Induction of Antigen-Specific Tumor Immunity by Genetic and Cellular Vaccines against MAGE: Enhanced Tumor Protection by Coexpression of Granulocyte-Macrophage Colony–Stimulating Factor and B7-1

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ABSTRACT

Background: A number of tumors express antigens that are recognized by specific cytotoxic T cells. The normal host immune responses, however, are not usually sufficient to cause tumor rejection. Using appropriate immunization strategies, tumor-specific antigens may serve as targets against which tumor-destructive immune responses can be generated. MAGE-1 and MAGE-3 are two clinically relevant antigens expressed in many human melanomas and other tumors, but not in normal tissues, except testis. Here, we have investigated whether DNA and cellular vaccines against MAGE-1 and MAGE-3 can induce antigen-specific anti-tumor immunity and cause rejection of MAGE-expressing tumors.

Materials and Methods: Mice were immunized against MAGE-1 and MAGE-3 by subcutaneous injection of genetically modified embryonic fibroblasts or intramuscular injection of purified DNA. Mice were injected with lethal doses of B16 melanoma cells expressing the corresponding MAGE antigens or the unrelated protein SIV tat, and tumor development and survival were monitored.

Results: Intramuscular expression of MAGE-1 and MAGE-3 by plasmid DNA injection and subcutaneous immunization with syngeneic mouse embryonic fibroblasts transduced with recombinant retroviruses to express these antigens induced specific immunity against tumors expressing MAGE-1 and MAGE-3. Both $CD4^+$ and $CD8^+$ T cells were required for anti-tumor immunity. Coexpression of granulocyte-macrophage colony–stimulating factor (GM-CSF) or B7-1 significantly increased anti-tumor immunity in an antigen-specific manner and resulted in a considerable proportion of mice surviving lethal tumor challenge.

Conclusions: Our results suggest that genetic and cellular vaccines against MAGE and other tumor antigens may be useful for the therapy of tumors expressing specific markers, and that GM-CSF and B7-1 are potent stimulators for the induction of antigen-specific tumor immunity.

INTRODUCTION

Recently, several genes encoding tumor antigens recognized by autologous $CD8^+$ T cells have been identified (1–10). Since these tumor antigens are recognized by cytotoxic T cells in vitro, they may serve as targets for tumor immunotherapy, provided that appropriate immunization enhances their recognition and generates effective cellular immune responses against tumor cells expressing these antigens in vivo. MAGE-1 and MAGE-3, two members of the MAGE gene family (11,12), are expressed by many human melanomas and other tumors (10,13,14), but not by normal tis-

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sues except for testis (7,15). MAGE-1 and MAGE-3 are found in the cytoplasm (16–18). Peptides derived from these proteins are presented on tumors in association with several different major histocompatibility (MHC) class I haplotypes and elicit specific cytotoxic T lymphocytes in patients (10,19–21). The high tumor specificity of these antigens and their expression in many different types of cancers make them attractive targets for tumor immunotherapy. It has previously been shown that melanoma patients immunized with a melanoma cell vaccine produce antibodies against MAGE-1 (22). In addition, MAGE-1–specific cytotoxic T lymphocytes have been generated after immunization with MAGE-1 peptide-pulsed antigen-presenting cells in melanoma patients (23) and in vitro (24). Inhibition of the growth of MAGE-1–expressing tumors in vivo by immunization has not, however, been documented.

By engineering nonimmunogenic mouse B16 melanoma cells to express MAGE proteins, we generated a mouse tumor system that allowed us to study whether immunization against MAGE antigens can inhibit or delay the growth of tumor cells expressing these antigens. Here we show that intramuscular expression of MAGE-1 and MAGE-3, and subcutaneous vaccination with genetically modified fibroblasts can induce specific anti-tumor immunity and rejection of tumor cells expressing these antigens. Stimulation of anti-tumor immunity was dependent on both $CD4^+$ and $CD8^+$ T cells. In addition, it could be significantly enhanced by coexpression of granulocyte-macrophage colony–stimulating factor (GM-CSF) or B7-1, resulting in a considerable proportion of long-term protected mice after lethal tumor challenge.

MATERIALS AND METHODS

Preparation and Analysis of Recombinant Retroviral Stocks

To generate recombinant viruses encoding MAGE-1, MAGE-3, murine GM-CSF, and B7-1, the respective coding sequences were introduced into the retroviral vector SFG. This vector is derived from MFG (25,26) by the introduction of two point mutations which create an *Nhe* I site and cause the premature termination of the gag ORF sequences present in MFG. The sequence around the gag ATG in SFG reads: ATGGGCCC GG*GCTAGC*CT. Disruption of the gag ORF reduces the potential for recombination events involving packaging cell sequences that may lead to helper virus formation under certain conditions (27). Forty micrograms of linearized plasmid were coelectroporated with 5 μg of pSV2neo into ψ-CRIP packaging cells (28). G418-resistant clones were picked and expanded, and the relative efficiencies of virus transmission by individual producer clones was assessed by infecting 3T3 cells with supernatant from producer clones and analyzing genomic DNA of infected cells for provirus integration using gene-specific probes.

