Plasmid DNA-based molecular cancer vaccines generally suffer from suboptimal immunogenicity. One of the key limitations is insufficient level of gene expression, which was surmounted in our approach by using the novel technique of in vivo plasmid electroporation-enhanced vaccination (electrovaccination). Electrovaccination with plasmids encoding the full-length autologous melanocyte antigen tyrosinase-related protein-2 induced limited melanocyte destruction in a subset of mice. Despite examples of vitiligo, vaccinated mice were not protected from a subsequent challenge of B16F10M melanoma cells. Novel constructs were then designed and submitted to a functional screen. Best performance was obtained when the relevant H-2Kb-restricted epitope SVYDFFVWL was placed into a context of sequences of the HLA-Cw3 molecule. After animals were electrovaccinated using this construct, direct enzyme-linked immunospot analysis of peripheral blood mononuclear cells indicated that very high numbers of T cells recognizing the specific tyrosinase-related protein-2 epitope were generated. CD8+ T cells isolated from the spleen also displayed a high degree of antigen-specific reactivity and vigorously reacted toward unmodified B16F10M cells. In vivo protective effects of this construct were demonstrated in mice using two different models; outgrowth of s.c. implanted B16F10M tumor cells was significantly delayed, and vaccinated mice developed no or only very few tumor nodules in an i.v. lung metastasis model. Thus, improved antigen vectors delivered by highly effective gene transfer methods may form the basis for future human applications.

INTRODUCTION

Since the cloning of the first human tumor antigens (1), antigen-specific vaccination has been considered as a promising therapeutic approach against tumors. The B16 melanoma model became a reference where such treatments can be tested (2). Similar to human cancers, B16 cells are very weakly immunogenic, and sublines exist with varying tumorigenic and metastatic capabilities (3, 4). Murine TRP2-2 has been identified as a tumor antigen in B16 melanoma upon expression cloning and screening with cytotoxic T cells (5).

One particular problem for antigen-specific vaccination is that many tumor antigens, including the melanocyte lineage-specific antigens of B16, are self proteins with enhanced expression in tumors. This inevitably raises the question of whether tolerance or clonal deletion would prevent effective immunizations against them (6). More recently, a number of reports have shown that immunization against selected self antigens is indeed possible and may result in protection against tumor challenge. Successful examples include autologous dendritic cells loaded with sources of tumor antigens (7, 8, 9) and various antigen-expressing recombinant live viral or bacterial vectors (10). The potential for elicitation of unwanted host responses, together with other safety, manufacturing, and economic issues, nevertheless reduces the attractiveness of these vaccines. In contrast, plasmid-based genetic immunization is an appealing strategy because it offers a cheap and stable antigen source. However, DNA vaccines are generally being considered as weak immunogens and thus require multiple improvements. Recent observations indicate that in vivo expression of encoded proteins is markedly augmented when plasmid injection is followed by subsequent electric pulses (11, 12). When the electroporated plasmids encoded viral antigens, increased antibody titers were observed (13).

In this study, we have investigated the effectiveness of the electroporation-enhanced in vivo plasmid DNA antigen delivery, or EV for short, in the stringent B16 melanoma model. The murine TRP-2 with a functional epitope displayed in H-2Kb context was chosen as the vaccine antigen. Although signs of broken tolerance were occasionally seen in the form of limited depigmentation, induction of high levels of antigen-specific T cells required profound modifications of the antigen structure. Significant antitumor immunity was achieved when these novel fusion antigen-encoding plasmids were applied in combination with EV.

MATERIALS AND METHODS

Cells. The murine thymoma cell line EL-4 was obtained from American Type Culture Collection (Manassas, VA). The murine melanoma B16F10 was a kind gift of R. Kircheis (Boehringer Ingelheim, Austria), from which the pigmented, TRP-1.2-expressing and in vivo aggressively growing B16F10M subline was subcultured in our laboratory. EL-4 cells were transfected with plasmids 624 and 626 by electroporation, and stable clones showing high expression of the relevant fusion proteins were obtained by chemical selection, FACS sorting, and limiting dilution. PBMCs were isolated by Ficoll-Hypaque density centrifugation from 250 μl of heparinized blood obtained from the retroorbital sinus. CD8+ T cells were isolated from spleen cell suspensions using antibody-coated paramagnetic particles (Miltenyi Biotech, Bergisch Gladbach, Germany).

