrate the effects of the vaccine on survival. The survival time in days for 4T1-treated (Fig. 3A), 4T1/SEB-treated (Fig. 3B), 4T1/A<sub>d</sub>/B7.1-treated (Fig. 3C), and 4T1/SEB+4T1/A<sub>d</sub>/B7.1-treated (Fig. 3D) animals was 35—52, 40—59, 47—54, and 41—74 days, respectively. Statistical analyses using the Tukey’s Honestly Significant Difference Test revealed that only the treatment with a 1:1 mixture of 4T1/A<sub>d</sub>/B7.1+4T1/SEB cells significantly increases the survival time of mice with established wild-type metastatic disease (P = 0.05). Treatment with either 4T1/SEB alone or 4T1/A<sub>d</sub>/B7.1 alone does not significantly increase survival. Therefore, therapy with this cell-based vaccine requires expression of all three molecules to extend mean survival time from 5–7.5 weeks for control-treated mice to 6–10.5 weeks for therapy-treated mice. Although this increase in survival time is relatively small, it is statistically significant and compelling because the immunotherapy was started at week 4 and untreated and/or 4T1-treated mice begin to die as early as 5 weeks after tumor challenge.

**Increase in Survival Correlates with Reduction of Metastatic Cancer.** To demonstrate that the increase in survival was due to a reduction of spontaneous metastatic cancer, lungs from therapy-treated animals were harvested and the number of clonogenic metastases was quantitated as described previously (3). Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 10<sup>6</sup> total cells of irradiated parental 4T1 (13 mice; 3), 4T1/A<sub>d</sub> (12 mice; 3), or 4T1/A<sub>d</sub>/B7.1 (7 mice; 3). To determine whether primary tumor size affects vaccine efficacy, statistical analyses using the Tukey’s Honestly Significant Difference Test was performed.

**Fig. 3. Immunotherapy of established wild-type spontaneous metastases with a mixture of MHC class II/CD80 and SEB transfectants increases survival.** Female BALB/c mice were challenged s.c. in the abdominal mammary gland with 7 × 10<sup>3</sup> live wild-type 4T1 cells. Primary tumors were measured and surgically resected 21 days after parental tumor challenge. Mice were treated every 3–5 days starting at day 28 with i.p. injections of 1 × 10<sup>6</sup> total cells of irradiated parental 4T1 (13 mice; A), 4T1/SEB (8 mice; B), 4T1/A<sub>d</sub>/B7.1 (7 mice; C), or a 1:1 mixture of 4T1/A<sub>d</sub>/B7.1 plus 4T1/SEB cells (14 mice; D). The 1:1 mixture of 4T1/A<sub>d</sub>/B7.1 plus 4T1/SEB therapy group is significantly different from the 4T1 control group (P = 0.05, Tukey’s Honestly Significant Difference Test).

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7 \times 10^3 \text{ wild-type 4T1 tumor cells. Starting 2 weeks after challenge, they were given i.p. injections of irradiated vaccine cells (1 \times 10^6 total/injection) twice a week until the day of sacrifice. At the time of sacrifice (6 weeks after the initial primary tumor challenge), primary TD of control-treated mice (i.e., mice given irradiated 4T1 cells) were comparable with TD of transfectant-treated animals (6.5–10.5 mm and 6.2–11.2 mm, respectively; two-tailed \( P = 0.61 \)). Therapy with the transfectants, therefore, does not reduce primary tumor growth, which concurs with our earlier studies using MHC class II or CD80 vaccines alone (3).

To assess the metastatic disease, lungs from the treated mice were removed, dissociated into single cell suspensions, and plated in culture medium containing 6-thioguanine to determine the number of clonogenic metastatic cells. Subsequently, these populations were essential for the therapeutic effect.

**Discussion**

SAgS, including SEB, have been previously recognized as potential reagents for up-regulating T lymphocyte responses against tumors. However, their use has been limited and they have not been combined with other factors that might optimize their therapeutic efficacy. For example, several studies describe redirected T-cell activation using SAgS coupled to tumor-specific monoclonal, anti-idiotypic, or bifunctional antibodies (17–19). SEB has also been administered systemically along with tumor cells, and SEB DNA has been inoculated intratumorally along with cytokine DNA to reduce primary tumor growth (20, 21). In addition, SAgS have been used to activate tumor-draining lymph node T cells \( \text{ex vivo} \) for adoptive transfer into tumor-bearing animals (22, 23). All of these approaches produce some reduction in primary tumor growth and/or decrease in metastatic lesions. However, the test settings have involved relatively small primary tumor and/or very small metastatic tumor burdens, which do not mimic the clinical situation. These results, taken together with the SEB transfected tumor vaccines presented in this study, show that SEB expression alone has only a modest effect on metastatic tumor progression. However, as shown in this study, the antitumor effect of SEB on highly advanced spontaneous metastases is more effective when combined with the cell-based vaccine containing MHC class II and CD80 molecules.