Preparation and Characterization of Transduced Embryonic Fibroblasts and Tumor Cell Lines

C57BL/6J embryonic fibroblasts expressing MAGE-1, MAGE-3, GM-CSF, and B7-1 were generated by infection with the corresponding recombinant retroviral stocks on three subsequent days for 4 hr in the presence of 8 μg/ml polybrene. Control embryonic fibroblasts were generated by infection with "empty" SFG virus. B16 tumor cells were infected twice with recombinant retroviruses encoding MAGE-1 and MAGE-3, respectively, and subcloned by limiting dilution. B16 cells expressing SIV tat were kindly provided by Connie Gee. Expression of the genes in infected cells was demonstrated by Northern blot analysis (MAGE-3), Western blot analysis (MAGE-1), enzyme-linked immunosorbent assay (ELISA) (GM-CSF), and flow cytometry (B7-1), respectively. Fibroblasts were tested for helper virus activity using the β-galactosidase mobilization assay and were found to be free of helper virus.

Immunizations

Infected embryonic fibroblasts were trypsinized, washed twice in 1× Hanks' balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY, U.S.A.) to eliminate traces of serum proteins and resuspended at $2–6 \times 10^6$ cells/ml in HBSS. For immunization, C57BL/6J female mice were injected subcutaneously with 0.5 ml of cell suspension. For intramuscular immunizations, the coding sequences of MAGE-1, MAGE-3, GM-CSF, and B7-1 were cloned into the expression vector pBK-CMV (Stratagene, La Jolla, CA, U.S.A.). Plasmid DNA was prepared and purified from endotoxins using kits from Qiagen Inc. (Chatsworth, CA). Mice were injected into both tibialis anterior muscles with 0.9% saline (control) or plasmid encoding the various proteins in 0.9% saline.

Tumor Injections

Mice were injected subcutaneously with B16 tumor cells expressing MAGE-1 or MAGE-3 in HBSS. Development of tumors was monitored every 2–3 days, and the mice were sacrificed when the sum of the two perpendicular tumor diameters reached 30 mm (a tumor volume of about 1.7 cm^3).

Characterization of T Cell Responses

Mice lacking CD4 (29) or CD8 (30) were immunized with genetically modified embryonic fibroblasts or DNA as described above and injected 2 weeks later with B16 cells expressing MAGE-1. Tumor development was monitored as described above.

RESULTS

Immunization with Embryonic Fibroblasts Expressing MAGE-1 and MAGE-3 Partially Protects Mice against Challenge with B16 Tumor Cells Expressing the Corresponding Antigens

Mice were injected subcutaneously with 3×10^6 embryonic fibroblasts engineered to express either MAGE-1 (EF-MAGE-1) or MAGE-3 (EF-MAGE-3) by using recombinant retroviruses (see Materials and Methods for details regarding the generation of antigen- and cytokine-expressing cell lines). Control mice were injected with fibroblasts transduced with "empty" retroviral vector (EF-SFG). Two weeks later the animals were challenged with 1.25×10^6 B16 melanoma cells expressing the corresponding antigens (10 times the minimal dose that caused 100% lethality in normal control mice) or with the same number of B16 cells expressing the immunologically unrelated protein, SIV tat. Tumor growth was monitored, and animals were killed when tumors reached a volume of about 1.7 cm^3 (see Materials and Methods). All mice injected with mock-infected fibroblasts (EF-SFG) developed tumors and had to be sacrificed 23.9 ± 1.5 days (mean ± SEM) after challenge with B16 melanoma cells expressing MAGE-1 (B16-MAGE-1) (Fig. 1A). In contrast, 30% of the mice immunized with fibroblasts expressing MAGE-1 (EF-MAGE-1) remained tumor free and survived for at least 92 days after tumor challenge (termination of experiment) (Fig. 1A). In three more experiments, 40–50% of EF-MAGE-1 injected mice remained tumor free (data not shown).

Mice immunized with MAGE-3 transduced embryonic fibroblasts (EF-MAGE-3) survived about twice as long (50.9 ± 4.3 days) as control mice injected with EF-SFG (24.0 ± 1.3 days) after a challenge with 1.25×10^6 B16-MAGE-3 tumor cells (Fig. 1B). Although all EF-MAGE-3 immunized mice eventually developed tumors, onset and progression of tumor growth were significantly delayed. Comparable results were obtained in three other experiments (data not shown). Vaccination with EF-MAGE-1 or EF-MAGE-3 did not induce any protection against tumor cells expressing the control antigen, SIV tat (B16 tat) (Fig. 1C), and immunization with EF-MAGE-3 did not induce protection against B16-MAGE-1 (Fig. 1A), or vice versa (Fig. 1B). This demonstrates that the subcutaneous cellular vaccines induced antigen-specific immune responses that were able to delay and/or prevent the growth of tumors expressing the corresponding antigens.