Plasmids and Immunizations. In keeping with Food and Drug Administration guidelines, all plasmids used for vaccinations carried the neomycin phosphotransferase gene (Kanr) for bacterial selection. CpG content and, consequently, immunogenicity of the plasmid backbone was therefore lowered (14). Full-length mTRP-2 was obtained by reverse transcription-PCR from mRNA of cultured mouse melanoma cells. The complete insert of pEGFP-C1 (B-D Clontech) was then exchanged for the TRP-2 cDNA to yield pKN. mTRP2. Plasmid pVAX.Cw3 was obtained by inserting a reverse transcription-PCR product of the full-length HLA-Cw*0303 coding sequence into pVAX1 (Invitrogen, Carlsbad, CA). Also in pVAX1, the modified human immunoglobulin kappa light chain leader sequence (MEAPAQLLFLLLWLP-DTIGE) was inserted (pVLL). Oligonucleotides encoding for the SVYDFFVWL epitope were then inserted downstream of the leader sequence, yielding pVLL_SVYD. Pairs of primers encoding for the SVYDFVW and TAYRYHLL (15) epitopes were used in SOE PCR on the pVAX.Cw3 template to generate pVAX.CS(VYD)C and pVAX.C/TAYRc. Oligonucleotides encoding for the SVYDDFFWV and TAYRYHLL epitopes were inserted in-frame into the multiple cloning site of pEGFP-C1 to yield plasmids 624 and 626. Plasmids were either purified on CCl gradients or custom manufactured by ELIM Pharmaceuticals (South San Francisco, CA) and contained <5 EU/mg endotoxin.
Mice. Female C57BL/6 mice, 8 weeks of age, were purchased from Harlan (Borcheu, Germany) and held under specific pathogen-free conditions. Mice were acclimated for 1 week before the start of the study. Mice were anaesthetized with Avertin, and required amounts of plasmids were diluted into 100 μl of 20 mM HEPES buffer (pH 7.4), and 50 μl were applied to both quadriceps femoris muscles. Immunization was followed immediately by electroporation of the injected area (80 V, three pulses of 60 ms with repoling) using an Electro Square Porator device (T820; BTX, San Diego, CA). For s.c. challenge, 3 × 10⁴ B16F10M cells were injected in 100 μl of PBS. Tumor volumes were measured three times a week. Mice that developed tumors of 1200 mm³ were sacrificed for ethical reasons. In the i.v. metastasis model, 8 × 10⁴ live B16F10M cells were injected s.c. Average tumor volumes are shown for each group of eight mice; bars, SE.

RESULTS

EV with Plasmids Encoding the Full-Length mTRP-2 Antigen Induces Limited Melanocyte Destruction but No Protection in a Stringent Mouse Melanoma Model. Electroporation was found previously by us and others to enhance expression of injected plasmids in vivo at least 10–100-fold (16). To test the efficiency of EV in a stringent mouse melanoma model, we have constructed an expression plasmid containing the autologous full-length mTRP2 under the control of the CMV promoter. Two vaccinations of 50 μg of plasmid DNA were applied at days 0 and 14. As a consequence, a few C57/B16 mice (3 of 23) displayed depigmentation, resulting in a characteristic salt-and-pepper-like pattern (Fig. 1, a and b). Histopathological examination of the depigmented skin areas taken from 1 of these mice showed absence of pigment in particular hair follicles (Fig. 1c). In contrast, all follicles were pigment loaded in the unaffected areas (Fig. 1d) of the same animal.

After similarly scheduled EVs, a s.c. challenge of 3 × 10⁴ live B16F10M cells (~30-fold tumorigenic dose) was applied at day 21. We found that despite the favorable expression-enhancing properties of the EV regimen, mice vaccinated with the full-length pKN.mTRP2 plasmid construct were not protected against the challenge. Vaccination with plasmid pVAX.SVYD, which encodes for the dominant mTRP-2 epitope (5, 9) fused to the immunoglobulin leader sequence, was similarly ineffective (Fig. 1e).