The modified tumor cells may function directly as APCs for the initial activation of tumor-specific CD8\(^+\) and CD4\(^+\) T cells following immunization. Previous studies demonstrate that both CD8\(^+\) and
CD4+ T lymphocytes are involved in immunity induced by MHC class II/CD80 vaccines (15) and that MHC class II/CD80 modified tumor cells function directly as APCs for the initial activation of tumor-specific CD4+ T cells (24). Direct presentation of antigen by tumor cells is possible because tumor cell expression of MHC class II molecules in the absence of invariant chain allows for presentation of endogenously synthesized tumor antigens by MHC class II molecules (24, 25). Because the vaccines express MHC class I, class II, CD80, and SEB molecules, antigen-specific and costimulatory signals will be efficiently delivered to CD8+ and CD4+ T cells. Likewise, because the activated CD8+ and CD4+ T cells are in close proximity to each other, there should be an efficient transfer of cytokines between CTLs and T helper cells (see Fig. 6, right).

Host-derived APCs are also likely to be involved in CD8+ and CD4+ T lymphocyte activation during vaccine therapy. Because MHC class II serves as a ligand for a sAg (10), it is likely that any host-derived class II+ cell will bind available SEB. The involvement of host APC is supported by the observation that SEB transfecteds alone, which do not express MHC class II, cause a modest therapeutic effect (Fig. 4, A versus B). Furthermore, other mouse tumor models have demonstrated that both a class I- and class II-restricted tumor-encoded antigen can be processed and presented indirectly by host-derived APCs (24, 26, 27). Taken together, it is likely that host-derived APCs, capable of migrating to lymph nodes, coordinately present SEB and tumor antigen to both CD8+ and CD4+ T cells (see Fig. 6, left).

SEB may also enhance vaccine efficacy because it induces an inflammatory response that stimulates immunity (28). Gene transfer techniques have demonstrated that in vivo expression of various sAg (SEA, SEB, and TSST-1) DNAs induces intense inflammatory responses (29). Although systemic administration of sAg (doses >500 μg) typically triggers T-cell release of cytokines such as tumor necrosis factor and lymphotixin that lead to cachexia (11), we did not see any adverse side effects in SEB-treated mice.

When a sAg, such as SEB, is coexpressed by the MHC class II/CD80 vaccine, additional activation and/or proliferation signals may be delivered to the specifically activated CD8+ and CD4+ T cells. Because SEB binds to the sides of MHC class II molecules and the T cell receptor while antigenic peptide binds within the MHC class II clef (30, 31), it is feasible that the sAg, tumor antigen-specific, and costimulatory signals are simultaneously received by the T cells. Whereas it is also possible that these signals are not coincident, several studies have shown that activation of T cells by SEB is facilitated or enhanced by B7/CD28 signaling (32–35). Controversy exists over the ability of costimulation to inhibit sAg-induced apoptosis, but one report demonstrates that lipopolysaccharide activation of B cells prevents sAg-induced deletion (36). Regardless of the precise kinetics in which the various activation signals are delivered, coordinate delivery of the three signals improves the efficacy of the vaccines to reduce spontaneous metastatic tumor growth. As a result, T-cell activation may be exceptionally efficient because both direct and indirect antigen presentation occur, thus yielding larger numbers of precisely those CD8+ and CD4+ tumor-specific cells that mediate tumor cell destruction.

New immunotherapies are routinely tested in experimental animal tumor systems. Although such experiments may provide “promising” therapeutic results, tumor regression in animal models does not necessarily predict successful treatment of tumors in human patients. There may be significant physiological and biochemical differences between animals and humans that preclude direct comparison of
Acknowledgments

We thank Drs. T. Iamonte-Armstrong and B. Bradley for assistance with statistical analysis and S. Mason for animal care.

References


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*Cancer Res* 2000;60:2710-2715.

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