Intramuscular Expression of Tumor Antigens MAGE-1 and MAGE-3 Induces Protective Tumor Immunity

Genetic immunization by intramuscular injection of purified DNA has been shown to induce both cellular and humoral immune responses (31,32). DNA immunization against human carcinoembryonic antigen (CEA) (33) or CD4 (34) induced protective immunity against challenge with tumor cells expressing the corresponding antigens. While both CEA and CD4 were expressed at the surface of tumor cells, many melanoma antigens including MAGE-1, MAGE-3, and tyrosinase (4,16–18) are intracellular proteins and may therefore be less immunogenic because they do not stimulate humoral immunity and are not or less amenable to uptake and presentation by host antigen-presenting cells. To address this question, intramuscular vaccination against the cytoplasmic MAGE proteins was tested for its ability to induce systemic anti-tumor immunity and result in rejection of MAGE-expressing tumors. The cDNAs for MAGE-1 and MAGE-3 were subcloned into the vector pBK-CMV in which transcription is controlled by the human cytomegalovirus (CMV) enhancer/promoter region. Plasmid DNAs encoding MAGE-1 (pCMV-MAGE-1) and MAGE-3 (pCMV-MAGE-3) were injected into muscles of mice, and the animals were challenged 2 weeks later with lethal doses of B16 melanoma cells. Two injections of 170 μg pCMV-MAGE-1 or pCMV-

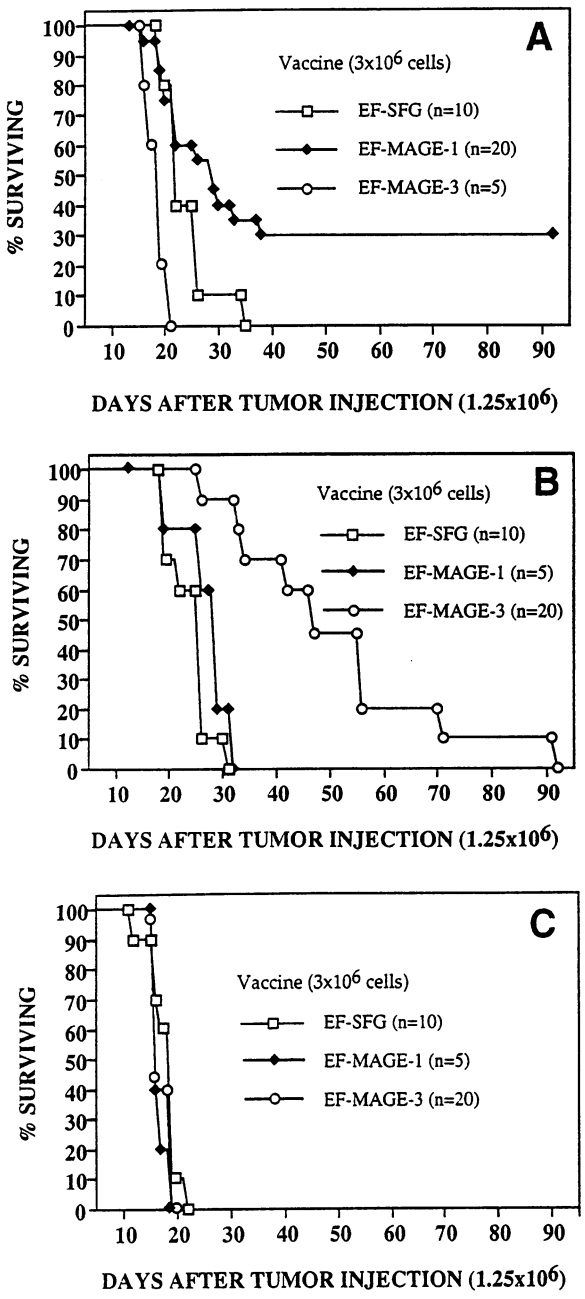

FIG. 1. Immunization against B16 tumor cells expressing MAGE-1 and MAGE-3 by subcutaneous injection of genetically modified embryonic fibroblasts

C57BL/6J mice (n = 5–20) were injected subcutaneously with 3×10^6 embryonic fibroblasts infected with control retroviral vector (EF-SFG, □) or retroviral vector encoding MAGE-1 (EF-MAGE-1, ◆) or MAGE-3 (EF-MAGE-3, ○). Two weeks later, animals were injected subcutaneously on the back with 1.25×10^6 B16 melanoma cells expressing (A) MAGE-1, (B) MAGE-3, or (C) SIV tat. Tumor development was monitored every 3 days and animals were sacrificed when the sum of the two tumor diameters reached 30 mm (a tumor volume of about 1.7 cm^3) or when tumors became ulcerated.