Optimized Vaccine Plasmid Constructs Obtained by Biological Screening. The inability of these vectors to protect against an autologous tumor challenge indicated that antigen structures had to be optimized. However, because B16F10M cells express only low levels of HLA class I molecules and in addition may switch off expression of the EV regimen, mice vaccinated with the full-length pKN.mTRP2 plasmid construct were not protected against the challenge. Vaccination with plasmid pVAX.SVYD, which encodes for the dominant mTRP-2 epitope (5, 9) fused to the immunoglobulin leader sequence, was similarly ineffective (Fig. 1e).

Histology. Skin tissues were fixed in 10% buffered formaldehyde and embedded in paraffin, and 5-μm sections were stained with H&E.

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A variety of constructs, encoding truncations, fusions, and targeted mutations of the antigen, were electroporated and tested for their protective effect against the transfected EL-4 cell lines. A particularly effective modification involved fusion of the dominant epitopes into the human HLA-Cw3 gene. HLA-Cw3 was described previously as a unique antigen that generated vast amounts of antigen-specific CD8+ T cells. The inability of these vectors to protect against an autologous tumor challenge indicated that antigen structures had to be optimized. However, because B16F10M cells express only low levels of HLA class I molecules and in addition may switch off expression of the TRP-2, we decided to establish a more robust tumor model to test novel vaccine plasmid constructs. EL-4 thymoma cells are syngeneic to C57/B16 mice and express high levels of H-2Kb, which is the allele presenting the dominant TRP-2-derived epitope (Fig. 2a). EL-4 cells were thus stably transfected with vectors that express SVYDFFVWl fused to the COOH-terminal end of EGFP (cell line ELA/#624; Fig. 2b). Another cell line (ELA/#626) that carries EGFP fused to the optimized TRP-1-derived epitope TAYRYHLL (15) was established in parallel (Fig. 2c).
CTLs (17), an observation that we could confirm with our EV protocols. Mice that were electrovaccinated with the plasmid pVAX.C(SVYD)C, encoding the TRP-2-derived epitope SVYDFFVWL in a Cw3 context, were subsequently protected against a lethal challenge with EL4#624 cells, which express the same epitope in a different context (Fig. 2d). These mice were, however, not protected against a challenge with the EL4#626 line, which carries the TRP-1-derived epitope. Similarly, mice electrovaccinated with the plasmid pVAX.C(TAYR)C were protected against EL4#626 cells but not against the EL4#624 line (Fig. 2e).

EV with Fusion-Sequence Plasmid Immunogens Generates High Levels of Specific T Cells. Induction of cellular immune responses is the consensus mode of action of cancer vaccines. To precisely determine the number of antigen-specific CD8+ T cells that were generated after EV, we have further refined the ELISpot method. Mice received two immunizations with various plasmids or were left untreated. PBMCs were isolated from a small amount of peripheral blood so that mice could be assessed individually without sacrificing them, and afterward could be used for additional tests. PBMCs were magnetically isolated from pooled spleen cell suspensions and restimulated in vitro. Effector cells were then cocultured with target cells and IFN-γ released into the supernatant was measured by ELISA. Cells obtained from mice that were electrovaccinated with pVAX.C(SVYD)C recognized with high efficiency the native and IFN-γ-pretreated B16F10M cells as well as the EL4#624 cell line carrying the corresponding TRP-2 epitope but not the irrelevant EL4 derivatives (Fig. 3c). In contrast, effector cells from pVAX.C(TAYR)C immunized mice recognized only the appropriate EL4#626 line but not the unmodified melanoma cells. Only after IFN-γ pretreatment was a small but significant recognition detectable (Fig. 3d). These effector cells were also simultaneously stained with peptide-loaded H2Kb:Ig fusion proteins (18) and with anti-CD8 mAb (Fig. 3e–h). More than 86% of the CD8+ T cells obtained from mice that were electrovaccinated with pVAX.C(SVYD)C bound H2Kb:1g–SVYDFFVWL (Fig. 3g), whereas they did not bind unloaded (Fig. 3e) or TAYRHYLL-pretreated (Fig. 3f) fusion proteins. This indicates that high affinity, TRP-2 epitope-specific CD8+ T cells are generated upon EV. The number of positive cells and intensity of staining with H2Kb:1g-TAYRHYLL was lower when similarly prepared, TAYRHYLL-specific CD8+ T cells were tested (Fig. 3h).