MAGE-3 expression plasmids significantly delayed tumor development and resulted in 80 and 20% of the mice surviving a challenge with 1.5×10^6 B16 tumor cells expressing the corresponding antigens (Fig. 2A). As with the cellular vaccines, immunization against MAGE-1 resulted in higher protection than immunization against MAGE-3, but the reason for this is unknown. Even a single intramuscular injection of 100 μg pCMV-MAGE-1 resulted in 37.5% survival after challenge with 2×10^6 B16-MAGE-1 tumor cells and was at least as efficient at inducing tumor protection as vaccination with MAGE-1–transduced embryonic fibroblasts (Fig. 2B). The fact that in this experiment only one out of eight (12.5%) mice immunized with EF-MAGE-1 cells survived may be due to the higher dose of tumor cells used for challenge (2×10^6) compared with the previous experiment (Fig. 1A; 1.25×10^6 B16-MAGE-1 cells). To our knowledge, these results are the first demonstration that genetic immunization can induce rejection of tumors expressing cytoplasmic antigens, although this approach has been used to immunize against intracellular antigens from viruses (32,35).

Granulocyte-Macrophage Colony–Stimulating Factor and B7-1 Significantly Enhance Tumor Protection

Vaccinating mice with irradiated tumor cells that secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to induce potent and long-lasting anti-tumor immunity against parental tumor cells (25,36). Because GM-CSF promotes the differentiation and maturation of dendritic cells (37), which are highly efficient antigen-presenting cells (38,39), it has been suggested that the induction of anti-tumor immunity by GM-CSF is due to enhanced tumor antigen presentation (25). This hypothesis, however, has not been directly verified experimentally. We therefore investigated whether coexpression of GM-CSF can enhance vaccination against tumor antigens by the routes described above. In addition, the effects of the T cell costimulatory molecule B7-1 (40) were studied. Normal embryonic fibroblasts and fibroblasts expressing MAGE-1 and MAGE-3 were infected with recombinant retroviruses encoding murine GM-CSF or B7-1. Comparable expression of antigens, GM-CSF, and B7-1 in singly and doubly infected fibroblasts was confirmed. Vaccination

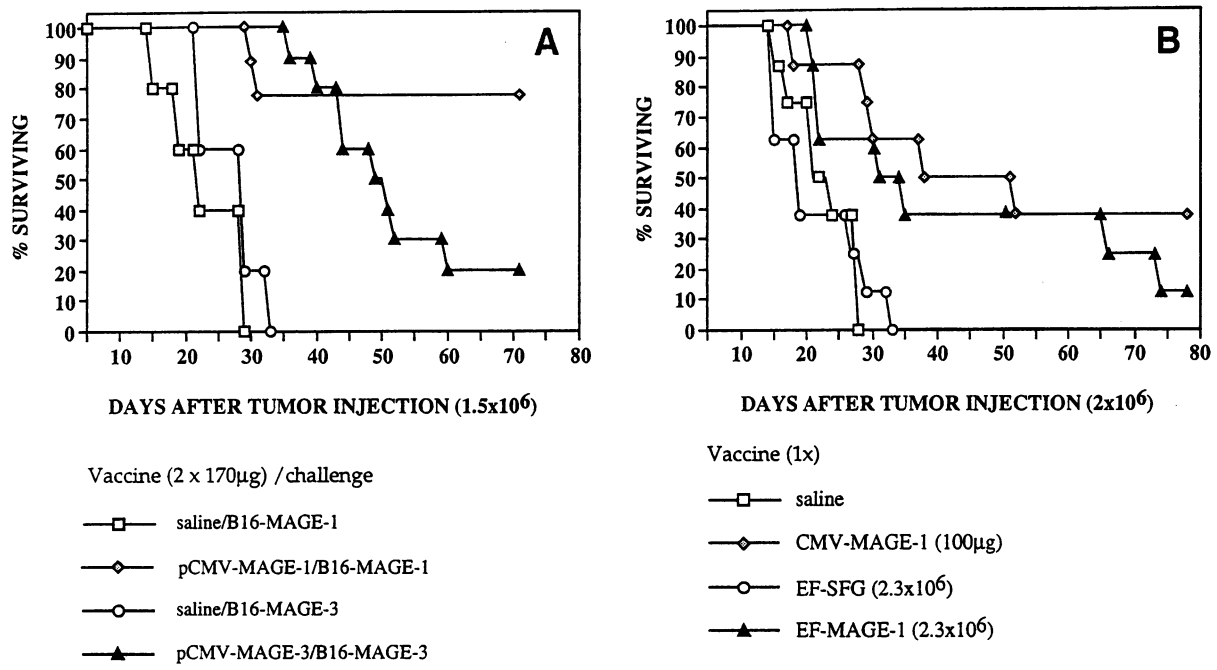

FIG. 2. Immunization against B16 tumor cells expressing MAGE-1 and MAGE-3 by intramuscular injection of plasmid DNA