Fusion-Sequence Plasmid Immunogens Protect against Outgrowth of Syngeneic Tumor Cells. Protective efficacy of the optimized constructs was examined in two different tumor challenge models. Animals were electrovaccinated on days 0 and 14 and challenged 1 week thereafter s.c. with 3 × 105 live B16F10M cells. Compared with control animals, tumor outgrowth was significantly delayed in the group of mice that had been immunized with the
pVAX.C(SVYD)C plasmid. In individual mice also, complete protection was achieved repeatedly (Fig. 4). Vaccination with plasmids encoding the two main constituents of pVAX.C(SVYD)C, i.e., the TRP-2 epitope (pVVL.SVYD) and the Cw3 molecule (pVAX:Cw3) alone, did not result in any significant growth delay over the untreated group.

In a lung metastasis model, mice were electrovaccinated three times and were then injected i.v. with 8 x 10^5 live B16F10M cells. Additional sentinel mice were included in these experiments and examined regularly. When their lung coverage approached 80%, generally at days 20–22 after tumor cell injection, the experiment was terminated. Mice that had been electrovaccinated with the pVAX.C(SVYD)C plasmid developed significantly fewer and smaller metastatic nodules, and some of them remained tumor free (Fig. 5, a, upper panel, and b). Lungs of each untreated animal were covered to a large extent with melanoma cells (Fig. 5a, lower panel). Immunization with the TRP-1-derived pVAX.C(TAYR)C plasmid also afforded some protection but clearly less than the TRP-2-based immunogen (Fig. 5b).

DISCUSSION

In vivo plasmid electroporation is a highly effective gene delivery system (19, 20, 21). We have thus asked whether the enhanced gene expression could be exploited for antitumor vaccinations. In fact, while these experiments were under way, promising reports appeared (22, 23). Our initial experiments confirmed that in certain cases, EV...
with an expression plasmid encoding for the autologous mouse TRP-2 can indeed break tolerance and provoke a limited autoimmunity, as seen by the loss of pigmentation in selected hair follicles of affected mice. However, in our hands, the same approach did not confer protection against a challenge with syngeneic melanoma cells.

Protection but no vitiligo upon mTRP-2 DNA vaccination was reported recently by Bronte et al. (24), but these investigators obtained their results by using F1, hybrid mice. Xenogenic human antigen analogues were also shown to improve antimalanoma activity in mice (15, 9). These approaches were not considered here, because for a future human vaccine composition, these results are difficult to interpret and use. It was thus a priority for us to remain in an uncompromised syngeneic model system. We have also abandoned the use of the β-lactamase bacterial selection marker for plasmid propagation, which in turn substantially decreased the number of potentially immunostimulatory CpG sequences in our vectors (25, 26). Other strategies, among them fusion constructs harboring potential enhancer or helper structures, were then considered. Recombinant EL-4 cell lines that expressed the relevant epitopes in addition to their high endogenous levels of MHC class I molecules were used to screen plasmids for their ability to prevent tumor outgrowth. Constructs that directed the expression of the autologous dominant mTRP-2 epitope fused into the human Cw3 molecule turned out to be the most effective ones. The recombinant mouse mastocytoma cell line P815 carrying the HLA-Cw3 molecule has been noted for its unique property of inducing high levels of CD8+ T cells in DBA/2 mice (17). Here we have replaced the H-2Kd-restricted dominant Cw3170-179 epitope with the epitopes derived from the TRP-1 and TRP-2 tumor antigen. In our hands, fusion constructs using these two epitopes generated far more antigen-reactive cells than unmodified constructs. Given an average 8–10% CD8+ T-cell content of PBMCs, the number of specific spots measured in the ELISpot assay corresponds to a frequency of 1 in 1000 to 1 in 300 in responder mice for the TRP-2 encoding pVAX.C(SVYD)C plasmid. Importantly, these numbers compare very favorably with the current best DNA prime-modified vaccinia virus Ankara boost protocols (27). In contrast, the unmodified full-length mTRP-2 or the dominant epitope coupled to a highly efficient leader sequence only occasionally induced low numbers of spots. After CD8+ T cells were isolated from the spleens of these mice, we further confirmed their specificity and found that although the TRP-2-specific CD8+T cells readily recognized the unmodified parental B16F10M cells, the TRP-1-specific cells did not. This was unexpected from published data (15). Possible explanations could be that the epitope product of the DNA vaccine physically or structurally differs from the synthetically prepared peptide, causing loss of affinity that was visible when peptide-loaded H-2Kd:Ig constructs were used for FACS analysis. More likely, the anchor-optimized TAYRYHLL functionally does not correspond with the natural peptide processed by unstimulated cells. The finding that B16F10M cells provoked a small but significant recognition after IFN-γ pretreatment points in this direction. With this background, it is not surprising that admixing the (in itself very immunogenic) pVAX.C(TAYYR)C vector to the pVAX.C(SVYD)C vector did not improve protection against the B16F10M challenge (data not shown).

As expected from the outstanding CTL data, EV with the pVAX.

**Fig. 4.** EV with fusion-sequence plasmid immunogens protects against s.c. tumor challenge. Groups of eight mice were electrovaccinated twice with the plasmid pVAX.C(SVYD)C (△), SVYDFFVWL epitope engineered in-frame into the human HLA-Cw3), with the plasmid pVLL.SVYD (●; SVYDFFVWL epitope downstream of the immunoglobulin leader), with the plasmid pVAX.Cw3 (○; full-length human HLA-Cw3 under CMV promoter), or were left untreated (◼). Average tumor volumes are shown after mice were challenged 1 week after the second vaccination with 3 × 10⁶ live B16F10M cells injected s.c.; bars, SE.

**Fig. 5.** EV with fusion-sequence plasmids can prevent tumor nodule formation in a B16 lung metastasis model. Mice were electrovaccinated at days 0, 7, and 14 with 50 μg of the indicated constructs. On day 21, 8 × 10⁵ B16F10M cells were injected i.v. Lungs were removed on day 43 and evaluated under a dissecting microscope. a, upper panel, lungs of mice that were electrovaccinated with the plasmid pVAX.C(SVYD)C are free of tumor nodules (left two lungs) or display only a few small metastatic nodules (arrowsheads). a, lower panel, lungs of untreated mice are covered to a large extent with melanoma cells. b, percentages of lung surfaces covered by melanoma metastases in untreated or electrovaccinated groups. Averages are shown, and the numbers of metastasis-free animals are reported in parentheses; bars, SD. Statistical comparison using Student’s t test yields P < 0.02 between untreated and pVAX.C(TAYYR)C plasmid-treated groups and P < 0.001 for untreated and pVAX.C(SVYD)C plasmid-treated groups.
C(SVYD)C fusion plasmid construct yielded considerable immunity against the wild-type B16F10M melanoma. Outgrowth of tumors was always significantly retarded, and although protection was incomplete, mice that remained tumor free were found frequently. Our results show clearly that a molecular vaccine against melanoma, based on in vivo electroporation of plasmids (EV), is an approach with great promise. Novel antigen structures that elicit superior CTL responses were generated. These can control tumor growth in a rigorous model system, which properly approximates the human situation. Nevertheless, further optimizations will be required before the promise of molecular cancer vaccines turns into reality.

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3. Zhu, D. Z., Cheng, C. F., and Pauli, B. U. Mediation of lung metastasis of murine melanoma, based on electroporation of plasmids (EV), is an approach with great promise. Novel antigen structures that elicit superior CTL responses were generated. These can control tumor growth in a rigorous model system, which properly approximates the human situation. Nevertheless, further optimizations will be required before the promise of molecular cancer vaccines turns into reality.
In Vivo Plasmid Electroporation Induces Tumor Antigen-specific CD8+ T-Cell Responses and Delays Tumor Growth in a Syngeneic Mouse Melanoma Model

Milena Kalat, Zaruhi Küpcü, Susanne Schüller, et al.


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