Mice were immunized by injections of plasmids into both tibialis anterior muscles or subcutaneously with embryonic fibroblasts and challenged subcutaneously with tumor cells 2 weeks after the last immunization. (A) Mice (n = 5/group) were injected twice separated by 2 weeks with 0.9% saline (□; ○), 170 μg (85 μg/muscle) of pCMV-MAGE-1 (◆), or 170 μg of pCMV-MAGE-3 (▲) and challenged with 1.5 × 10^6 B16 tumor cells expressing MAGE-1 (□; ◆) or MAGE-3 (○; ▲). (B) Mice (n = 8/group) were injected once with 0.9% saline (□), 100 μg (50 μg/muscle) of pCMV-MAGE-1 (◆), 2.3 × 10^6 control embryonic fibroblasts (○), or 2.3 × 10^6 embryonic fibroblasts expressing MAGE-1 (▲). Animals were challenged with 2 × 10^6 B16-MAGE-1.

of mice with fibroblasts coexpressing MAGE-1 and GM-CSF (EF-MAGE-1-GM-CSF) or MAGE-1 and B7-1 (EF-MAGE-1-B7-1) resulted in 50 and 40% survival after challenge with 2 × 10^6 B16-MAGE-1 tumor cells, while only 10% of the animals immunized with fibroblasts expressing MAGE-1 alone (EF-MAGE-1) survived (Fig. 3A). The survival rates after challenge with 2.5 × 10^6 B16-MAGE-3 melanoma cells of mice immunized with EF-MAGE-3-GM-CSF, EF-MAGE-3-B7-1 and EF-MAGE-3 were 60, 40, and 20, respectively (Fig. 3B). Reducing the tumor challenge to 10^6 B16-MAGE-3 cells, a dose that was still highly lethal for control-immunized mice, resulted in 100% survival in the former two groups of animals, while 40% of mice vaccinated with EF-MAGE-3 alone survived under these conditions (Fig. 3C). The same vaccines did not protect mice against challenge with 10^6 B16-tat cells (Fig. 3D). Furthermore, fibroblasts expressing GM-CSF or B7-1 alone failed to induce protective immunity against B16-MAGE-3 (Figs. 3 B and C). These results demonstrate that GM-CSF increases tumor protection by enhancing immune responses against the tumor antigens MAGE-1 and MAGE-3. B7-1 most likely enhanced tumor protection by costimulation of tumor-antigen–specific T cells (40).

In order to test whether GM-CSF and B7-1 also enhance anti-tumor immunity after intramuscular immunization, mice were injected twice with different amounts of DNA encoding tumor antigen MAGE-1, in the absence or presence of 100 μg of pCMV-GM-CSF or 100 μg of pCMV-B7-1. Control mice received two injections of saline. Mice were challenged 2 weeks after the second immunization with 2.5 × 10^6 B16 cells expressing MAGE-1 or with B16 cells expressing SIV tat. Fifty percent of the mice injected twice with 100 μg pCMV-MAGE-1 survived this challenge for at least 75 days (termination of experiment), and neither GM-CSF nor B7-1 increased tumor protection after immunization with 100 μg of pCMV-MAGE-1. However, when the amount of MAGE-1 antigen was reduced, the immune-stimulatory effects of GM-

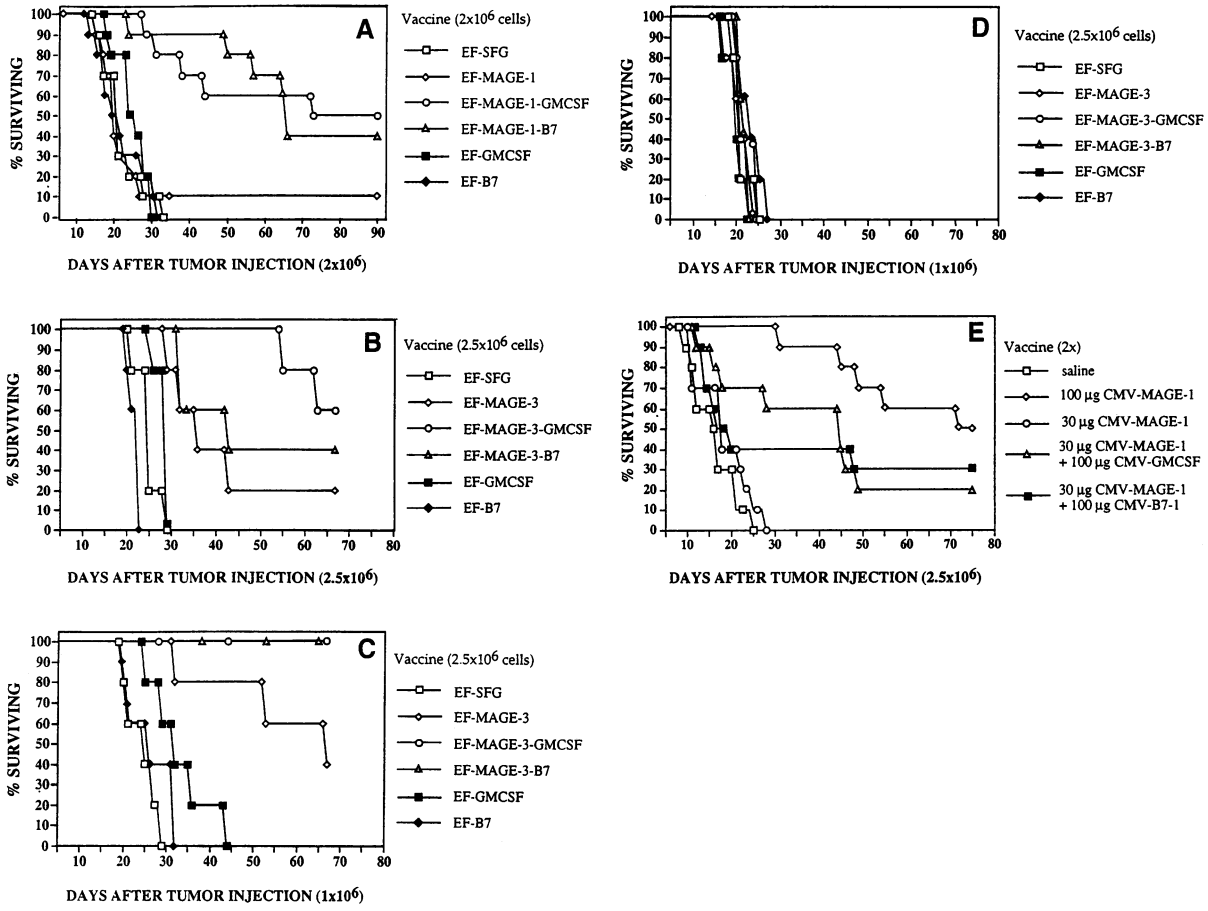

FIG. 3. Effects of granulocyte-macrophage colony–stimulating factor (GM-CSF) and B7-1 on immunization with genetically modified embryonic fibroblasts and naked DNA

(A) Mice (n = 10/group) were injected subcutaneously with 2 × 10^6 fibroblasts expressing MAGE-1 (◇), MAGE-1 and GM-CSF (○), MAGE-1 and B7-1 (△), GM-CSF (■), B7-1 (◆), or mock-infected fibroblasts (□), and challenged 2 weeks later with 2 × 10^6 B16-MAGE-1 melanoma cells. (B through D) Mice (n = 5/group) were injected subcutaneously with 2.5 × 10^6 fibroblasts expressing MAGE-3 (◇), MAGE-3 and GM-CSF (○), MAGE-3 and B7-1 (△), GM-CSF (■), B7-1 (◆), or mock-infected fibroblasts (□). Animals were challenged subcutaneously 2 weeks later with 2.5 × 10^6 B16-MAGE-3 (B), 1 × 10^6 B16-MAGE-3 (C), or 1 × 10^6 B16 tat (D) melanoma cells. (E) Mice (n = 10/group) were injected twice separated by 2 weeks with 0.9% saline (□), 100 µg of pCMV-MAGE-1 (◇), 30 µg of pCMV-MAGE-1 (○), 30 µg of pCMV-MAGE-1 and 100 µg of pCMV-GMCSF (△), or 30 µg of pCMV-MAGE-1 and 100 µg of pCMV-B7-1 (■). Animals were challenged 2 weeks after the second immunization with 2.5 × 10^6 B16-MAGE-1 cells.

CSF and B7-1 became apparent. As shown in Fig. 3E, none of the animals immunized twice with 30 µg of pCMV-MAGE-1 survived; all animals of this group developed tumors within about 10 days similarly to saline-injected control mice. In contrast, two injections of 30 µg of pCMV-MAGE-1 together with 100 µg of pCMV-GM-CSF or 100 µg of pCMV-B7-1 caused a significant delay in tumor development and resulted in 20 and 30% long-term surviving animals, respectively (Fig. 3E). None of the mice survived a challenge with B16 cells expressing SIV tat protein, and immunization with pCMV-GM-CSF alone or pCMV-B7-1 alone did not induce any tumor protection against B16-MAGE-1. Comparable effects of GM-CSF and B7-1 were observed when mice were vaccinated against B16-MAGE-3 with pCMV-MAGE-3 (data not shown). Taken together, these experiments show that intramuscular immunization by DNA injection induces antigen-specific tumor protection in a dose-dependent manner and that coinjection of plasmids encoding GM-CSF or B7-1 converts undetectable immune responses after

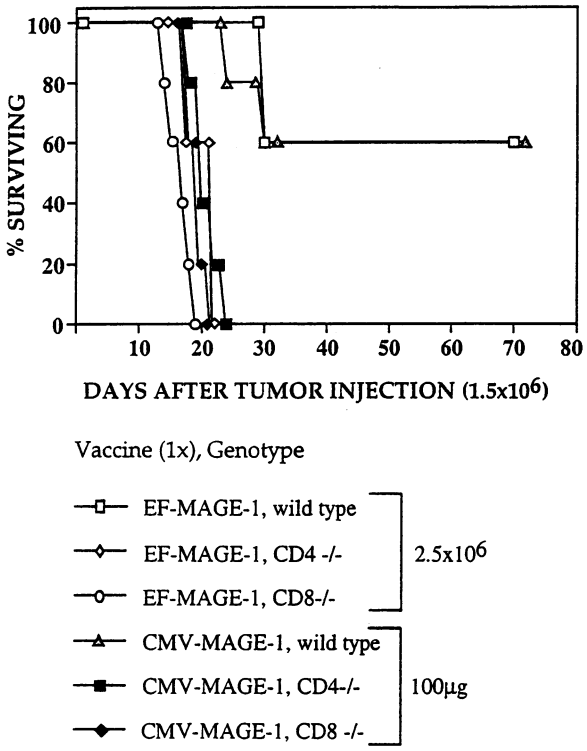

FIG. 4. Requirement of $CD4^+$ and $CD8^+$ T cells for tumor rejection

Mice were immunized once subcutaneously with 2.5×10^6 embryonic fibroblasts expressing MAGE-1 (EF-MAGE-1) or intramuscularly with 100 μg of plasmid pCMV-MAGE-1 and injected 2 weeks later with 1.5×10^6 B16-MAGE-1 cells. Wild type: normal C57BL/6 mice; $CD4^{-/-}$; CD4-deficient C57BL/6 mice; $CD8^{-/-}$: CD8-deficient C57BL/6 mice.

injection of a low amount of antigen DNA to appreciable tumor protection.

T Cell Responses Mediating Tumor Rejection

To determine whether helper T and cytotoxic T cells are required for tumor protection, normal C57BL/6 mice as well as $CD4^{-/-}$ (29) and $CD8^{-/-}$ (30) mice that had been backcrossed to the same genetic background were immunized once with either embryonic fibroblasts expressing MAGE-1 or intramuscularly with pCMV-MAGE-1, in the presence or absence of GM-CSF or B7-1, and injected 2 weeks later with 1.5×10^6 B16-MAGE-1 melanoma cells. Neither $CD4^{-/-}$ nor $CD8^{-/-}$ mice were protected against tumor challenge (Fig. 4), indicating that both $CD4^+$ and $CD8^+$ T cells are required for tumor protection.

DISCUSSION

The isolation and characterization of tumor-specific and tumor-associated antigens that elicit specific cytotoxic T lymphocytes in patients has caused widespread interest in exploiting these molecules as potential targets for tumor immunotherapy (41). MAGE-1 and MAGE-3 are two clinically important members of the MAGE gene family, which are expressed in many melanomas and other human tumors, but not in normal tissues, except for some cells of the testis. Their high tumor specificity and expression in a variety of tumors makes them attractive targets for cancer immunotherapy. By transducing B16 melanoma cells with retroviruses encoding MAGE-1 and MAGE-3, we generated a mouse tumor system that allowed us to study whether immunization of syngeneic C57BL/6 mice against MAGE antigens could elicit protective responses against tumors expressing these antigens. We chose the B16 tumor model because B16 cells are nonimmunogenic (25) and introduction of MAGE antigens into B16 melanoma cells did not alter their growth rate or tumorigenicity in any way as compared to parental B16 cells. Thus, although MAGE antigens are foreign to the mouse, B16-MAGE melanoma cells were never rejected spontaneously and grew indistinguishably from wild-type B16 cells (data not shown). Here we investigated whether genetic immunization against MAGE-1 and MAGE-3 can induce specific anti-tumor immunity and cause rejection of B16 melanoma cells expressing these antigens, and compared this method to an ex vivo approach using genetically modified syngeneic fibroblasts as a vaccine. We show that both intramuscular expression of MAGE-1 and MAGE-3 by direct injection of plasmid DNA and subcutaneous injection of syngeneic embryonic fibroblasts expressing these antigens induces antigen-specific immune responses that delay and/or prevent the development of tumors expressing the corresponding antigens in a significant fraction of mice. In contrast, no protection was obtained against the same parental B16 cells expressing a different antigen, SIV tat. Both $CD4^+$ and $CD8^+$ T cells were required for tumor protection since immunized "knock-out" mice lacking these molecules developed tumors within a similar period as mock-immunized wild-type mice. Intramuscular immunization was at least as efficient as antigen-expressing fibroblasts at inducing protective immunity. To our knowledge, our results are the first demonstration that

genetic immunization can induce rejection of tumors expressing cytoplasmic antigens.

In our hands, DNA vaccines did not affect the progression of day 7–established tumors (5 mm). There may be several reasons for this. Firstly, the amount of antigen expression in the muscle may be too low to induce sufficient immune responses against an established tumor burden. Secondly, because both the B16 and CT26 tumors used in these studies grew very quickly (mice in control groups had to be sacrificed between 15 and 20 days after tumor injection), there was no time for the booster immunizations which had increased tumor protection in the tumor challenge model (data not shown). Finally, it has been shown that established tumors can produce factors that suppress an antitumor immune response (42–45) and it is conceivable that such a mechanism may have been involved in our system as well.

Anti-tumor immunization by both the intramuscular and subcutaneous route was enhanced by GM-CSF and B7-1, resulting in fewer mice with tumors, delayed or arrested tumor growth, and significantly increased overall survival. Vaccination of mice with GM-CSF–secreting irradiated B16 melanoma cells has previously been shown to induce anti-tumor immunity against normal B16 melanoma cells. This effect has been attributed to enhanced tumor antigen presentation by GM-CSF by promoting the differentiation and maturation of dendritic cells (25). It has, however, been difficult to demonstrate that GM-CSF indeed enhances immune responses to specific tumor antigens, since the same (tumor) cells were used for both vaccination and challenge, and the antigen had not been defined on these tumors. In contrast, in our model MAGE-1 and MAGE-3 are likely to be the only antigens common to the modified fibroblasts, injected muscle cells, and B16 melanoma cells. Consequently, the increased tumor protection obtained by coexpressing GM-CSF should be the result of enhanced immune responses against these antigens. The fact that GM-CSF alone (in the absence of tumor antigen) failed to induce protective immunity against B16-MAGE-1 or B16-MAGE-3, and that protection was specific for tumors expressing MAGE-1 or MAGE-3, suggests that GM-CSF indeed causes increased tumor protection by enhancing the presentation of and immune responses against these tumor antigens. A similar effect of GM-CSF on intramuscular immunization against the rabies virus G protein was demonstrated previously (46). Taken together, the results strongly support the hypothesis that enhanced tumor protection after coexpression of GM-CSF is due to increased immune responses against specific tumor antigens, presumably mediated by enhanced antigen presentation by dendritic cells.

The stimulation of tumor protection by coexpressing B7-1 was most likely due to increased costimulation of MAGE-specific T cells (40,47, 48). This notion is supported by a recent study which showed that mice immunized with a mixture of recombinant vaccinia viruses encoding the human carcinoembryonic antigen (CEA) and B7-1 have enhanced CEA-specific T cell responses and antitumor immunity as compared to mice immunized with either vaccinia virus alone (49). B7-1 has been shown to stimulate the development of Th1 type helper T cells (50) which secrete cytokines that induce cellular immunity (51) believed to be important for tumor rejection. However, the enhancement of antitumor immunity by B7-1 in our experiments may be more likely caused by direct costimulation of antigen-specific cytotoxic T cells (47,48), since embryonic fibroblasts and muscle cells do not express MHC class II molecules. Alternatively or in addition, expression of B7-1 by embryonic fibroblasts and muscle cells may have mediated their recognition and lysis by natural killer cells, as has been shown for B16 cells expressing high levels of B7-1 (52). This could have resulted in increased release of MAGE antigens for uptake and presentation by bone marrow–derived antigen-presenting cells, a mechanism that has been implicated in the generation of anti-tumor immunity caused by tumor cell vaccines (53).

Since MAGE antigens are not expressed in normal human tissues, except testis, it is unlikely that tolerance against them has been established (54). This is consistent with the presence of MAGE-specific cytotoxic T lymphocytes (CTLs) in melanoma patients expressing certain MHC class I haplotypes (10,19–21). Coexpression of GM-CSF and B7-1 with MAGE-1 and MAGE-3 in autologous antigen-presenting cells of patients may boost the immune response of these lymphocytes against the MAGE antigens and may be exploited as a tumor therapy. This is supported by the recent demonstration that immunization of melanoma patients with autologous antigen-presenting cells pulsed in vitro with a MAGE-1 nonapeptide stimulated tumor-reactive CTLs in situ (23). Our results further suggest that investigation of intramuscular immunization against MAGE antigens as a therapy against melanomas

and other MAGE-expressing tumors in humans may be warranted in patients with slow tumor progression that would allow for multiple immunizations, or in combination with other immunotherapies. Genetic immunization, if proven safe, would have the advantage of circumventing the isolation and ex vivo manipulation of the patient's antigen-presenting cells or tumor-infiltrating lymphocytes. In addition, mixing of plasmids encoding tumor antigens and cytokines to enhance anti-tumor immunity could be easily accomplished. The significance of the fact that GM-CSF and B7-1 augmented tumor immunity only for low amounts of antigen-encoding DNA is unclear. The importance of this effect depends on whether the amount of antigen DNA that can be delivered is limiting and on the intrinsic immunogenicity of the antigen. The methods described in this work should be applicable to other tumor antigens such as the E6 and E7 proteins expressed by human papilloma virus-induced cervical carcinomas (55), HER-2/neu and prostate-specific antigen, which are overexpressed in ovarian (56) and prostate (57) cancers, respectively, as well as the melanoma antigens MART-1, tyrosinase, gp100 (54), BAGE (2), and GAGE (9).

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